FACTORS RELACIONATS AMB EL MANTENIMENT DE LA INFLAMACIÓ I L'OCLUSIÓ VASCULAR. IMPLICACIONS EN L'EVOLUCIÓ DELS PACIENTS AMB ARTERITIS DE CÈL·LULES GEGANTS.

Tesi presentada per

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Life would be very simple
if the signal and response were connected by a single linear pathway,
but this seems never to be the case.
Rather, multiple branching, overlapping and partially redundant pathways control the behavior of the cell. Probably such complicated networks are necessary to confer stability and resilience on the extraordinarily complex machinery of a cell.

Human Molecular Genetics
Strachan T.

Agraïments

AGRAÏMENTS (per ordre d'aparició)

Sempre m'he estimat més donar les gràcies personalment i en el seu moment. Ara és una mica complicat fer-ne una llista perquè m'han ajudat molt i moltes persones, no voldria descuidar-me ningú, vosaltres sabeu qui sou, o sigui que gràcies a tots.

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(Encara tinc l'esperança que siguin gens d'expressió tardana)

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Abreviatures

ABREVIATURES

aa – aminoàcids

ACG - Arteritis de cèl·lules gegants (= GCA)

AP-1 - *Activator protein S-1*

CML – Leucèmia mieloide crònica (*Chronic Mieloid Leukemia)*

CCL2 – Chemokine CC ligand 2 (= MCP-1)

EC - Cèl·lules endotelials

ECE – Enzim convertidor de l'endotelina (*Endothelin converting enzyme*)

ECM – Matriu extracel·lular (*Extracellular matrix*)

ERK – Quinasa regulada per senyals extracel·lulars (*extracellular signal-regulated kinase*).

ET-1 – Endotelina-1

FGF-2 – Factor de creixement per a fibroblasts (*Basic fibroblast growth factor*)

GCA - Arteritis de cèl·lules gegants (*Giant Cell Arteritis*)

GIST - Tumor estromal gastrointestinal

HIF - Factor induïble per la hipoxia (*Hypoxia inducible factor*)

ICAM-1 - *Intracellular adhesion molecule-1*

IC50 - Concentració d'inhibició del 50%

IFN-γ – Interferó γ

IL-1 – Interleucina -1

MAPK - *Mitogen-activated protein kinase*

MCP-1 – Proteïna quimiotàctica per a monòcits *(Monocyte chemoattractant protein-1)* (= CCL2)

MMP – Metal·loproteïnases

NFκB – Factor de transcripció nuclear kappa B (*Nuclear factor kappa B)*

NO – Òxid nítric

PDGF - Factor de creixement derivat de plaquetes (*Platelet-derived growth factor*)

PI3k - Fosfatidilinositol 3' quinasa

SCF - Factor de creixement de cèl·lules mare (*Stem cell factor*)

SH2 - Domini d'homologia a Src (Src *homology 2 domain*)

SMC - Cèl·lules de múscul llis (S*mooth muscle cells*)

TGFβ – Factor de creixement transformant β (*Transforming Growth Factor β*)

TNFα – Factor de necrosi tumoral α (*Tumor Necrosis Factor α*)

Tyr – Tirosina

VCAM-1 - Molècula d'adhesió de cèl·lules vascular-1 (*Vascular cell adhesion molecule*)

VEGF - Factor de creixement endotelial vascular (*Vascular endothelial growth factor*)

VSMC - Cèl·lules de múscul llis vasculars (*Vascular smooth muscle cells*)

Introducció

1. INTRODUCCIÓ

Les vasculitis són un grup heterogeni de síndromes caracteritzades per la inflamació de la paret dels vasos sanguinis. Les seves manifestacions clíniques depenen del calibre i localització dels vasos lesionats i del tipus d'inflamació que hi té lloc. Així, l'espectre clínic pot fluctuar des de quadres autolimitats de caràcter benigne amb escasses manifestacions, freqüentment cutànies, fins a greus processos inflamatoris amb repercussió sistèmica, complicats freqüentment amb fenòmens isquèmics viscerals.

1.1. L'ARTERITIS DE CÈL·LULES GEGANTS (ACG)

1.1.1. DEFINICIÓ

L'ACG és una arteritis sistèmica de caràcter granulomatós que afecta vasos de calibre mitjà i gran, preferentment les branques de la caròtida, com són l'artèria temporal superficial, l'artèria oftàlmica i les artèries ciliars posteriors, però també l'aorta i altres de les seves branques majors. A diferència de l'arteritis de Takayasu, on quasi tots els pacients presenten símptomes per l'afectació de les grans branques aòrtiques, a l'ACG només el 10-15% dels pacients els presenten (Cid et al., 2007).

L'histopatologia típica de la malaltia va ser descrita per Horton l'any 1932. Es caracteritza per la presència d'infiltrats inflamatoris mononuclears que predominen en la proximitat de la lamina elàstica interna, constituïts per limfòcits i macròfags, que en aproximadament el 50% dels casos contenen cèl·lules gegants multinucleades. A diferència d'altres arteritis, a l'ACG és rara la presència de necrosi fibrinoide. Freqüentment s'observa neoangiogènesi, produint una extensió de la xarxa vascular més

enllà de la seva localització normal restringida a l'adventícia cap a la mitja i íntima arterial.

D'etiologia desconeguda, la seva patogènia és immune tot i que l'antigen o antígens que desencadenen aquesta resposta encara no han estat identificats. El reclutament i l'activació de macròfags constitueix un del punts més importants de la seva patogènia. La destrucció produïda pels macròfags del teixit elàstic arterial és un fenomen rellevant, així com la producció d'altres factors promotors de la proliferació de la neoíntima, responsable a través de l'obliteració de la llum, de les manifestacions isquèmiques de la malaltia. El procés s'acompanya d'una important repercussió sistèmica caracteritzada per una forta reacció de fase aguda i símptomes generals de malaltia poc específics. D'altra banda, un important percentatge dels pacients presenten un quadre de polimiàlgia reumàtica. Té major incidència en dones que en homes. La mitjana d'edat en el moment de l'aparició de la malaltia es situa al voltant dels 70 anys (Cid et al., 1998).

La morbilitat més greu associada a aquesta malaltia es relaciona amb fenòmens d'isquèmia distals a l'estenosi luminal de les artèries inflamades i en menor mesura, amb la formació d'aneurismes per la debilitació de la paret arterial i ruptura aòrtica (Garcia-Martinez et al., 2008). Tot i que, la mortalitat en els pacients amb biòpsia positiva augmenta durant els quatre primers mesos després d'iniciar el tractament, la supervivència no sembla veure's afectada per l'aparició de la malaltia (Nordborg, Nordborg & Petursdottir, 2000).

1.1.2. MANIFESTACIONS CLÍNIQUES

Les manifestacions clíniques d'aquesta malaltia es poden classificar en :

1. Símptomes arterítics secundaris a la inflamació vascular focal i isquèmia a nivell cranial : alteracions visuals, claudicació mandibular, cefalea, engruiximent arterial, dolor local, etc.
2. Símptomes sistèmics : astènia, anorèxia, pèrdua de pes i febre
3. Símptomes polimiàlgics : dolor i rigidesa a les articulacions proximals de cintures.

Les artèries ciliars posteriors són la principal font d'aportament sanguini del nervi òptic. L'oclusió d'aquestes artèries produeix la neuropatia òptica isquèmica anterior arterítica (A-AION). Ocasionalment, també es pot produir l'oclusió de l'artèria retinal central, en ambdós casos, la pèrdua de visió és habitualment irreversible (Hayreh, 2000). La pèrdua de visió és una de les complicacions isquèmiques de l'ACG més temudes, succeeix aproximadament en el 15-20 % dels pacients. Les alteracions visuals transitòries estan presents en aproximadament el 15% dels pacients.

Tot i que, els símptomes esmentats poden variar en cada pacient, s'observen dos subgrups de malalts clarament diferenciats en funció de la seva resposta inflamatòria. D'una banda, els **pacients amb una resposta inflamatòria dèbil** tenen més risc de desenvolupar fenòmens isquèmics derivats de la malaltia (Cid et al., 1998). Aquests pacients expressen menys citocines proinflamatòries en el sèrum i en les biòpsies arterials. A més, tenen menys expressió de molècules d'adhesió endotelial i la resposta angiogènica és més dèbil (Cid et al., 2000). En aquests casos, els pacients assoleixen més

ràpidament una remissió sostinguda de la malaltia, pateixen menys recurrències i requereixen una menor dosi acumulada de corticosteroides.

D'altra banda, els **pacients amb una forta resposta inflamatòria** pateixen una malaltia més refractària. Els nivells de citocines proinflamatòries en sèrum i en teixit són més alts (Hernandez-Rodriguez et al., 2003). A les lesions arterials, s'observa més neovascularització (Cid et al., 2002) i més expressió de molècules d'adhesió. Aquests pacients tenen menys complicacions isquèmiques però requereixen més temps de tractament i pateixen més recurrències (Hernandez-Rodriguez et al., 2002).

1.1.3. HISTOPATOLOGIA DE LA LESIÓ

Els components bàsics de la paret dels vasos sanguinis són les cèl·lules endotelials (EC), les cèl·lules de múscul llis (SMC) i les proteïnes de matriu extracel·lular (ECM) que inclouen elastina, col·lagen i glicosaminoglicans. Les tres capes concèntriques : íntima, mitja i adventícia, estan clarament definides en els vasos més grans, particularment, a les artèries (figura 1). A les artèries no patològiques, l'íntima consisteix en una sola capa de cèl·lules endotelials i el teixit connectiu subendotelial és mínim, però pot augmentar amb l'edat. Està separada de la làmina mitja per una membrana elàstica densa anomenada làmina elàstica interna. Les capes de SMC de la mitja que es troben a prop de la llum del vas reben l'oxigen i els nutrients per difusió directa des de la llum, mentre que les parts més externes en els vasos grans i mitjans es nodreixen gràcies a petites arterioles que es localitzen al voltant del vas, anomenades *vasa vasorum*, literalment vasos dels vasos. El límit extern de la làmina mitja en la majoria de les

artèries està ben definit per la làmina elàstica externa. Al seu voltant la làmina adventícia consisteix en teixit connectiu amb fibres nervioses i el *vasa vasorum*.

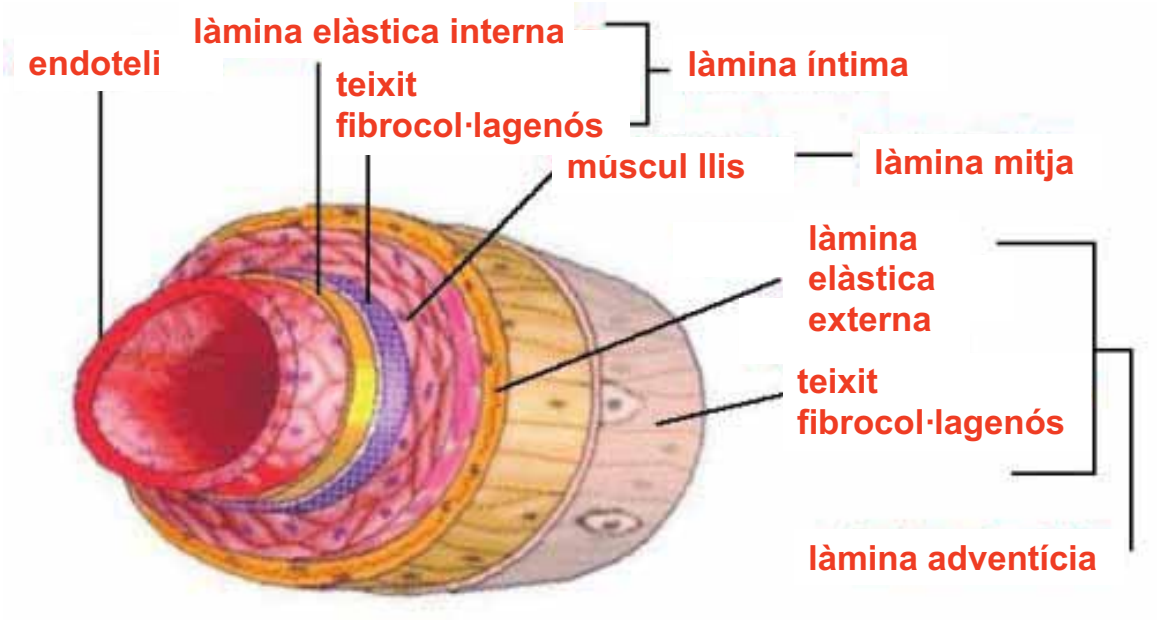
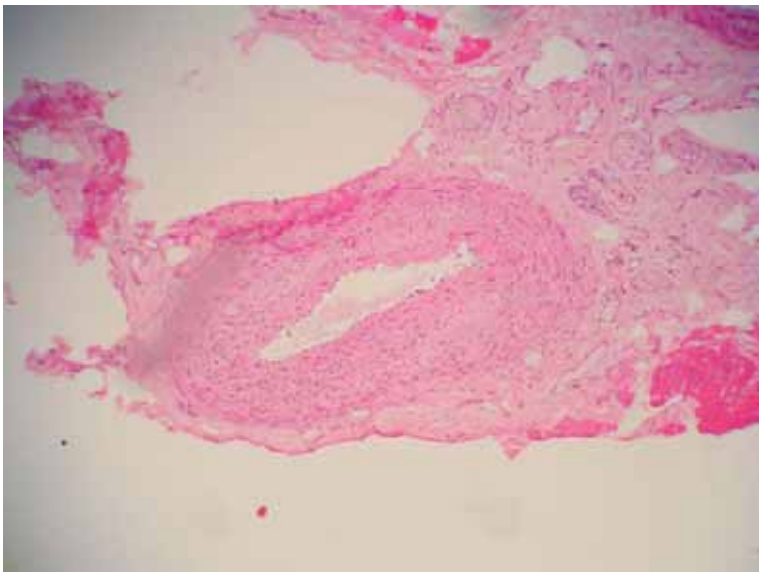


Figura 1. Esquema de l'estructura d'una artèria

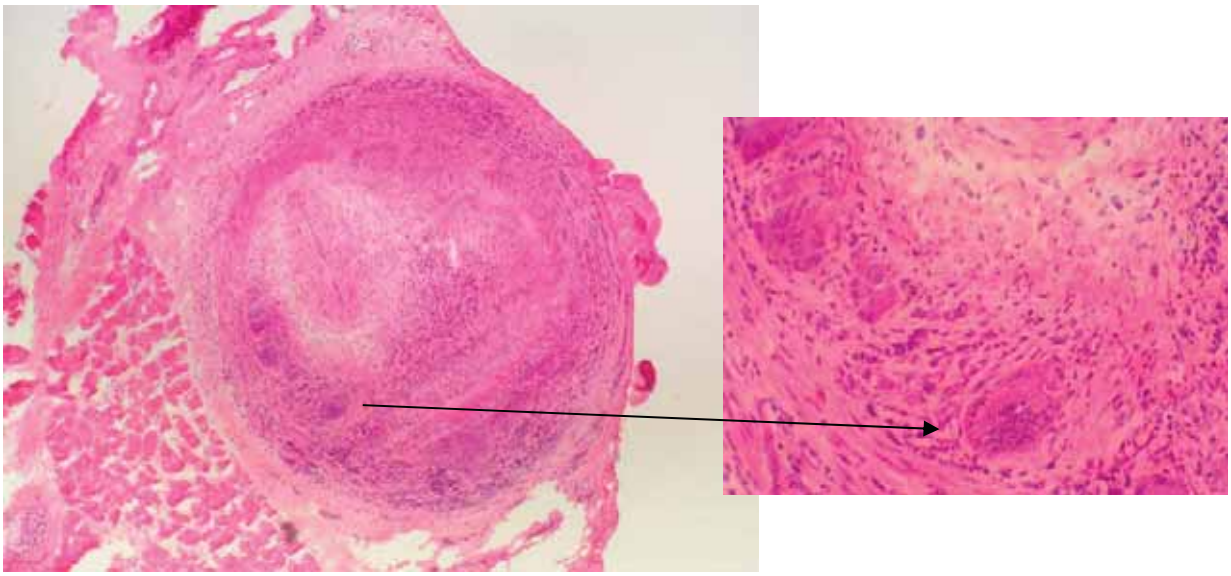
La biòpsia de l'artèria temporal és la prova que permet la confirmació histopatològica de la malaltia. La possible falsa negativitat de la biòpsia s'atribueix al caràcter segmentari de la malaltia.

L'ACG es pot definir com una arteritis linfomonocitària, amb freqüent formació de granulomes, en els que en el 50 % dels casos són apreciables cèl·lules gegants. Donat que l'ACG debuta a edats avançades, cal diferenciar els canvis histopatològics de l'ACG dels canvis propis de l'envelliment, com poden ser la hiperplàsia intimal o possibles calcificacions. L'ACG típica consisteix en una arteritis granulomatosa amb predomini de cèl·lules mononuclears i presencia de cèl·lules gegants, localitzades principalment al voltant de la membrana elàstica interna, amb destrucció d'aquesta, eixamplament de la capa íntima amb estenosi de la llum arterial i presència de neoangiogènesi. La infiltració

de polimorfonuclears amb necrosi fibrinoide és un fenomen molt rar i faria pensar en un altre tipus de vasculitis (figura 2).



A.



B.

Figura 2. Artèria temporal negativa (A) i positiva per ACG (B), tinció hematoxilina-eosina. Detall de les cèl·lules gegants multinucleades.

1.1.3.1. INFILTRAT INFLAMATORI

Les troballes histopatològiques i immunohistoquímiques suggereixen la presència d'una reacció immune cel·lular. L'infiltrat inflamatori està format majoritàriament per macròfags i cèl·lules T. Les cèl·lules de l'estirp **monòcit-macròfag (CD68+)** estan presents predominantment a la regió interna de la capa mitja i a la proximitat de la làmina elàstica interna, que es troba fragmentada. Les cèl·lules T que infiltren la paret són majoritariament (70-90%) **limfòcits col·laboradors CD4+** (Cid et al., 1989).

Es postula que la resposta inflamatòria s'inicia a la capa adventicial i l'arribada de l'infiltrat inflamatori es produeix a través dels *vasa vasorum*. Es suposa que el procés inflamatori s'inicia quan els limfòcits T són activats per la presentació d'un antigen o antígens desconeguts per part de les cèl·lules dendrítiques. Les **cèl·lules dendrítiques** són cèl·lules derivades de moll d'os que s'han trobat en teixits epitelials i limfoides. Funcionen com a cèl·lules presentadores d'antigen per a limfòcits T *naïve* i són importants per l'inici de la resposta immune adaptativa (Abbas & Sharpe, 2005). A l'ACG, les lesions arterials presenten aquests tipus de cèl·lules a les capes adventícia i mitja on activen als limfòcits T (Cid et al., 1989; Weyand et al., 2005).

Els limfòcits CD4+ participen en una **resposta tipus Th1** produint grans quantitats d'interferó-γ (IFNγ) que desencadena l'activació dels macròfags. Els macròfags que infiltren la paret arterial participen en la destrucció tissular produint dany oxidatiu i segregant metal·loproteïnases (MMP) i alhora produeixen citocines proinflamatòries i factors angiogènics com la interleucina 1β (IL-1β), tumor necrosi factor α (TNFα) i interleucina 6 (IL-6). Els macròfags també secreten citocines profibròtiques (TGF β,

PDGF) que poden conduir a l'oclusió vascular i les conseqüents complicacions isquèmiques.

A les lesions vasculars de l'ACG, els limfòcits CD4+ s'expandeixen de forma clonal i activen els fagòcits (monòcits i neutrofils) per una via dependent d'IFNγ generant la lesió granulomatosa (Weyand, Ma-Krupa & Goronzy, 2004). Els fagòcits també contribueixen a la inflamació vascular, secretant proteïnes S100 (complex S100A8/ S100A9 i S100A12) (Foell & Roth, 2004).

1.1.3.2. CÈL·LULES ENDOTELIALS (EC)

Les cèl·lules endotelials formen una sola capa de cèl·lules que limita tot el sistema cardiovascular i col·lectivament s'anomena endoteli. La integritat funcional i estructural endotelial és fonamental per el manteniment de l'homeostasi de la paret vascular i de la funció circulatòria normal. Les EC són les úniques que contenen els cossos de Weibel-Palade, són organuls de 1 μm d'amplada i 3 μm de llargada, que contenen substàncies vasoactives com la histamina, el factor von Willebrand (vWF), la P-selectina, l'osteoprotegerina (OPG) i l'endotelina (ET), entre d'altres. Les cèl·lules endotelials poden ser identificades immunohistoquímicament amb anticossos contra PECAM-1 (CD31, una proteïna localitzada a les unions interendotelials), CD34 i vWF, entre d'altres.

L'endoteli és un teixit multifuncional que participa activament en les interaccions sang-teixit. Com es tracta d'una membrana semipermeable, l'endoteli controla la transferència de molècules a través de la paret vascular. Les EC participen en el manteniment d'una interacció sang-teixit no-trombogènica produint molècules

anticoagulants, antitrombòtiques i reguladors fibrinolítics, com la prostaciclina, trombomodulina, molècules *heparin-like* i l'activador de plasminogen. I també poden produir molècules protrombòtiques com el factor vWF, *tissue factor* i l'inhibidor de l'activador de plasminogen. Les EC també modulen el flux sanguini i la resistència vascular, fabricant vasoconstrictors com l'endotelina (ET) i l'ACE (*angiotensin-converting enzyme*); i vasodilatadors com l'òxid nítric (NO) i les prostaciclines (Luscher, 2001).

Les cèl·lules endotelials regulen reaccions immunes i inflamatòries produint: IL-1, IL-6, quimiocines, molècules d'adhesió (VCAM-1, ICAM, E-selectina i P-selectina) i antigens d'histocompatibilitat de classe II (Cid, 2002). A més, poden controlar el creixement cel·lular d'altres tipus de cèl·lules, especialment les SMC, ja que produeixen factors estimuladors del creixement com PDGF, CSF, FGF i inhibidors com el TGFβ (Hirschi et al., 1999).

L'endoteli pot respondre a diferents estímuls fisiològics ajustant les seves funcions habituals, la funció endotelial normal es caracteritza per l'habilitat del vas de respondre apropiadament als diferents estímuls. L'endoteli també pot adquirir noves propietats (induïbles), un procés anomenat activació endotelial. Com a inductors d'aquest procés podem trobar citocines (TNFα) i productes bacterians (LPS), els quals causen inflamació i shock sèptic.

La disfunció endotelial es defineix per un fenotip alterat que perjudica la vasoreactivitat o bé indueix una superfície que es trombogènica o anormalment adhesiva a les cèl·lules inflamatòries, i que pot ser responsable, al menys en part, per l'inici de lesions vasculars.

1.1.3.3. CÈL·LULES DE MÚSCUL LLIS (VSMC)

Les cèl·lules de múscul llis vasculars (*vascular smooth muscle cells*, VSMC) són l'element cel·lular predominant de la làmina mitja. Són les responsables de la vasoconstricció i dilatació en resposta a estímuls fisiològics o farmacològics. També sintetitzen col·lagen, elastina i proteoglicans, i elaboren factors de creixement i citocines. Després d'un dany vascular, tenen la capacitat de migrar fins a la lesió i augmentar la seva proliferació. Així, les SMC són elements importants en la reparació vascular que es produeix en alguns processos patològics.

Les activitats migratòries i proliferatives de les SMC estan regulades per promotors i inhibidors del creixement. Entre els promotors es troben el PDGF, trombina, FGF i IL-1. Entre els inhibidors trobem heparà sulfats, NO i TGFβ. Altres reguladors inclouen el sistema renina-angiotensina (ex: angiotensina II), catecolamines, el receptor d'estrogen i osteopontina (un component de l'ECM) (Berk, 2001; Yancopoulos et al., 2000).

1.1.3.4. MATRIU EXTRACEL·LULAR (ECM)

La matriu extracel·lular (*extracellular matrix,* ECM) està formada per un conjunt de proteïnes fibroses i glicoproteïnes organitzades en xarxes ordenades i associades a les cèl·lules vasculars que les produeixen. Proporcionen una estructura mecànica resistent i alhora elàstica, i poden regular el comportament de les cèl·lules vasculars (proliferació, migració, supervivència).

A la capa íntima, l'ECM subendotelial està enriquida en proteoglicans i àcid hialurònic (HA). La capa mitja està formada per SMC envoltades d'elements elàstics,

col·lagens i proteoglicans. I la capa adventícia està constituïda per col·lagen fibrilar, fibroblasts i el *vasa vasorum*. L'ECM de cada capa proporciona propietats diferents al vas.

Els col·làgens estan formats per una hèlix de tres cadenes polipèptiques α, cadascuna amb una seqüència repetida gly-x-y. Als vasos sanguinis, el tipus I i III són els predominants i s'uneixen formant fibres que enforteixen el vas. Dins les membranes basals, sota les EC i envoltant SMC, es localitzen els col·làgens del tipus IV i VIII que no són fibrilars i formen xarxes tridimensionals.

L'elastina forma fibres elàstiques necessàries per resistir les pulsacions del flux sanguini, així com els canvis de pressió. La fibrilina és una glicoproteïna que dóna suport a l'acoblament d'aquestes fibres elàstiques.

Els proteoglicans i HA són molècules hidrofíliques que representen el tercer component de l'ECM. Els proteoglicans consisteixen en una proteïna central amb un o més polisacarids i regulen l'estructura i la permeabilitat del teixit connectiu. L'HA és una gran molècula formada per disacàrids repetits que pot unir moltes molècules d'aigua, formant un gel viscós que permet resistir forces de compressió.

La fibronectina i la laminina són glicoproteïnes que formen connexions amb altres ECM i amb cèl·lules vasculars. La fibronectina (≈ 450kDa) és una glicoproteïna que forma dímers amb ponts disulfur i que pot unir col·lagen, fibrina i proteoglicans a través de dominis específics, així com cèl·lules vasculars a través d'integrines específiques (Raines, 2000).

1.1.4. TRACTAMENT DE L'ARTERITIS DE CÈL·LULES GEGANTS

Actualment, els corticosteroides són el tractament d'elecció per els pacients amb ACG, ja que normalment aconsegueixen un ràpid alleujament dels símptomes i prevenen la pèrdua de visió i altres possibles complicacions isquèmiques. Tot i així, les necessitats terapèutiques varien molt entre els pacients. En alguns casos s'aconsegueix una remissió permanent de la malaltia en pocs mesos, mentre que d'altres sofreixen recurrències i requereixen dosis de corticosteroides durant períodes més llargs(Pipitone et al., 2006). El tractament perllongat amb corticosteroides ha estat associat amb nombrosos efectes secundaris:

- efectes adversos cardiovasculars : hipertensió, hiperlipidèmia
- musculoesquelètics : miopatia, osteoporosi, fractures vertebrals per compressió, fractures de maluc
- gastrointestinals : úlcera pèptica, sagnat gastrointestinal superior
- endocrins : intolerància a la glucosa, exacerbació de la diabetis
- oftàlmics : cataractes, glaucoma
- immunològics : increment en el risc de patir infeccions

Els efectes iatrogènics d'aquest tractament i el fet que alguns malalts no responguin correctament obliga a la recerca d'altres tractaments que es converteixin en una alternativa terapèutica, o bé en un tractament adjuvant que ens permeti disminuir la dosi acumulada de corticosteroides. Algunes de les opcions estudiades han estat :

- Àcid acetilsalicílic : donat que dosis baixes d'aspirina prevenen fenòmens isquèmics en malalts amb aterosclerosi, Nesher et al van realitzar un estudi retrospectiu amb 166

pacients amb ACG en el que van concloure que dosis baixes d'aspirina disminueixen la freqüència de fenòmens isquèmics, inclouen pèrdua de visió i accidents cerebro-vasculars (Nesher et al., 2004).

- Metotrexat (MTX) inhibeix la tetrahidrofolat deshidrogenasa. Té propietats antiinflamatòries i immunosupressores, i ha estat utilitzat en el tractament d'artropaties inflamatòries, com l'artritis reumatoide. En un estudi clínic en el que s'administrava MTX en combinació amb prednisona, l'efecte del MTX no va arribar a ser significatiu per a controlar la malaltia ni per disminuir la dosi acumulada de glucocorticoides (Hoffman et al., 2002). En un estudi estadístic recent que agrupa diferents estudis previs (metaanàlisi), els autors conclouen que el MTX té un efecte modest (Mahr et al., 2007) però que en casos refractaris de la malaltia es pot utilitzar per reduir la dosi de corticosteroides i els consegüents efectes secundaris.

- Inhibidors del TNFα : recentment s'ha realitzat un estudi multicentric (USA, UK, Bèlgica, Itàlia i Espanya) amb infliximab, un anticòs monoclonal neutralitzant del TNFα, ja que estudis preliminars han demostrat que el TNFα es troba sobreexpressat en les lesions de l'ACG (Hernandez-Rodriguez et al., 2004). Es van analitzar 44 pacients de nou diagnòstic, dels quals 16 van iniciar el tractament amb glucocorticoides més placebo i 28 glucocorticoides més infliximab. Els autors van concloure que l'infliximab no disminuïa el nombre de recurrències i que no constituïa una millora significativa (Hoffman et al., 2007).

Aprofundir en el coneixement dels mecanismes moleculars i cel·lulars que es produeixen durant el desenvolupament de la malaltia, ens permetrà descobrir noves

dianes terapèutiques i dissenyar teràpies dirigides més efectives i amb menys efectes secundaris.

1.2. MECANISMES D'OCLUSIÓ VASCULAR

Els estudis histològics de les biòpsies d'artèria temporal mostren diferents graus d'hiperplàsia intimal, però no s'ha trobat una correlació clara amb els fenòmens isquèmics. De fet, l'artèria temporal és necessària per al diagnòstic però les complicacions isquèmiques més greus es produeixen per l'afectació d'altres artèries en les que desconeixem el grau d'hiperplàsia intimal. D'altra banda, no es poden descartar la intervenció d'altres mecanismes d'oclusió vascular, com són la trombosi i el vasospasme.

1.2.1. HIPERPLÀSIA INTIMAL

La hiperplàsia intimal ha estat relacionada amb l'increment de factors de creixement i citocines proinflamatòries que es produeixen durant el desenvolupament de la malaltia. La formació d'una neoíntima sovint és la conseqüència d'un procés de reparació del teixit. Un cop solucionat el dany vascular disminueixen les molècules que havien desencadenat aquest procés (factors de creixement i factors profibròtics). En canvi, a les lesions de pacients amb ACG, aquests mediadors moleculars es mantenen elevats, el procés inflamatori esdevé crònic i la hiperplàsia intimal continua evolucionant fins a produir una oclusió parcial ó total del vas.

En el procés d'hiperplàsia intimal participen diferents mecanismes en els que intervenen els diferents components cel·lulars. L'infiltrat inflamatori secreta citocines i factors de creixement que afectaran el comportament de les SMC. Les SMC activades pateixen un canvi de fenotip, del fenotip contràctil (*spindle-shaped cell*) a un fenotip secretor (epiteloide)(Rzucidlo, Martin & Powell, 2007). Les SMC contràctils expressen alts nivells de proteïnes contràctils com la miosina i nivells baixos d'α-actina. Mentre que

les SMC secretores expressen alts nivells d'α-actina, de proteïnes d'ECM i nivells baixos de miosina. En general, les SMC contràctils no són tan proliferatives ni migratòries com les secretores. En les lesions arterials de l'ACG, les SMC activades migren cap a la llum del vas, on augmenten la seva proliferació i la secreció de proteïnes d'ECM. Així doncs, la neoíntima no només està formada per SMC sinó també per un dipòsit de proteïnes d'ECM. Anteriorment, hem comentat quines són les proteïnes d'ECM que es troben en els vasos i també definirem quins són els factors moleculars que es troben implicats a l'ACG (secció 1.3) que poden jugar un paper en la formació de la hiperplàsia intimal.

1.2.2. TROMBOSI

Malgrat que els pacients amb ACG tenen un nombre elevat de plaquetes i també es troben elevats el PF4 (*platelet factor* 4) i el BTG (*platelet-derived factors β-thromboglobulin*) (Vrij et al., 2000), la trombosi no sembla una de les causes més probables dels fenòmens isquèmics que es produeixen en el decurs de la malaltia, ja que en els estudis histopatològics no s'observen troballes que suggereixin fenòmens trombòtics. A més, la presència d'anticossos anti-fosfolípids i factors trombofílics no es correlaciona amb els fenòmens isquèmics relacionats amb la malaltia en els malalts amb ACG (Espinosa et al., 2001).

1.2.3. ESPASME VASCULAR

L'espasme vascular és un fenomen d'oclusió vascular que també cal tenir en compte, especialment en el cas de pacients que presenten fenòmens isquèmics transitoris com per exemple, l'amaurosi fugax, una pèrdua de visió transitòria, que ens porta a

pensar en una possible oclusió espasmòdica. Per estudiar aquest mecanisme cal analitzar la presència de vasoconstrictors en el desenvolupament de la malaltia. El PDGF i l'endotelina es plantegen com a bons candidats d'inducció de l'espasme vascular per les seves propietats vasoconstrictores. S'ha demostrat que el PDGF es troba sobreexpressat en les lesions arterials a l'ACG (Kaiser et al., 1998), en canvi, no hi ha estudis sobre l'expressió de l'endotelina i aquest serà un dels objectius d'aquesta tesi.

1.3. FACTORS IMPLICATS EN LA INFLAMACIÓ I L'OCLUSIÓ VASCULAR A L'ARTERITIS DE CÈL·LULES GEGANTS.

La major part de manifestacions clíniques i complicacions isquèmiques que pateixen els pacients amb ACG són atribuïbles als potents efectes biològics de les citocines i els factors de creixement que es produeixen durant el procés inflamatori. De tota manera, és difícil saber quina és la contribució de cada una d'aquestes molècules al procés inflamatori i al desenvolupament de l'oclusió vascular en l'ACG. A continuació, definirem algunes molècules que es troben sobreexpressades a les lesions arterials de l'ACG i les accions que poden produir en el context d'altres malalties. També comentarem si l'augment d'una d'aquestes molècules s'ha associat a alguna manifestació clínica, com per exemple el PDGF es correlaciona amb complicacions isquèmiques a l'ACG (Kaiser et al., 1998). Però per confirmar quin paper tenen realment en l'ACG caldrien experiments funcionals, per exemple el bloqueig selectiu d'una aquestes molècules en un model animal. Com hem esmentat anteriorment, l'ACG té una etiologia desconeguda i no disposen d'un model animal, de fet en aquesta tesi es desenvolupa un model de cultiu d'artèria temporal, en el que es poden realitzar estudis funcionals com per exemple el bloqueig selectiu de PDGF amb imatinib mesylate (veure resultats). Per tant, la informació que tenim fins al moment i us comentarem a continuació són: quines molècules es troben sobreexpressades en l'ACG, la seva correlació amb alguna manifestació clínica i les seves funcions, estudiades en altres malalties, que podrien contribuir a la inflamació i l'oclusió vascular en l'ACG.

D'acord amb la diferenciació funcional de tipus Th1, els limfòcits CD4+ que infiltren la paret vascular produeixen **interferó γ** (IFN-γ). Aquesta citocina és crucial per

l'activació dels macròfags, tant en la resposta immune innata com en la resposta immune adaptativa mediada per cèl·lules. A més, l'IFNγ pot actuar indirectament potenciant els efectes d'altres factors, com per exemple els del PDGF, ja que l' IFNγ augmenta l'expressió de mRNA del receptor α de PDGF en macròfags i en cèl·lules THP-1 (línia monocitària), tot i que, no l'augmenta en SMC humanes (Morelli et al., 2006). L'expressió d'IFN-γ es troba elevada en les biòpsies d'artèria temporal de pacients amb ACG, i existeix una correlació positiva entre aquesta expressió i els pacients amb símptomes isquèmics (Weyand & Goronzy, 2003; Weyand et al., 1997).

La reacció inflamatòria sistèmica tan característica de l'ACG (febre, perdua de pes, malestar, anèmia i augment en la producció de proteïnes de fase aguda) es relaciona amb l'elevada producció de **citocines proinflamatòries** a les lesions (Interleucina-1β, Interleucina-6 i el factor de necrosi tumoral α (TNFα)).

L' **Interleucina-1** produïda principalment pels fagòcits mononuclears activats és una citocina crucial en la resposta inflamatòria. Les dues isoformes (α i β) s'uneixen als mateixos receptors i tenen efectes biològics similars, que inclouen la inducció de molècules d'adhesió, estimulació de la producció de quimiocines per les EC i pels macròfags, estimulació de la síntesi dels reactants de fase aguda al fetge, i febre. L'IL-1β també podria participar en el desenvolupament de l'oclusió vascular ja que és un potent factor de creixement per les VSMC (Dinarello, 1996). L'expressió de l'IL-1β a les lesions arterials de pacients amb ACG es troba elevada tant a nivell de mRNA com a nivell proteic (Hernandez-Rodriguez et al., 2004).

L'**interleucina-6** (IL-6) és una citocina produïda per molts tipus cel·lulars, que inclouen fagòcits mononuclears activats, EC, fibroblasts i SMC. Té funcions tant en la

resposta immune innata com en l'adaptativa, per exemple l'IL-6 estimula la síntesi de les proteïnes de fase aguda pels hepatòcits però també estimula la proliferació dels limfòcits B productors d'anticossos. S'ha demostrat que l'IL-6 es troba elevada en les lesions arterials en l'ACG (Emilie et al., 1994). Els pacients amb complicacions isquèmiques tenen menys expressió d'IL-6 tant en sèrum com en el teixit arterial. Una possible explicació d'aquest fet podria ser l'existència d'un mecanisme compensatori en el que l'IL-6 contraresta els efectes isquèmics degut a les seves propietats com a promotora de l'angiogènesi (Hernandez-Rodriguez et al., 2003).

El **factor de necrosi tumoral (TNFα)** és una citocina produïda principalment pels fagòcits mononuclears activats que estimula el reclutament de neutròfils i monòcits als llocs d'infecció i activa aquestes cèl·lules per eradicar els microorganismes. Entre les funcions del TNFα destaquem que estimula les EC per augmentar l'expressió de molècules d'adhesió, indueix macròfags i EC per augmentar la secreció de quimiocines i promou l'apoptosi de cèl·lules diana. Durant el procés d'infecció, el TNFα és produït en grans quantitats i té efectes a nivell sistèmic, que inclouen febre, síntesi de proteïnes de fase aguda a fetge i caquexia. La producció de grans quantitats de TNFα pot causar trombosi intravascular i shock. A l'ACG, el TNFα i els seus receptors es troben elevats a les lesions arterials (Field, Cook & Gallagher, 1997; Hernandez-Rodriguez et al., 2004). Aquest fet, juntament amb les potents funcions proinflamatòries, van fer pensar que el TNFα podia jugar un paper clau en el procés inflamatori. Tot i així, un assaig clínic que estudiava l'ús d'infliximab com a tractament adjuvant de l'ACG no va demostrar que el bloqueig de TNFα tingués efectes beneficiosos (descrit apartat 1.1.4 tractament). Així doncs, el fet que una molècula es trobi sobreexpressada a les lesions i pugui tenir unes

determinades funcions en altres malalties no significa que aquestes funcions siguin extrapolables a l'ACG.

En el procés inflamatori, també poden intervenir **molècules quimiotàctiques** com el *Monocyte Chemoattractant Protein* – 1 (MCP-1 ó **CCL2).** El CCL2 és una quimiocina per a monòcits i limfòcits Th1 activats que s'expressa en les SMC i EC de la paret arterial. S'ha demostrat que en les lesions dels pacients amb ACG augmenta la expressió de CCL2 a nivell de RNA i a nivell proteic, fet que s'associa a la persistència de la malaltia (Cid et al., 2006).

A més d'aquestes citocines i quimiocines proinflamatòries, també s'ha demostrat que els macròfags produeixen **factors de creixement amb capacitat fibrogènica** com el factor de creixement derivat de plaquetes (PDGF), el Transforming Growth Factor β (TGFβ) i el Fibroblast Growth Factor (FGF-2). Alguns estudis preliminars suggereixen que aquests factors podrien contribuir a l'oclusió vascular i les subsegüents complicacions isquèmiques (Kaiser et al., 1998).

El **PDGF** és un potent factor de creixement per les VSMC, i alhora és un potent quimiotàctic. Aquests dos fets juntament amb altres funcions fibrogèniques, el converteixen en un bon candidat per ser un dels factors implicats en el desenvolupament de l'hiperplàsia intimal en l'ACG. En parlarem molt més extensament en el següent apartat (1.3.1).

El **TGFβ** és una citocina produïda per les cèl·lules T activades, per fagòcits mononuclears i altres cèl·lules. Algunes de les seves funcions principals són inhibir la proliferació i la diferenciació de les cèl·lules T, inhibir l'activació dels macròfags i contrarestar els efectes de les citocines proinflamatòries. El TGF-β1 i TGF-βR2 han estan

implicats en la maduració dels vasos ja que inhibeixen la proliferació i migració de les EC i alhora indueixen la diferenciació de les SMC i estimulen la producció d'ECM. A més en alguns processos patològics, s'ha observat que les EC activades poden augmentar l'expressió de bFGF, PDGF-B i TGF-β1 per induir el creixement de les SMC i l'engruiximent del vas(Carmeliet, 2000). Les propietats profibrogèniques del TGF-β (proliferació SMC, estimulació d'ECM) el converteixen en un bon candidat com a factor implicat en la formació de la hiperplàsia intimal. A més, estudis realitzats al nostre laboratori han demostrat que l'expressió del TGF-β es troba elevada en les lesions arterials dels pacients amb l'ACG i està associat a complicacions isquèmiques.

El **Fibroblast Growth Factor** acídic i el FGF bàsic són mitògens molt potents per les SMC, s'expressen en les EC i en les SMC, respectivament. Així doncs, el bFGF (FGF-2) actua de forma autocrina i l'aFGF de forma paracrina per les SMC. Existeixen 4 isoformes de bFGF, tres es troben al nucli (24, 22 i 21 kDa) i una és secretada (18 kDa). De receptors de FGF també hi ha quatre tipus (110-130 kDa), el FGFR-1 és el principal tipus expressat en les SMC arterials humanes en proliferació. Tant el FGFR-1 com el FGFR-2 són presents a les SMCs. A les plaques ateroscleròtiques, el bFGF no s'ha trobat elevat però el FGFR-1 s'ha vist elevat en processos de dany vascular (Berk, 2001). S'ha demostrat que el FGF-2 s'expressa en les lesions arterials de pacients amb ACG (Kaiser et al., 1999). En aquest estudi, no s'analitzaven les seves accions com a estimulador del creixement de les SMC, i per tant, la seva possible implicació en el procés d'oclusió vascular. Els autors investigaven possibles candidats com a factors angiogènics a l'ACG i arribàvem a la conclusió que l'expressió de FGF-2 no es correlaciona amb angiogènesi,

en canvi, demostren una associació entre l'angiogènesi i l'expressió del Vascular Endothelial Growth Factor (VEGF).

Això ens porta a un tercer tipus de molècules, hem resumit breument molècules implicades en el procés inflamatori i molècules pro-fibrogèniques com a possibles causants de l'oclusió vascular, però a les lesions arterials també s'observen formació de nous vasos (angiogènesi). Algunes de les molècules ja esmentades també tenen capacitats **pro-angiogèniques**, com l'IL-6, el FGF-2 i el PDGF, però a continuació explicarem breument les característiques de dues molècules proangiogèniques que també podrien jugar un paper a l'ACG: el VEGF i l'angiogenina.

El **VEGF** és un conegut promotor del creixement i la supervivència de les EC. Indueix una potent resposta angiogènica en una gran varietat de models *in vivo* (Ferrara, Gerber & LeCouter, 2003). El fet que sigui un factor de permeabilitat vascular, li confereix un paper important en els processos inflamatoris (Dvorak et al., 1995). No només té una gran influència sobre les EC, també s'ha demostrat que promou quimiotaxi en els monòcits (Clauss et al., 1990). Existeixen 4 isoformes de 121, 165, 189 i 206 aa, la $VEGF_{165}$ és la predominant, pot ésser secretada però també una part significativa roman unida a la superficie cel·lular i a l'ECM, d'on poden ser alliberada amb un tall al C-terminal per la plasmina (Ferrara et al., 2003). Estudis sobre l'estabilització de nous vasos en l'angiogènesi han mostrat que les EC secreten PDGF-B probablement en resposta al VEGF i aquest fet facilita el reclutament de les cèl·lules murals (Jain, 2003). Com s'ha esmentat anteriorment el VEGF es troba expressat en les artèries de pacients amb ACG i la seva expressió es correlaciona amb angiogènesi (Kaiser et al., 1999).

L'**angiogenina** és un agent inductor de neovascularització *in vivo* en models experimentals com són la membrana corioalantoidea de l'embrió del pollastre (Riordan & Vallee, 1988) i la cornea de conill (Fett et al., 1985), ja que promou la invasió de les EC (Hu, Riordan & Vallee, 1994) . L'humana té 14 kDa, comparteix el 35 % de la seqüència d'aminoàcids amb la ribonucleasa pancreàtica humana, i ha conservat residus essencials en el lloc actiu. Per induir l'angiogènesi cal un lloc actiu enzimàtic funcional i un domini d'unió cel·lular. L'angiogenina s'uneix a les cèl·lules endotelials i cèl·lules de múscul llis vasculars. A més, l'angiogenina s'uneix estretament a l'inhibidor de ribonucleases, una proteïna intracel·lular que aboleix la seva activitat angiogènica (Hatzi, Bassaglia & Badet, 2000). Recentment , s'ha demostrat que l'Actibind, una ribonucleasa extracel·lular de l'*Aspergillus niger*, té propietats antiangiogèniques perquè inhibeix la diferenciació tubular de les EC induïda per l'angiogenina (Roiz et al., 2006).

1.3.1. FACTOR DE CREIXEMENT DERIVAT DE LES PLAQUETES (PDGF)

El **PDGF** i l'endotelina són les dues molècules que s'han estudiat més profundament en aquesta tesi i en parlarem amb una mica més de detall. El PDGF és un potent factor de creixement per les VSMC, i alhora és un potent quimiotàctic en altres malalties vasculars(Hafizi et al., 1998). A més, en alguns estudis preliminars realitzats en les SMC que havien aïllat d'artèria temporal de malalts amb ACG, el PDGF també va ser demostrar ser un potent mitogen. També s'ha demostrat que el PDGF està elevat en les lesions de l'ACG (Kaiser et al., 1998). Aquests motius converteixen el PDGF en un bon candidat per ser un dels factors implicats en el desenvolupament de l'hiperplàsia intimal en l'ACG.

1.3.1.1. INTRODUCCIÓ

El factor de creixement derivat de plaquetes (PDGF = *Platelet-derived growth factor*) és un del principals mitògens per fibroblasts, cèl·lules de múscul llis (SMC) i altres cèl·lules d'origen mesenquimal.

El PDGF es troba emmagatzemat als gränuls α de les plaquetes i és secretat quan les plaquetes són activades, per exemple per trombina. Estudis recents han mostrat que el PDGF també pot ser sintetitzat per altres tipus de cèl·lules, incloent macròfags, EC, fibroblasts, SMC i algunes línies tumorals (Heldin & Westermark, 1999).

1.3.1.2. ESTRUCTURA DEL PDGF

El PDGF és una proteïna de 30 kDa hidrofílica, termoestable i carregada positivament (pI=9.8-10). Consisteix en dues cadenes polipeptídiques unides per ponts disulfur formant homodímers (AA, BB) o heterodímers (AB).

Els gens que codifiquen per les cadenes A i B es troben als cromosomes 7 i 22, respectivament. Estan organitzats de forma similar en 7 exons, l'exó 1 codifica el pèptid senyal, el 2 i 3 codifiquen les seqüències precursores que seran eliminades durant el processament, i el 4 i 5 produiran la majoria de la proteïna madura. L'exó 6 codifica per la seqüència C-terminal que serà eliminada durant la maduració de la cadena B (14 kDa, 140 aminoàcids), en canvi, per produir la cadena A es poden donar variants diferents, amb i sense la seqüència codificada en l'exó 6 (16 kDa, 124 aminoàcids). I l'exó 7 és majoritàriament no codificant.

S'ha descrit algunes variants de PDGF-A i presenten un 60% de homologia amb PDGF-B. El PDGF-B és idèntic al producte de l'oncogen *sis (simian sarcoma virus)*. Les dues cadenes presenten vuit residus de cisteïna (Cys) molt conservats, i l'espai característic que es troba entre les Cys s'ha observat també en membres de la família dels VEGFs (*vascular endothelial growth factor*-A, -B, -C, -D i *placental growth factor*).

Les cadenes A i B del PDGF són sintetitzades com a molècules precursores, es dimeritzen en el reticle endoplasmàtic i en el complex de Golgi passen per un processament proteolític en l'extrem N-terminal, i en el cas de la cadena B, també en el C-terminal.

A les dues cadenes, l'exó 6 codifica per una seqüència bàsica que permet la interacció amb components de la matriu extracel·lular i també pot produir la seva retenció

dins la cèl·lula productora (Raines & Ross, 1992). El PDGF es pot unir a diferents tipus de col·lagen (Somasundaram & Schuppan, 1996) però probablement el principal component de la matriu que uneix PDGF és l'heparà sulfat (Lustig et al., 1996). Així, el precursor de la cadena B pot quedar retingut a la matriu, i després de la maduració, quan la seqüència de retenció C-terminal és eliminada, augmenta la difusió de la molècula. En el cas de la cadena A, la variant curta, sense C-terminal, pot difondre més fàcilment a través dels teixits i afectar a teixits més llunyans. En canvi, la variant llarga de la cadena A només pot estimular en l'ambient més proper. La disponibilitat del PDGF emmagatzemat a la matriu pot ser regulada per la proteòlisi de les molècules de matriu (Field et al., 1996). El PDGF també pot interaccionar amb proteïnes solubles, per exemple PDGF-BB pot unir-se a α_2-microglobulina (Bonner & Osornio-Vargas, 1995).

Recentment, s'han descobert dues isoformes més (PDGF-C i -D), només formen homodímers, i per la seva similitud estructural es poden considerar un subgrup dins la família dels PDGFs (Bergsten et al., 2001; Li & Eriksson, 2003).

1.3.1.3. RECEPTORS DE PDGF

Les diferents isoformes del PDGF interactuen amb dos receptors tirosina quinasa estructuralment relacionats(α i β) amb diferent afinitat i produeixen funcions biològiques diferents (Hafizi et al., 1998). Cada cadena del PDGF conté dos epítops simètrics d'unió al receptor, cadascun construït amb estructures de les dues cadenes.

Els receptors α i β són glicoproteïnes transmembrana amb pesos moleculars d'aproximadament 170 i 180 kDa, respectivament. Extracel·lularment, cada receptor conté 5 dominis similars a immunoglobulines, i intracel·lularment, conté un domini

tirosina quinasa amb una seqüència característica inserida d'uns 100 aa sense activitat catalítica. L'estructura dels receptors de PDGF és similar a la dels receptors de CSF-1 (*colony stimulating factor*) i de SCF (*stem cell factor*). El gen del receptor α està localitzat al cromosoma 4q12, prop del gens del receptor de SCF i del receptor 2 del VEGF. El gen del receptor β es troba al cromosoma 5 a prop del receptor de CSF-1 (Heldin & Westermark, 1999).

Els dímers de PDGF s'uneixen a les dues cadenes del receptor simultàniament i forma un pont que indueix la dimerització del receptor, formant homo- o heterodímers. El receptor α pot unir cadenes A i B amb alta afinitat, mentre que el receptor β només s'uneix a cadenes B. Així, PDGF-AA activa receptors αα, PDGF-AB pot activar αα i αβ, i PDGF-BB pot activar les tres combinacions de receptor (αα, αβ i ββ)(Hart et al., 1988) (figura 3).

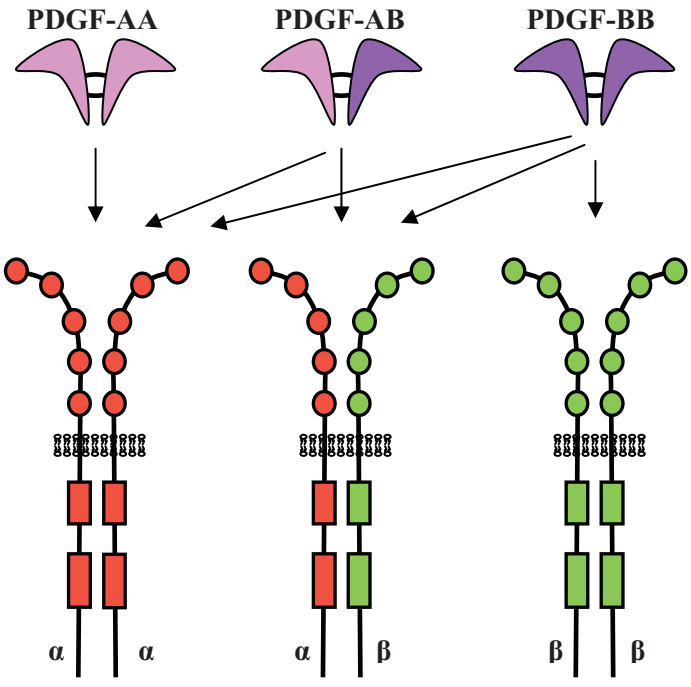


Figura 3. Interacció del PDGFs amb els seus receptors.

A més, de l'efecte pont del PDGF, el dímer de receptor també s'estabilitza per interaccions directes entre els dominis Ig 4 de cada receptor. Els tres dominis més externs són els epítops d'unió al lligand, el Ig 2 sembla ser el més important.

La dimerització del receptor juxtaposa els dominis intracel·lulars del receptor i permet l'autofosforilació en *trans* dels residus tirosina (Tyr 849 en el receptor α i Tyr 857 en el β). L'autofosforilació del receptor genera llocs d'unió (*docking sites*) per varies molècules que activen diferents vies de senyalització intracel·lulars.

Les funcions produïdes per PDGF depenen del tipus de receptor que iniciï la senyalització. Els homodímers αα i ββ transdueixen senyals mitogènics molt potents. Respecte a la quimiotaxi, el receptor β produeix una estimulació potent tant en la forma d'homodímer com d'heterodímer amb el receptor α. En canvi, l'homodímer αα no intervé en la quimiotaxi, al menys en alguns tipus cel·lulars, com les VSMC (Koyama et al., 1998). El fet que el PDGF-AB tingui un major efecte mitogènic i quimiotàctic en cèl·lules que expressen ambdós receptors, fa pensar que l'heterodímer αβ pot tenir propietats úniques.

Les clàssiques cèl·lules diana de PDGF són els fibroblasts i les SMC que expressen ambdós receptors, però generalment amb nivells més alts de receptor β. L'expressió de receptor no és constant, per exemple, VSMC estimulades amb bFGF incrementen selectivament l'expressió de receptor α (Schollmann et al., 1992). En alguns casos, la regulació del receptors depèn del tipus cel·lular per exemple : l'IFNγ augmenta l'expressió de mRNA del receptor α de PDGF en macròfags i en cèl·lules THP-1 (línia monocitària), en canvi no l'augmenta en SMC humanes (Morelli et al., 2006).

1.3.1.4. VIES DE SENYALITZACIÓ DE PDGF

L'activació del receptor desencadena diversos efectes biològics relacionats amb la regulació de la proliferació, la progressió del cicle cel·lular, l'apoptosi, la supervivència i la migració cel·lular.

Un gran nombre de proteïnes amb domini SH2 (*Src homology 2 domain*) s'uneixen als receptors α i β dels PDGFs. El domini SH2 és un motiu conservat d'uns 100 aminoàcids que pot unir una tirosina fosforilada en un context específic. Algunes d'aquestes molècules de transducció de senyal són enzims com la fosfatidilinositol 3' kinasa (PI3k), la fosfolipasa C γ (PLCγ), la família de tirosina quinases Src, etc. Altres molècules com Grb2, Grb7, Nck, Shc i Crk funcionen com molècules adaptadores, connectant el receptor amb molècules catalítiques que es troben per sota en la via de senyalització. També membres de la família Stat s'uneix als receptors de PDGF, són factors de transcripció que quan es fosforilen, dimeritzen i es transloquen al nucli (Heldin & Westermark, 1999) (veure taula 1).

Taula 1: Molècules amb dominis SH2 que s'uneixen al receptor de PDGF α ó β (Ronnstrand & Heldin, 2001). Les Tyr que reconeixen els diferents dominis SH2 estan indicades així com el seu paper en la senyalització:

Molècula	**Tipus de molècula**	**PDGF R α**	**PDGF R β**	**Paper en la senyalització**
Src, Yes, Fyn	Tirosina quinasa citoplasmàtica	Tyr572, Tyr574	Tyr579, Tyr581	Mitogenicitat
PI3 quinasa	Quinasa de lípids	Tyr731, Tyr742	Tyr740, Tyr751	Mitogenicitat, quimiotaxi
RasGAP	Proteïna amb activitat GTPasa	No s'uneix	Tyr 771	Mitogenicitat, quimiotaxi
SHP-2	Tirosina fosfatasa	Tyr720, Tyr754	Tyr763, Tyr1009	Mitogenicitat, quimiotaxi
PLC-γ	Lipasa	Tyr988, Tyr1018	Tyr1009, Tyr1021	Mitogenicitat, quimiotaxi
Grb2	Adaptadora	No s'uneix	Tyr716	Mitogenicitat, quimiotaxi
Nck	Adaptadora	No s'uneix	Tyr751	?
Grb10	Adaptadora	?	Tyr771	Mitogenicitat, quimiotaxi
Shc	Adaptadora	No s'uneix	Tyr579, Tyr740 Tyr751	Mitogenicitat, quimiotaxi
Grb7	Adaptadora	No s'uneix	Tyr775	?
Crk	Adaptadora	Tyr762	No s'uneix	?
Stat5	Factor de transcripció	?	Tyr579, Tyr581 Tyr775	?

Breument introduirem algunes de les vies de senyalització activades pels receptors de PDGF més ben caracteritzades (Figura 4).

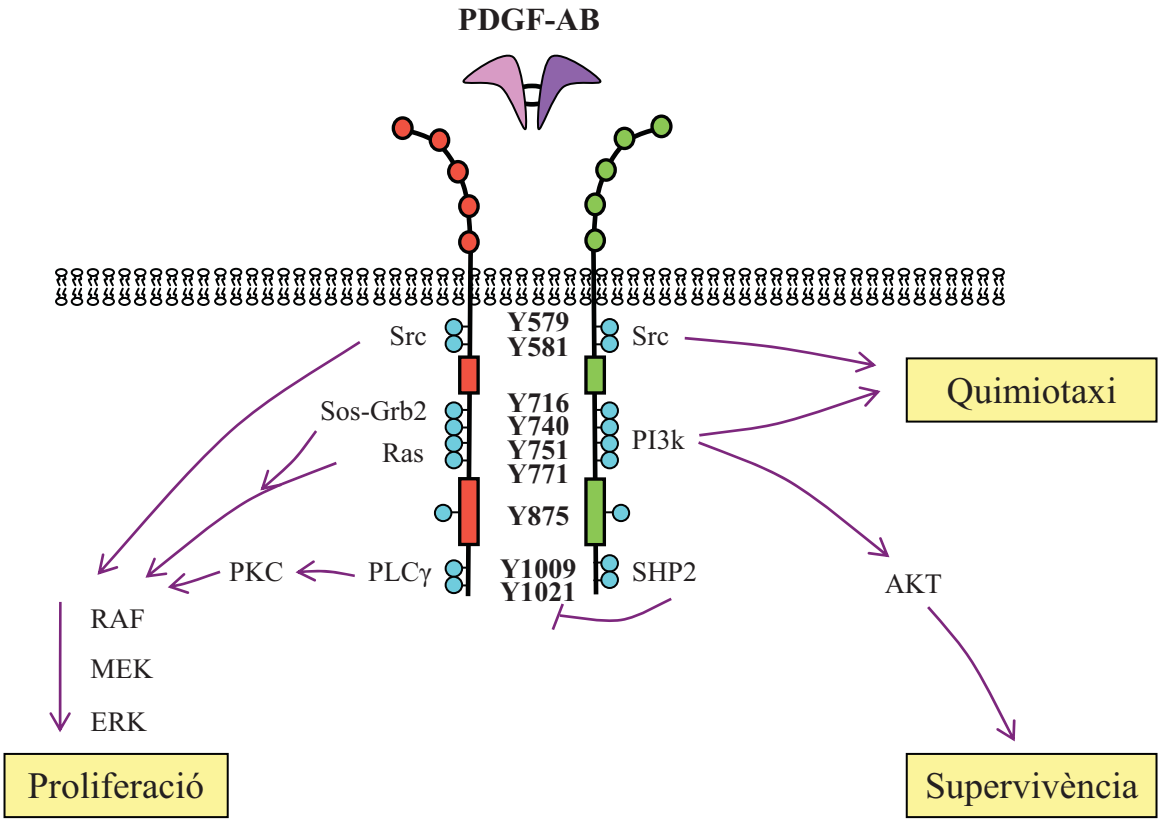


Figura 4. Principals vies de senyalització del PDGF.

1.3.1.4.1. PI 3 QUINASA

Els membres de la família de tirosina quinases de PI3k que són activats pels receptors de PDGF consisteixen en una subunitat reguladora, p85, i una subunitat catalítica, p110. La unió de PI3k a les tirosines fosforilades Tyr740 i Tyr751 del receptor condueix a l'activació de PI3k. El principal substrat és el fosfatidil inositol 4,5-bifosfat ($PI(4,5)P_2$) que passa a $PI(4,5)P_3$. La PI3k juga un paper central en la senyalització intracel·lular perquè pot activar un gran nombre de molècules efectores implicades en diverses respostes biològiques, com la reorganització de l'actina, quimiotaxi, proliferació cel·lular i antiapoptosi.

PI3k activa la serina/treonina quinasa PKB (*protein kinase B*), també anomenada Akt perquè l'oncogen v-Akt es va identificar en el retrovirus AKT8 en la soca de ratolins AKR amb una alta incidència de leucèmies. Akt interacciona amb els fosfolípids $PI(4,5)P_3$ generats per PI3k, produint el reclutament d'Akt a la part interna de la membrana plasmàtica, on serà fosforilada per PDK-1 (*3'-phosphoinositide-dependent kinase*)(Blume-Jensen & Hunter, 2001) (figura 5).

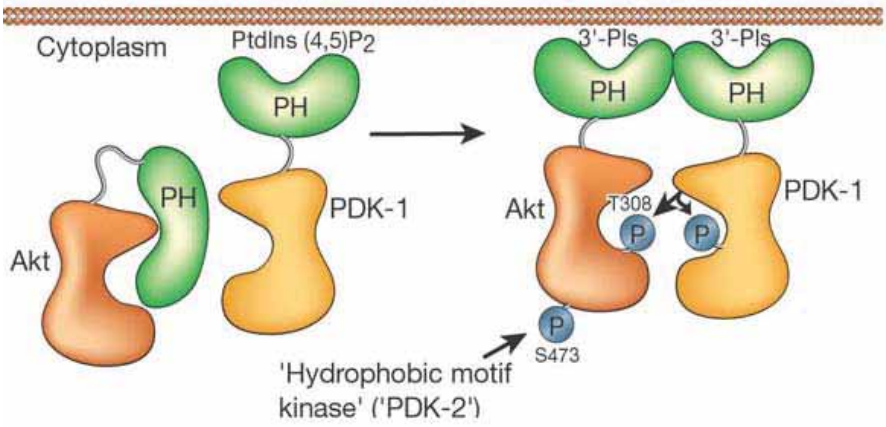


Figura 5. Reclutament d'Akt i fosforilació per PDK-1.

Les tres isoformes d'Akt (α,β,γ) contenen un domini PH (*pleckstrin-homology*) N-terminal, un domini quinasa amb un lloc de fosforilació Thr308 i aprop del C-terminal, un domini regulador molt conservat amb un lloc de fosforilació Ser473. La fosforilació de Thr308 és un prerequisit per l'activació d'Akt però és necessària també la fosforilació de Ser473 per aconseguir l'activació total d'Akt.

Akt promou supervivència cel·lular mitjançant diversos factors de transcripció i inhibint l'apoptosi (Kaplan-Albuquerque et al., 2003). Entre els mecanismes antiapoptòtics mediats per Akt s'ha observat l'augment en l'expressió de Bcl-2, i també de c-myc via E2F (Coffer, Jin & Woodgett, 1998).

1.3.1.4.2. Src

Els membres de la família de tirosina kinases de Src (*Rous sarcoma virus*) es caracteritzen per la presència d'un domini SH3 i un SH2 a més del domini catalític. A la configuració inactiva, un residu de Tyr fosforilada (527 en ratolí, 530 en la c-Scr humana) a l'extrem C-terminal forma una interacció intermolecular (Blume-Jensen & Hunter, 2001).

L'activació de Src depèn de la unió del domini SH2 a la Tyr 579 receptor autofosforilat de PDGF produeix la fosforilació de la Tyr 416 i que alhora es produeixi la desfosforilació de la Tyr 527 a l'extrem C-terminal (figura 6). Src intervé en la resposta mitòtica del PDGF, ja que anticossos bloquejant Src inhibeixen el creixement cel·lular. Tot i així, la unió directa entre Src i el receptor de PDGF no és imprescindible per la senyalització mitòtica, ja que mutants Y579F del receptor β tenen activitat mitogènica.

Els membres de la família Src han estat implicats en la reorganització del citoesquelet, i probablement estan relacionats amb la migració induïda per PDGF.

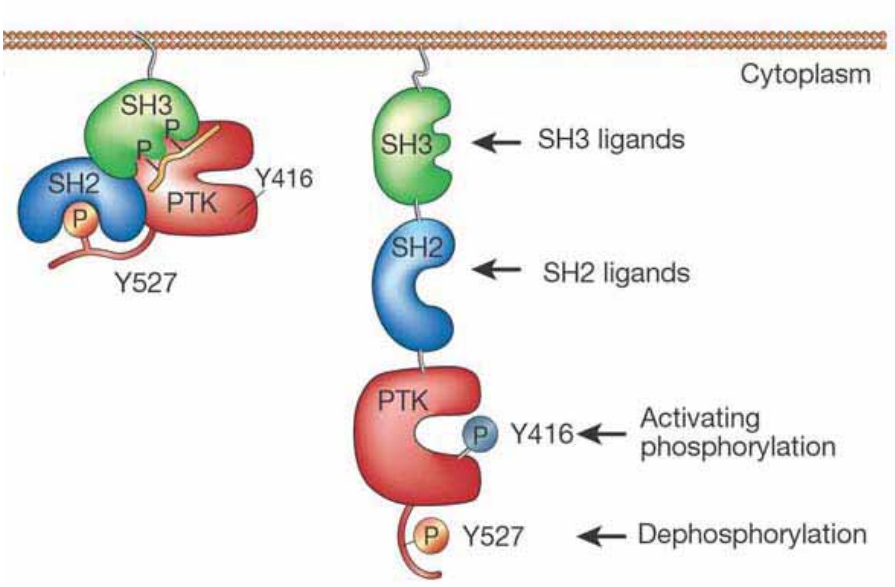


Figura 6. Activació de Src en la senyalització del receptor del PDGF.

1.3.1.4.3. Grb2/Sos

Grb2 (*growth factor receptor bound* 2) és una molècula adaptadora amb un domini SH2 i dos SH3, els darrers intervenen en la unió a Sos, que activarà Ras. El domini SH2 de Grb2 es pot unir directament a la Tyr716 del receptor autofosforilat de PDGF o indirectament via Shc (*SH2 domain and collagen-like*) o SHP-2. L'activació de Ras és molt important per diferents respostes cel·lulars, ja que Ras activada s'uneix a la serina/treonina kinasa Raf-1 que inicia l'activació de la cascada de MAPK (*mitogen-activaded protein kinase*), una via implicada en la estimulació del creixement cel·lular, migració i diferenciació (Bornfeldt et al., 1997).

1.3.1.4.4. SHP-2

SHP-2 és una tirosina fosfatasa amb dos dominis SH2, ambdós necessiten estar units a residus de Tyr fosforilada per activar completament l'activitat catalítica. SHP-2 pot desfosforilar els receptors de PDGF i substrats d'aquests receptors. Així, pot modular negativament la senyalització de PDGF, generant un mecanisme de retroalimentació negativa.

1.3.1.4.5. COOPERACIÓ AMB SENYALITZACIÓ VIA INTEGRINA

La majoria dels tipus cel·lulars que responen al PDGF són dependents d'ancoratge, és a dir, que el seu creixement depèn del contacte amb les molècules de matriu que envolten la cèl·lula mitjançant receptors transmembrana anomenats integrines. La unió de les integrines a molècules específiques d'ECM genera la formació d'adhesions focals on interaccionen un gran nombre de molècules com Src, PI3k i Ras. La senyalització per integrines incrementa la proliferació cel·lular induïda per factors de creixement, la migració cel·lular i en alguns casos pot evitar l'apoptosi (Sundberg et al., 2003). Les fibres del col·lagen suprimeixen la síntesi de DNA induïda per PDGF en SMC arterials, probablement suprimint l'activitat E-Cdk2 via integrina (Motamed et al., 2002). Altres proves de cooperació són l'existència d'una subfracció de receptors β de PDGF altament fosforilada formant complexos amb integrines $\alpha_v\beta_3$ (Schneller, Vuori & Ruoslahti, 1997). A més, la unió d'integrines β_1 en fibroblasts cultivats sobre col·lagen ó fibronectina causa la fosforilació transitòria dels receptors β de PDGF en absència de PDGF (Sundberg & Rubin, 1996). D'altra banda, s'ha observat que PDGF estimula la síntesi de col·lagen per unió a integrines $\alpha_2\beta_1$ en fibroblasts (Kirchberg et al., 1995).

1.3.2.5. FACTORS QUE REGULEN LA SÍNTESI DE PDGF

En un context inflamatori, s'ha observat que TNFα indueix l'expressió de PDGF-A en cultius de fibroblasts humans (Battegay et al., 1995). D'altra banda, el TGFβ combinat amb l'angiopoietina (Ang-1) regula negativament l'expressió de PDGF en cèl·lules HUVEC (*human umbilical vein endothelial cells*) (Nishishita & Lin, 2004).

L'interferó-γ (IFNγ) augmenta l'expressió de mRNA del receptor α de PDGF en macròfags i en cèl·lules THP-1 (línia monocitària), tot i que, no l'augmenta en SMC humanes (Morelli et al., 2006). En un altre estudi es demostra que l'IFN-γ promou el creixement de les SMC mitjançant la regulació positiva dels receptors β del PDGF (Tellides et al., 2000).

1.3.2.6. FUNCIONS DEL PDGF EN EL SISTEMA VASCULAR

Al contrari que altres citocines, el PDGF no és alliberat a la circulació. La vida mitjana després d'una administració intravenosa és de menys de dos minuts. El PDGF s'uneix a varies proteïnes del plasma i també a l'ECM, fet que facilita la seva concentració local.

El PDGF està relacionat amb la regulació del to vascular. D'una banda, indueix vasoconstricció de diferents tipus de vasos sanguinis (Berk et al., 1986) i d'altra banda, l'estimulació de les EC per PDGF-BB indueix la relaxació de l'aorta de conill via alliberament de prostaglandines (Yamawaki et al., 2000).

Un altre efecte del PDGF sobre el sistema vascular és que disminueix l'agregació plaquetària. És un mecanisme de control per retroalimentació negativa : després de l'agregació plaquetària, s'allibera el contingut dels gränuls α de les plaquetes, inclouen el

PDGF, llavors es produeix una activació del receptors α de PDGF a les plaquetes i disminueix l'agregació plaquetària (Vassbotn et al., 1994).

Els receptors de PDGF s'expressen a les cèl·lules endotelials on el PDGF ha demostrat tenir un efecte angiogènic, tot i que, és un efecte més dèbil que el produït pels FGFs o VEGFs. El PDGF no sembla ser important en la formació inicial dels vasos sanguinis, excepte en alguns casos específics com les cèl·lules microvasculars cardíaques (Kaminski et al., 2001). Però la cadena B de PDGF produïda pels capil·lars pot jugar un paper important en el reclutament de pericits que es requereix per promoure la integritat estructural dels vasos sanguinis.

El PDGF actua sobre diferents tipus de cèl·lules implicades en la reparació de teixits. Estimula la proliferació i la migració de fibroblasts i de SMC, i la migració de neutròfils i macròfags. A més, el PDGF estimula la producció de varies molècules de la matriu extracel·lular, com la fibronectina, col·lagen, proteoglicans i àcid hialurònic. També estimula la secreció de col·lagenasa en fibroblasts, suggerint un important paper en la fase de remodelatge en la reparació de teixits. Alguns estudis immunohistoquímics han demostrat que fibroblasts i SMC en teixits normals contenen nivells baixos de receptors de PDGF, mentre que durant el procés inflamatori el receptor β és regulat positivament (Reuterdahl et al., 1991).

1.3.1.7. PAPER DEL PDGF A LA MALALTIES VASCULARS

El PDGF s'expressa a nivells baixos a les artèries d'adults sans, la seva expressió s'incrementa en conjunció amb la resposta inflamatòria-fibroproliferativa que caracteritza algunes malalties vasculars com l'arteriosclerosi (Ross et al., 1990).

En el context de l'arteriosclerosi s'han realitzat molts estudis per intentar esbrinar el paper del PDGF. Tot i que, l'ACG és una malaltia totalment diferent, si que podem trobar alguns paral·lelismes i aprendre sobre els mecanismes d'acció del PDGF. Un problema comú a les dues malalties és l'engruiximent de l'íntima, en experiments de cateterització de la caròtida de rates, el tractament amb anticossos neutralitzants del PDGF va inhibir l'engruiximent de l'íntima (Ferns et al., 1991). El paper del PDGF en aquestes lesions podria ser estimular la migració de les SMC des de la capa mitja fins la íntima, on estimularia la proliferacio i la producció de proteïnes de matriu extracel·lular (Raines, 2004).

En el cas de l'ACG, l'expressió de PDGF no ha estat estudiada en profunditat però en un estudi immunohistoquímic de 8 artèries ACG positives es van trobar sobreexpressats el PDGF A i el B. Paral·lelament es van realitzar immunohistoquímiques de les cèl·lules CD68+ (macròfags i cèl·lules gegants) i es va observar una localització topogràfica similar, però caldria un estudi amb microscòpica confocal per determinar si existeix colocalització (Kaiser et al., 1998).

1.3.1.8. IMATINIB MESYLATE : INHIBIDOR DEL RECEPTOR DEL PDGF

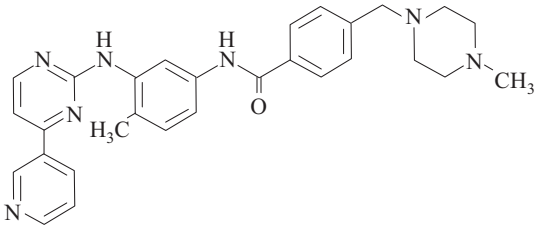
1.3.1.8.1. DEFINICIÓ

Aquest fàrmac és un inhibidor selectiu de les proteïnes quinases : Bcr-abl, els receptors de PDGF i c-Kit (*stem cell factor* receptor). Inicialment, els enzims proteïna quinasa no van ser considerats com a bones dianes terapèutiques donada la seva ubiqüitat i al paper crític que tenen en diversos processos fisiològics. Tot i així, l'arribada de

l'imatinib mesylate com a prototip dels inhibidors de senyals de transducció (STI) va demostrar que aquests inhibidors podien ser específics i esdevenir agents terapèutics efectius. L'imatinib és específic perquè aquestes quinases són notablement diferents en la regulació de la seva catàlisi, tot i que, comparteixen dominis catalítics molt conservats en seqüència i estructura. El lloc d'unió de l'ATP es troba entre dos lòbuls d'un plec de la quinasa. Aquest lloc, juntament amb les seqüències menys conservades dels voltants, ha estat l'objectiu en el disseny d'inhibidors que aprofita aquestes diferències entre estructura i conformació per aconseguir selectivitat.

Imatinib mesylate [també conegut com a Gleevec® (USA), Glivec® (Europa), STI-571 ó CGP57148] ha demostrat una utilitat clínica eficaç en el tractament de la leucèmia mielode crònica, dels tumors estromals gastrointestinals (GIST) i d'alguns tumors més infreqüents amb mutacions oncogèniques dels receptors del PDGF.

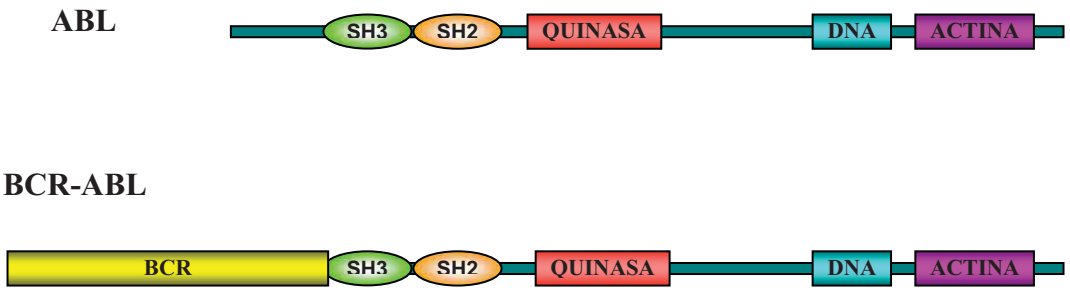
Imatinib mesylate va ser desenvolupat a partir d'una sèrie de compostos identificats a la recerca de inhibidors de la proteïna quinasa C (Capdeville et al., 2002). El



compost inicial va ser la fenilaminopirimidina que va ser modificada per augmentar l'activitat cel·lular, la solubilitat i la biodisponibilitat (Schindler et al., 2000). Imatinib mesylate ocupa el lloc d'unió a l'ATP de la proteïna quinasa Bcr-Abl, inhibint competitivament la fosforilació de les molècules efectores que es formen part de la cascada de senyalització de Bcr-Abl (Savage & Antman, 2002).

1.3.1.8.2. ACTIVITAT

Druker i col·laboradors van demostrar que l'imatinib mesylate suprimia la proliferació *in vitro* de les cèl·lules BRC-ABL-positives de la leucèmia mieloide crònica (CML) mentre que els progenitors hematopoètics normals no van ser afectats (Druker et al., 1996).



Aquest compost també és un inhibidor efectiu d'altres tirosina quinases com el c-Kit (CD117) (stem cell factor receptor) i els receptors de PDGF (Druker et al., 2001). El factor de cèl·lules mare (*stem cell factor* SCF) s'uneix al receptor tirosina quinasa c-Kit de cèl·lules mare pluripotencials. Les cèl·lules estromals del moll d'os i del timus poden produir SCF en forma soluble ó unit a membrana. El SCF és una proteïna necessària en l'hematopoesi, en els primers passos del desenvolupament dels limfòcits T en el timus i en el desenvolupament dels mastòcits.

L'imatinib mesylate inhibeix l'activitat tirosina quinasa d'ambdós receptors PDGFR-α and PDGFR-β (Buchdunger et al., 2000). En algunes malalties mieloproliferatives cròniques, la translocació [t(5;12)(q33;p13)] produeix una proteïna de fusió, ETV6-PDGFRB, que és el receptor β de PDGF activat sense lligand. Aquesta proteïna de fusió també és sensible a la inhibició per imatinib (Apperley et al., 2002).

Recentment s'ha trobat casos de GIST amb una baixa expressió de c-Kit que, tot i així, responien al tractament amb imatinib, suggerint un mecanisme d'acció alternatiu (Borg et al., 2004). Es va observar que quan es produeix la senyalització de c-Kit a les cèl·lules dendrítiques s'inhibeix l'activació de les cèl·lules NK(*natural killer).* El tractament amb imatinib, inhibint la senyalització de c-Kit, millora l'activació de les NK i incrementa la seva producció d'interferó γ. En concordança amb aquests resultats es va observa que en els pacients de GIST tractats amb imatinib es produeix un augment en la producció d'IFNγ en les cèl·lules NK que es correlaciona amb un increment en la resposta antitumoral (Borg et al., 2004).

En estudis amb ratolins, l'imatinib activa les cèl·lules IKDC (*interferon-producing killer dendritic cell*), un tipus de cèl·lula que es pot comportar com una cèl·lula dendrítica (secretant IL-12 i interferó α) i en funció dels estímuls rebuts es pot comportar com una NK, produint IFNγ. Les cèl·lules IKDC no expressen c-Kit i es desconeix per quin mecanisme l'imatinib potencia la seva funció *in vivo* (Smyth, 2006).

1.3.1.8.3. ESPECIFICITAT

L'imatinib mesylate és molt específic, amb unes concentració d'inhibició del 50% (IC50s) de 188 nM per c-Abl, 413 nM per c-Kit, i de 386 nM per PDGFR-β, al contrari dels IC50s de >10.000 nM per la majoria de les altres tirosina quinasa cel·lulars (Manley et al., 2002). L'imatinib mesylate ha mostrat un important efecte antitumoral en leucèmies Bcr-Abl-positives [Philadelphia chromosome-positive t(9:22)(q34;q11)], GISTs, amb mutacions activadores Kit, i en una varietat de càncers amb alteracions en el sistema del receptor del PDGF.

1.3.1.8.4. FARMACOLOGIA

L'imatinib té una alta biodisponibilitat oral. El pic de concentració en plasma es produeix a les 4 hores. La seva vida mitjana en humans és de 13 a 16 hores. Es metabolitza en el fetge (principalment via citocrom P450-3A4). Les concentracions en plasma suficients per arribar al IC50 poden ser obtingudes a dosis ≈ 400 mg un cop al dia. Els nivells en sèrum oscil·len en un rang de 1.46 a 4.6 μM (Druker et al., 2001).

1.3.1.8.5. EFECTES SECUNDARIS

L'imatinib és un fàrmac ben tolerat en els pacients amb CML, segons ha demostrat un estudi de seguiment durant 5 anys, realitzat amb 553 pacients tractats amb imatinib versus 553 tractats amb IFNα i *cytarabine* (Druker et al., 2006). Els efectes secundaris predominants són normalment lleus i consisteixen en edema/retenció de líquids (60%), rampes musculars (49%), diarrea (45%), nàusea (50%), dolor musculoesquelètic (47%), granissada i altres problemes de la pell (40%), dolor abdominal (37%), cansament (39%), dolor articular (31%) i cefalàlgia (37%). Els efectes secundaris de grau 3 o 4 són neutropènia (17%), trombocitopènia (9%), anèmia (4%) i elevació d'enzims hepàtics (5%). En aquest estudi, només es va donar un cas d'insuficiència cardíaca congestiva relacionada amb el tractament (<1%) (Druker et al., 2006). Segons l'estudi de Cohen MH *et al.* menys del 5% van deixar el tractament per causa dels efectes secundaris (Cohen et al., 2002).

En els pacients amb GIST, el tractament amb imatinib també va ser ben tolerat, tot i que, l'edema, diarrea i cansament van ser freqüents (Demetri et al., 2002). Recentment, també s'han descrit algunes alteracions en el metabolisme als ossos. En pacients amb

CML i GIST tractats amb imatinib, s'ha observat hipofosfatèmia associada amb nivells sèrics baixos de 25-hidroxivitamina D, 1,25-dihidroxivitamina D i calci. Una possible explicació seria que la inhibició del receptor de PDGF afecta la formació i resorció de l'os (Berman et al., 2006). Cal tenir en compte aquest punt , ja que l'ACG té més prevalencia en dones i la mitjana d'edat és aproximadament 70 anys.

1.3.1.8.6. RESISTÈNCIES

Tot i l'espectacular èxit assolit per l'imatinib mesylate, els reptes actuals són com maximitzar la resposta i com lluitar contra les resistències. Els mecanismes que generen les resistències inclouen l'augment de proteïnes de resistència a múltiples fàrmacs, inactivació funcional de l'imatinib mesylate, amplificació del gen Brc-Abl o mutacions, i pèrdua de la diana, la quinasa Bcr-Abl (Gorre et al., 2001; Weisberg & Griffin, 2000).

Les proves més convincents donen suport al paper de les mutacions en l'aparició de resistències. De fet, les mutacions en la quinasa Bcr-Abl han estat detectades en més d'un 90% de pacients que van recaure després de la resposta inicial (Hochhaus et al., 2002; Shah et al., 2002). Continua la recerca en aquest camp, amb el disseny de nous fàrmacs que puguin ser útil en cas de mutacions que confereixin resistència, com per exemple : Dasatinib (BMS-354825, Bristol-Myers Squibb) que, a diferència de l'imatinib, s'uneix a la proteïna de fusió BCR-ABL tant en la seva forma activa com inactiva (Talpaz et al., 2006).

1.3.2. EL SISTEMA DE L'ENDOTELINA

Els fenòmens isquèmics transitòris que pateixen un subgrup del nostres pacients, com per exemple la ceguesa transitòria (amaurosi fugax), són difícils d'explicar com a conseqüència d'un engruiximent de l'íntima i més aviat suggereixen una oclusió vascular deguda a fenòmens vasospàstics. Per aquest motiu, vam decidir analitzar l'expressió de l'ET, que és una molècula amb una gran capacitat vasoconstrictora.

1.3.2.1. INTRODUCCIÓ

L'Endotelina (ET-1) és un pèptid de 21 aminoàcids identificat a l'any 1988 en el sobrenedant d'un cultiu d'EC aòrtiques de porc (Yanagisawa et al., 1988). Té un pes molecular de 2492, amb un C-terminal hidrofòbic i dos ponts disulfur intramoleculars (Cys^1-Cys^{15} i Cys^3-Cys^{11}). L'ET-1 és un potent vasoconstrictor, de fet, comparteix un alt grau d'homologia en la seqüència i estructura amb la família de les sarafotoxines (SRT) aïllades del verí de la serp *Atractaspis engaddensis* (Figura 7).

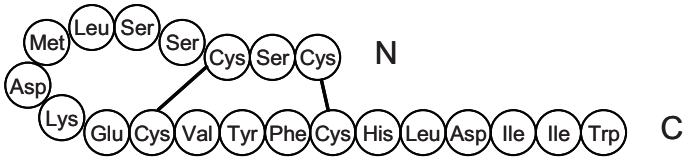


Figura 7. Seqüència d'aminoàcids de l'ET-$1_{(1-21)}$.

Dins d'aquell mateix any, 1988, es van identificar dos pèptids estructuralment relacionats que es diferencien en 2 i 6 aminoàcids, anomenats ET-2 i ET-3, respectivament. L'ET-1 és la isoforma predominant (Luscher & Barton, 2000). Actualment, també s'han identificat una endotelina de 31 aminoàcids.

A més dels seus efectes cardiovasculars, les ETs estan involucrades en desenvolupament embrionari, broncoconstricció, carcinogènesi, i funció endocrina i gastrointestinal.

1.3.2.2. BIOSÍNTESI DE L'ENDOTELINA

L'ET-1 és secretada per les EC i les VSMC, i per una gran varietat d'altres cèl·lules com els macròfags, mastòcits, miòcits cardíacs, neurones, hepatòcits, cèl·lules epitelials de l'intestí i ronyó, etc (Mawji & Marsden, 2003; Miyauchi & Masaki, 1999).

La concentració local en la paret vascular és igual o superior a 100 vegades la concentració en el plasma, degut en part al fet que el 80% de l'ET-1 és secretada en el costat basal de l'EC (Kedzierski & Yanagisawa, 2001).

La vida mitjana de l'ET-1 al plasma és de 4 a 7 minuts, així les cèl·lules vasculars poden ajustar ràpidament la producció d'ET-1 quan es requereix la regulació del to vascular. L'ET-1 secretada per les EC es dirigeix cap a les VSMCs, on s'uneix a receptors específics i produeix vasoconstricció (Levin, 1995).

El gen de l'ET-1 es troba al cromosoma 6p23-p24, la regió promotora té les típiques seqüències reguladores CAAT i TATA, i alguns elements addicionals en *cis* que proporcionen llocs de regulació als diferents estímuls. Entre ells, un lloc d'unió a la proteïna GATA-2, crucial en la producció d'ET-1 basal, també un lloc AP-1 on s'uneixen

c-fos i *c-jun*. Mitjançant aquests i altres promotors *upstream*, una gran varietat de factors de creixement i proteïnes vasculars poden modular la transcripció del gen de l'ET-1, aprofundirem en la regulació de l'endotelina en l'apartat 1.3.2.5.

La **preproendotelina-1** té 203 aminoàcids i és processada per una furina convertasa generant una prohormona de 39 aminoàcids, anomenada *big* ET-1, la qual és secretada i circula en el plasma (Figura 8). La *big* ET-1 té cent vegades menys potència que l'ET-1, però la seva concentració en el plasma és suficientment alta en certes condicions, com insuficiència cardíaca, que la seva conversió extracel·lular a ET-1 podria ser biològicament important (Levin, 1995).

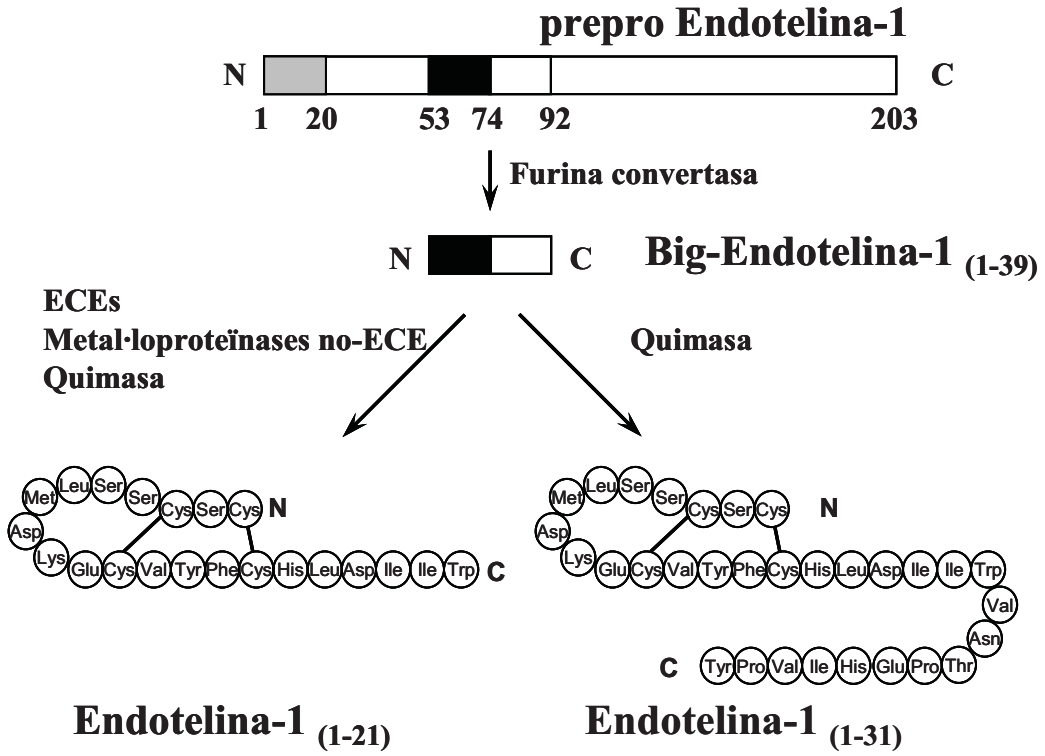


Figura 8. Biosíntesi de l'endotelina-1.

La *big* ET-1 és processada a ET-1_{1-21} mitjançant un tall entre Trp_{21}-Val_{22} produït per l'**enzim convertidor d'endotelina (ECE-1)**, o bé, per l'ECE-2. Els ECEs són metal·loproteïnases dependents de Zinc, unides a membrana amb homologia estructural amb l'endopeptidasa 24.11 (NEP) i amb el grup de proteïnes del grup sanguini Kell. ECE-1 i ECE-2 comparteixen un 59% d'homologia i són metal·loproteïnases sensibles al fosforamidó amb especificitat per la big ET-1. Existeix un tercer ECE, l'ECE-3, que té especificitat per la big ET-3 (Russell & Davenport, 1999). L'ECE-1 té un pic d'activitat a pH neutre i processa la big ET intracel·lularment i en la superfície cel·lular. ECE-2 té un pH òptim de 5.8 i és probable que actuí intracel·lularment.

A més d'aquestes proteases, existeixen enzims no identificats que també poden realitzar aquest darrer pas, ja que ratolins sense ECE-1 ni ECE-2 tenen nivells significatius d'endotelina madura (Kedzierski & Yanagisawa, 2001). L'endotelina també es pot produir per vies independents dels ECE, s'ha identificat una no-ECE metal·loproteïnasa i una altra quimasa en les VSMC. A més, la quimasa pot tallar la big ET-1 en el pont Tyr_{31}-Gly_{32}, formant ET-1_{1-31} (Figura 9).

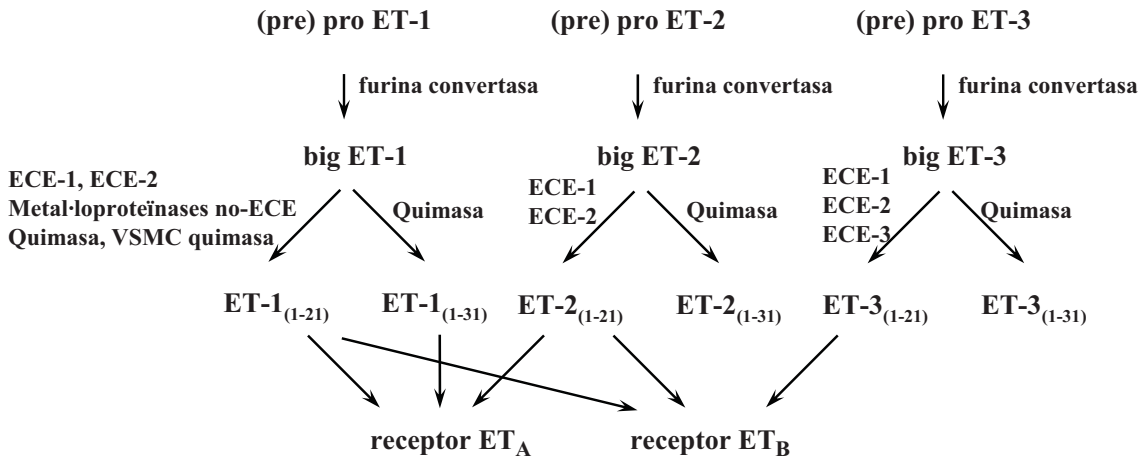


Figura 9. Biosíntesi de l'endotelines i interaccions amb els receptors.

L'ECE-1 és l'ECE predominant en humans. Existeixen quatre isoformes (ECE-1a, ECE-1b, ECE-1c i ECE-1d) codificades en un sol gen i que es diferencien en els seus dominis N-terminal citoplasmàtics. Gràcies a estudis amb formes solubles d'ECE-1 i a models experimentals, es considera que els domini extracel·lular conté el lloc catalític de l'activitat ECE.

Els ECEs han estat localitzats en cèl·lules endotelials i de múscul llis, cardiomiòcits i macròfags. L'expressió d'ECE-1 està regulada per mecanismes dependents de la proteïna quinasa C, receptors ET_B, el factor de transcripció ets-1 i citocines.

S'han establert dos tipus de vies de secreció de l'ET: la via constitutiva i la regulada. En la constitutiva, les EC alliberen ET de forma basal contribuint al manteniment de to vascular. Mentre que a la via regulada intervenen els cossos de Weibel-Palade que són vesícules on s'emmagatzemen substàncies vasoactives com la histamina, el factor von Willebrand, la P-Selectina i també l'ET (Russell & Davenport, 1999). Existeixen diferents estímuls que poden produir la desgranulació dels cossos de Weibel-Palade : físics, com l'estirament mecànic, o químics com el Phorbol 12-myristate 13-acetate que activa la proteïna quinasa C.

El catabolisme de l'ET es duu a terme mitjançant els receptors B, particularment en el pulmó, on el 80% de l'ET-1 que passa a través d'aquest òrgan queda retinguda (Kedzierski & Yanagisawa, 2001).

1.3.2.3. RECEPTORS DE L'ENDOTELINA

L'ET activa dos receptors amb 7 dominis transmembrana que estan acoblats a proteïnes G_i i van de 45000 a 50000 daltons. En humans, els **receptors ET_A i ET_B** comparteixen un 63% d'homologia i estan codificats en gens diferents localitzats en els cromosomes 4 i 13, respectivament. Cada receptor consisteix en un domini amino-terminal extracel·lular inusualment llarg, 7 dominis hidrofòbics que travessen la membrana i un domini carboxi-terminal intracel·lular. La cua C-terminal i el tercer *loop* citoplasmàtic contenen alguns llocs de fosforilació possibles. Moltes de les respostes induïdes per l'endotelina, com la generació de segons missatgers, l'alliberament de Ca^{2+} intracel·lular, la contracció i l'activació de MAPK són produïdes amb la interacció de vies sensibles a la toxina pertussis i vies insensibles. Per tant, hi ha varies proteïnes G implicades, com G_i, G_s i G_q. Per exemple, G_i i G_q acoblen els receptors d'ET amb la fosfolipasa C (PLC) i també formen part de la cascada de senyals que porta a l'activació de creixement cel·lular i diferenciació (Neylon, 1999).

1.3.2.4. VIES DE SENYALITZACIÓ DE L'ENDOTELINA

La unió de l'ET a receptors ET_A activa la fosfolipasa C, fet que porta a l'acumulació de inositol 1,4,5-trifosfat (IP_3) i diacilglicerol (DG). L'IP_3 augmenta la concentració de Ca^{2+} intracel·lular i provoca una vasoconstricció de llarga durada. Aquesta vasoconstricció persisteix després que l'ET-1 hagi estat eliminada del receptor, probablement perquè la concentració de Ca^{2+} intracel·lular roman elevada (Clarke et al., 1989). L'òxid nítric escurça la durada de la vasoconstricció ja que accelera el retorn de la

concentració de Ca^{2+} intracel·lular als valors basals. El diacilglicerol i el calci estimulen la proteïna quinasa C, la qual intervé en l'acció mitòtica de l'ET-1 (Levin, 1995).

L'activació dels receptors A també indueix proliferació cel·lular en diferents teixits. En canvi, l'activació dels B estimula l'alliberament de NO que estimula la guanilat ciclasa a les SMC conduint a la relaxació. L'activació dels receptors B també s'associa a l'alliberament de prostaciclines, prevé l'apoptosi, i inhibeix l'expressió d'ECE-1 en les EC. Els receptors B també intervenen en la *clearance* pulmonar d'ET-1 circulant i la reabsorció d'ET-1 per les EC (Luscher & Barton, 2000) (Figura 10).

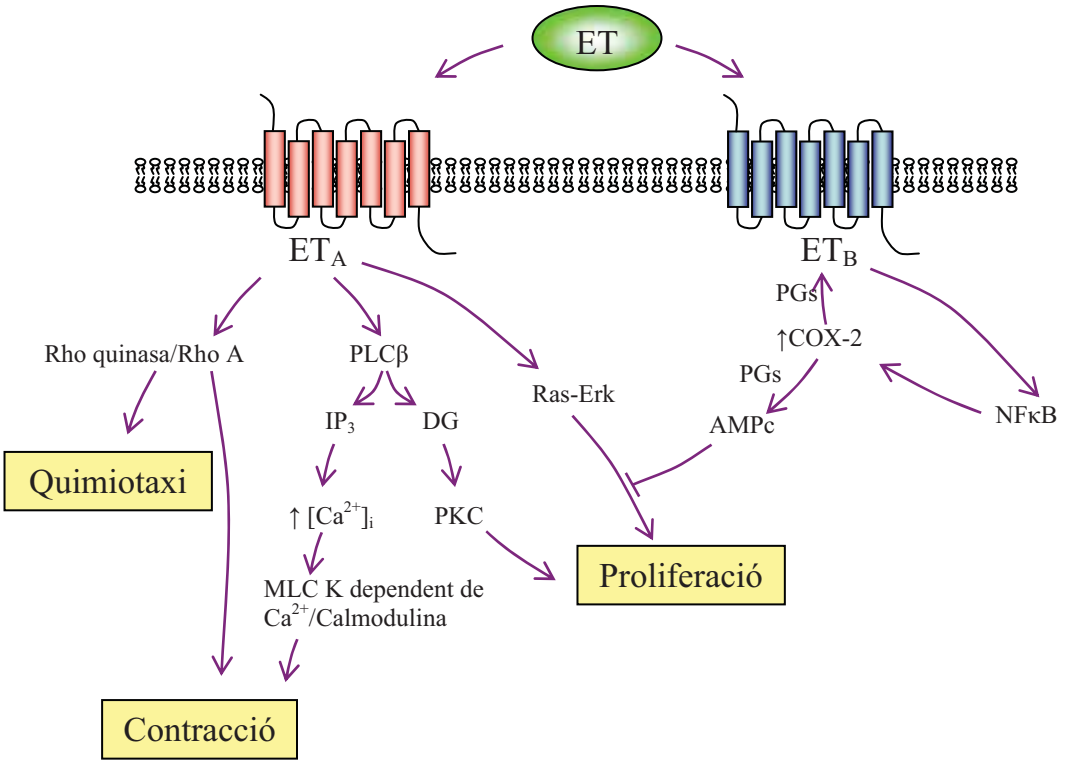


Figura 10. Principals vies de senyalització de l'ET.

Els receptors ET_A tenen 10 vegades més afinitat per l'ET-1 que per l'ET-3. Aquests receptors transmeten l'acció de vasoconstricció, tot i que, els receptors ET_B contribueixen a aquesta acció en certs territoris vasculars.

En la vasculatura, els receptors A estan expressats abundantment en els VSMC i els miòcits cardíacs mentre que els B s'han localitzat en EC, SMC i macròfags. A les artèries són més abundant els A, mentre que a les venes ho són més el B. Tot i que el nivell de receptor B en SMC pot augmentar en algunes patologies, com per exemple, la hipertensió arterial (Kedzierski & Yanagisawa, 2001).

1.3.2.5. FACTORS QUE REGULEN LA SÍNTESI D'ET

La síntesi d'ET està regulada per factors físico-químics com distensió pulsàtil, *shear stress*, i pH. La biosíntesi de l'ET-1 és estimulada per factors de risc cardiovascular com nivells elevats de LDL oxidades, glucosa, deficiència d'estròg ens, obesitat, cocaïna, edat, i mediadors procoagulants com la trombina (Luscher & Barton, 2000).

A més, l'expressió d'ET-1 s'incrementa amb el tractament de les EC amb diferents factors de creixement i citocines (**TGFβ**, **TNFα**, IGF-I, EGF, VEGF i FGF-2), insulina i amb substàncies vasoactives com la norepinefrina i l'angiotensina II (Levin, 1995; Miyauchi & Masaki, 1999; Schiffrin, 2005). La inducció de la síntesi d'ET per **TGFβ** no només activa la transcripció sinó que també estabilitza el seu RNA missatger (Lee SD et al. 2000). La **hipòxia** aguda i crònica indueix el promotor de l'ET-1 mitjançant l'element de resposta d'unió a HIF (*hypoxia inducible factor*) i es potencia per la unió de p300/CBP, AP-1 i GATA-2 (Figura 11) (Mawji & Marsden, 2003) .

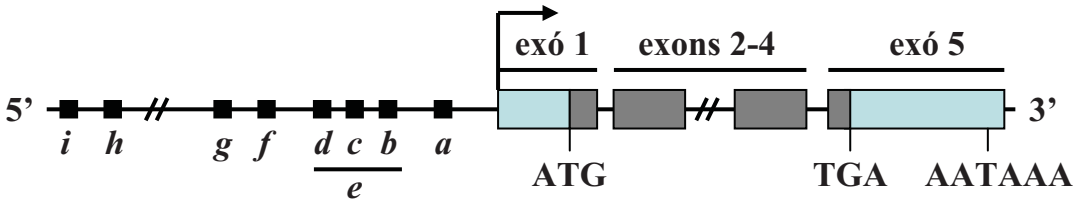


Figura 11 : Estructura del gen humà de l'ET-1 i regulació en cèl·lules endotelials. Els elements en *cis* del promotor responen als següents factors de transcripció : ***a*** = Vezf1/DB1, ***b*** = AP-1, ***c*** = HIF, ***d*** = GATA-2, ***e*** = p300/CBP (via ***b,d***), ***f*** = EC- *enhancer/complex*, ***g*** = element de resposta a la trombina, ***h*** = NFκB, ***i*** = *shear response* i ***j*** = 3'-UTR RNP complex. L'inici de transcripció (nucleotid +1) s'indica amb una fletxa horitzontal, l'ATG i el TGA marquen l'inici i el final de la traducció, i l'AATAAA és el lloc de poliadenilació (Mawji & Marsden, 2003).

Els inhibidors de la síntesi d'ET són l'òxid nítric (NO), prostaciclines i pèptids natriürètics atrials (Luscher & Barton, 2000). Les cèl·lules endotelials exposades a 17β-estradiol mostren una disminució en l'expressió basal d'ET i atenua la inducció per trombina i angiotensina II (Morey et al., 1998). L'activació de PPARγ (*peroxisome proliferator-activated receptor gamma*) també atenua la resposta a trombina (Delerive et al., 1999). Les estatines, fàrmacs inhibidors de la HMG-CoA reductasa, també disminueixen la producció basal d'ET i la induïda per LDL oxidades (Hernandez-Perera et al., 1998).

La regulació de la producció de receptors d'endotelina sovint és paral·lela a la regulació de les endotelines. Per exemple, la hipòxia produeix una estimulació ràpida de la producció d'ET-1 i dels receptors ET_A a les EC (Kourembanas et al., 1991). L'EGF, FGF-2, cAMP i estrogens augmenten els receptors ET_A en alguns teixits, mentre que PDGF, TGFβ i angiotensina II disminueixen els receptors ET_A (Levin, 1995).

1.3.2.6. FUNCIONS EN EL SISTEMA CARDIOVASCULAR

A més dels efectes vasoconstrictors i mitogènics, l'ET estimula la producció de citocines i factors de creixement com el VEGF, FGF-2 i epiregulina (Luscher & Barton, 2000).

L'ET-1 té activitat profibròtica ja que estimula la producció de proteïnes de matriu extracel·lular i fibronectina; i alhora potencia l'efecte del TGFβ i PDGF (Hafizi et al., 2004).

D'altra banda, l'ET produeix dany vascular per altres mecanismes que inclouen l'augment de l'estrès oxidatiu que porta a l'activació de gens redox-sensibles, l'estimulació de NFκB i l'AP-1 (*activator protein S-1*), la regulació positiva de VCAM-1 (*vascular cell adhesion molecule-1*), d'ICAM-1 (*intracellular adhesion molecule-1*), CCL2 (MCP-1, *monocyte chemoattractant protein-1*) i altres mediadors que atrauen a macròfags i neutròfils cap a la paret vascular (Schiffrin, 2001). Aquest fet, juntament amb la inducció de factors de creixement, desencadena un augment de la resposta inflamatòria i l'oclusió vascular.

La incubació de SMC amb ET-1 durant temps llargs causa una marcada regulació negativa dels receptors de l'endotelina, per internalització del complex lligand-receptor dins els lisosomes, l'ambient acídic promou la dissociació del lligand. A les VSMCs, l'ARNm dels receptors ET_A es regula positivament per cAMP i negativament per dexametasona.

1.3.2.7. PAPER DEL SISTEMA DE L'ENDOTELINA A LES MALALTIES VASCULARS

El paper de l'ET en el sistema cardiovascular ha estat analitzat en diferents malalties vasculars. En la insuficiència cardíaca crònica es va observar que els pacients tenien elevada l'ET-1 en plasma, els seus efectes a llarg termini són hipertròfia i dany cel·lular del miocardiòcits (Sakai et al., 1996).

També tenen l'ET-1 elevada en sèrum, els pacients amb espasme arterial cerebral (Fujimori A et al 1990). A més, a nivell experimental s'ha observat una millora dels símptomes amb l'administració intravenosa de bosentan, un inhibidor d'ambdós receptors de l'ET (Shigeno et al., 1995).

A la hipertensió arterial pulmonar, els nivells d'ET en plasma també es troben incrementats i es correlaciona amb la gravetat de la malaltia (Miyauchi & Masaki, 1999).

S'han realitzat pocs estudis que estudiïn l'ET a les vasculitis. S'ha estudiat en el cas de l'arteritis de Takayasu, aquesta vasculitis també afecta els vasos grans i a nivell histopatològic és molt similar a l'ACG, tot i que demogràficament afecta pacients més joves i amb més cronicitat. Els pacients amb l'arteritis de Takayasu tenen valors d'ET-1 en plasma elevats i correlaciona positivament amb la velocitat de sedimentació eritrocitària (ESR), els autors conclouen que l'ET podria intervenir en la progressió de l'engruiximent luminal dels vasos afectats (Akazawa et al., 1996).

En el cas de l'ACG només tenim constància d'un estudi amb quatre pacients, diagnosticats amb biòpsia d'artèria temporal positiva per ACG. Els quatre malalts presentaven uns valors elevats d'ET-1 en plasma respecte valors de referència de constrols sans (Pache et al., 2002). Pensem que el sistema de l'ET no ha estat estudiat més

en profunditat en els malalts amb ACG i que aquesta molècula podria jugar un paper en la malaltia.

Hem comentat una sèrie de molècules que podrien estar implicades en els mecanismes d'oclusió vascular. Estudis preliminars ens van fer concloure que el PDGF és un bon candidat com a promotor de la hiperplàsia intimal. D'altra banda, els fenòmens isquèmics transitoris que pateixen un subgrup del nostres pacients, com per exemple la ceguesa transitòria (amaurosi fugax), suggereixen una oclusió vascular deguda a fenòmens vasospàstics. Per aquest motiu, vam decidir analitzar l'expressió de l'ET, que és una molècula amb una gran capacitat vasoconstrictora.

Hipòtesi

2. HIPÒTESI

En l'Arteritis de Cèl·lules Gegants (ACG), el 15-20% dels malalts pateixen complicacions isquèmiques greus com són la ceguesa o l'ictus. Es desconeixen els mecanismes immunopatològics que produeixen el desenvolupament l'oclusió vascular. Els estudis d'aquesta tesi es centren en el comportament de les cèl·lules miointimals aïllades d'artèria temporal humana (HTAMC) perquè són claus en els possibles mecanismes d'oclusió vascular, com la hiperplàsia intimal i els espasmes vasculars. Aprofundir en el coneixement de quins estímuls hi intervenen ens permetrà dissenyar teràpies dirigides que millorin el tractament actual amb glucocorticoides. En aquest context establim les següents hipòtesis de partida :

1. L'artèria temporal és el teixit diana en el que s'ha basat els estudis immunopatològics sobre l'ACG (immunohistoquímica, PCR). Pensem que desenvolupar un nou mètode de cultiu *ex vivo* d'artèries temporals podria tenir dues possibles aplicacions: 1. l'aïllament de SMC per realitzar estudis *in vitro* que ens permetran acostar-nos més al context real de la lesió arterial i 2. ser un model funcional en el que es podrien analitzar els efectes de nous agents terapèutics.
2. Existeixen diferents estímuls en aquest context inflamatori que podrien estar intervenint en el comportament de les HTAMC. Entre ells, els activadors més potents de la proliferació, la migració i la producció de matriu extracel·lular podrien ser promotors de la hiperplàsia intimal i per tant, esdevenir dianes terapèutiques adients.
3. Factors vasospàstics com l'endotelina podrien tenir un paper important a l'oclusió vascular i estar implicats en les complicacions isquèmiques transitòries que

pateixen amb freqüència els pacients amb ACG abans de sofrir complicacions irreversibles. Si es demostrés que el sistema de l'endotelina està sobreexpressat en els malalts, esbrinarem quins són els mecanismes que regulen aquest sistema.

Objectius

3. OBJECTIUS

1. Aïllar i caracteritzar les cèl·lules miointimals a partir de biòpsies d'artèria temporal dels pacients amb ACG (HTAMC) per acostar-nos més al context de la lesió, ja que els estudis anteriors, en altres malalties vasculars, s'han realitzat amb SMC d'origen aortic o provinents de línies comercials.
2. Estudiar quins estímuls presents a les lesions inflamatòries i amb capacitat de modular respostes biològiques en cèl·lules mesenquimals (PDGF-AB, FGF-2, VEGF, EGF, TGFβ, CCL2, IL-6, IL-1β) poden regular les diferents funcions de les HTAMC implicades en la inflamació i la hiperplàsia intimal : proliferació, migració, producció de proteïnes de matriu extracel·lular i secreció de mediadors proinflamatoris.
3. Inhibir la senyalització del PDGF en les HTAMC *in vitro* mitjançant l'imatinib mesylate, un inhibidor del receptor de PDGF. Analitzar l'acció de l'imatinib mesylate sobre les accions estimulades per PDGF a les HTAMC.
4. Confirmar l'acció d'aquest fàrmac sobre un model *ex vivo* de cultiu d'artèria temporal.
5. Quantificar els nivells d'endotelina en els pacients amb ACG, en sèrum i en el teixit arterial, i la seva relació amb el desenvolupament de les complicacions isquèmiques.
6. Analitzar l'expressió dels components del sistema de l'endotelina (ET, ECE i ambdós receptors A i B) a nivell d'ARNm i a nivell proteic en les lesions arterials de l'ACG.
7. Investigar els mecanismes que intervenen sobre la regulació de l'endotelina *in vitro* en HUVEC i en HTAMC.
8. Estudiar l'efecte del tractament amb glucocorticoides sobre el sistema de l'ET.

Resultats

4.1.Model de cultiu d'artèria temporal i pulmonar humana

Donat que no es disposa d'un model animal de la malaltia, hem desenvolupat un **mètode de cultiu d'artèria temporal *ex vivo*** que ens permet analitzar tota la complexitat d'aquest sistema, ja que conté *ex vivo* tots els tipus cel·lulars de l'artèria i a més les cèl·lules de l'infiltrat inflamatori.

Figura 12. Cultiu de biòpsia d'artèria temporal al 7 dies de cultiu.

En aquest mètode de cultiu col·loquem una secció de biòpsia sobre Matrigel®, que és una membrana basal reconstituïda extreta del sarcoma de ratolí Engelbreth-Holm-Swarm (EHS), un tumor ric en proteïnes d'ECM. El principal component és la laminina, seguit per collagen IV, proteoglicà heparà sulfat, entactina i nidogen. També conté TGF-β, FGF i activador del plasminogen tissular (www.bdbiosciences.com). El Matrigel® proporciona un ancoratge imprescindible per el creixement de les SMC, i alhora conté factors de creixement i proteïnes d'ECM necessaris per l'inici del creixement cel·lular.

La biopsia d'artèria temporal és necessària per el diagnostic de la malaltia i és el teixit diana en el que s'ha basat els estudis immunopatològics sobre l'ACG (immunohistoquímica, PCR). Aquest nou mètode de cultiu *ex vivo* d'artèries temporals té dues possibles aplicacions:

1. l'aïllament de cèl·lules miointimals per realitzar estudis *in vitro* que ens permetran acostar-nos més al context real de la lesió arterial. El creixement de les SMC s'inicia als 6-7 dies, fins a arribar a formar un cultiu confluent. Aquestes cèl·lules poden ser tripsinades i sembrades en flascons, sobreviuen més de 10 cops aquest procés, tot i que, els experiments es van realitzar entre el 3è i el 6è cop, per evitar una possible desdiferenciació de les cèl·lules.

2. ser un model funcional en el que es poden analitzar els efectes de nous agents terapèutics. El model de cultiu d'artèria temporal desenvolupat en aquesta tesi és el model experimental més proper a la lesió arterial dels pacients amb ACG, ja que no es disposa d'un model animal. Els resultats obtinguts amb aquest mètode ens proporcionen informació sobre la funció que desenvolupa cada molècula individualment i poden analitzar si pot esdevenir una bona diana terapèutica.

Aquesta tècnica també s'ha utilitzat per cultivar artèries de petit calibre provinents de pulmó. El creixement de les cèl·lules miointimals pulmonars es va produir de forma similar i ens va permetre realitzar estudis de proliferació (resultats addicionals- 1). El PDGF, a diferència de l'ET, va demostrar ser un potent mitògen per aquestes cèl·lules, i l'imatinib va inhibir totalment l'efecte del PDGF. Pensem que aquest model de cultiu pot ser útil també en l'estudi d'altres malalties vasculars..

Imatinib mesylate inhibits* in vitro *and* ex vivo *biologic responses related to vascular occlusion in giant-cell arteritis.

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IMATINIB MESYLATE INHIBITS *IN VITRO* AND *EX VIVO* BIOLOGIC RESPONSES RELATED TO VASCULAR OCCLUSION IN GIANT-CELL ARTERITIS

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Short title: Imatinib inhibition of PDGF effects on intimal hyperplasia in GCA

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ABSTRACT

Objectives: Ischemic complications occur in 15-20% of patients with giant-cell arteritis (GCA). The aim of our study was to explore the effect of mesenchymal growth factors expressed in GCA lesions on myointimal cell responses related to the development of intimal hyperplasia and vessel occlusion.

Methods and results: We developed a method to obtain primary human temporal artery derived myointimal cells (HTAMC) based on the culture of temporal artery sections on Matrigel. Among the factors tested (PDGF-AB, FGF-2, VEGF, EGF, TGFβ, CCL2, IL-6, IL-1β), PDGF exhibited the strongest activity in inducing HTAMC proliferation and migration. As assessed by protein array, immunoassay and quantitative real-time PCR, PDGF stimulated matrix proteins (collagen-I, collagen-III and fibronectin) as well as CCL2 and angiogenin production by HTAMC. Imatinib-mesylate inhibited PDGF-mediated activation of signaling pathways (Src, ERK and Akt phosphorylation) related to cell motility and survival, efficiently resulting in inhibition of PDGF-induced HTAMC responses. Myointimal cell outgrowth from cultured temporal artery sections from patients with GCA, where multiple interactions take place, was also efficiently reduced by imatinib.

Conclusion: Among several mediators produced in GCA, PDGF has the highest vaso-occlusive potential. PDGF may also contribute to disease perpetuation by stimulating the production of angiogenic factors (angiogenin) and chemoattractants (CCL2). Imatinib-mesylate strongly inhibits PDGF-mediated responses, suggesting a therapeutic potential to limit vascular occlusion and ischemic complications in large-vessel vasculitis.

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INTRODUCTION

Giant-cell arteritis (GCA) is a chronic inflammatory disease involving large and medium-sized vessels[1,2,3]. Vascular remodeling in response to inflammation leads to intimal hyperplasia resulting in lumen occlusion and ischemia of supplied tissues[4-7]. In GCA, the most frequent and devastating ischemic events occur in territories supplied by carotid and vertebral artery branches and include ischemic optic neuritis and stroke[8]. About 10-15% of patients also develop an occlusive vasculopathy involving large and medium-sized vessels, particularly the aortic arch and limbs[9]. These vascular occlusive complications may occur during the follow-up of patients who underwent a satisfactory response of classical disease-related symptoms to corticosteroid therapy[9]. Moreover, when large-vessel stenoses occur, response to steroids is less complete and more unpredictable and patients may require angioplasty or derivative surgery[9]. Vascular occlusion is even more frequent in Takayasu's disease, another large-vessel granulomatous vasculitis[10]. Although GCA and Takayasu's disease differ in their demographic and ethnic distribution, histopathologic features are nearly identical, and both diseases may share immunopathogenic mechanisms[2, 11].

Under the influence of a variety of signals, vascular smooth muscle cells (VSMC) react to injury by evolving from their quiescent, contractile phenotype to proliferating, migratory and extracellular matrix (ECM)-secreting myointimal cells, key players in the development of intimal hyperplasia and vessel occlusion[4, 7, 12, 13]. Expression of several factors able to stimulate proliferation of mesenchymal cells has been demonstrated in GCA lesions. These include platelet-derived growth factor (PDGF), transforming growth factorβ (TGFβ), interleukin-1β (IL-1β), IL-6, fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factor (VEGF), among others[2,3,5,14-16]. Some of these factors have been also detected in Takayasu's disease[2,11]. Most of our current understanding of the pathogenic mechanisms leading to GCA comes from studies performed in temporal artery biopsies[2,3,5,6,7,14-16]. To gain a better understanding of the mechanisms leading to vascular occlusion in GCA, we developed a system to obtain and culture myointimal cells from temporal arteries (HTAMC) in order to assess the effects of various mediators on biologic responses related to the development of intimal hyperplasia such as proliferation, migration, and ECM production.

Among the factors tested, PDGF exhibited the strongest activity in our system. Given that imatinib-mesylate, an inhibitor of the tyrosine kinase activity of the oncogenic protein BCR/ABL generated in chronic myeloid leukemia, may also inhibit PDGF receptor-mediated signaling[18-20], we tested the effect of this compound on PDGF-induced responses in our model. We found that imatinib inhibited HTAMC biologic responses related to the development of intimal hyperplasia. Our findings suggest that imatinib might be a therapeutic option to limit occlusive vasculopathy in large-vessel vasculitis.

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MATERIAL AND METHODS

Reagents

Recombinant human TGFβ, IL-1β, IL-6, epidermal growth factor (EGF), monocyte chemoattractant protein-1 (CCL2/MCP-1), FGF-2, VEGF, purified PDGF-AB, and neutralizing mAb against PDGF-receptor α (clone 35248) were obtained from R&D Systems (Minneapolis, MN). Imatinib-mesylate (Gleevec®) was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland).

Isolation and culture of myointimal cells from human temporal arteries

Serial, 1 mm thick, sections from temporal artery fragments, obtained for diagnostic purposes, were placed onto ice-cold reconstituted basement membrane Matrigel™ (BD Biosciences, San Jose CA) and cultured in DMEM-10% fetal bovine serum (FBS) at 37ºC in 5% CO_2. HTAMC sprouted from the explanted sections within 1 week (figure 1A) and reached confluence in 3 weeks. Cells were then released with trypsin-EDTA (Invitrogen), transferred to uncoated flasks, and split 1:2 upon confluence. The myointimal cell phenotype[21] was verified by confirming expression of α-smooth muscle actin by flow cytometry and type I and III collagens by RT-PCR as described below. In all the experimental conditions tested, cell viability was confirmed by trypan blue exclusion.
The study was approved by the IRB of the Hospital Clínic of Barcelona and all patients signed informed consent.

Flow-cytometry analysis of α-smooth muscle actin expression

Cells were harvested with trypsin-EDTA, gently fixed with 4% paraformaldehyde for 1 hour at 4ºC, permeabilized with 0.2% Tween20 in PBS, and incubated with a mAb anti-α-smooth muscle actin (clone 1A4, Dako, Glostrup, Denmark) for 30 min at 4ºC, and, subsequently, with a goat anti-mouse antibody (AlexaFluor488, Molecular probes, Eugene OR) for 30 min at 4ºC. Both antibodies were used at 1/100 dilution. Cells were analyzed with a fluorescence-activated cell sorter (FACScan, Becton Dickinson, San Jose, CA).

Proliferation Assays

Proliferation was assessed as previously described[6,16]. Briefly, HTAMC were plated in 96-well plates at 4000 cells/well, in DMEM with 10% FBS and incubated at 37ºC in 5%CO_2 for 1 to 6 days, with or without addition of the above listed growth factors. These were used at concentrations ranging from 5-50 ng/mL, selected on the basis of previously published data and preliminary experiments. At several time-points, cells were fixed and stained with 0.2% crystal violet (Sigma-Aldrich) in 20% methanol for 10 minutes. Wells were washed, air-dried, and solubilized in 1% SDS. Optical density was measured at 600 nm wavelength.

In some experiments, HTAMC proliferation was also assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT) assay (Promega, Madison, WI), according to the instructions of the manufacturer.

Migration assay

HTAMC migration was measured in 48-well microchemotaxis Boyden chambers with 0.1% gelatin-coated, 10μM pore polyester filters (Poretics, Osmonics Inc.). HTAMC were placed at 5,000 / well in the upper chambers. Growth factors were loaded at various concentrations in the bottom chamber of quadruplicate wells. After

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6h-incubation in 5% CO_2 at 37°C, cells were removed from the upper surface and filters were fixed with methanol and stained with hematoxylin. Cells in 5 randomly selected fields /well were counted under a microscope at 125x magnification.

Western-Blot analysis

Assessment of protein phosphorylation by Western-blot was performed as described[22]. Briefly, cell lysates were obtained in modified RIPA buffer and supplemented with protease inhibitors (Complete, Boehringer Mannheim, Mannheim, Germany) and Na_3VO_4 at 200 µM. Twenty µg of protein per lane were subjected to SDS-PAGE (8%) and blotted onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). Blocked membranes were incubated overnight at 4ºC with rabbit anti-human Src, Akt, or ERK phosphorylated at different residues. Immunodetection was performed with HRP-conjugated goat anti-rabbit antibody at 1:2000 dilution. Blots were stripped and re-probed with rabbit anti-human Src, Akt, and ERK antibodies. All primary antibodies were from Cell Signaling Technology (Beverly, MA) and were all used at 1:1000 dilution.

Assessment of protein secretion by protein array and immunoassay

Quantimatrix™ ELISA kit (Chemicon, Temecula, CA) was used to measure fibronectin in HTAMC supernates and cell lysates. A custom RayBio® Human Cytokine Antibody Array (RayBiotech, Inc. Norcross, GA) was designed to compare release of 30 inflammatory mediators of interest in the supernates of PDGF-stimulated versus control HTAMC. CCL2 and angiogenin concentrations in HTAMC supernates were quantified with specific enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Reverse Transcription and Real-Time PCR

Total RNA was extracted from cultured HTAMC cells using TRIzol® Reagent (Invitrogen). One microgram of total RNA was reverse transcribed using Archive kit (Applied Biosystems, Foster City, CA). Collagen I (COL1A1) and III (COL3A1) mRNA were measured by real-time quantitative PCR with specific pre-developed Taqman gene expression assays from Applied Biosystems. Detailed methodological aspects have been depicted in previous publications[3,5,6,16]. Fluorescence was detected with ABI PRISM 7900 Sequence Detection system and results were analyzed with the Sequence Detection Software v. 1.6.3 (Applied Biosystems). All samples were normalized to the expression of the endogenous control GUSb. Comparative C_T method was used to assess the relative gene expression of COL1A1 and COL3A1.

HTAMC outgrowth from *ex vivo* cultured temporal artery sections from patients with GCA

Temporal artery sections obtained from 5 untreated patients with biopsy-proven GCA and 8 controls were cultured on Matrigel™ in the presence or in the absence of imatinib mesylate. We also tested in this system a similar size fragment of an aortic aneurysm surgically excised from a patient with GCA treated with corticosteroids for 4 years. This specimen showed mild remaining inflammatory infiltrates in the media. Daily photographs were taken from day 3 to day 17. Outgrowth of HTAMC was scored by 2 investigators blinded to the conditions tested, as follows: 1= visible outgrowth of scattered cells; 2= cells sprouting from the entire ring; 3= cells expanding at least 2 times the length of 2; and 4= cells covering the entire surface of the well. Agreement

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was achieved in 96 % of measurements. When there was discrepancy in the first assessment, consensus was reached after re-evaluation.

Statistical analysis

Quantitative data were compared with the Mann-Whitney U test. The Kruskal-Wallis test was used for multiple comparisons.

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RESULTS

Culture and characterization of HTAMC

After the first passage, HTAMC were able to grow on uncoated tissue culture flasks with no addition of growth factors other than FBS (figure 1B). Flow cytometry analysis revealed a homogeneous cell population with >90% of cells expressing α-smooth muscle actin (figure 1C). HTAMC doubled in 5-6 days (figure 1D) and were used for experiments between passages 3 and 6. Cells survived for at least 10 doublings with no apparent changes in cell morphology, phenotype, or growth rate. No apparent differences in morphology or growth rate could be observed between cells obtained from positive biopsies and those obtained from normal temporal arteries, indicating that proliferative and migratory activities relevant to the generation of intimal hyperplasia that occur in inflamed arteries are not intrinsic and mainly depend on stimuli present in the microenvironment.

Comparative effects of mesenchymal cell growth factors on *in vitro* HTAMC proliferation and migration

We next tested the effect of PDGF-AB, IL-1β, IL-6, CCL2, FGF-2, TGFβ, VEGF, and EGF on HTAMC proliferation and migration. These factors were considered relevant candidates to provide the microenvironmental stimuli necessary for myointimal cell activation because of their known ability to stimulate mesenchymal cell proliferation and their previously demonstrated expression in GCA lesions[5,14-16]. Among the factors tested, PDGF elicited the highest stimulation of HTAMC proliferation, in a dose-dependent manner (figures 2A and 2B). FGF-2, IL-1β, TGF β, and EGF, also induced HTAMC proliferation but to a lesser extent (figures 2A and 2C). PDGF-induced HTAMC proliferation was abrogated by a blocking mAb against the PDGF-receptor α (figure 2 B), confirming the specificity of the response.

Given that the development of intimal hyperplasia requires not only myointimal cell proliferation but also migration towards the lumen, we explored the effects of the above growth factors on HTAMC migration in Boyden chambers. As shown in table 1, PDGF had the strongest activity in inducing HTAMC migration. Maximum activity was obtained at concentrations of 10-20 ng/mL. EGF also increased cell migration but to a lesser extent. At the range of concentrations tested, the remaining factors did not stimulate HTAMC motility (table 1).

Inhibition of PDGF-induced HTAMC growth and migration by imatinib mesylate

As shown in figure 2B, the effect of PDGF on cell proliferation was inhibited with a function-blocking monoclonal antibody against PDGF receptor α. Since imatinib inhibits tyrosine kinase activity of the PDGF receptor, we tested its ability to inhibit PDGF-induced activation of signaling pathways related to cell survival, proliferation, and migration in HTAMC, given the relevance of these responses in the development of intimal hyperplasia. As shown in figure 2D, treatment with PDGF resulted in an efficient activation of Akt, a pivotal enzyme transducing anti-apoptotic signals[23] , and Akt phosphorylation was strongly inhibited by imatinib. PDGF also increased ERK1/2 and p60 Src activation, crucial pathways in cell proliferation and migration[24,25] which decreased upon imatinib treatment.

We next assessed whether the inhibitory effects of imatinib on PDGF–activated signaling pathways resulted in an efficient reduction of HTAMC responses. As displayed in figures 3A-C, imatinib efficiently inhibited PDGF-induced cell growth and

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motility. No inhibitory effect of imatinib was observed on spontaneous, FGF-2, or EGF-induced HTAMC proliferation or migration (figure 3B and data not shown).

Imatinib mesylate inhibits PDGF-induced extracellular matrix production by HTAMC

Since neointima formation requires, not only myointimal cell proliferation and migration but also excessive matrix deposition, we next investigated the impact of imatinib on PDGF-induced ECM production by HTAMC. Among potentially relevant matrix proteins, we investigated fibronectin, given its key function in supporting integrin-dependent cell proliferation and migration. We also focused on collagen I and collagen III, the major structural collagens of blood vessels. As shown in figure 3D-F, PDGF significantly up-regulated soluble and cell-associated fibronectin, as well as collagen I and collagen III production by HTAMC. These effects were efficiently reduced by imatinib.

Imatinib mesylate inhibits PDGF-induced HTAMC responses related to persistence of inflammatory activity

We investigated additional PDGF-mediated responses potentially contributing to amplify vascular inflammation in GCA. We designed an antibody-based protein array with 30 inflammatory mediators to screen for PDGF-induced molecules in HTAMC. Mediators screened were selected according to their previously demonstrated expression in GCA or their role in the regulation of inflammatory, angiogenic, or fibrogenic responses, given that these are considered relevant in disease perpetuation or vascular occlusion (figure 4A and 4B). Among the surveyed factors, PDGF significantly up-regulated CCL2 and angiogenin production by HTAMC in a dose-dependent manner and this increased expression was abrogated by imatinib (figures 4C and 4D).

Imatinib mesylate strongly reduces HTAMC outgrowth from *ex vivo* cultured temporal artery explants

PDGF may not be the only factor contributing to intimal hyperplasia in GCA. In order to assess whether the inhibitory effects of imatinib on PDGF-induced HTAMC responses could be overcome by other factors present in GCA lesions, we explored the effect of imatinib on HTAMC outgrowth from cultured temporal artery sections from 5 patients with GCA and 8 controls. As shown in figure 5, imatinib efficiently reduced, but not abrogated, HTAMC outgrowth from cultured artery sections, a complex system including multiple cell interactions and mediators. No significant differences were found between normal and GCA specimens, indicating that PDGF is a significant factor in inducing myointimal cell differentiation and growth in response to both inflammatory and physical (sectioning) injury.

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DISCUSSION

We successfully developed a new method to expand myointimal cells derived from thin arterial sections, using reconstituted basement membrane Matrigel™ as an anchorage-providing system. Our method has the advantage of requiring just a thin arterial section, which is very important since the major portion of the specimens excised must be processed to rule out or confirm the histopathologic diagnosis of GCA. This system may be also applied to efficiently grow myointimal cells obtained from other vascular beds, when there is paucity of tissue or the vessels are small. Most of the studies investigating smooth muscle cell biology have been performed with cells of aortic origin or aortic explants[26,27]. Obtaining cells from targeted vessels is important because, VSMCs derived from different vascular territories may have different phenotypes and functional responses[28].

In this study, we took advantage of this model to explore the effects of several growth factors known to be expressed in GCA lesions, on HTAMC responses related to the development of intimal hyperplasia[2,3,5,6,14-16]. Among the factors tested, PDGF exhibited the highest activity in stimulating HTAMC proliferation and migration. PDGF has been demonstrated to have mitogenic and chemotactic activitiy on cultured aortic VSMC, aortic explants and in *in vivo* models of arterial injury in other territories[[26,27,29-31]. Based on these findings PDGF was proposed as a candidate factor involved in the development of intimal hyperplasia in GCA[4]. Its expression by activated macrophages was demonstrated subsequently in GCA lesions and was shown to be associated with the development of cranial ischemic complications and clinically symptomatic large vessel stenoses[14]. Our findings functionally demonstrate that PDGF is a major growth factor for smooth muscle cells derived from arteries involved by GCA lesions.

Imatinib mesylate was specifically designed as an ATP-competitive inhibitor of the constitutive tyrosine kinase activity of the fusion protein BCR/ABL generated in chronic myeloid leukemia[18-20]. Imatinib was subsequently found to also inhibit the tyrosine kinase activity of c-kit, the receptor for stem cell factor (SCF), and PDGF receptor[19,20]. Imatinib is an effective therapy for patients with chronic myeloid leukemia, and patients with gastrointestinal stromal tumors bearing oncogenic mutations in the c-kit or PDGF receptors[19,20]. Given that imatinib has been used in a large number of patients with these conditions with an excellent safety profile[19,32,33], we investigated whether imatinib could inhibit PDGF-mediated responses related to the development of intimal hyperplasia in HTAMC. Imatinib did not show any effect on resting cells, but strongly inhibited PDGF-induced Akt, Src, and ERK1/2 phosphorylation, resulting in an efficient abrogation of PDGF-induced HTAMC proliferation and migration. PDGF also induced production of fibronectin and collagens I and III, the major structural collagens of blood vessels. Through integrin–mediated signals, fibronectin is able to support proliferation and migration of both inflammatory cells and VSMC[13,22]. Treatment with imatinib significantly reduced PDGF-induced fibronectin, collagen I, and collagen III production, suggesting that imatinib might be useful in reducing intimal hyperplasia in GCA.

In addition to promote fibrogenic responses, PDGF has pro-inflammatory effects by stimulating certain NFκB-mediated responses including chemokine production by VSMC[26-34]. PDGF also has angiogenic activity in various models and induces angiogenic factors such as VEGF and FGF-2[17, 35,36]. Together, PDGF and PDGF-induced angiogenic molecules, may contribute to inflammation-induced angiogenesis,

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an important mechanism supporting disease persistence in chronic inflammatory diseases.

A survey of PDGF-induced molecules in HTAMC revealed significant up-regulation of CCL2 and angiogenin. CCL2 is also an angiogenic molecule and a potent chemoattractant for monocytes and Th1 lymphocytes, the main components of inflammatory infiltrates in large-vessel vasculitis [5,37]. We have recently shown that increased CCL2 expression is associated with persistence of disease-activity in GCA[5]. Although not fully characterized for biologic functions, angiogenin has angiogenic activity in several systems[38]. We found that imatinib efficiently inhibited PDGF-induced CCL2 and angiogenin production by HTAMC. Together, these findings suggest that, besides reducing intimal hyperplasia, imatinib may inhibit PDGF-driven proinflammatory activities potentially contributing to disease persistence in large-vessel vasculitis.

PDGF may not be the only factor promoting myointimal cell proliferation and migration in GCA. To assess whether blocking PDGF effects could be compensated in vivo by other factors, the inhibitory effect of imatinib was tested in a temporal artery culture system. Imatinib substantially reduced but not abrogated HTAMC outgrowth from cultured temporal artery explants from patients with biopsy-proven GCA. A similar effect was observed in an aortic specimen. Thus, imatinib had a strong effect in a complex system containing all the cell components and mediators participating in the development of GCA lesions. The fact that imatinib does not interfere with other factors and, accordingly, did not completely suppress HTAMC outgrowth from GCA specimens is important given that a certain extent of neointima formation may be necessary to reinforce the vessel wall, preventing dilatation and rupture. Imatinib also reduced myointimal cell outgrowth from normal temporal arteries. Taken together these findings indicate that PDGF has a major, but not exclusive, role in driving vascular response to both inflammatory and physical injury.

In summary, imatinib inhibits cell responses related to the development of intimal hyperplasia in HTAMC and in a temporal artery organ culture suggesting a potential therapeutic benefit in preventing the progression of vascular occlusion in stenosing large vessel vasculitis. However, in the absence of *in vivo* data, any therapeutic implication must be considered with caution. Testing the effects of imatinib in animal models of large vessel inflammation[39,40] would add valuable information about the potential of imatinib to reduce intimal hyperplasia in vivo. Since there are no true animal models for GCA or Takayasu disease, the efficacy and safety of imatinib in reducing vascular occlusion in these diseases can only be assessed in clinical trials.

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ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Isolation, culture, and identification of human temporal artery myointimal cells (HTAMC). A) Primary HTAMC outgrowth from temporal artery rings cultured on Matrigel™ for 10 days (100x magnification). B) Typical appearance of confluent HTAMC grown on uncoated flasks (200x) C) Expression of α-smooth muscle actin by flow cytometry. D) Kinetics of HTAMC growth in DMEM+10%FBS (MTT assay).

Figure 2. Effect of mesenchymal growth factors on HTAMC proliferation. A) Proliferation of serum-starved HTAMCs incubated for 6 days with the displayed growth factors at 10 ng/mL. B) Inhibition of HTAMC by a function-blocking mAb against the PDGF-receptor α, added at 10 μg/mL, 60 min before exposure to PDGF (10 ng/mL). C) Dose response of several growth factors on serum-starved HTAMC proliferation at day 6. Proliferation was assessed by crystal violet staining. Experiments were repeated 3 times and one representative experiment is shown. In all figures, symbols/bars represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Kruskal-Wallis test. D) Effect of imatinib on PDGF-induced activation of Akt, ERK, and Src in HTAMC. Western-blot detection of Akt, ERK, and p60Src, phosphorylated at the indicated residues, in control or PDGF-stimulated HTAMC, in the presence or absence of imatinib. Subconfluent HTAMC were serum-starved for 24 h, pre-incubated with imatinib (1μM) for 60 min, and treated with PDGF-AB (10ng/mL) for 20 min. Blots are representative of 3 independent experiments.

Figure 3. Effect of imatinib on PDGF-induced HTAMC proliferation, migration and extracellular matrix production. A) Cell proliferation in response to PDGF in the absence and in the presence of imatinib (MTT assay). Cells were serum-deprived for 24h, pre-incubated with imatinib (1μM) for 60 min, and exposed to PDGF-AB (10ng/mL) for 5 days. B) Cell proliferation in response to FGF-2 (10 ng/mL) in the absence and in the presence of imatinib. C) Cell migration as described in the Methods section. D) Fibronectin concentration in the supernates of serum-free, PDGF-stimulated HTAMC in the presence or in the absence of imatinib. To detect cell-associated fibronectin in addition to that released to the medium, fibronectin content was also measured in cell lysates. Cell lysates and supernates were collected after 3 days of exposure to PDGF with or without imatinib. Fibronectin concentration was normalized to cell content, assessed by crystal violet staining. Detection was performed in duplicate wells. The experiment was performed 3 times with equivalent results. E) collagen I and F) collagen III mRNA concentrations in HTAMC cultured as in A and collected after 24h.

Figure 4. Effects of PDGF on CCL2 and angiogenin production by HTAMC and inhibition by imatinib. A) Design of the array membrane where 30 mediators are detected in duplicate spots. B) Mediators were detected by protein array in serum-free supernates of HTAMC with or without exposure to PDGF for 3 days. An increase in CCL2 and angiogenin production can be observed. Exogenously added PDGF is also detected. C and D) CCL2 and angiogenin detection in the supernates of HTAMC incubated for 1 and 3 days, respectively, with increasing concentrations of PDGF in the presence or in the absence of imatinib (1μM). Results were normalized to cell number.

Figure 5. Imatinib inhibits HTAMC outgrowth from cultured temporal artery sections from patients with GCA. A) Temporal artery sections exposed at the indicated

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concentrations of imatinib and cultured on Matrigel™ for 15 days . HTAMC outgrowth reaches a visual score of 4, 3, 2, and 1 at imatinib concentrations of 0, 5 , 10, and 15 μM, respectively. Phase-contrast microscopy (100x). B) HTAMC outgrowth from normal (solid bars) and GCA involved (white bars) temporal artery sections exposed to various imatinib concentrations, measured at day 15. C) Effect of imatinib mesylate on myointimal cell outgrowth from an aortic fragment of a GCA-related aortic aneurysm.

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TABLE 1: Migration of HTAMC (cell number/field) in response to increasing concentrations of growth factors (ng/mL).

	0	2.5	5	10	20
PDGF	44 ± 5.3	68.7 ± 6.3*	87.63 ± 4.6**	101.4 ± 5**	122.4 ± 3.9**
EGF	44 ± 5.3	66.8 ± 1.7*	77.8 ± 6.2*	79.3 ± 3.4**	71 ± 4.6*
VEGF	48.6 ± 3.5	41.6 ± 1.6	48 ± 3.5	50.7 ± 2.1	47.4 ± 2.1
CCL2	48.6 ± 3.5	47.3 ± 4.2	37.1 ± 2.3	47.3 ± 3.2	49.6 ± 3.3
IL-1β	52.4 ± 2.9	58.5 ± 4	59.4 ± 4	54.2 ± 4.1	48.5 ± 4.1
FGF-2	53.7 ± 2.4	57.1 ± 5.1	64.6 ± 2.6	65.1 ± 3	58.8 ± 3.6

* $p < 0.05$; **$p < 0.01$ by Kruskal-Wallis test

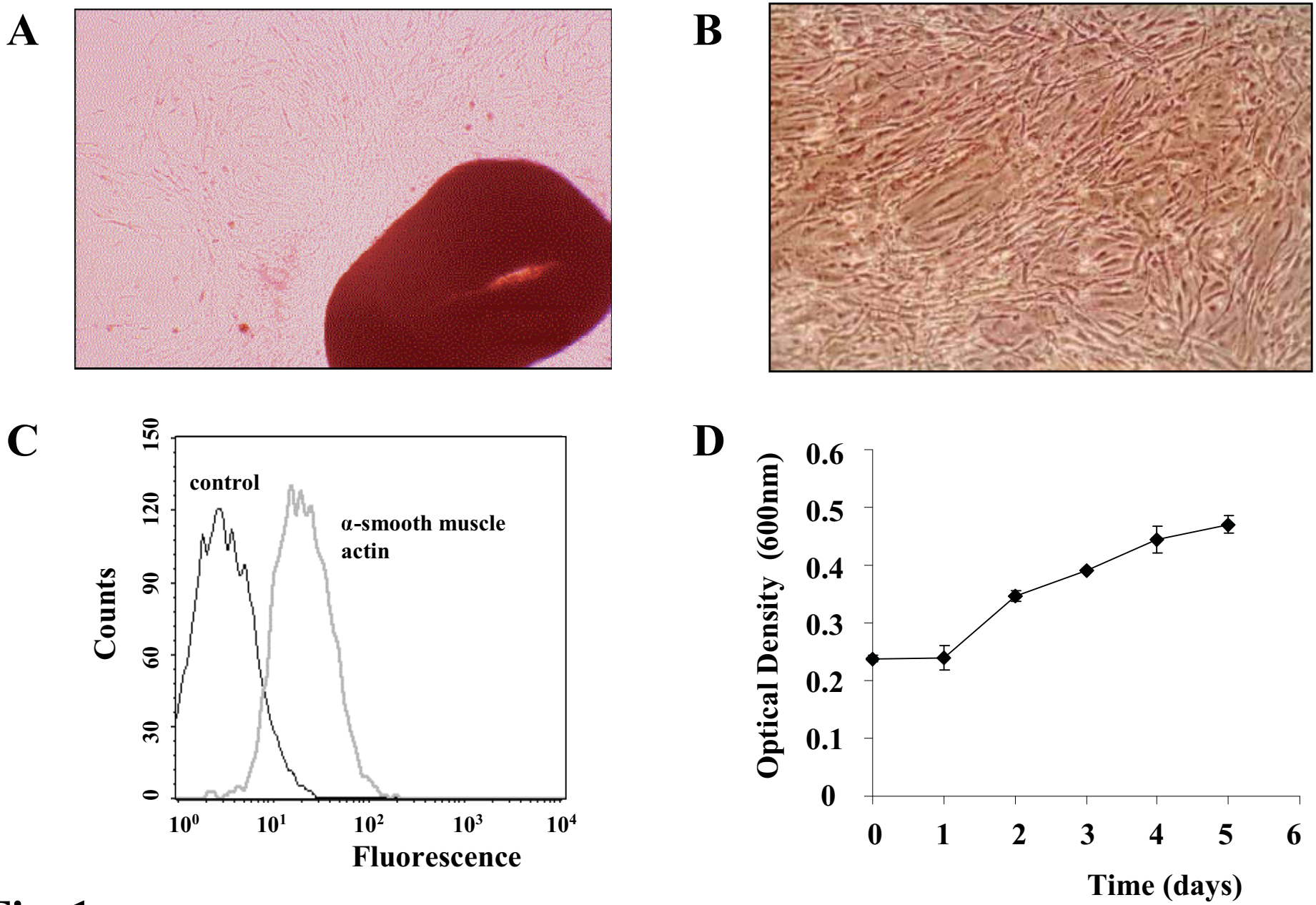

Fig. 1

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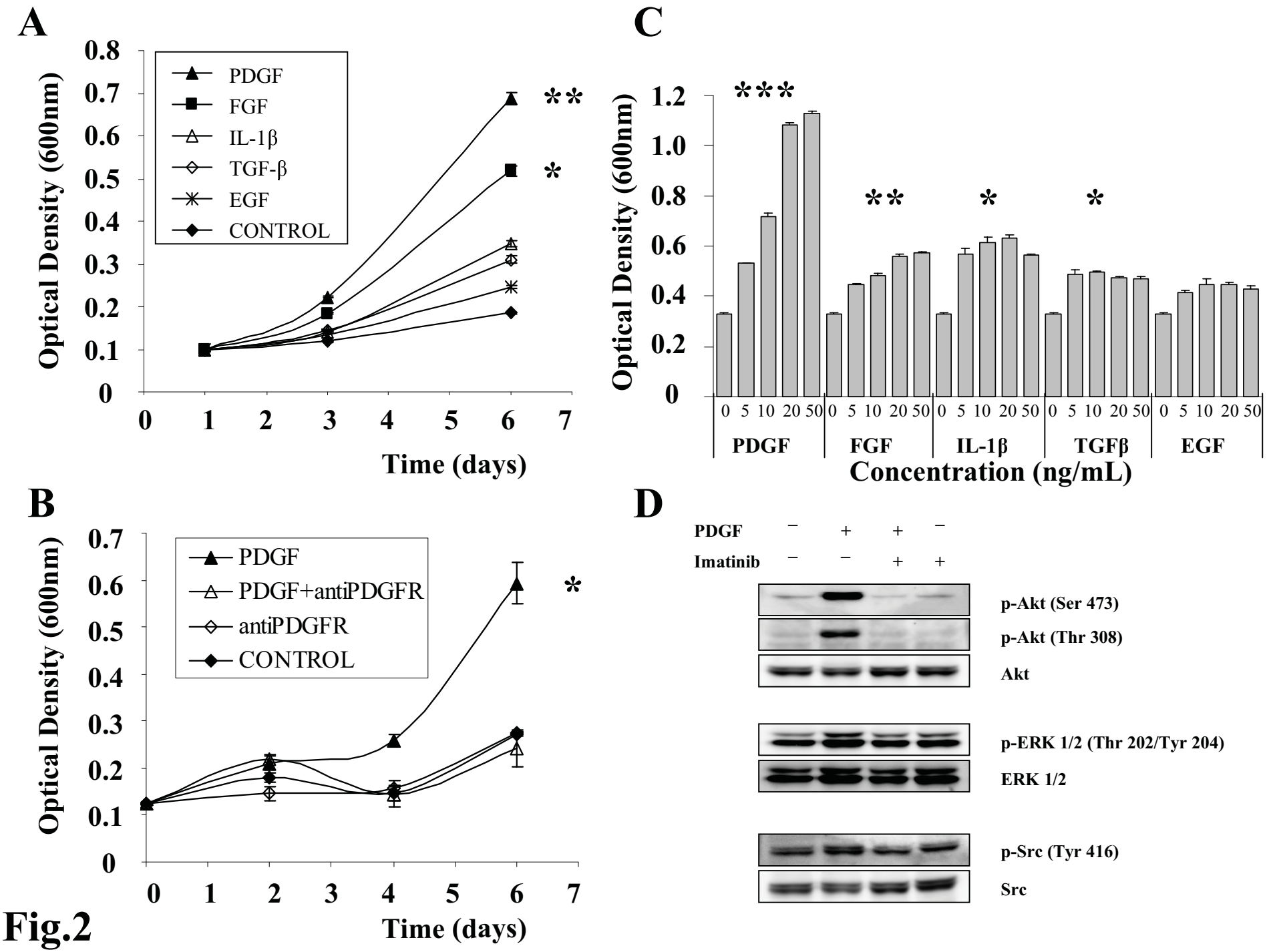

Fig.2

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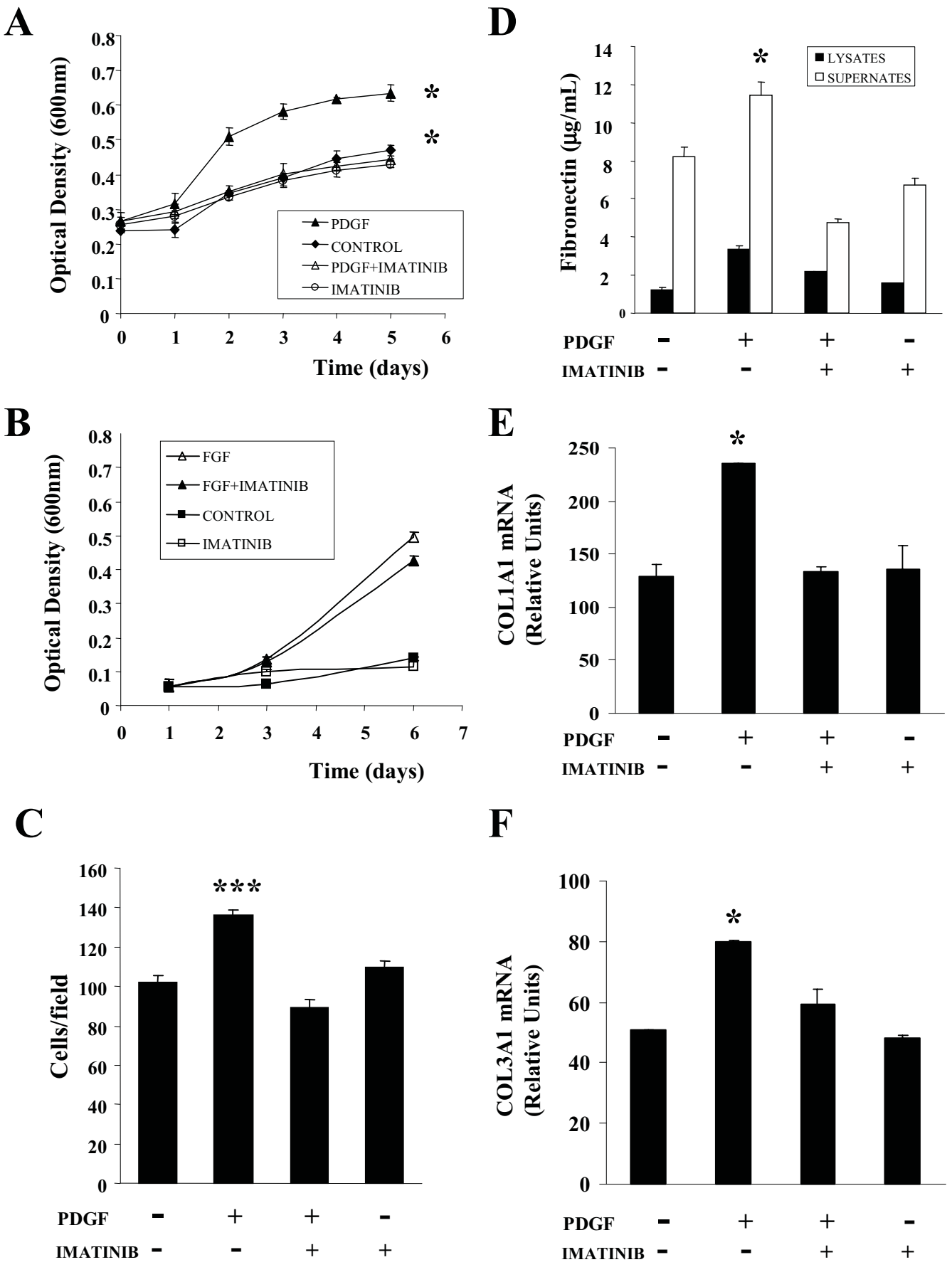
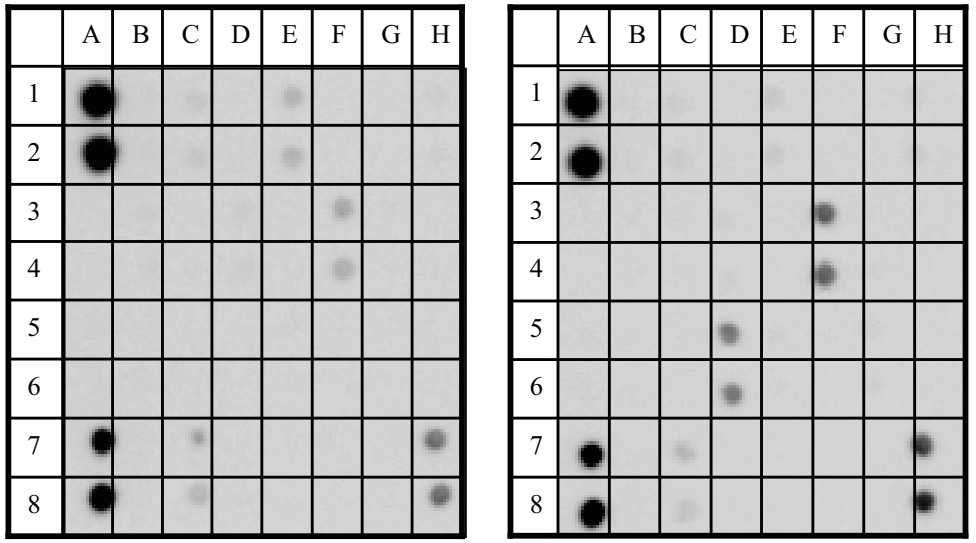


Fig. 3

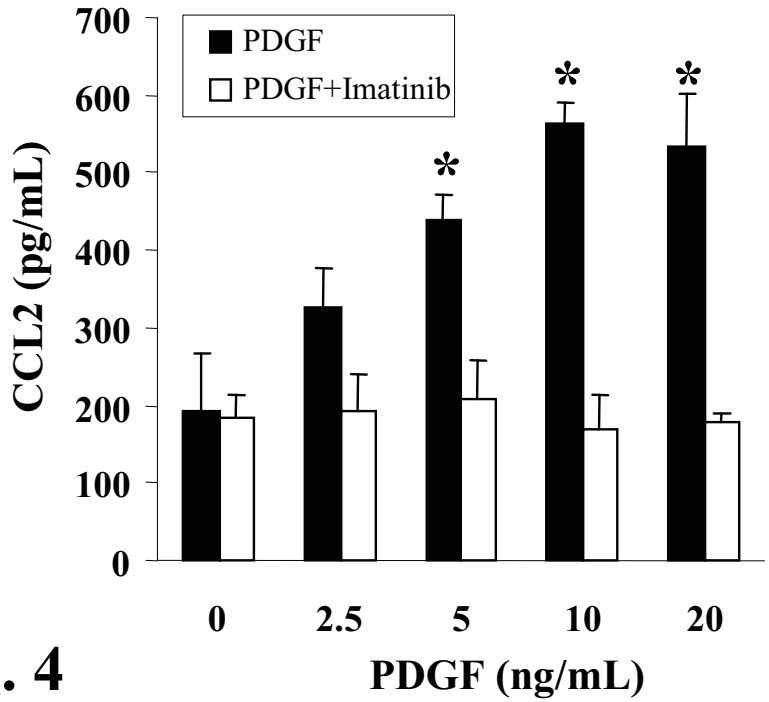
A

	A	B	C	D	E	F	G	H
1	POS	MIP-1β	TGF-β1	ICAM-1	OPG	SDF-1	IGF-I	FGF-2
2								
3	IL-1β	GM-CSF	TGF-β2	BMP-4	OSM	**ANGIOGENIN**	Ang-2	NEG
4								
5	IL-1ra	IL-10	TGF-β3	**PDGF**	LIF	EGF	HGF	VEGF
6								
7	IL-6	IL-6 sR	IL-8	TRAIL sR3	IP-10	TNF-α	IFN-γ	**CCL2**
8								

B



C



D

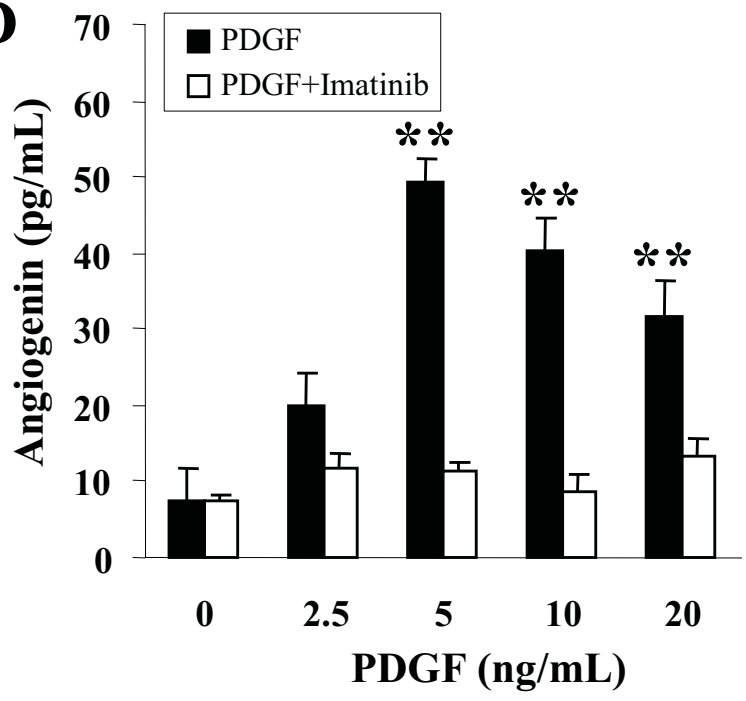


Fig. 4

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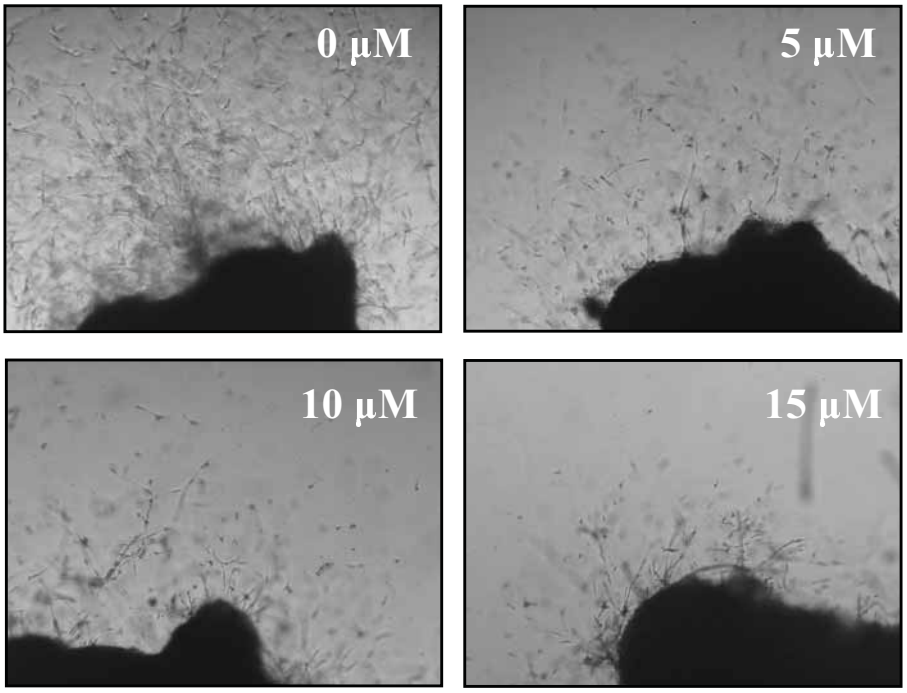
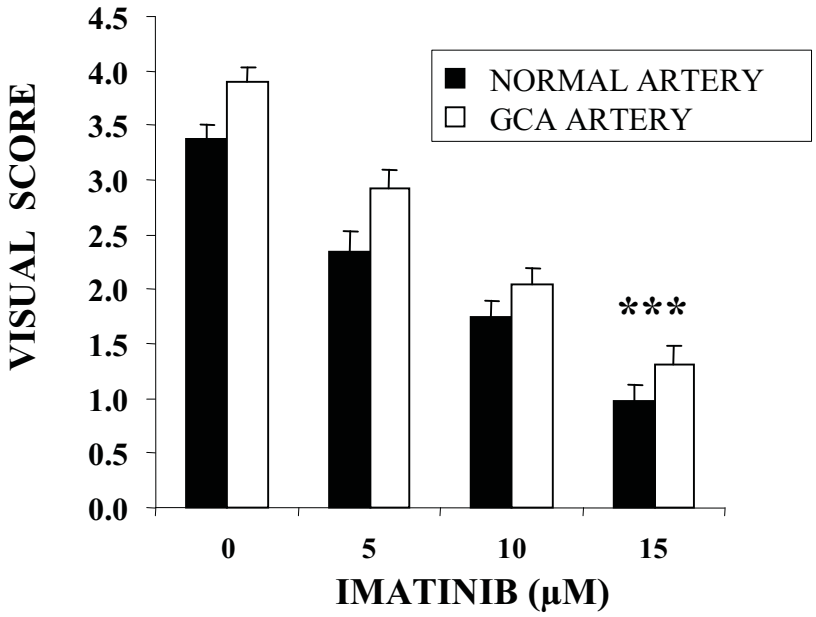
A



B



C

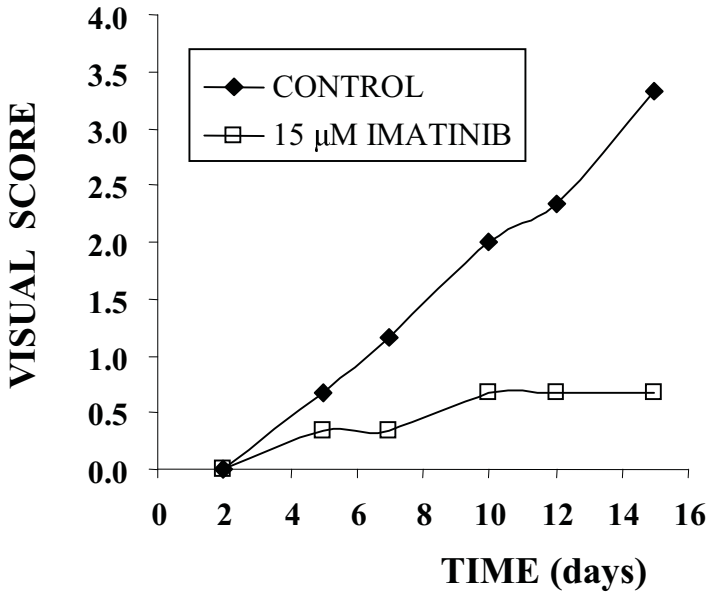


Fig. 5

Imatinib mesylate inhibits* in vitro *and* ex vivo *biologic responses related to vascular occlusion in giant-cell arteritis.

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RESUM DE RESULTATS

1. Hem desenvolupat un nou mètode d'**obtenció de cèl·lules miointimals** (HTAMC) a partir de biòpsies **d'artèries temporals** dels pacients amb ACG. Es va confirmar l'homogeneïtat de tipus cel·lular analitzant l'expressió d'α-actina per citometria de flux i la producció de col·lagen tipus I per realtime-PCR.
2. Hem analitzat l'efecte de diferents estímuls (PDGF-AB, FGF-2, VEGF, EGF, TGFβ, CCL2, IL-6, IL-1β) sobre la proliferació i migració de les HTAMC. El **PDGF és el principal agent mitogènic**, seguit pel FGF-2, l'IL-1β, el TGFβ i l'EGF. Respecte als experiments de migració, només el **PDGF i l'EGF** demostren una acció **quimiotàctica** sobre aquestes cèl·lules.
3. Donat que el PDGF ha resultat ser el factor que més influencia el comportament de les HTAMC, hem estudiat els efectes de l'imatinib mesylate (Gleevec®), un inhibidor del receptor del PDGF, sobre les HTAMC. Hem demostrat que l'**imatinib mesylate bloqueja les vies de senyalització del PDGF** en aquestes cèl·lules, analitzant per Western blot les fosforilacions d'Akt, Erk i Src produïdes

per l'estimulació per PDGF i la inhibició d'aquestes fosforilacions quan pre-tractavem les HTAMC amb imatinib abans de la incubació amb PDGF.

4. **L'imatinib mesylate inhibeix completament la proliferació i la migració** de les HTAMC estimulades amb el PDGF .
5. El **PDGF incrementa l'expressió dels col·làgens tipus I i tipus III** i també augmenta la producció de **fibronectina** en les HTAMC. L'**imatinib mesylate inhibeix** l'estimulació en la producció de proteïnes de matriu extracel·lular.
6. **PDGF estimula la secreció de CCL2 i d'angiogenina** de forma dosi-dependent en les HTAMC. L'**imatinib mesylate contraresta** també aquests efectes del PDGF.
7. Donat que no es disposa d'un model animal de la malaltia, el cultiu de seccions d'artèria temporal sobre Matrigel® és un mètode que ens permet analitzar tota la complexitat d'aquest sistema, ja que conté *ex vivo* tots els tipus cel·lulars de l'artèria més l'infiltrat inflamatori. Als sis o set dies, s'inicia el creixement de les SMC fins a arribar a formar un cultiu confluent. **L'imatinib mesylate inhibeix el creixement de les SMC a partir de l'artèria** de manera proporcional a la dosi emprada.

CONCLUSIONS

1. El mètode de cultiu d'artèria sobre Matrigel® permet aïllar i caracteritzar **cèl·lules miointimals a partir d'artèries temporals humanes (HTAMC).**
2. El comportament de les HTAMC *in vitro* està influït per **diversos factors** que es troben sobreexpressats en les lesions arterials de l'ACG, per exemple el FGF-2 estimula la proliferació i l'EGF estimula la quimiotaxi. Probablement el comportament d'aquestes cèl·lules *in vivo* és el resultat d'un balanç de diferents estímuls, per tant, poden existir vàries dianes biològiques i caldrà plantejar-se el disseny de **teràpies dirigides i combinades**.
3. Entre els factors estudiats, el **PDGF** és l'estímul més important a nivell de proliferació i de migració. L'imatinib mesylate bloqueja la senyalització intracel·lular induïda per PDGF en aquestes cèl·lules, fet que comporta la inhibició dels efectes proliferatius i quimiotàctics del PDGF. Per tant, **l'imatinib podria ser terapèuticament útil per contrarestar el procés d'hiperplàsia intimal** que es produeix en les artèries de calibre mitjà i gran en l'arteritis de cèl·lules gegants.
4. L'engruiximent de la paret de les artèries es deu a la proliferació i migració de les HTAMC però també a l'augment de proteïnes de matriu extracel·lular. El PDGF té funcions profibròtiques, ja que estimula la producció de proteïnes d'ECM en les HTAMC (col·làgens i fibronectina). Per tant**, l'imatinib podria disminuir el dipòsit excessiu de proteïnes d'ECM que es produeix en el procés d'engruiximent de l'artèria**.

5. Altres efectes del PDGF sobre aquestes cèl·lules són l'augment en la secreció de CCL2 i angiogenina, participant així en la perpetuació del procés inflamatori i en la formació de nous vasos. L'**imatinib** inhibeix també aquests efectes *in vitro,* per tant **podria disminuir la inflamació i l'angiogènesi** que es produeix en el desenvolupament de la lesió.

6. En un cultiu de l'artèria temporal, on es troben tots els components implicats (diferents tipus cel·lulars, factors solubles i ECM), el tractament amb **imatinib mesylate inhibeix significativament el creixement de les HTAMC a partir de l'artèria temporal**. Aquest model de cultiu *ex vivo* és el més proper a la situació *in vivo*, donat que no es disposa d'un model animal de la malaltia, només un assaig clínic podria comprovar que l'imatinib pot ser útil *in vivo* per disminuir l'oclusió vascular i la progressió de la malaltia.

The Endothelin System in Giant-Cell Arteritis.

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Increased Levels of the Endothelin System in Giant Cell Arteritis Lesions.

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Abstract

Objective - Giant Cell Arteritis (GCA) is a chronic disease characterized by intimal hyperplasia of large and medium arteries. Fifteen to 20 % of patients develop severe ischemic complications. The aim of our study was to characterize the endothelin (ET) system expression in GCA patients in order to investigate the relationship between ET system and ischemic complications, and to analyze its response to corticosteroid therapy.

Methods - ELISA, Western blot, real-time PCR

Results - We found that GCA patients with ischemic complications have elevated plasma ET-1 levels. Consistently, GCA patients showed a significant increase in ET in the temporal artery. Endothelin converting enzyme (ECE-1) and both ET receptors (ET_AR and ET_BR) tissue levels were also increased. Surprisingly, when we quantified mRNA expression of ET-1, ECE-1 and both receptors in temporal arteries we found that transcripts were significantly downregulated in GCA patients. In order to investigate this mechanism, we performed *in vitro* studies with HUVEC and with primary human myointimal cells obtained from temporal arteries (HTAMC), ET did not downregulate its own expression, in contrast, PDGF and IL-1β downregulated ET expression in a dose dependent manner. Corticosteroid therapy efficiently reduced ECE-1 and ET_AR values at protein level and partially recovered ET-1, ECE-1 and ET_BR mRNA tissue expression. Accordingly, *in vitro* studies demonstrated dexametasone upregulates ET and ECE-1 expression.

Conclusion - GCA patients had a significant increase in ET system components at protein level but their expression was dramatically downregulated. With corticosteroid administration, ET-1 tissue levels remained elevated.

Word count : 246 (<250)

KEY WORDS: Ischemia - Inflammation - Endothelin - Glucocorticoids.

Introduction

Giant cell (temporal) arteritis (GCA) is a chronic granulomatous vasculitis preferentially targeting large- and medium-sized arteries. Most of the classic disease manifestations result from symptomatic involvement of the carotid artery branches. Typical symptoms include headache, jaw claudication, scalp tenderness, and a variety of aches in the craniofacial area[1]. Cranial ischemic complications, in particular permanent visual loss, constitute the most feared aspects of this vasculitis, permanent visual loss is still present in 7-14% of patients[2]. We have previously shown that cranial ischemic events frequently appear relatively early in the course of the disease and tend to cluster in certain patients, suggesting that these individuals would be more prone to suffer ischemic complications when developing GCA[3,4]. Permanent visual loss often develop despite appropriate corticosteroid therapy[5].

The endothelin family of peptides (ET-1, ET-2 and ET-3) is generated in a variety of tissues and act primarily as paracrine and autocrine factors. The major isoform in the cardiovascular system, ET-1, is generated from a precursor, big ET-1, through cleavage by a specific endothelin-converting enzyme (ECE)[6]. ET-1 is the only family member produced in endothelial cells (EC) and vascular smooth muscle cells (VSMC)[7]. ET-1 has major influence on the function and structure of the vasculature as it favors vasoconstriction through activation of specific ET_A and ET_B receptors on VSMC[8]. The role of the ET system in GCA remains to be elucidated.

Therefore, the aim of the present study was to characterize the implications for ET system in the pathogenesis of GCA. We also investigated *in vitro* mechanisms that can modulate ET expression in HUVEC and isolated myointimal cells from GCA lesions (HTAMC). Furthermore, we studied the effect of corticosteroid treatment on this system.

Patients and Methods

Patients

Circulating plasma ET-1 levels were determined in 61 biopsy-proven GCA patients with an average age of 77 yr (range 58-91) and 16 control donors average age of 73 yr (range 60-87). ET system tissue levels were quantified in 15 GCA patients with an average age of 77 yr (range 64-91) and 10 histologically normal temporal arteries from patients with an average age of 78 yr (range 58-88). In order to investigate corticosteroid effect, we studied 7 treated patients who had received prednisone (1 mg/kg per day) for an average of 8 days (range 3–20) at the time of the temporal artery excision.

The gene expression study group consisted of 35 untreated patients with an average age of 77 yr (range 58–91) with biopsy-proven GCA. Nine patients suffered severe ischemic complications with established amaurosis. We also studied 18 patients who had received prednisone (1 mg/kg per day) for an average of 8 days (range 3–20). As control samples we included 16 histologically normal temporal arteries from patients with an average age of 73 yr (50-87) in whom a surrogate diagnosis was obtained. The ultimate diagnoses in these patients were isolated polymyalgia rheumatica (5 patients), self-limited constitutional symptoms with anaemia (4 patients), non-vasculitic ischaemic optic neuropathy (2 patients), headache associated with cutaneous infection (2 patients), chronic otitis media (1 patient), slowly resolving pneumonia (1 patient) and headache associated with persistent pyelonephritis (1 patient).

Determination of ET levels

ET-1 plasma levels were determined using Parameter ELISA kit (R&D Systems, Inc. Minneapolis, MN) according to the manufacturer's protocol. This kit exhibits cross-reactivity with other ET peptides as follows: ET-2 45%; ET-3 14%; and big ET <1%. ET-1 tissue levels were assessed by ELISA kit (Biomedica Medizinprodukte GmbH,

Vienna).This kit exhibits cross-reactivity with other ET peptides as follows: ET-2 100%; ET-3<5%; and big ET <1%.

Western Blot Analysis

Tissue extracts (25 μg) were resolved on 10% reducing SDS-PAGE gels and blotted onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). Protein expression was analyzed using antibodies against the following epitopes: ECE-1 (R&D Systems, Inc. Minneapolis, MN), ET_AR (BD Biosciences Pharmingen, Franklin Lakes, NJ) and ET_BR (Abcam, Cambridge UK).

Quantitative real-time RT-PCR Analysis

RNA isolation. Surgically excised temporal artery biopsies were embedded in optimal cutting temperature (OCT), quickly frozen in isopentane prechilled in liquid nitrogen and stored at –80°C until used. Total RNA was obtained from 100 serial sections (20 μm thick) per sample using TRIzol® Reagent (Invitrogen).

cDNA synthesis. Total RNA (1 μg) was reverse transcribed to cDNA using the Archive kit (Applied Biosystems, Foster City, CA) employing random hexamer primers in a final volume of 100 μL. Reaction conditions were carried out according to the manufacturer's recommendations. Samples were stored at –20°C until use.

Real-time quantitative PCR. mRNAs were measured by real-time PCR using specific Pre-Developed TaqMan gene expression assays from Applied Biosystems as previously described (ref). Fluorescence was detected with ABI PRISM 7900 Sequence Detection system and results were analyzed with the Sequence Detection Software v. 1.6.3 (Applied Biosystems). All samples were normalized to the expression of the endogenous control GUSb. Results were analyzed with the Sequence Detection Software v. 2.3 (Applied Biosystems). Comparative C_T method was used to assess the relative gene expression and values were expressed as relative units.

Primary Culture of human temporal artery myointimal cells (HTAMCs) and HUVEC

As described previously (ref), HTAMCs were isolated from remnant segments of temporal artery biopsies obtained for diagnostic purposes, cultured in DMEM medium supplemented with 10% FBS. Myofibroblast cell phenotype was verified by confirming expression of α-smooth muscle actin by flow cytometry and type I collagen expression by rt-PCR.

Statistics

Data are expressed as mean±SEM. Statistical significance was estimated with Mann-Whitney test. A probability value <0.05 was considered significant.

Results

Elevated plasma ET levels in GCA patients with ischemic complications

Circulating ET levels were similar in GCA patients than in age and sex-matched healthy donors (1.1121 ± 0.039 pg/mL n=61 *vs.* 1.1191 ± 0.061 pg/mL n=16). However, among GCA patients we found a significant increase of circulating ET-1 levels in GCA patients with ischemic complications (1.2048 ± 0.634 pg/mL, n=25) *versus* GCA patients without ischemic events (1.0478 ± 0.4747 pg/mL, n=36) ($p<0.05$) (Figure1).

Endothelin System is increased at protein level in GCA Lesions

We next investigated ET levels in temporal artery biopsies by ELISA. As depicted in Figure 2, GCA lesions showed a significant increase in ET (0.835 ± 0.193 fmol/mg total protein, n=24) *versus* negative biopsies (0.403 ± 0.117 fmol/mg total protein, n=22; p=0.028). Accordingly, we found significant higher levels of ECE-1, ET_AR and ET_BR in GCA lesions (n=20) *versus* negative arteries (n=15) (Figure 2 B-D). No significant differences were observed between GCA patients with severe ischemic complications and patients without ischemic events.

Endothelin System mRNA expression is downregulated in GCA Lesions

We next studied the mRNA expression of preproET-1, ECE-1, ET_A and ET_B receptors in temporal arteries from patients with GCA compared with control subjects. Surprisingly, transcript analyses indicated a substantial decrease in ET axis mRNA in positive GCA lesions compared with negative arteries (ET-1: non-GCA, 9.9±2.85, n=16 relative units *vs.* GCA, 2.03±0.29 n=37, p=0.000; ECE-1: non-GCA, 21.5±1.87 n=17 *vs.* GCA, 6.1±0.66, n=34, p=0.000; ET_AR: non-GCA, 20.2±1.96 n=16 *vs*. GCA, 7.3±1.15, n=38, p=0.000; ET_BR: non-GCA, 32.5±3.46 n=15 *vs.* GCA, 8.8±1.53, n=34 p=0.000)(Figure 3). No significant difference was observed in the ET system expression in patients with ischemic complications. A significant correlation was found between ET-1

expression and ECE-1 expression (*R*= 0.813, p=0.000), suggesting a coordinated regulation.

PDGF and IL1β downregulate *in vitro* ET-1 mRNA expression in myointimal cells from GCA arteries (HTAMC)

These data strongly support a downregulation mechanism in GCA lesions. To evaluate whether overexpressed molecules in GCA lesions may downregulate ET system expression *in vitro*, we stimulated primary HTAMC with dose-response of ET-1, Transforming Growth Factor (TGFβ), Platelet-Derived Growth Factor (PDGF), interleukin-1 (IL-1β), interleukin-6 (IL-6) and Tumor Necrosis Factor α (TNFα)[9]. ET did not regulate its own expression (Figure 4 A). Among the GCA mediators, IL-6 did not downregulate ET expression, TGFβ and TNFα significantly upregulated ET expression. PDGF and IL-1β efficiently reduced ET-1 expression in a dose dependent manner (Figure 4 B-F). We also examined whether these GCA mediators may downregulate ET expression in endothelial cells (HUVEC). None of them regulated negatively ET mRNA expression. TGFβ, TNFα and IL-1β increased ET expression in HUVEC.

Corticosteroid therapy partially modulates ET system expression

Corticosteroids are the treatment of choice for patients with GCA. To analyze the effect of corticosteroid therapy on the ET system, we compared protein level in arteries from treated GCA patients. We did not observed significant differences in ET level between treated versus untreated patients; both groups maintained an elevated ET level (Figure 5A). However, ECE and ET_AR were efficiently decreased in treated versus untreated patients, reaching normal values. The decrease in ET_BR levels in treated patients were not significant (Figure 5 B-D).These findings indicate that, corticosteroid treatment (average 8 days) only decreased some components of the ET system, but ET levels remained elevated. We also studied three patients who were biopsied before the treatment and after six months of corticosteroid therapy. As shown in Figure 5 (E-F), ET and ECE-1 levels were significantly decreased after six months of treatment.

We found at mRNA level a complete downregulation in untreated GCA patients compared with control donors. To investigate corticosteroid effect on ET system expression, we studied mRNA levels of 35 out of the 53 patients who had received no treatment at the time of the temporal artery excision, whereas 18 had received prednisolone at 1 mg/Kg for an average of 8 days (range 3–20). Treated patients showed significant higher expression ET-1, ECE-1 and ET_BR mRNAs although it did not reach control values. ET_AR expression did not show differences between treated and untreated patients, remaining downregulated (Figure 6 A, D).

Interestingly, *in vitro* studies showed that ET and ECE expression was upregulated in HTAMCs treated with dexametasone in a dose-dependent manner (Figure 6 E-F). ET_AR and ET_BR expression did not show to be modulated by dexametasone (data not shown). Dexametasone-induced increase in ET-1 and ECE-1 expression is consistent with the levels of expression found in treated patients.

Discussion

Endothelin is a pathogenic mediator with a number of deleterious effects, including vasoconstriction, fibrosis, vascular hypertrophy and inflammation. Clinical investigations have associated overexpression of ET with various systemic rheumatic diseases, including SSc, Raynaud's phenomenon, SLE and Takayasu arteritis[10]. ET-1 is the only family member produced in endothelial cells and vascular smooth-muscle cells. ET-1 should be regarded more as a paracrine than as an endocrine hormone[7, 11]. Plasma endothelin-1 measurements are nevertheless useful, because plasma concentrations have been found to correlate well with the severity of disease, such as congestive heart failure[12], pulmonary arterial hypertension[13, 14] and atherosclerotic disease[15, 16]. We analyzed whether the ET plasma level correlate with the development of ischemic complications in patients with GCA and we found that ET is significantly elevated in these patients. Increase in plasma ET levels is consistent with studies in Takayasu arteritis[17].

ET-1 plays an important role in the maintenance of vascular tone and pathological states such as ischemia. A functional ET system is crucial for controlling vascular resistance[18, 19]. ET may play a role in the pathogenesis of cardiovascular diseases through multiple mechanisms, ET has a substantial role in the regulation of cellular proliferation and survival[20], cellular matrix production[21] and neovascularization[22]. Recent studies in vascular remodeling in the internal mammary artery graft showed that elevated distribution of collagen indicative of fibrosis coupled with high levels of ET-1 and ET_A expression suggests altered VSMC regulation is fundamental to the remodeling process[23]. Therefore, the increased levels of the ET system may contribute to vasoconstriction and intimal hyperplasia in GCA lesions.

ET is involved in the development of inflammation in the vessel wall in atherosclerosis given that ET-1 activated NFκB in human THP-1 monocytes[24]. The biologic effects of ET-1 in inflammatory process also include the function and survival of dendritic cells[25]. ET system may be pathologically involved in the development of inflammatory infiltrates in GCA.

We provide the first evidence that in biopsy-proven GCA patients, the ET system is elevated in GCA lesions; however it is dramatically downregulated at the mRNA level.

These findings suggest a negative regulation of the ET system components in GCA in order to prevent the progression of the vascular occlusion. To elucidate the mechanism that generates this downregulation, we investigated *in vitro* ET system mRNA regulation. Inhibitors of ET-1 synthesis include nitric oxide, prostacyclin, atrial natriuretic peptides and estrogens[8]. Among the factors tested (ET-1, TGFβ, TNFα, PDGF-AB, IL-1β and IL-6), none of them achieved ET mRNA downregulation in endothelial cells. TGFβ and TNFα stimulated ET mRNA expression in HTAMC and HUVEC which is consistent with previous studies with EC[26, 27]. We found that PDGF-AB and IL-1β can negatively regulate ET expression in myointimal cells. PDGF-AB and IL-1β are overexpressed in GCA lesions, therefore, these results argue that they may be involved in ET downregulation that we observed in GCA lesions. Although studies in rat VSMC showed that PDGF-AA stimulated mRNA ET[28] it is well-know that PDGF isoforms trigger different functions because they interact with different receptors (α and β). Besides, our *in vitro* studies were assessed with primary human miointimal cells. Recently, it has been demonstrated that IL-1 β decreased ET mRNA and ET secretion in human myometrial cells[29].

Analyzing the effect of corticosteroids therapy, we found that treatment decreased ECE and ET_AR at protein level to normal values, but it did not modify ET levels. While at RNA level, we demonstrated that treated patients only partially recover ET-1, ECE-1 and ET_BR tissue expression. Interestingly, *in vitro* studies showed that dexametasone can upregulate ET-1 and ECE-1 expression in HTAMC. This result is consistent with Kanse SM and colleagues studies who demonstrated that glucocorticoids induced ET release from VSMC of rat and rabbit aortas[30]. Corticosteroids can modulate ET system expression and have a positive effect on some ET system components reaching normal values (ECE and ET_AR) but ET levels remain elevated, therefore, a complementary treatment should be necessary.

Figure Legends

Figure 1. Circulating levels of ET-1 (in pg/mL) in GCA patients with severe ischemic complications were significantly higher (p=0.013).

Figure 2. ET system components were elevated in GCA patients. **A.** ET elevated in GCA by ELISA. **B.** Representative ECE, ET_AR and ET_BR immunoblot with 4 negative biopsies and 5 GCA-positive biopsies. Actin was used to demonstrate loading equivalence. **C-E.** Densitometric analysis of protein expression of ECE-1, ET_AR and ET_BR determined by Western blot.

Figure 3. ET system expression was significantly downregulated in GCA patients ($p<0.05$). **A.** ET mRNA expression; **B.** ECE-1 mRNA expression; **C.** ET_AR mRNA expression and **D.** ET_BR mRNA expression was analyzed by real-time PCR.

Figure 4. *In vitro* Regulation of ET system in HUVEC and in HTAMC. **A-B.** ET and IL-6 did not modify ET expression. **C-D.** TGFβ and TNFα upregulated ET expression. **E-F.** ET-1 expression was significantly downregulated with PDGF and IL-1β in HTAMC but not in HUVEC.

Figure 5. Effect of corticosteroid therapy on ET system. **A.** Tissue ET levels remain elevated in treated GCA patients. **B-C.** ECE-1 and ET_AR recovers normal values ($p<0.05$). D. ET_BR decreased in treated patients. **E-F.** Three patients were biopsied before treatment (1) and after six months of corticosteroid therapy (2), ET and ECE decreased in the three cases while ET_AR and ET_BR decreased in a lesser extent.

Figure 6. Analysis ET system mRNA expression in treated patients. **A-B.** ET and ECE-1 expression was upregulated in treated patients. **C**. ETAR expression in treated patients is not significant increased. **D** ETBR expression was upregulated with corticosteroid therapy. **E-F.** ET and ECE were upregulated in a dose-dependent manner in HTAMC and HUVEC treated with dexametasone.

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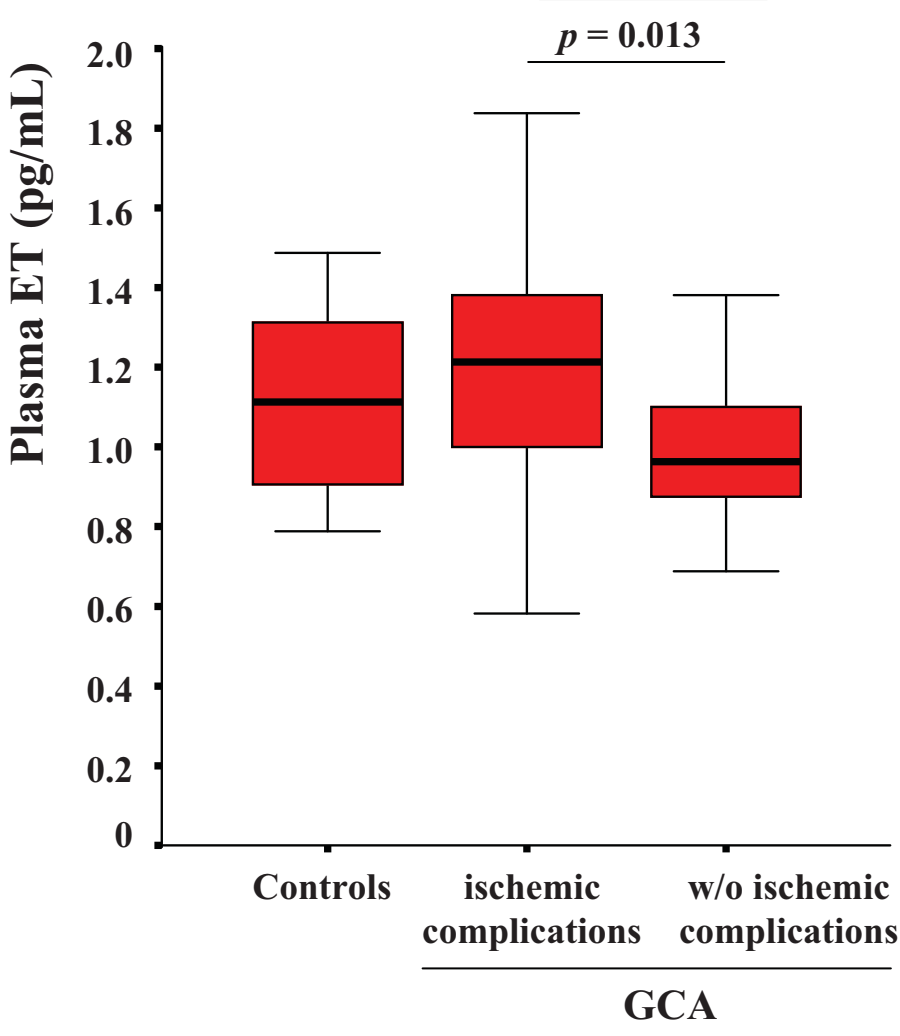
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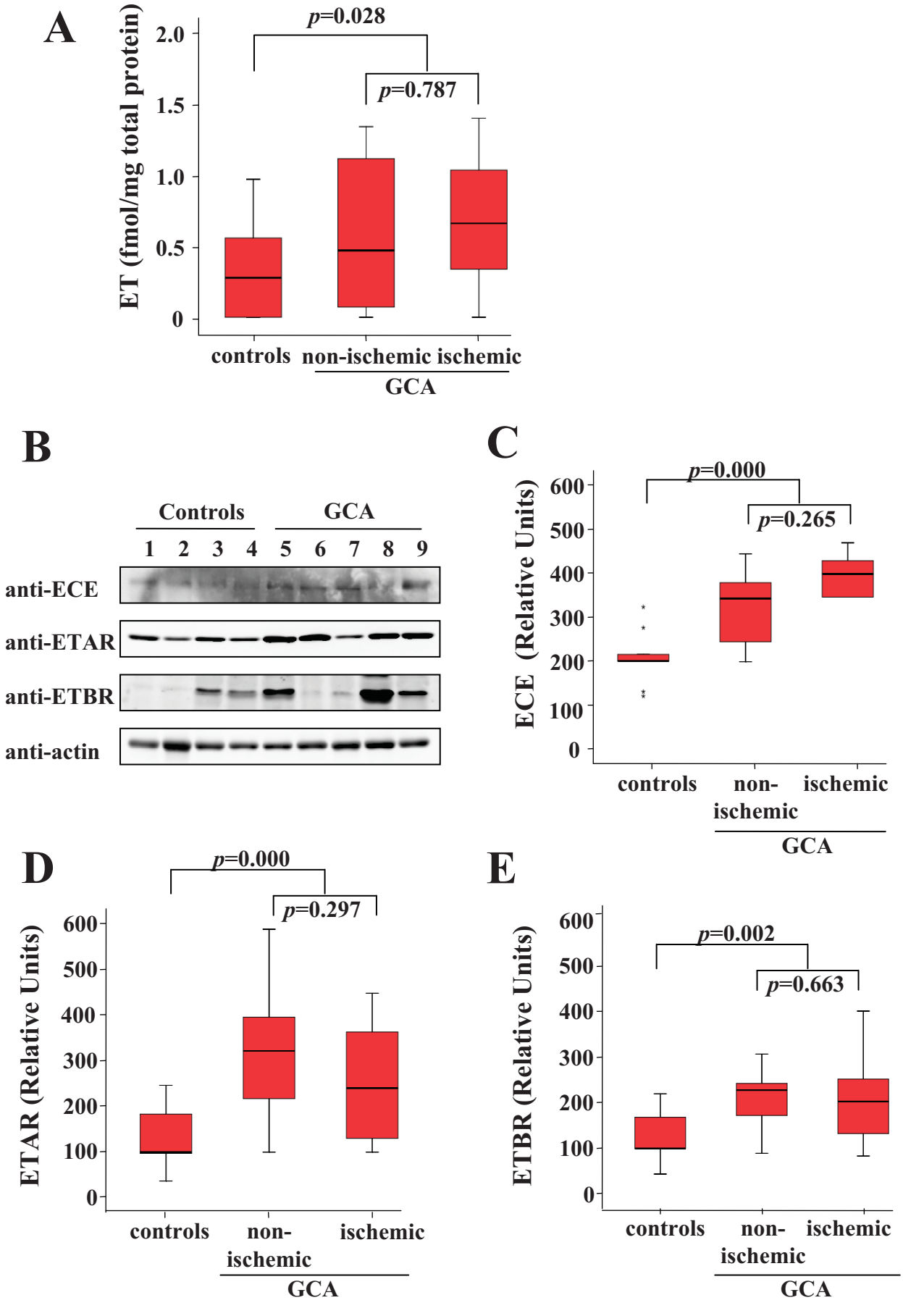
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Controls
ischemic complications
w/o ischemic complications
GCA

Fig. 1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1.5
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controls
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GCA
1 2 3 4 5 6 7 8 9
anti-ECE
anti-ETAR
anti-ETBR
anti-actin
C
p=0.000
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ECE (Relative Units)
600
500
400
300
200
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controls
non-ischemic
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p=0.000
p=0.297
ETAR (Relative Units)
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controls
non-ischemic
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p=0.002
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controls
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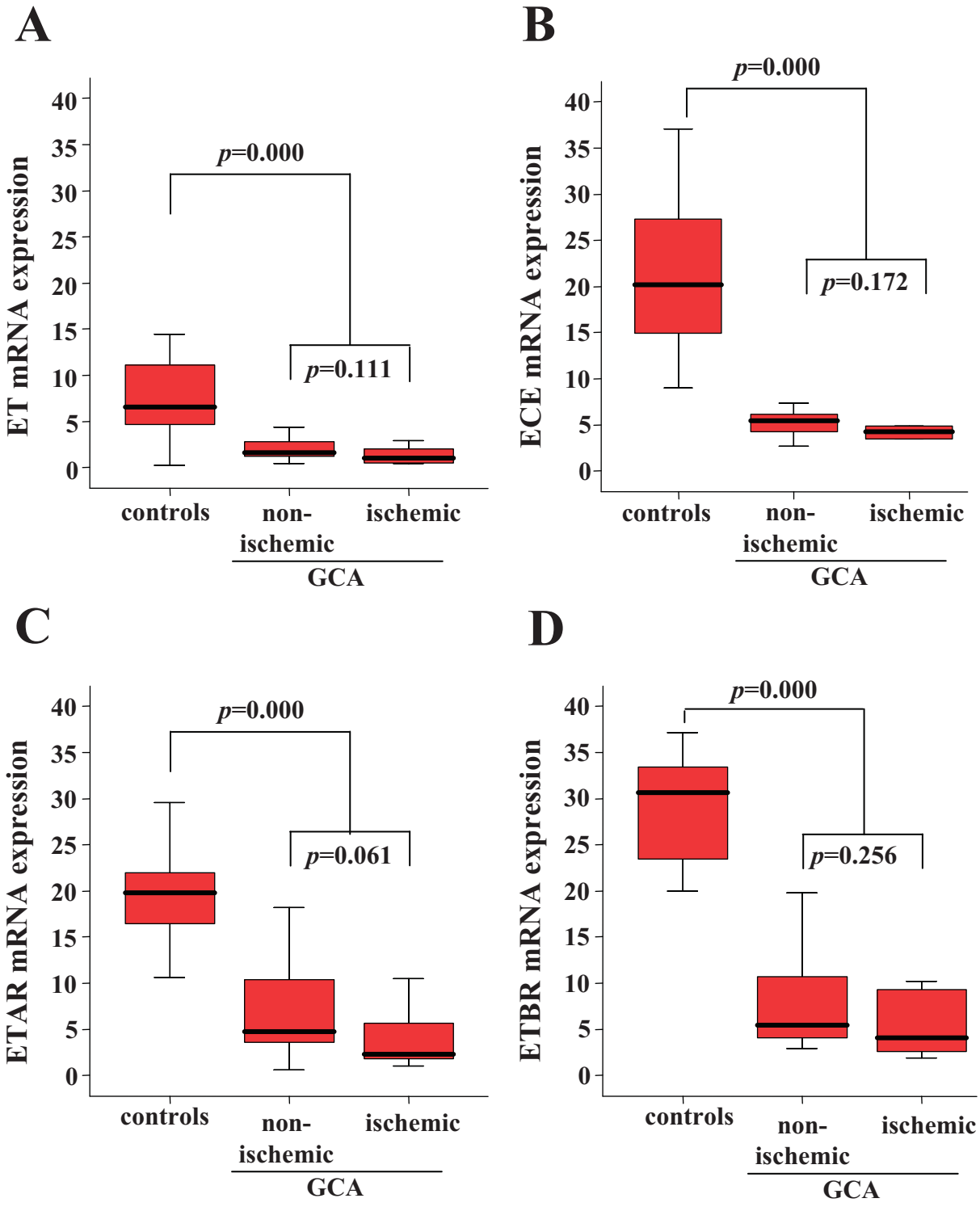
ischemic
GCA

Fig. 2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30
25
20
15
10
5
0
p=0.000
p=0.111
controls
non-ischemic
ischemic
GCA
B
ECE mRNA expression
p=0.000
p=0.172
controls
non-ischemic
ischemic
GCA
C
ETAR mRNA expression
p=0.000
p=0.061
controls
non-ischemic
ischemic
GCA
D
ETBR mRNA expression
p=0.000
p=0.256
controls
non-ischemic
ischemic
GCA

Fig. 3

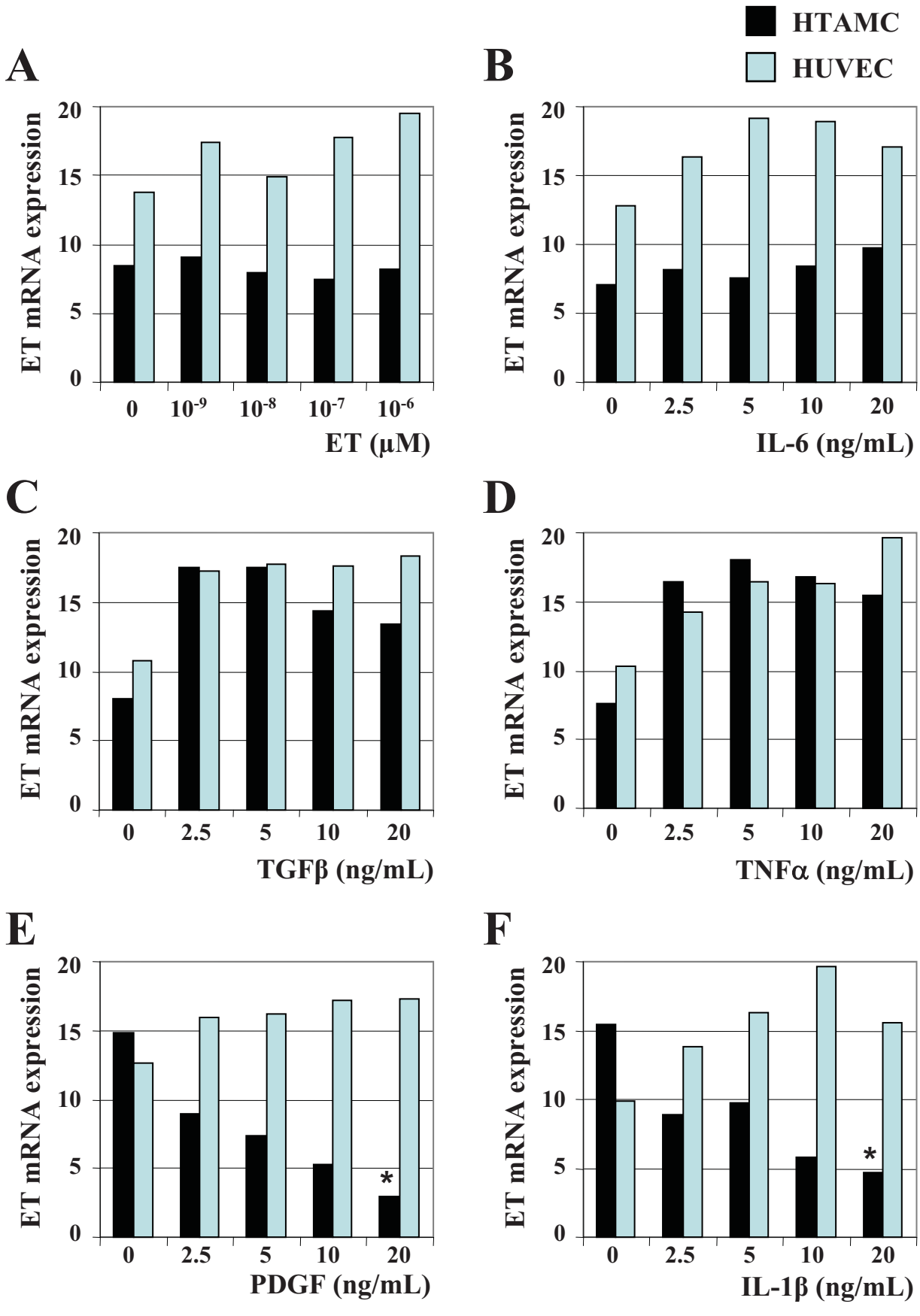
HTAMC
HUVEC
A
B
C
D
E
F
ET mRNA expression
0
5
10
15
20
0
10-9
10-8
10-7
10-6
ET (μM)
2.5
5
10
20
IL-6 (ng/mL)
TGFβ (ng/mL)
TNFα (ng/mL)
PDGF (ng/mL)
IL-1β (ng/mL)
*

Fig. 4

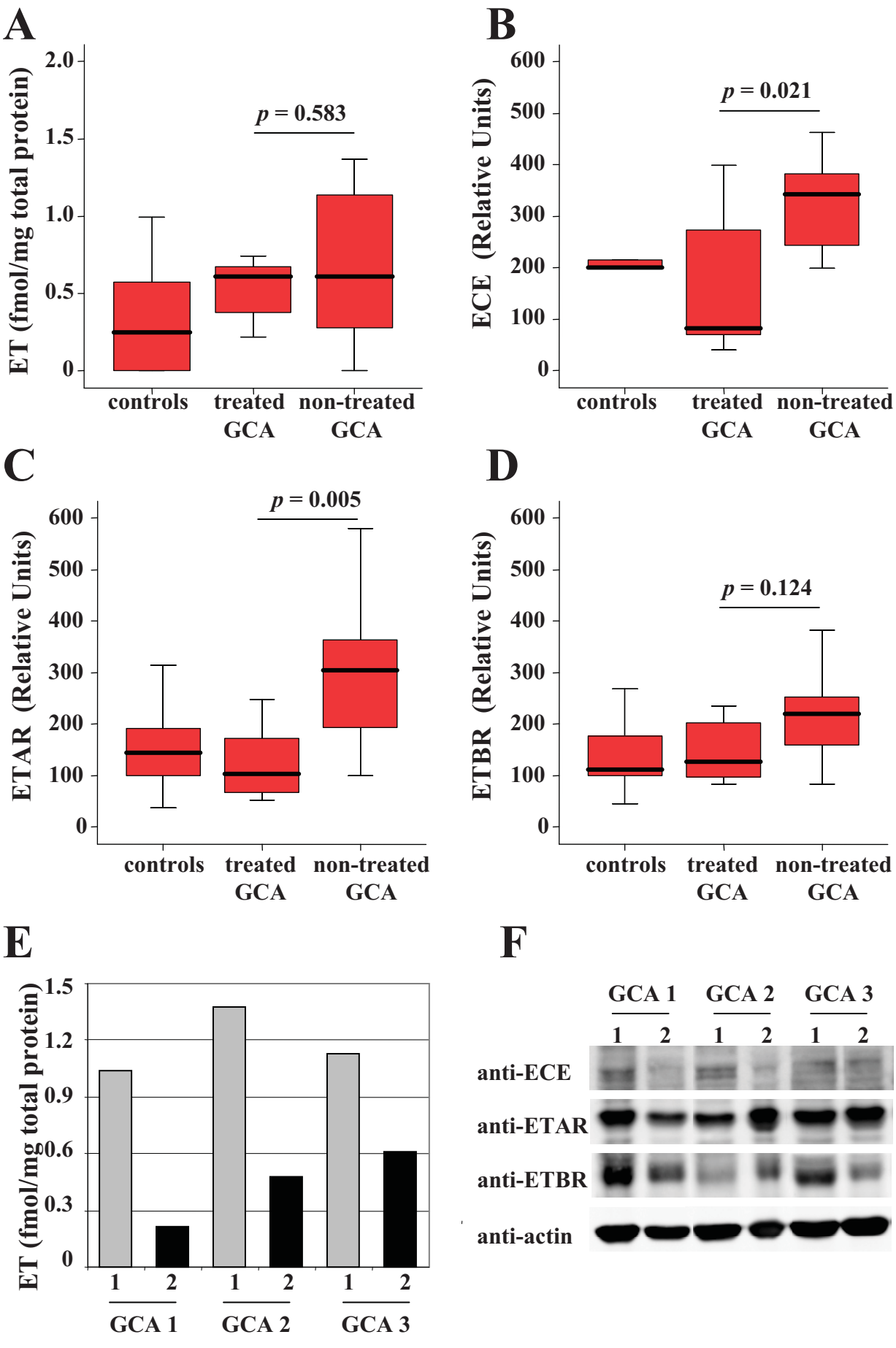
A
ET (fmol/mg total protein)
2.0
1.5
1.0
0.5
0
p = 0.583
controls
treated GCA
non-treated GCA
B
ECE (Relative Units)
600
500
400
300
200
100
0
p = 0.021
controls
treated GCA
non-treated GCA
C
ETAR (Relative Units)
600
500
400
300
200
100
0
p = 0.005
controls
treated GCA
non-treated GCA
D
ETBR (Relative Units)
600
500
400
300
200
100
0
p = 0.124
controls
treated GCA
non-treated GCA
E
ET (fmol/mg total protein)
1.5
1.2
0.9
0.6
0.3
0
1
2
1
2
1
2
GCA 1
GCA 2
GCA 3
F
GCA 1
GCA 2
GCA 3
1
2
1
2
1
2
anti-ECE
anti-ETAR
anti-ETBR
anti-actin

Fig. 5

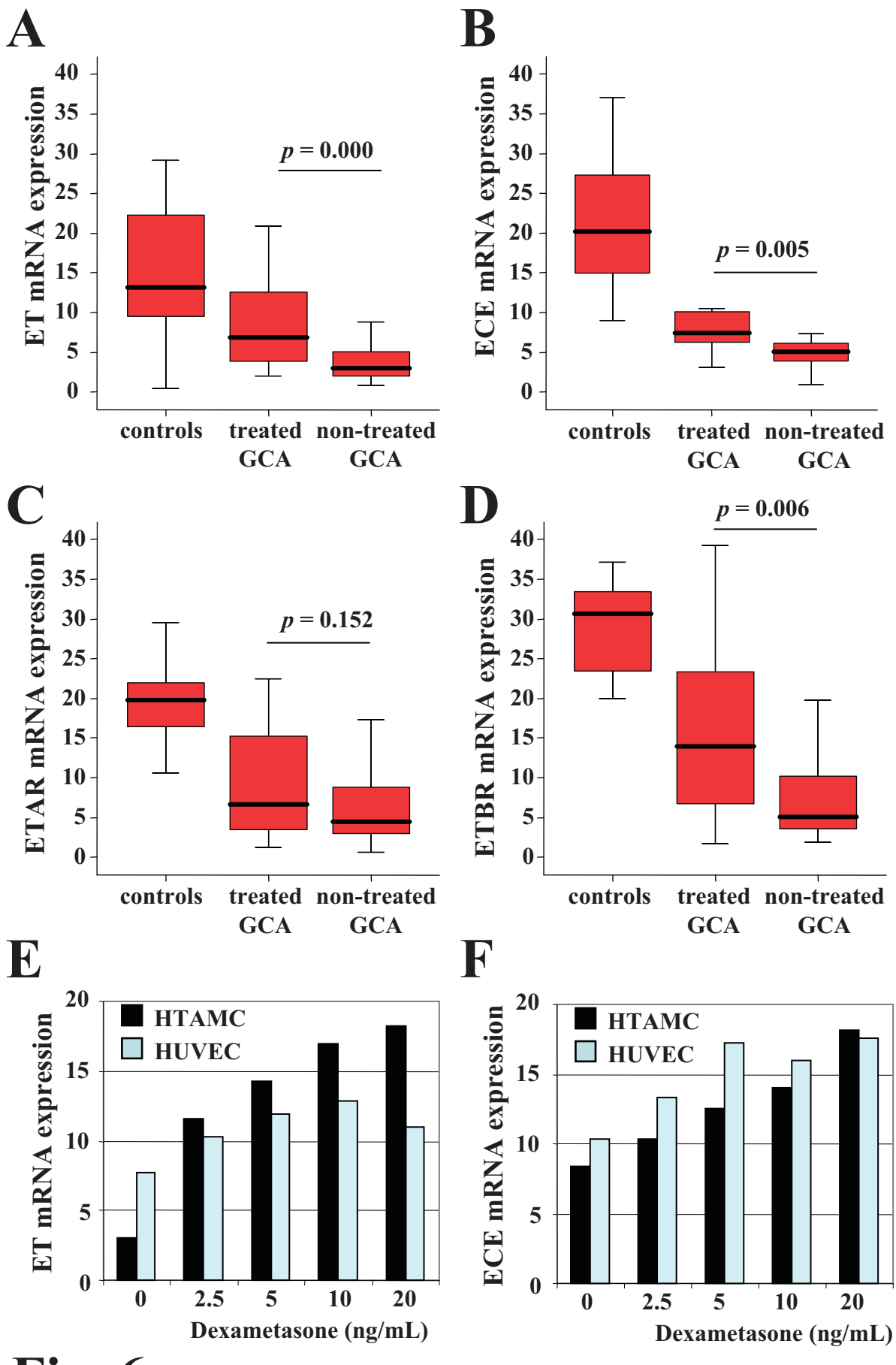
A
ET mRNA expression
40
35
30
25
20
15
10
5
0
p = 0.000
controls
treated GCA
non-treated GCA
B
ECE mRNA expression
p = 0.005
C
ETAR mRNA expression
p = 0.152
D
ETBR mRNA expression
p = 0.006
E
ET mRNA expression
HTAMC
HUVEC
0
2.5
5
10
20
Dexametasone (ng/mL)
F
ECE mRNA expression

Fig. 6

The Endothelin System in Giant-Cell Arteritis.

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RESUM DE RESULTATS

1. Els pacients amb ACG que pateixen **complicacions isquèmiques** tenen l'endotelina **(ET-1) en plasma significativament més elevada** respecte als pacients que no tenen complicacions isquèmiques.
2. **A nivell proteic,** les biòpsies d'artèria temporal dels pacients amb ACG tenen nivells **més alts de tots els components del sistema de l'ET** (ET-1, ECE-1 i ambdós receptors A i B).
3. Mentre que a nivell d'expressió d'ARNm en l'artèria temporal, els pacients amb ACG tenen nivells **més baixos d'expressió d'ARNm** de tots els components del sistema de l'ET.
4. Davant d'aquesta **regulació negativa** de tot el sistema, ens plantegem l'existència d'un mecanisme de retroalimentació negativa regulada per l'excés del producte, en aquest per l'excés d'ET. Els experiments *in vitro* amb cèl·lules endotelials i miointimals demostren que **l'ET no regula negativament** la seva pròpia expressió.

5. Aquest fet ens porta a investigar si altres factors sobrexpressats a les lesions arterials (TGFβ, PDGF, Il1β, IL6 i TNFα) poden aconseguir aquesta regulació negativa de l'ET. Cap d'aquestes molècules regula negativament l'expressió d'ET en les EC. En canvi, el **PDGF i l'IL1β regulen negativament** l'expressió de l'ARNm de **l'ET en HTAMC**.

6. El tractament amb **glucocorticoides**, durant 8 dies de mitjana**,** no aconsegueix disminuir el nivell d'ET ni de ETBR a nivell proteic, com observem quan comparem biòpsies d'artèries de malalts tractats *versus* no tractats. Però **els nivells d'ECE-1 i ETAR disminueixen fins a valors similars als controls.**

7. A nivell d'expressió d'ARNm, els pacients amb ACG tenen nivells més baixos que els controls i el tractament amb **glucocorticoides fa que els nivells d'ET, ECE-1 i ETBR pugin significativament**, tot i que, es mantenen regulats negativament respecte als controls. L'ETAR segueix aquesta tendència però no arriba a ser significativa.

8. En concordança amb els resultats trobats en els pacients tractats, en els experiments *in vitro* amb HTAMC, la **dexametasona augmenta** el nivell d'expressió de l'ARNm de l'**ET-1** i de l'**ECE-1**, però no dels receptors.

CONCLUSIONS

1. **L'endotelina** es troba elevada en el plasma de pacients amb fenòmens isquèmics, per tant, **pot tenir un paper en el desenvolupament de complicacions vasculars oclusives que es produeixen a l'ACG**.
2. Les proteïnes del **sistema de l'endotelina** (ET, ECE-1 i ambdós receptors A i B) es troben **elevades** en les lesions arterials dels pacients amb ACG.
3. En canvi, els nivells d'ARNm són més baixos en les artèries de pacients amb l'ACG, fet que ens fa pensar que s'està produint una **regulació negativa del sistema**.
4. Quan analitzem aquest mecanisme de **regulació negativa *in vitro*** observem que no està causat per la pròpia ET ni en les EC ni en les HTAMC. En canvi, en les HTAMC trobem que el **PDGF i l'IL-1β** poden desencadenar aquesta resposta. Així, a la lesió arterial PDGF i ILβ podrien estar jugant un paper protector de l'oclusió vascular, ja que aconsegueixen regular negativament l'ET que és un dels més potents agents vasoconstrictors.
5. Els **glucocorticoides disminueixen només** els nivells proteics **d'ECE-1 i ETAR** fins valors similars als controls, però **no** aconsegueixen disminuir els nivells d'**ET-1** ni **ETBR**.
6. D'altra banda, els **glucocorticoides augmenten l'expressió** del sistema en la lesió arterial fins a valors significativament més propers als controls, malgrat que no s'assoleixen els valors trobats a les biòpsies negatives.

7. Per tant, els **glucocorticoides**, que són el tractament d'elecció de l'ACG, participen en la regulació del sistema. Un tractament de 8 dies de mitjana disminueix, a nivell proteic, l'ECE-1 i ETAR però **no disminueix els nivells d'ET-1, que és la molècula amb activitat funcional vasoconstrictora**. Fet que fa pensar en la necessitat de noves aproximacions terapèutiques i en tractaments combinats.

Discussió

DISCUSSIÓ

L'arteritis de cèl·lules gegants (ACG) és una malaltia inflamatòria d'etiologia desconeguda, es desconeix l'antigen o l'agent causal que l'origina, per tant, cal **aprofundir en el coneixement dels processos que la desenvolupen**, cal esbrinar quin paper juga cada tipus cel·lular, quines molècules participen en la senyalització intercel·lular, quins estímuls reben de l'entorn extracel·lular, quines cascades de senyalització intracel·lular s'activen davant aquests estímuls i finalment quins efectes patològics es produeixen. En els nostres estudis, tota aquesta informació dels malalts amb ACG, la comparem amb persones sanes de característiques demogràfiques similars i també la comparem amb pacients que ja han estat tractats, per esbrinar quines diferències són significatives i entre elles, quines participen a la malaltia. Trobar una molècula diferencialment expressada no significa que sigui la causant de l'inici de la patologia, pot ser una conseqüència i, en aquest cas, el seu bloqueig no representaria una millora. Per demostrar causalitat calen models funcionals. No es disposa d'un model animal de la malaltia, i per aquest motiu hem desenvolupat un cultiu d'artèries temporals ex vivo que ens permet analitzar el bloqueig de diferents molècules, com per exemple el bloqueig del receptor del PDGF amb l'imatinib mesylate.

El nostre grup de recerca ha dut a terme una llarga sèrie de treballs de recerca a diferents nivells, hem estudiat mecanismes moleculars i cel·lulars a limfòcits (Cid et al., 2000; Segarra et al., 2005) i a les cèl·lules endotelials(Cid, 2002; Hernandez-Rodriguez et al., 2003). Dins d'aquest context neix el primer treball que es fonamenta en **l'estudi del comportament de les cèl·lules de múscul llis** (SMC) en l'ACG. El paper d'aquestes

cèl·lules en l'ACG no havia estat estudiat en profunditat fins el moment. Tot i que, l'observació d'una important hiperplàsia intimal en un gran nombre de biòpsies d'artèria temporal dels pacients amb ACG, suggeria un augment de la proliferació de les SMC preexistents i probablement una migració des de la túnica mitja fins a la íntima.

Els coneixements que es tenien inicialment de les SMC provenen d'estudis en altres malalties vasculars i sovint són línies cel·lulars comercialitzades obtingudes a partir d'aorta. Existeix una **gran heterogeneïtat de SMC** depenent del territori vascular (Hao, Gabbiani & Bochaton-Piallat, 2003; Li et al., 2001; Turner et al., 2007) i a més es produeixen diferències associades amb l'edat avançada, que es el cas dels pacients amb ACG (Orlandi et al., 2006). Per tant, era important aproximar-nos el màxim possible al teixit diana, i aïllar les SMC directament de les biòpsies de l'artèria temporal dels pacients. A la bibliografia que teníem al nostre abast, no s'havia publicat cap mètode anterior per obtenir aquestes cèl·lules. Així doncs, vam dissenyar un **mètode de cultiu d'artèria temporal *ex vivo*** en el que una secció de biòpsia es col·loca sobre Matrigel®, una membrana basal reconstituïda. El Matrigel® proporciona un ancoratge imprescindible per el creixement de les SMC, i alhora conté factors de creixement i proteïnes d'ECM necessaris per l'inici del creixement cel·lular, que s'observa als 6-7 dies. Aquestes cèl·lules poden ser tripsinades i sembrades en flascons, sobreviuen més de 10 cops aquest procés, tot i que, els experiments es van realitzar entre el 3è i el 6è cop, per evitar una possible desdiferenciació de les cèl·lules.

S'ha observat que les SMC tenen una gran plasticitat en alguns processos patològics d'altres malalties cardiovasculars, com a l'aterosclerosi, on poden adoptar un

fenotip activat, que té com a marcadors l'expressió α-SM-actina (α-*smooth muscle actin*) i la producció de col·lagen tipus I. Les SMCs en aquest estat d'activació en un context remodelat vascular se les anomena cèl.lules miointimals (Owens, Kumar & Wamhoff, 2004). Per confirmar l'homogeneïtat del nostre cultiu primari, vam analitzar l'expressió d'α-SM-actina per citometria de flux i va ser superior al 90 % de cèl·lules. També vam demostrar que les SMC que havien obtingut a partir d'artèries temporal **(HTAMC** = *human temporal artery myointimal cells*) produïen ARNm de col·lagen tipus I, utilitzant tècniques de *realtime*-PCR.

Un cop obtingudes i caracteritzades, el nostre primer objectiu va ser esbrinar quins estímuls poden influir en el comportament d'aquestes cèl·lules a nivell de **proliferació**. Vam seleccionar algunes molècules que sabem que es troben sobreexpressades en l'ACG, com són : PDGF, IL-6, IL-1β i CCL2 (MCP-1) i altres que modulen el comportament de les SMC en altres malalties com són TGFβ, ET-1, FGF-2 (bFGF), VEGF i EGF. L'estímul més potent a nivell de proliferació va ser el PDGF. Fet que concorda amb resultats en altres tipus de SMC (Hughes et al., 1996) i estudis en altres malalties (Raines, 2004). A la lesió arterial, altres molècules també poden estar intervenint en les funcions d'aquestes cèl·lules, el comportament final és un balanç de diversos factors. Així, en els estudis de proliferació *in vitro* , vam observar que FGF-2, IL-1β, TGFβ i EGF també són inductors del creixement en les HTAMC. La resta de factors estudiats no van mostrar un efecte clar sobre el creixement, i cap d'ells va mostrar un efecte inhibitori. Tractant-se de cèl·lules que no havien estudiades específicament, hi poden haver diferències amb altres estudis amb altres SMCs, com per exemple, el que fet que el **TGFβ estimuli la proliferació**. El TGFβ és un factor pleiotròpic però sovint es

considera un factor profibròtic que estimula un fenotip menys proliferatiu (Khanna, 2004; Su et al., 2007) i implicat en la diferenciació de les SMC (Chen et al., 2006; Sinha et al., 2004). Tot i així, hi ha estudis en els que el TGFβ juga un paper activador del creixement en funció de la dosi (Stouffer & Owens, 1994).

Un tema controvertit és **l'efecte proliferatiu de l'ET-1** en les SMCs. L'ET-1 sovint s'associa a la formació de la hiperplàsia intimal i alguns estudis han demostrat que estimula la proliferació de les SMC administrada sola (Hafizi et al., 1999; Hirata et al., 1989) o només en combinació amb el PDGF (Yang, Krasnici & Luscher, 1999). En canvi, altres estudis no troben un efecte proliferatiu i més secretor (Hafizi et al., 2004). Aquestes discordances poden ser degudes als diferents tipus cel·lulars emprats. En els nostres estudis, no hem detectat que l'ET tingui un efecte estimulant de la proliferació amb cap dels tres mètodes utilitzats: quantificant el nombre total de cèl·lules amb cristall violeta, ni amb assaigs de viabilitat amb MTT; ni amb assaigs d'incorporació de desoxibromouridina al DNA de cèl·lules en creixement (BrdU) (resultats addicionals-2).

Als estudis de **migració** amb la cambra de Boyden, el **PDGF** va demostrar ser el més potent agent quimiotàctic per aquestes cèl·lules, fet que concorda amb estudis fets amb altres models (Bornfeldt et al., 1995; Hauck, Hsia & Schlaepfer, 2000). L'**EGF també pot activar la migració** significativament, aquest fenomen no havia estat descrit però si s'havia associat l'activació del receptor de l'EGF amb la inducció de pro-MMP2 en les SMC arterials (Kodali et al., 2006). En un estudi a nivell experimental, van induir la hiperplàsia intimal a la caròtida de la rata fent passar un catèter *balloon*, van bloquejar el receptor de l'EGF amb anticossos neutralitzants i van observar una disminució en la

proliferació de les SMC i de la hiperplàsia de l'íntima en la rata (Chan et al., 2003). Estudis com aquest han demostrat un efecte de l'EGF sobre la proliferació, en el nostre estudi hem demostrat que l'EGF també té propietats quimiotàctiques sobre les HTAMC, i aquest podria constituir un altre mecanisme amb el que l'EGF col·labora en la formació de l'hiperplàsia intimal.

Del ventall de factors estudiats, el PDGF va ser l'estímul mes potent sobre la proliferació i la migració. A més, el PDGF es troba sobreexpressat en les lesions arterials en l'ACG (Kaiser et al., 1998). Aquestes característiques ens van portar a estudiar quines altres funcions té sobre aquestes cèl·lules i quines possibilitats existeixen per bloquejar els seus efectes.

Primerament, vam demostrar que **l'imatinib mesylate (Gleevec®)** bloquejava eficaçment les vies de senyalització activades pel PDGF a les HTAMC. Seguidament, vam comprovar que els efectes proliferatius i quimiotàctics del PDGF també són abolits amb el pretractament de les HTAMC amb l'imatinib. A més, quan vam incubar seccions de l'artèria *ex vivo* amb l'imatinib, vam aconseguir inhibir el creixement de les SMC de forma dosi dependent. Per tant, el bloqueig selectiu del PDGF és suficient per disminuir molt significativament la proliferació de les SMC. Tot i així, l'artèria conté altres estímuls proliferatius que no són inhibits per l'imatinib i la proliferació no s'atura completament. Aquest fet pot ser convenient perquè un cert grau de proliferació és necessària per la regeneració del teixit, ja que si no es podria produir una dilatació excessiva de l'artèria, formant aneurismes, i fins i tot, es podria produir la ruptura del vas.

El model de cultiu d'artèria temporal desenvolupat en aquesta tesi és el model experimental més proper a la lesió arterial dels pacients amb ACG, ja que no es disposa d'un model animal. Aquests resultats a nivell experimental, ens fan pensar que els efectes inhibidors de l'imatinib podrien ser útils per controlar la hiperplàsia intimal que es desenvolupa en la majoria de pacients amb ACG. Tot i així, aquestes conclusions només es poden validar amb un assaig clínic, dirigit especialment al subgrup de malalts en els que el tractament amb glucocorticoides no es prou eficaç per evitar l'oclusió vascular.

D'altra banda, en aprofundir en altres funcions del PDGF sobre les cèl·lules miointimals vam observar que el PDGF té efectes fibrogènics ja que augmenta l'expressió de col·lagen tipus I i III i la producció de fibronectina. El dipòsit excessiu de proteïnes de matriu extracel·lular és, juntament amb la proliferació i la migració de les SMC, una de les causes de l'engruiximent de l'artèria. Per tant, **l'imatinib contraresta les accions fibrogèniques del PDGF en els estudis *in vitro***. A més, recentment s'ha publicat un estudi en el que l'imatinib inhibeix el dipòsit excessiu de matriu en rates en els que se'ls ha provocat una esclerodèrmia. Els autors conclouen que l'imatinib inhibeix l'acció del PDGF i també del TGFβ (Distler et al., 2007). El mecanisme d'inhibició de l'imatinib sobre algunes tirosina quinases (receptors del PDGF, c-abl, brc-abl, c-kit) ha estat molt ben caracteritzat. En canvi, no es coneix com pot inhibir els efectes del TGFβ, els autors especulen que es deu a la inhibició de la proteïna c-abl, que segons s'ha descrit recentment forma part d'una via de senyalització del TGFβ (Wilkes & Leof, 2006). De tota manera, les vies de senyalització de TGFβ clàssiques es componen d'un seguit d'intermediaris intracel·lulars (Smad) que independentment de l'abl, activen l'expressió dels gens del col·lagen (Shi & Massague, 2003; ten Dijke & Arthur, 2007). En

experiments fets al nostre laboratori, l'imatinib no inhibia els efectes del TGFβ en les HTAMC, ni a nivell de proliferació ni tampoc inhibia l'augment en l'expressió de col·lagen tipus I i III (resultats addicionals-3). Per tant, els efectes antifibròtics de l'imatinib observats en les rates amb esclerodèrmia poden ser deguts a la **inhibició conjunta de PDGF i TGFβ**, o potser només del PDGF. De tota manera, són resultats en un model animal que demostren que l'imatinib té activitat antifibròtica i que contribueix a fer pensar que l'imatinib podria ser útil per disminuir la hiperplàsia intimal de l'ACG.

En el cas de l'esclerodèrmia, pensem que l'imatinib podria ser especialment útil, no només perquè pot inhibir l'acció del PDGF sinó també perquè s'ha demostrat que els pacients presenten autoanticossos contra el receptor del PDGF que funcionen com a agonistes i desencadenen els mateixos processos patològics que el PDGF (Baroni et al., 2006). En aquest treball, els autors recomanen l'ús d'anticossos neutralitzants però havien trobat que el 22% dels pacients tenen autoanticossos que reconeixen diferents canvis conformacionals del receptor. Per tant, l'ús d'anticossos neutralitzants en aquest casos no seria útil. En canvi, l'imatinib bloqueja el receptor del PDGF perquè competeix pel lloc d'unió de l'ATP en la part intracel·lular del receptor, per tant, pensem que seria una bona opció encara que es produïssin canvis conformacionals en el receptor del PDGF (Lozano, Segarra & Cid, 2006)(inclós a l'annex).

La participació de les cèl·lules miointimals en el procés inflamatori i l'angiogènesi

Les cèl·lules endotelials havien estat considerades com a cèl·lules únicament estructurals però aquest punt de vista s'ha ampliat i actualment es consideren cèl·lules que participen activament en els mecanismes immunopatològics (Michiels, 2003) per exemple modificant els seus receptors a la membrana plasmàtica o segregant molècules vasoactives com l'ET. De manera similar, les cèl·lules miointimals sovint es consideren cèl·lules estructurals i s'estudia la seva proliferació i les seves accions profibrotíques perquè poden contribuir a la hiperplàsia intimal, però pensem que aquestes cèl·lules també poden intervenir en el procés inflamatori. Amb l'objectiu d'investigar quines molècules podien segregar-se induïdes pel PDGF en les HTAMC, vam explorar 30 possibles molècules candidates amb l'ajut d'un *array* de proteïnes. Vam observar que el **PDGF estimulava la secreció de CCL2** (MCP-1**) i angiogenina i inhibia la secreció l'osteoprotegerina** (OPG). L'*array* de proteïnes és semiquantitatiu i per tant, cal quantificar aquests resultats per ELISA. Vam confirmar que les diferències eren significatives i proporcionals a la dosi de PDGF. A més, l'imatinib inhibia totalment aquests canvis en la secreció.

L'augment de **CCL2** en els sobrenedants de HTAMC estimulades amb PDGF concorda amb un estudi amb SMC que estimulades amb PDGF-BB augmentava els nivells de l'expressió de CCL2 per estabilització del seu ARNm (Liu, Poon & Taubman, 2006). Aquests resultats obren un nou ventall d'accions del PDGF en la malaltia. Fins el moment, hem associat el PDGF amb l'augment de migració, proliferació i producció d'ECM, efectes que condueixen al desenvolupament de la hiperplàsia intimal. Però el fet

que el PDGF augmenti una citocina proinflamatòria com és el CCL2 té altres implicacions en el desenvolupament de la lesió arterial. L'augment del CCL2 implica un reclutament dels monòcits i macròfags, que invaeixen la paret arterial i alliberen substàncies proinflamatòries i factors de creixement, entre elles el propi PDGF, formant-se així un mecanisme de retroalimentació positiva que podria contribuir a la persistència del procés inflamatori. Aquest tipus de mecanismes poden ser els causants de que la inflamació esdevingui crònica i explicarien perquè el tractament amb glucocortidoides sol ser tan perllongat, de mitjana calen de 8 a 12 mesos des del diagnòstic per aconseguir reduir la dosi inicial (40-60mg/dia) a una dosi de manteniment (5-7.5mg/dia) (Nordborg & Nordborg, 2003; Proven et al., 2003).

D'altra banda, el fet que el **PDGF augmenti la producció d'angiogenina** no havia estat descrit anteriorment, almenys en els estudis que hem revisat. No es coneix quina és l'expressió d'angiogenina en les lesions arterials dels malalts amb ACG, però sí que han estat estudiats alguns mecanismes d'angiogènesi que es produeixen en el desenvolupament de la malaltia. El *vasa vasorum* és el conjunt de vasos que alimenten les cèl·lules arterials més allunyades de la llum del vas. Quan es desenvolupa la hiperplàsia intimal augmenta el nombre de capes d'aquestes cèl·lules, que necessiten un aportament sanguini. Es considera aquest fenomen de formació de nous vasos com a un mecanisme que intenta contrarestar la hipòxia produïda per la hiperplàsia intimal. També podria ser una conseqüència de l'alliberament de substàncies proangiogèniques durant el procés d'inflamació. Entre les molècules que s'han demostrat que poden estimular **l'angiogènesi en l'AGC**, es troba l'IL6. L'IL6 es troba sobreexpressada en les lesions d'ACG i té propietats angiogèniques sobre les EC, per tant, és un dels estímuls que pot produir

angiogenèsi en l'ACG (Hernandez-Rodriguez et al., 2003). El PDGF també és un agent proangiogènic perquè les seves propietats proliferatives i quimiotàctiques sobre les SMC fan que intervingui en la formació i consolidació de nous vasos. En el nostre estudi, hem demostrat que el PDGF també pot tenir una acció proangiogènica indirecta, induint l'expressió d'altres factors proangiogènics com l'angiogenina. Aquest resultat obre les portes a una nova línia de recerca per esbrinar quins són els nivells d'expressió de l'angiogenina en les lesions arterials de l'ACG i quin és el seu paper en les complicacions isquèmiques de la malaltia.

La utilització de l'*array* de proteïnes també ens va permetre observar que el **PDGF disminuïa la secreció de l'osteoprotegerina (OPG).** Aquest resultat es va quantificar per ELISA amb concentracions creixents de PDGF i es demostra que la inhibició en la secreció d'OPG és dosi-dependent. Aquest resultat es va comprovar a nivell d'expressió d'ARNm per *real-time* PCR (resultats addicionals- 4). Són uns resultats força sorprenents ja que un estudi anterior fet amb VSMC concloïa que el PDGF induïa l'OPG (Zhang et al., 2002). Aquesta discordança pot ser deguda a diversos factors : les cèl·lules utilitzades en aquell estudi vam ser d'aorta humana comercialitzades, mentre que en el nostre cas vam utilitzar SMC primàries; aquests autors van utilitzar la proteïna recombinant PDGF-BB i nosaltres l'heterodímer PDGF-AB purificat de plaquetes humanes; també els temps d'incubació són més breus (4-6 hores) mentre que en el nostre cas, la reducció en la secreció d'OPG s'observa les 72 hores. En l'estudi de Zhang et al., van detectar un augment en l'expressió de l'ARNm de l'OPG a les 6 hores i una disminució a les 12 hores. En el nostre cas, l'ARNm disminuïa a les 24 hores proporcionalment a la concentració de PDGF (resultats addicionals - 4). Les implicacions

d'aquesta regulació negativa en les lesions arterials de l'ACG són difícils de dilucidar. L'OPG és un receptor soluble membre de la superfamília de receptor de TNF, que s'uneix al RANKL (*receptor activator of NF-κB ligand*) impedint que s'uneix al seu receptor RANK ancorat a la membrana. El sistema OPG/RANKL/RANK és clau per la regulació de la fisiologia de l'os i recentment també s'ha associat a malalties cardiovasculars (Hofbauer & Schoppet, 2004). D'altra banda, l'OPG també pot segrestar una molècula pro-apoptòtica anomenada TRAIL (*TNF-related apoptosis inducing ligand*) que comparteix un 35% d'homologia amb el RANKL. Per aquest motiu, l'OPG es considera anti-apoptòtica (Secchiero et al., 2007). Aquestes són dues de les funcions més conegudes de l'OPG, però desconeixem quin paper juga en l'ACG i quines implicacions poden derivar-se del fet que el PDGF pugui regular negativament l'expressió de l'OPG en les HTAMC.

A més, aquestes cèl·lules miointimals tenen una secreció basal important d'IL-6 (resultats addicionals-5) i també poden respondre a altres estímuls com per exemple el receptor soluble de l'IL6 incrementant la secreció de l'IL8, una potent citocina proinflamatòria i proangiogènica. Aquests resultats suggereixen que les HTAMC podrien contribuir en l'angiogènesi i el manteniment de les cascades proinflamatòries que produeixen en les lesions arterials de l'ACG (figura 13).

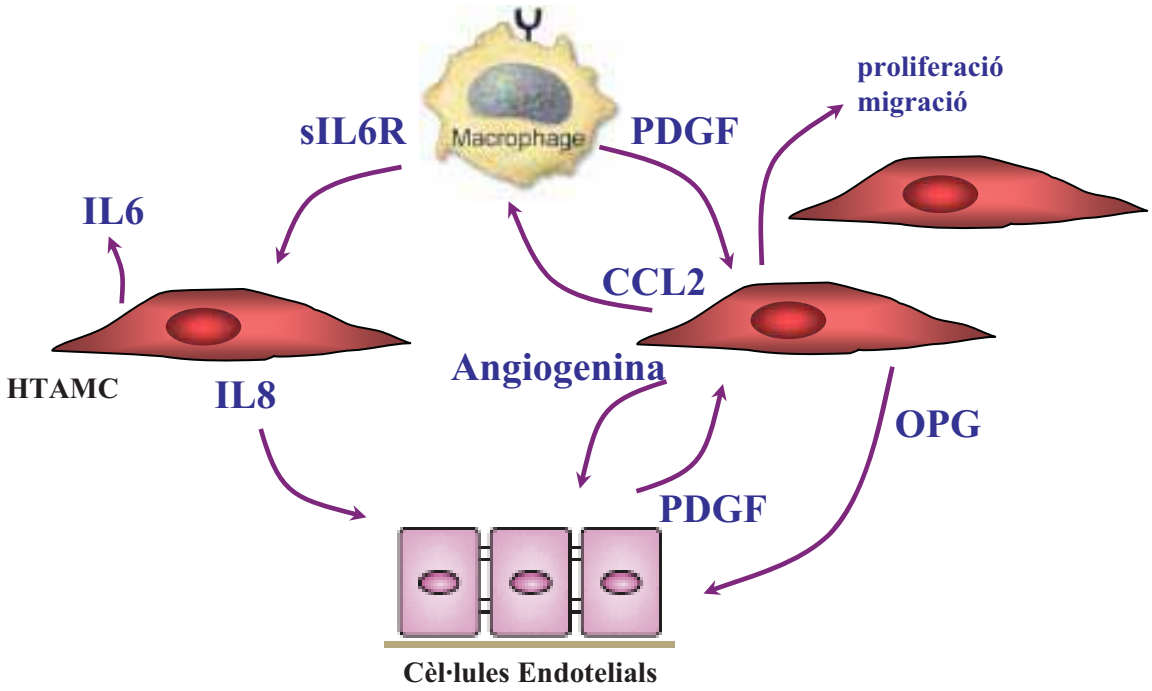


Figura 13. Participació de les HTAMC en la perpetuació del procés inflamatori i en l'angiogènesi.

L'imatinib mesylate com a agent terapèutic en l'arteritis de cèl·lules gegants

L'arteritis de cèl·lules gegants (ACG) és la vasculitis més freqüent en persones de més de 50 anys. Essent una malaltia d'etiologia desconeguda però amb implicació del sistema immunitari, **el tractament** d'elecció actual són els glucocorticoides, per les seves característiques immunosupressores. Normalment s'inicia amb algunes dosis inicials altes i després es disminueix progressivament en funció de les necessitats terapèutiques de cada pacient. Sovint són tractaments llargs que duren mesos i fins i tot, anys. Alguns dels problemes amb el tractament amb glucocorticoides són sobretot els **efectes secundaris que s'acumulen en un tractament de llarga duració** (descrits en profunditat en la secció 1.7. de la introducció), especialment tractant-se majoritàriament de pacients d'edat avançada. Aquest fet ens planteja la necessitat de trobar nous agents terapèutics que ens permetin substituir l'actual, o si més no, ajudar a disminuir la dosi acumulada dels nostres pacients (tractaments adjuvants).

Un altre motiu per a la recerca de noves dianes biològiques és l'existència d'un **subgrup de pacients** ens els que el tractament amb glucocorticoides **no és prou eficaç**. En aquests casos, la malaltia contínua progressant malgrat el tractament i arriba a tenir greus conseqüències com l'estenosi de grans vasos i els aneurismes aòrtics. En l'ACG es desconeix quin és la causa d'aquesta manca d'eficàcia, però en malalties com l'artritis reumatoide greu, s'han descrit freqüents casos de resistència als glucocorticoides (Chikanza, 2002). Aquesta resistència pot ser deguda a una disminució en l'expressió del receptor α ó un augment en el β, ó bé una activació de la MAPK que fosforila el receptor de glucocorticoides inhibint la seva senyalització (Rhen & Cidlowski, 2005).

Davant la necessitat de trobar noves aproximacions terapèutiques, hem explorat quines molècules podrien ser les dianes biològiques més adients. El PDGF ha destacat per la seva capacitat de provocar múltiples respostes relacionades amb la progressió de la lesió. Els nostres resultats a nivell experimental han estat molt encoratjadors, demostrant que l'imatinib pot inhibir els efectes estudiats del PDGF relacionats amb la progressió de la hiperplàsia intimal i amb la persistència de la malaltia. També en un model *ex vivo*, cultivant seccions d'artèries, hem comprovat que l'imatinib redueix molt significativament el creixement cel·lular. Aquest model ha permés bloquejar selectivament la funció del PDGF i suggereix que el PDGF pot tenir un paper important en els mecanismes que realment s'estan produint *in vivo* en els malalts amb ACG i l'ús de l'imatinib podria ser terapèuticament útil (figura 14).

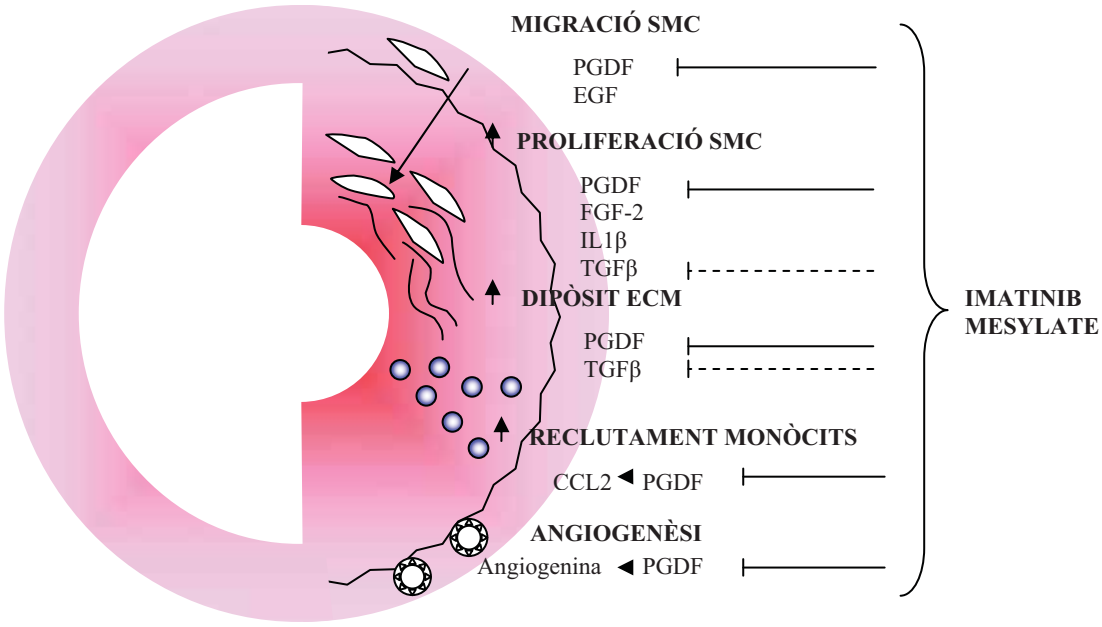


Figura 14. Resum d'efectes que l'imatinib mesylate podria produir en les lesions arterials de l'ACG

Existeixen estudis experiementals en el context d'altres malalties vasculars en els que s'ha administrat **l'imatinib per frenar la progressió de la hiperplàsia intimal**. Per exemple en models animals de l'aterosclerosi, el tractament amb imatinib protegia la integritat de la paret vascular, disminuint el creixement de les SMC i la disrupció de la capa elàstica (Boucher et al., 2003). En un altre estudi, utilitzant conills hipercolesterolèmics com a model animal, es va observar que l'administració d'imatinib no era suficient per reduir el creixement de la neoíntima a les 6 setmanes i recomanaven una inhibició conjunta del VEGF-C (Leppanen et al., 2004).

D'altra banda, també s'ha demostrat que **l'imatinib té altres propietats anti-inflamatòries** que podrien ser útils en l'ACG. L'imatinib inhibeix la capacitat funcional dels monòcits (Dewar et al., 2005) i afecta al desenvolupament i funció de les cèl·lules dendrítiques (Appel et al., 2004). També s'ha descrit que inhibeix c-fms (*macrophage colony-stimulating factor M-CSF receptor*) (Dewar et al., 2005). A més, hi ha estudis que relacionen l'imatinib amb fenòmens antiangiogènics ja que interfereix amb l'estabilització dels vasos (Rocha, Azevedo & Soares, 2007).

Abans de plantejar-se la utilització de l'imatinib en l'ACG, és important analitzar quina és la toxicitat d'aquest fàrmac en un tractament perllongat. Recentment, s'ha publicat el seguiment de pacients amb CML (leucèmia mielode crònica) tractats amb l'imatinib durant 5 anys (Druker et al., 2006) on no es van observar efectes secundaris majors. I en un darrer estudi en el que es valorava el tractament de la CML durant 6 anys (Hochhaus et al., 2007) es va considerar que l'imatinib havia estat molt ben tolerat. Entre

els efectes adversos més greus, destaquen que la cardiotoxicitat derivada del tractament és una complicació molt infreqüent.

En resum, hem demostrat que l'imatinib pot inhibir respostes de les HTAMC relacionades amb la progressió de la hiperplàsia intimal, de la inflamació i de l'angiogènesi. L'imatinib també és eficaç en el model de cultiu d'artèria temporal *ex vivo* i en models experimentals d'altres malalties vasculars. A més l'imatinib té altres propietats anti-inflamatòries i és un tractament que ha estat ben tolerat en altres malalties. Per tant, pensem que l'imatinib podria ser útil per disminuir la hiperplàsia intimal i la inflamació que es produixen en l'arteritis de cèl·lules gegants.

El sistema de l'endotelina en l'arteritis de cèl·lules gegants

En el segon estudi ens vam plantejar esbrinar quin és el paper de l'endotelina en l'arteritis de cèl·lules gegants. El primer pas va ser estudiar els nivells d'endotelina (ET) en el plasma dels pacients amb ACG. Vam quantificar els nivells d'ET circulant en plasma i tot i que, no es van observar diferències entre els malalts i els controls, els pacients que patien fenòmens isquèmics tenien significativament més ET circulant que els que no tenien aquestes complicacions isquèmiques. En altres malalties vasculars, aquest paràmetre s'associa amb la severitat de la malaltia (Best & Lerman, 2000; Lerman et al., 1991; Miyauchi & Masaki, 1999). En el cas de l'arteritis de Takayasu, l'ET circulant també es va trobar elevada (Akazawa et al., 1996). Existeix un estudi amb només quatre pacients amb ACG en el que els autors troben elevats els nivells d'ET en plasma respecte a valors de referència obtinguts en el seu laboratori (Pache et al., 2002). Aquestes dades feien pensar que el sistema de l'ET podia tenir un paper en l'ACG.

El següent pas va ser estudiar els nivells del sistema de l'ET (ET-1, l'enzim activador de l'ET = ECE-1, i ambdós receptors A i B) en el teixit diana, és a dir, en les biòpsies de l'artèria temporal. De fet, l'ET es considera més una hormona paracrina que endocrina, ja que les EC segreguen la major part de l'ET que produeixen en direcció a les SMC que les envolten (Levin, 1995). Vam observar que a les lesions arterials dels pacients amb ACG, els nivells proteics de tots els components del sistema de l'ET (ET, ECE, ETAR i ETBR) eren significativament més elevats. El subgrup de pacients amb complicacions isquèmiques no presentaven un increment del sistema respecte els pacients que no pateixen aquestes complicacions. Per tant, el sistema de l'ET està sobreexpressat

en els malalts amb ACG però no es correlaciona amb un augment dels fenòmens isquèmics, possiblement degut al fet que els estudis s'han realitzat en l'artèria temporal, que és un dels teixits diana que poden biopsiar, però els fenòmens isquèmics més greus són causats per l'oclusió d'altres artèries no biopsiables, com per exemple les artèries ciliars posteriors. Les implicacions clíniques d'aquest excés d'ET en les lesions arterials dels pacients amb ACG poden ser molt diverses tenint en compte l'ampli ventall d'efectes que pot generar l'ET. Possiblement, el principal efecte de l'ET és una ràpida i potent vasoconstricció que podria ésser la causant dels fenòmens isquèmics transitoris a l'ACG com l'amaurosi fugax. Però l'ET també podria contribuir a l'oclusió vascular mitjançant altres mecanismes, com per exemple els seus efectes profibròtics augmentant l'expressió del CTGF (*connective tissue growth factor*)(Rodriguez-Vita et al., 2005).

Sorprenentment, quan vam analitzar l'expressió del sistema a nivell de ARNm, vam observar que les biòpsies de pacients amb ACG tenien nivells més baixos de tots els components del sistema (ET, ECE, ETAR i ETBR) que les biòpsies negatives. Davant d'aquests resultats, ens vam plantejar l'existència d'un mecanisme de retroinhibició provocat pels alts nivells d'ET. Per comprovar aquesta hipòtesi de treball a nivell experimental, vam analitzar la regulació de l'expressió de l'ET en cèl·lules endotelials (HUVEC) i cèl·lules miointimals de l'artèria temporal (HTAMC). En ambdós casos, l'ET no va regular negativament la seva pròpia expressió. El pas següent va ser estimular les cèl·lules amb altres molècules que es troben sobreexpressades en les lesions arterials : TNFα, TGFβ, PDGF, IL1β i IL6. A les HUVEC, cap d'elles disminueix els nivells d'ARNm de l'ET. De fet, TNFα, TGFβ i IL1β augmenten aquests nivells, concordant amb altres estudis amb aquestes cèl·lules (Kurihara et al., 1989; Marsden & Brenner,

1992). En el cas de les cèl·lules miointimals, vam trobar dues molècules PDGF i IL1β que poden regular negativament l'expressió d'ET d'una manera dosi dependent. El PDGF no s'ha descrit abans com a repressor de l'expressió de l'ET. Aquesta relació no ha estat estudiada en profunditat però existeix un estudi en el que el PDGF-AA estimulava l'expressió d'ET en SMC de rata (Hahn et al., 1990). Aquesta discordança pot ser deguda que es tracta d'una isoforma diferent de PDGF, en el nostre estudi és PDGF-AB, i també a que es va realitzar en cèl·lules no humanes. Respecte a l'IL1β, un estudi cèl·lules miometrials humanes ha demostrat que l'IL1β pot regular negativament l'expressió d'ET (Breuiller-Fouche et al., 2005). És molt interessant el fet que en estudis amb cocultius d'EC i SMC, s'hagi observat que les SMC poden inhibir l'expressió de l'ARNm de l'ET en les EC (Di Luozzo, Bhargava & Powell, 2000). Els autors conclouen que aquesta repressió depèn d'un augment en l'activitat de l'òxid nítric sintetasa (NOS) en les EC, ja que un inhibidor de la NOS (L-NAME) reverteix el fenomen. Desafortunadament, no troben el mecanisme de com les SMC estimulen l'activitat de NOS en les EC i no analitzen les citocines segregades al medi.

Les principals conclusions d'aquest estudi sobre el sistema de l'ET en l'ACG són que els components d'aquest sistema estan significativament elevats en els pacients i que el tractament amb glucocorticoides, durant 8 dies de mitjana, disminueix l'ECE i l'ETAR fins a valors similars als controls, però tot i així, els nivells d'ET es mantenen tan elevats com en el grup de pacients no tractats. Si l'ET contribueix al desenvolupament de les lesions isquèmiques i no disminueix amb el tractament, aquests fets podrien explicar perquè un aproximadament 4% de pacients continuen desenvolupant pèrdua visual durant les primeres setmanes de tractament (Cid et al., 2007). Per tant, l'ET es converteix en una

possible diana terapèutica que no està sent bloquejada amb el tractament amb glucocorticoides.

Per aprofundir en l'estudi dels mecanismes immunopatològics que produeixen la progressió de la inflamació i l'oclusió vascular a l'ACG hem analitzat les funcions de les cèl·lules miointimals aïllades d'artèria temporal humana (HTAMC). Hem demostrat que el seu comportament pot ser influenciat per diferents factors, entre ells ha destacat el PDGF com un dels més potents estimuladors de respostes que poden conduir a la hiperplàsia intimal i la persistència de la inflamació. Per aquest motiu, hem analitzat el bloqueig de la seva senyalització amb l'imatinib i hem observat que l'imatinib inhibeix els efectes del PDGF *in vivo* i *ex vivo*. Per tant, pensem que l'imatinib podria tenir un efecte terapèutic en disminuir la hiperplàsia intimal i la inflamació a l'ACG.

D'altra banda, els fenòmens isquèmics transitoris probablement no es deuen al desenvolupament de la hiperplàsia intimal sinó a fenòmens vasospàstics produïts per factors vasoconstrictors. En aquest cas, el sistema de l'endotelina podria jugar un paper en aquest mecanisme d'oclusió vascular, ja que tots els components de sistema (ET, ECE, ETAR i ETBR) es troben significativament elevats en els pacients amb ACG.

Les nostres dades experimentals indiquen que el PDGF i l'endotelina poden estar implicats en la progressió de les lesions arterials dels pacients amb ACG i pensem que aquests resultats poden ser útils pel disseny d'assaigs clínics de teràpies més dirigides.

Conclusions

CONCLUSIONS

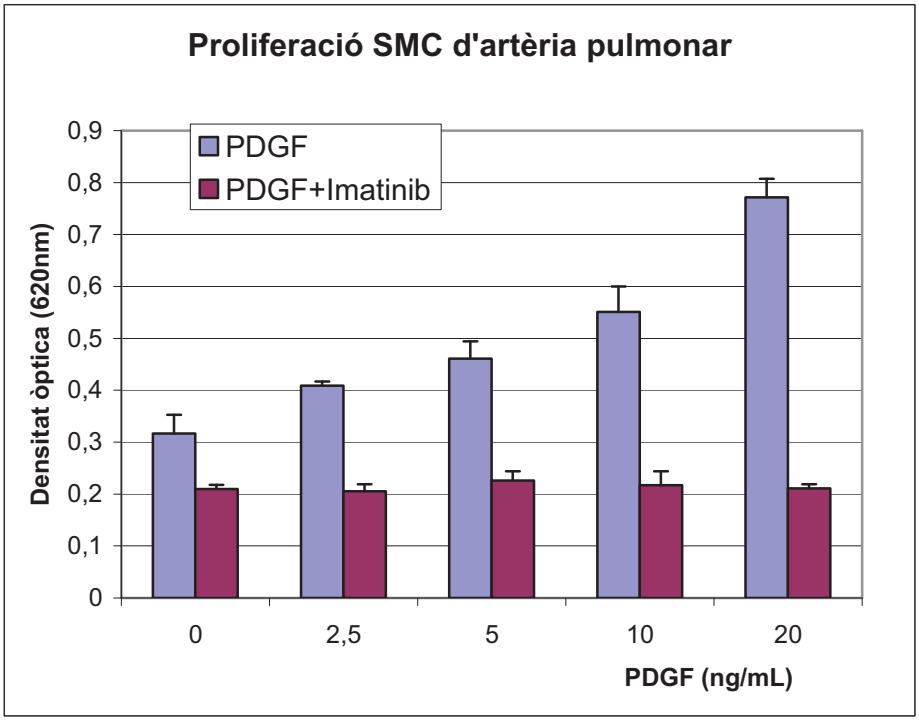
1. El mètode de cultiu d'artèria sobre Matrigel® permet aïllar i caracteritzar **cèl·lules miointimals a partir d'artèries temporals humanes (HTAMC)** i també és un **model funcional en el que poden provar nous agents terapèutics.**
2. Entre els factors estudiats, el **PDGF** és el més important a nivell de proliferació i de migració. L'**imatinib inhibeix** els efectes del PDGF en els estudis ***in vitro*** realitzats amb les HTAMC. A més. en el model de cultiu de l'artèria temporal, on diferents factors expressats en l'ACG poden estar actuant sobre el creixement de les HTAMC, l'**imatinib mesylate inhibeix significativament el creixement de les HTAMC a partir de l'artèria temporal *ex vivo.*** Fet que demostra que el PDGF té un paper important sobre els creixement de les HTAMC i que el seu bloqueig amb imatinib podria **ser útil per disminuir el procés d'hiperplàsia intimal en l'arteritis de cèl·lules gegants.**
3. L'imatinib inhibeix les funcions profibròtiques del PDGF en HTAMC. Per tant**, l'imatinib podria disminuir el dipòsit excessiu de proteïnes d'ECM que es produeix en el procés d'engruiximent de l'artèria**.
4. D'altra banda, l'**imatinib** inhibeix també la secreció de molècules proinflamatòries i proangiogèniques induïdes per PDGF *in vitro,* per tant **podria disminuir la inflamació i l'angiogènesi** que es produeix en el desenvolupament de la lesió arterial a l'ACG.
5. Quan analitzem el sistema de l'ET en l'ACG troben que els components d'aquest sistema (**ET, ECE, ambdós receptors A i B) estan significativament elevats** en els pacients. El tractament amb **glucocorticoides** (8 dies de mitjana) només

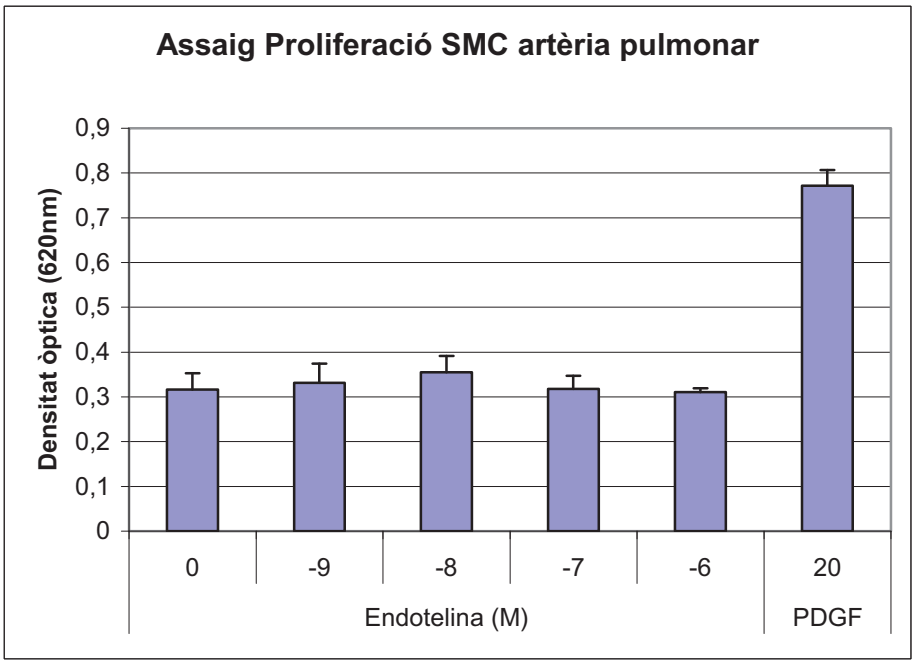
aconsegueix disminuir alguns dels components (ECE i ETAR) fins a valors similars als controls, però tot i així, **els nivells d'ET es mantenen elevats,** similars als dels pacients no tractats. Per tant, l'ET es converteix en una possible diana terapèutica que no està sent bloquejada amb el tractament actual amb corticoides.

6. Donat que no es disposa d'un model animal de la malaltia, només un assaig clínic podria comprovar la repercussió *in vivo* aquests resultats i la utilitat del bloqueig de l'endotelina en reduir el risc residual de ceguesa una vegada instaurat el tractament amb corticoides.

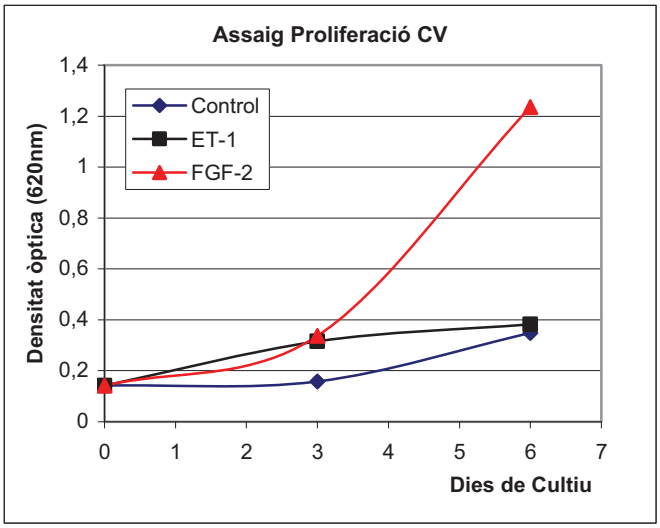
Resultats addicionals

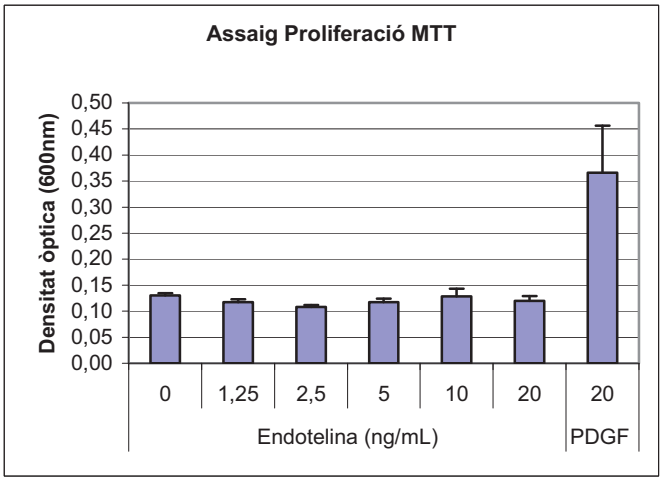
1. Resultats de la proliferació SMC d'artèria pulmonar

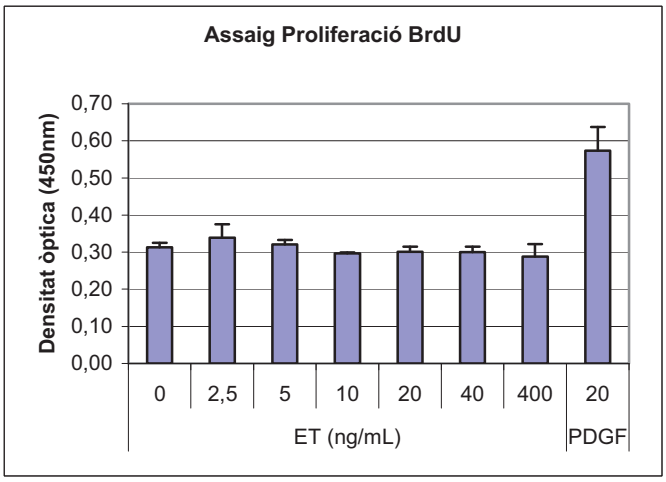




2. L'endotelina no augmenta la proliferació de les HTAMC







3. L'imatinib mesylate no inhibeix els efectes de TGFβ sobre les HTAMC

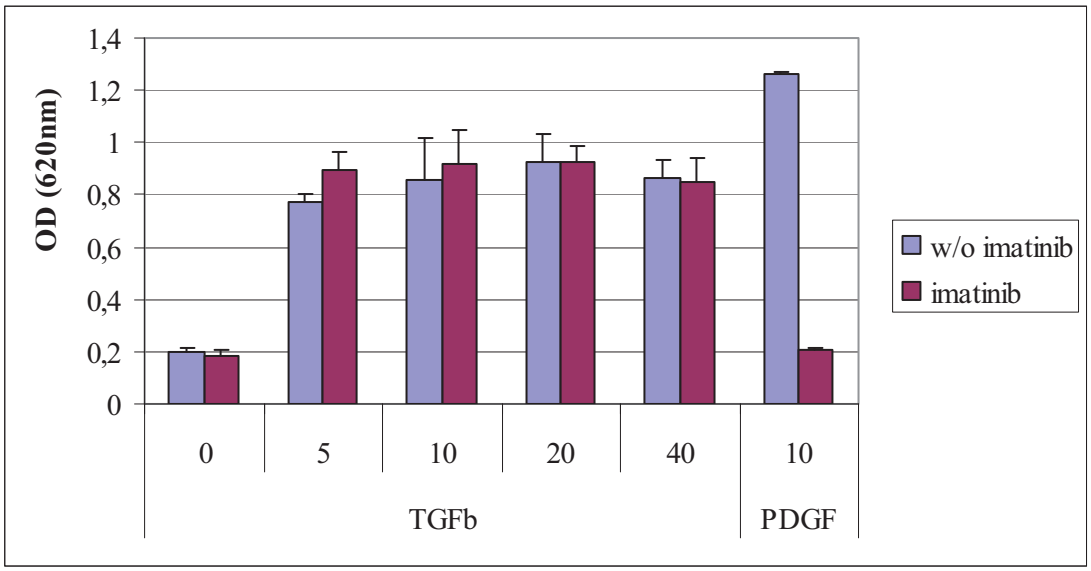


Figura 1. L'imatinib no inhibeix l'efecte proliferatiu del TGFβ sobre les SMCs (assaigs de proliferació quantificant el nombre total de cèl·lules per cristall violeta).

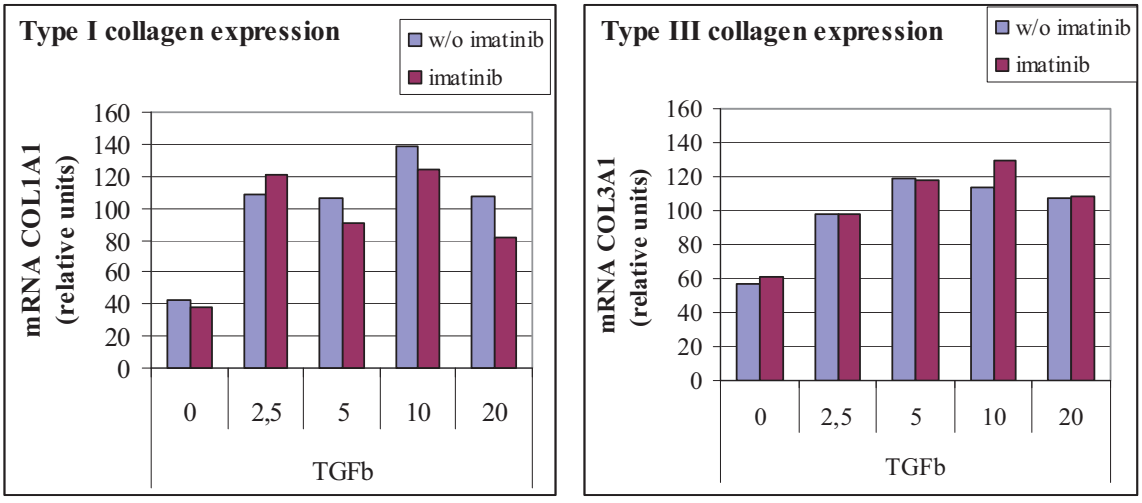


Figura 2. L'imatinib no inhibeix l'augment en l'expressió de col·lagen tipus I i III que produeix el TGFβ sobre les SMCs (quantificació relativa de l'ARNm per PCR a temps real).

4. PDGF disminueix l'osteoprotegerina (OPG) a nivell d'ARNm i a nivell proteic sobre les HTAMC

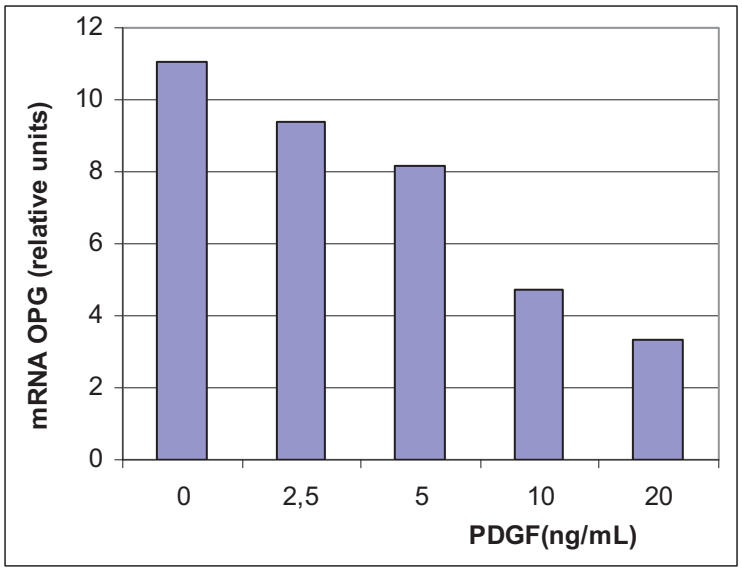


Figura 1. PDGF reprimeix l'expressió de l'ARNm de l'OPG sobre les HTAMC (quantificació relativa de l'ARNm per PCR a temps real).

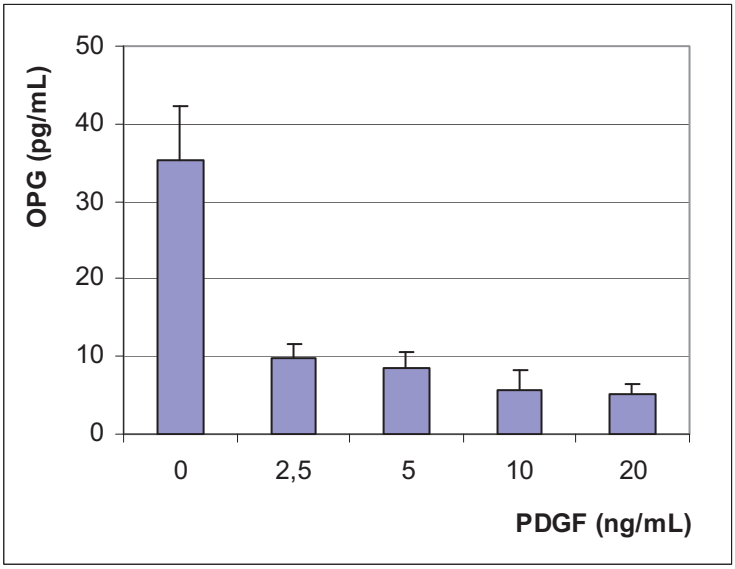
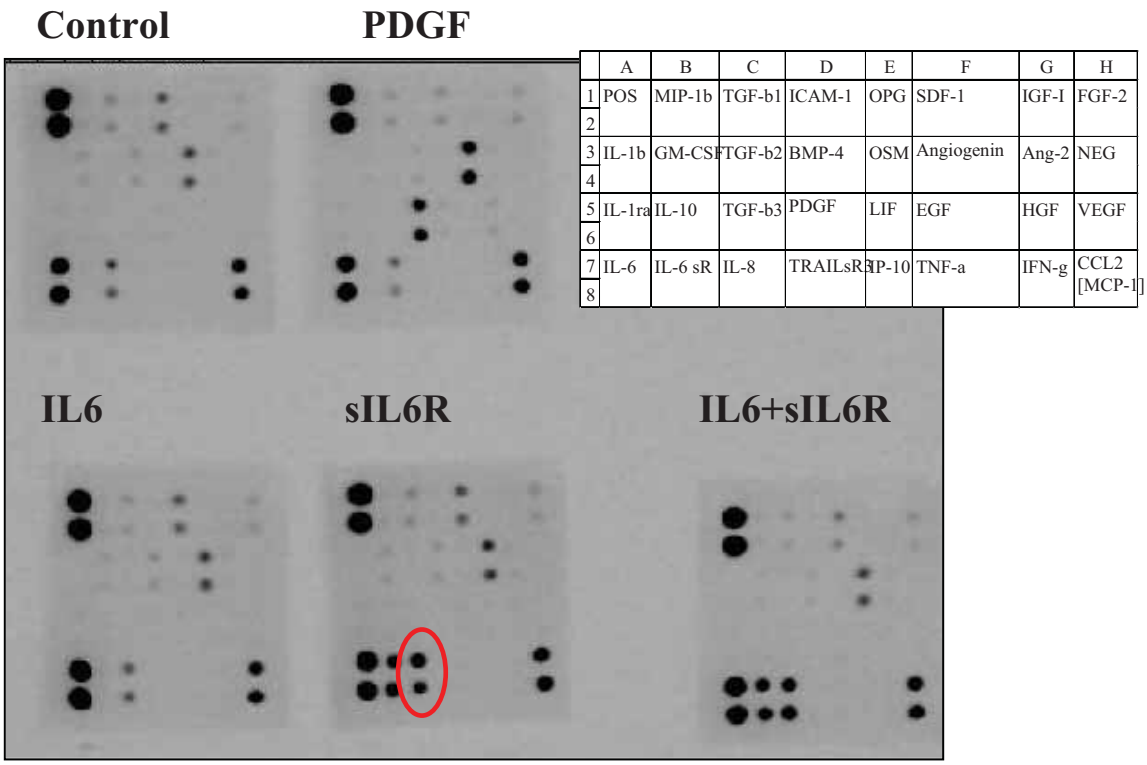


Figura 2. PDGF dismueix la secreció de l'OPG sobre les HTAMC (quantificació per ELISA).

5. Participació de les HTAMC en l'angiogènesi i el procés inflamatori.



	A	B	C	D	E	F	G	H
1	POS	MIP-1b	TGF-b1	ICAM-1	OPG	SDF-1	IGF-I	FGF-2
2								
3	IL-1b	GM-CSF	TGF-b2	BMP-4	OSM	Angiogenin	Ang-2	NEG
4								
5	IL-1ra	IL-10	TGF-b3	PDGF	LIF	EGF	HGF	VEGF
6								
7	IL-6	IL-6 sR	IL-8	TRAILsR3	IP-10	TNF-a	IFN-g	CCL2 [MCP-1]
8								

El receptor soluble de l'IL6 estimula la secreció de l'IL8. Cal destacar que la important secreció de l'IL6 de les HTAMC no estimulades.

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Annex

ANNEX

Altres treballs addicionals relacionats amb el tema de la tesi en els quals he participat :

ARTICLES ORIGINALS

García-Martínez A, Hernandez-Rodriguez J, Arguis P, Paredes P, Segarra M, **Lozano E**, Nicolau C, Ramírez J, Lomeña F, Josa M, Pons F and Cid MC. *Development of Aortic Aneurysm/Dilation During the Followup of Patients With Giant Cell Arteritis: A Cross-Sectional Screening of Fifty-Four Prospectively Followed Patients.* ARTHRITIS & RHEUMATISM (ARTHRITIS CARE & RESEARCH) 2008; 59(3): 422-430

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ORIGINAL ARTICLE

Development of Aortic Aneurysm/Dilatation During the Followup of Patients With Giant Cell Arteritis: A Cross-Sectional Screening of Fifty-Four Prospectively Followed Patients

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Objective. **Giant cell arteritis (GCA) may involve the aorta. Retrospective studies have demonstrated a higher prevalence of aortic aneurysm among patients with GCA compared with the general population. We investigated the prevalence of aortic aneurysm in a cohort of patients with biopsy-proven GCA using a defined protocol and assessed whether persisting low-grade disease activity is associated with higher risk of developing aortic aneurysm.**

Methods. **Fifty-four patients with GCA (14 men and 40 women) were cross-sectionally evaluated after a median followup of 5.4 years (range 4.0–10.5 years). The screening protocol included a chest radiograph, abdominal ultrasonography scan, and computed tomography scan when aortic aneurysm was suspected or changes with respect to the baseline chest radiograph were observed. Clinical and laboratory data, corticosteroid requirements, and relapses were prospectively recorded.**

Results. **Twelve patients (22.2%) had significant aortic structural damage (aneurysm/dilatation), 5 of them candidates for surgical repair. Aortic aneurysm/dilatation was more frequent among men (50%) than women (12.5%; relative risk 3.5, 95% confidence interval 1.53–8.01, $P = 0.007$). At the time of screening, patients with aneurysm/dilatation had lower serum acute-phase reactants, lower relapse rate, and needed shorter periods to withdraw prednisone than patients without aortic structural damage.**

Conclusion. **There is a substantial risk of developing aortic aneurysm/dilatation among patients with GCA. Our data do not support that aneurysm formation mainly results from persistent detectable disease activity. Additional factors including characteristics of the initial injury or the target tissue may also determine susceptibility to aortic aneurysm/dilatation.**

INTRODUCTION

Giant cell arteritis (GCA) is a granulomatous vasculitis affecting large and medium-sized vessels. The most common vascular symptoms of the disease (headache, jaw claudication, scalp tenderness) derive from inflammatory involvement of the craniofacial arteries, but other vascular territories may also be affected (1,2).

Aortic inflammation in patients with GCA was first described in the late 1930s/early 1940s (3) and sporadically reported thereafter (4,5). The prevalence of aortitis in GCA is unknown but appears to be remarkable. Systematic necropsy studies performed by Ostberg in 1972 disclosed aortic inflammation in 12 (92%) of 13 patients with GCA (4). Due to the lack of appropriate imaging techniques able

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to detect aortic inflammation in living individuals, the clinical relevance of aortic involvement has been neglected for years. Currently, computerized tomography or magnetic resonance imaging can detect thickening, increased mural contrast enhancement, and, possibly, edema in the aortic wall (6–8), but available data are still limited. ^{18}F-fluorodeoxyglucose (FDG) uptake measured by positron emission tomography (PET) scan is emerging as a useful method to assess inflammatory activity in large vessels. In recent studies, increased aortic FDG uptake has been detected in approximately 50–60% of untreated patients, decreasing after 3–6 months of corticosteroid treatment (9–11).

Aortic inflammation appears to be frequent in GCA but remains asymptomatic unless structural damage leads to aneurysm, dissection, or aortic valve dysfunction. All of these events may have relevant clinical consequences and increase mortality in patients with GCA (5,12). They may appear early in the course of the disease or, more frequently, as delayed complications.

The prevalence of aortic structural damage related to GCA is unknown given that the occurrence of aortic complications has only been evaluated in retrospective, chart-review studies encompassing long periods. Reported prevalences range from 9.5% to 18% (13–15). These studies include patients diagnosed over very extended periods (20–50 years) including times when awareness of GCA was lower, treatment delay was longer, recommended corticosteroid doses were lower, duration of corticosteroid regimens were more brief, and life expectancy was much shorter (3,4). These factors may all influence both the intensity of aortic inflammation and the detection of clinically apparent complications.

Corticosteroid treatment usually elicits satisfactory relief of symptoms as well as normalization of acute-phase reactants in patients with GCA. However, when corticosteroids are tapered, relapses are frequent and persistent mild to moderate elevation of inflammatory markers can be observed in a substantial proportion of patients in clinical remission, suggesting subclinical activity (16–18). Corticosteroid tapering and withdrawal are currently guided by assessment of clinical activity and acute-phase reactants, mainly erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Based on the reported finding of inflammatory lesions in surgical or necropsy specimens from patients with aneurysm or dissection, there is some concern regarding whether or not persistent subclinical activity may eventually lead to the development of these complications (4,19,20). To date, it is not known whether or not persistent disease activity or persistent elevation of inflammatory markers is associated with a higher risk of developing delayed complications such as aortic aneurysm or dissection.

The goal of our study was 1) to investigate the prevalence and distribution of aortic aneurysm/dilatation detected with a defined screening protocol in a series of 54 patients with biopsy-proven GCA who were prospectively evaluated and treated and 2) to investigate factors associated with the development of this complication, particularly whether persistent subclinical inflammatory activity or a smoldering/relapsing course is associated with higher incidence of aneurysm formation.

PATIENTS AND METHODS

Patient selection. Between September 1995 and July 2001, 125 patients were diagnosed with biopsy-proven GCA at our department (Internal Medicine, Hospital Clínic, Barcelona, Spain). Seven were subsequently treated and followed at other departments/institutions, 16 died during followup, 5 were transferred to nursing homes for advanced dementia, and 38 were lost or had incomplete followup for a variety of reasons, including moving to other regions or not returning for periodic visits by study physicians after successful corticosteroid withdrawal. During the planned study period (2000–2005), 59 patients had already completed or would complete a prospective followup of at least 4 years and were considered eligible for aneurysm screening. This period was arbitrarily selected on the basis that aneurysm is considered to be a delayed complication. Five of the 59 patients declined participation due to advanced age or comorbidities and the remaining 54 agreed to participate and were included.

All patients were prospectively treated and followed by the investigators according to a defined protocol. All patients received an initial prednisone dosage of 1 mg/kg/day (up to 60 mg/day) for 1 month. Subsequently, prednisone was tapered 10 mg/week. Reduction below 20 mg/day was slower and individualized. A further reduction to a maintenance dosage of 10 mg/day was attempted over a 2-month period. If tolerated, reduction to 7.5 mg/day was attempted after 3 months and maintained for 3 additional months. A maintenance dosage of 5 mg/day was attempted for 6 months. If patients were asymptomatic with normal acute-phase proteins and ESR $<$40 mm/hour, tapering at an approximate rate of 1 mg per 3 months was attempted until discontinuation. If patients responded well but elevation of acute-phase proteins persisted, the maintenance dosage of 5 mg/day was maintained for 1 year before attempting withdrawal. If the ESR increased to $>$40 mm/hour, the corticosteroid dose was held for 2 months and if no clinical symptoms appeared, tapering was attempted again. Relapse was defined as reappearance of disease-related symptoms. Persistent malaise and anemia with elevation of acute-phase reactants were also considered relapses if they were not attributable to other causes after detailed evaluation and if they resolved after increasing steroids. When a relapse occurred, prednisone dosage was increased by 10 mg/day above the previous effective dose. Clinical findings and laboratory values at the time of diagnosis were prospectively recorded. These included ESR, CRP level, haptoglobin, α_2-globulin, blood cell counts, and liver function tests by usual automatized systems.

Screening protocol. Patients were screened once between 2000 and 2005 at their regular followup visits. Patients underwent a medical interview, complete physical examination, routine blood tests, and detection of serum concentration of proinflammatory cytokines (interleukin-6 [IL-6], tumor necrosis factor α, and IL-18). These were

determined by immunoassay (R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. Chest radiography was performed in all patients and carefully compared with that performed at the time of diagnosis. When aortic dilatation or changes with respect to the baseline radiograph were suspected, a contrast-enhanced spiral chest computed tomography (CT) scan was performed. The diameter of the aorta was measured at 3 different levels (ascending aorta, aortic arch, and descending aorta). Significant aortic structural damage was considered when an aortic aneurysm was found (defined as focal dilatation of the aortic wall) or when the aortic wall was diffusely dilated with a diameter >4 cm in the ascending aorta or at least 4 cm in the aortic arch and descending aorta. The aortic diameter at the same levels was measured in 28 consecutively selected age- and sex-matched individuals who underwent a chest CT scan for melanoma or gastric cancer as routine followup. The abdominal aorta was evaluated by ultrasonography. Prednisone requirements and relapse rate were prospectively recorded in all patients.

FDG uptake assessment by PET scan. To assess whether aneurysm development could be related to detectable subclinical inflammation, FDG uptake was evaluated by PET scan in 11 patients with GCA (7 with and 4 without aortic aneurysm or dilatation, all confirmed by CT scan), in 4 age- and sex-matched controls randomly selected among patients undergoing evaluation for cancer staging, and in 3 patients with noninflammatory thoracic aortic aneurysm who were scheduled for surgery. In these latter patients, the noninflammatory nature of the aortic aneurysm was confirmed by histopathologic examination after surgical repair. Two of the patients had aortic aneurysm secondary to myxoid degeneration of the aortic valve and 1 had severe atherosclerosis. Funding limitations precluded extension of PET scan study to the entire series.

After a fasting period of 6 hours and after verifying a blood glucose concentration <120 mg/ml, 370 MBq of FDG was injected intravenously and PET/CT was performed with a Biograph (Siemens Medical Solutions, Enlargen, Germany). Whole-body images from the base of the skull to mid-femur were acquired 50 minutes after the radiotracer injection. CT parameters were 50 mA, 130 kV, and 8-mm sections. Iterative reconstruction was performed and attenuation correction was based on CT. Attenuation-corrected and nonattenuation-corrected images were evaluated by 2 independent investigators. The maximum standard uptake value (SUV) and the median SUV were obtained from a zone of interest drawn on sagittal slices over the thoracic aorta. The study was approved by our local ethics committee and all patients gave informed consent.

Immunohistochemistry and gelatin zymography. Serial 4–6-μm cryostat sections from a surgically removed aortic segment from a patient with GCA were air dried and fixed with cold acetone. Sections were incubated with a polyclonal rabbit anti-human matrix metalloprotease 2 (MMP-2; Chemicon, Temecula, CA) at 1:500 dilution or a mouse monoclonal anti-human MMP-9 (clone GE-213; Chemicon) at 1:1,000 dilution. Immunoglobulins obtained from the same species as the primary antibodies were used as negative controls at the same concentrations. Immunodetection was carried out with an HRP-labeled polymer conjugated to secondary antibodies (EnVision kit from Dako, Carpinteria, CA).

Elastic fibers were stained with 1% Shikata's orcein (Scharlau Chemie, Barcelona, Spain) in 70% ethanol. Gelatin zymography of tissue extracts from a normal temporal artery, a temporal artery with active GCA lesions, and a surgically excised GCA-related thoracic aortic aneurysm was performed as described (21).

Statistical analysis. Mann-Whitney U test and Student's *t*-test, when applicable, were applied to quantitative data. Kruskal-Wallis test was used for multiple comparisons. Fisher's exact test was used for contingency tables.

Table 1. Description of aortic characteristics in patients with aortic structural damage

Patient	Aortic characteristics
1	Diffuse dilatation of aortic root and ascending aorta, maximum diameter of 5.7 cm. Moderate aortic insufficiency secondary to dilatation. Surgical repair declined because of age and concomitant diseases.
2	Diffuse dilatation of thoracic aorta with maximum diameter of 6 cm in ascending aorta. The aneurysm was surgically repaired. The histology showed moderate inflammation in adventitia and scattered inflammatory foci in the media layer.
3	Aneurysm of ascending aorta and aortic root with maximum diameter of 7.3 cm and severe aortic insufficiency. Surgical repair refused because of age and concomitant diseases.
4	Dilatation of ascending aorta with maximum diameter of 5 cm and important dilatation of aortic arch. Moderate aortic insufficiency. The patient refused surgical repair.
5	Aneurysm of ascending aorta with maximum diameter of 5.8 cm. Moderate aortic insufficiency secondary to dilatation. The aneurysm was surgically repaired. The histology showed moderate atherosclerosis with moderate chronic inflammation in the intima and adventitia.
6	Aneurysm of ascending aorta with maximum diameter of 5 cm.
7	Aneurysm of ascending aorta with maximum diameter of 4.8 cm.
8	Aneurysm of abdominal aorta (5.1 × 3.1 × 2.9 cm).
9	Dilatation of the ascending aorta (4.5 cm).
10	Dilatation of the ascending aorta (4.2 cm).
11	Dilatation of the aortic arch (4 cm).
12	Dilatation of the aortic arch and the descending aorta (4 cm).

Table 2. Clinical data at baseline of patients with and without aortic abnormalities*

	Altered aorta (n = 12)	Normal aorta (n = 42)	*P*
Sex, male/female	7/5	7/35	0.007
Age, median (range) years	76 (70–89)	79 (63–91)	NS
Followup, median (range) years	5.4 (4–8.5)	5.5 (4–10.5)	NS
Duration of symptoms, median (range) weeks	10 (2–52)	16 (1–104)	NS
Cranial symptoms			
Headache	75	83	NS
Jaw claudication	25	50	NS
Scalp tenderness	25	57	NS
Ischemic events	0	19	NS
Systemic symptoms			
Polymyalgia rheumatica	42	52	NS
Fever	25	43	NS
Weight loss	58	52	NS
Vascular risk factors			
Smoking	8	5	NS
Hypertension	75	79	NS
Diabetes	17	12	NS
Hypercholesterolemia	17	57	0.021

* Values are the percentage unless otherwise indicated. Vascular risk factors have been determined at baseline or during proper followup. NS = not significant.

The time required to achieve a stable maintenance prednisone dosage <10 mg/day and the time until definitive corticosteroid withdrawal were analyzed by the Kaplan-Meier survival analysis and compared by the log rank test.

RESULTS

Prevalence and characteristics of aortic structural damage in patients with GCA. Changes in the screening chest radiograph led to the performance of a chest CT scan in 28 (52%) patients. Significant structural abnormalities in the thoracic aorta were confirmed in 11 patients. In the remaining 17, suspected changes observed in the radiograph were positional or due to aortic elongation or hiatal hernia. No thoracic aortic aneurysm was found among controls and only 2 had an ascending aorta diameter >4 cm. Aortic diameters among individuals considered not to have aortic dilatation tended to be higher in patients with GCA at the level of the descending aorta when compared with controls (median 2.5 cm, range 2.1–3.6 versus median 2.3 cm, range 2–2.8; $P = 0.018$). No significant differences were found in the other segments. This finding indicates that a low degree of structural damage leading to slight diffuse dilatation is common in patients with GCA.

Ultrasonography revealed abdominal aortic aneurysm in only 1 patient. Overall, 12 (22.2%) patients developed significant structural aortic damage (aneurysm or dilatation) during a median followup of 5.4 years (range 4–10.5 years).

A brief description of the abnormalities detected is shown in Table 1. In 5 patients surgery was recommended because of the size of the aneurysm or resulting aortic valve insufficiency. Two of these patients underwent successful surgical repair of the aneurysm. One patient refused intervention. In the remaining 2 patients, surgery was eventually declined because of advanced age and comorbidities. Incidentally, the screening protocol led to the discovery of a thoracic hydatid cyst in 1 patient, lung cancer in 1 patient, hypernephroma in 1 patient, and ovarian mucinous cystadenoma in 1 patient.

Clinical findings associated with the development of significant aortic structural damage. No significant differences in age, duration of followup, or initial clinical manifestations were found between patients with and without aortic structural damage. The prevalence of traditional cardiovascular risk factors did not differ among patients with or without aortic structural damage except for hypercholesterolemia, which, surprisingly, was more frequent among patients who did not develop aortic structural damage (relative risk [RR] 0.29, 95% confidence interval [95% CI] 0.081–1.062, $P = 0.021$). In our series, significant aortic structural damage was detected in 50% of men but only 12.5% of women (RR 3.5, 95% CI 1.529–8.014, $P = 0.007$) (Table 2). Interestingly, patients who later developed aortic aneurysm/dilatation tended to have lower concentrations of acute-phase reactants at the time of diagnosis compared with patients who did not develop significant aortic damage (Table 3). When the overall intensity of the acute-phase response was evaluated combining clinical and analytical abnormalities as reported (22), aneurysm/dilatation was significantly more frequent among patients with a weak systemic inflammatory reaction (RR 1.7, 95% CI 1.166–2.626, $P = 0.046$) (Figure 1A). This was unexpected given that patients with strong acute-phase response usually have more resistant disease (22).

At the time of screening, all patients were in clinical remission. Twenty-seven were in stable remission without therapy and 27 still required low doses of corticosteroids (median dosage 3.75 mg/day, range 1.25–12.5). No significant differences in clinical outcome during followup were observed between patients with and without aortic abnor-

Table 3. Laboratory parameters at baseline and at the time of evaluation*

	Altered aorta	Normal aorta	*P*
At diagnosis			
ESR, mm/hour	87 ± 24	95 ± 28	NS
CRP, mg/dl	6.5 ± 5.7	10 ± 9.3	NS
Haptoglobin, gm/liter	3.2 ± 1.27	4 ± 1.73	NS
Hemoglobin, gm/dl	117 ± 18	110 ± 15	NS
Alkaline phosphatase, units/liter	227 ± 80	283 ± 212	NS
GGT, units/liter	66 ± 107	48 ± 51	NS
Proteins, gm/liter	68 ± 8	68 ± 7	NS
Albumin, gm/liter	34 ± 5	35 ± 5	NS
α_2-globulin, gm/liter	8.4 ± 2.5	10.2 ± 3.6	NS
Platelet count, $\times 10^9$/liter	292 ± 52	341 ± 110	NS
At screening			
ESR, mm/hour	18 ± 9	34 ± 14	0.001
CRP, mg/dl	0.9 ± 1.4	1.1 ± 0.8	NS
Haptoglobin, gm/liter	1.38 ± 0.62	1.73 ± 0.51	NS
Hemoglobin, gm/dl	141 ± 17	128 ± 12	0.005
IL-6, pg/ml	13 ± 8	24 ± 39	NS
TNFα, pg/ml	30 ± 19	32 ± 13	NS
IL-18, pg/ml	290 ± 132	288 ± 159	NS

* Values are the mean ± SD unless otherwise indicated. ESR = erythrocyte sedimentation rate; NS = not significant; CRP = C-reactive protein; GGT = gamma glutamyl transpeptidase; IL-6 = interleukin-6; TNFα = tumor necrosis factor α; IL-18 = interleukin-18.

malities. Contrary to what was expected, patients with aneurysm/dilatation did not show a smoldering or relapsing course that might indicate stronger persisting inflammatory activity. In fact, as shown in Figure 1B, aneurysm/dilatation tended to be more frequent among patients who did not have recurrences compared with those who had a relapsing course (RR 2.9, 95% CI 1.214–7.965, $P = 0.05$). No significant differences in cumulated prednisone dosages during the first year (mean ± SD 6.3 ± 1.3 gm versus 6.2 ± 1.8; $P = 0.86$) or in the time required to reach a maintenance daily prednisone dosage <10 mg were observed between patients with or without significant aortic structural damage. However, patients with aortic structural damage needed shorter periods to withdraw prednisone therapy than patients without aortic structural damage (Figures 1C and 1D). At the time of screening, no significant differences in proinflammatory cytokine concentrations were observed between patients with or without aortic structural damage. Nevertheless, patients with aortic structural damage had significantly lower ESR ($P = 0.001$) and higher concentrations of hemoglobin ($P = 0.005$) than patients without significant aortic structural damage (Table 3). Although these data should be confirmed in larger series, they suggest that persistent subclinical inflammatory activity is not the major determinant of aneurysm formation in patients with GCA and that other factors may be involved.

FDG positron emission tomography. None of the patients examined showed remarkable FDG uptake by aortic tissue, as has been reported in active disease (9–11). Accurate measurement of maximal and median SUV did not show significant differences between patients with GCA-related aneurysm and patients with GCA with no aneurysm, controls, or patients with noninflammatory aneurysm (Figure 2). The intensity of uptake was much lower than that reported in active patients and similar to that found in patients in remission or patients with atherosclerotic lesions. Although the number of patients examined was small, these findings suggest that, in appropriately treated patients, the development of aneurysm is not mainly related to major differences in persistent, detectable, local inflammatory activity.

Histopathologic examination of aortic specimens. Surgically removed specimens showed inflammatory infiltrates in the adventitial layer in both 2 patients with GCA and 3 patients with noninflammatory aneurysm. Scattered inflammatory foci were seen in the media only in patients with GCA and in the patient with severe atherosclerosis. No dense granulomatous lesions or giant cells were observed. Remaining foci of inflammatory cells immunostained positive for MMP-9 and MMP-2 (Figure 3A). MMP-2 expression by vascular smooth muscle cells was also observed. Elastic fibers were markedly disrupted in areas with remaining inflammatory cells, but also in many additional areas devoid of inflammatory infiltrates (Figure 3B). Gelatin zymography of tissue extracts revealed MMP-9 gelatinolytic signal in the temporal artery with active inflammatory lesions, whereas in a normal temporal artery and in a GCA-related aneurysm MMP-9 gelatinolytic signal was faintly detectable. MMP-2 gelatinolytic signal was observed both in active GCA lesions and in GCA-related aneurysm (Figure 3C).

DISCUSSION

Systematic screening of a cohort of 54 patients with biopsy-proven GCA demonstrated that 12 (22.2%) patients had

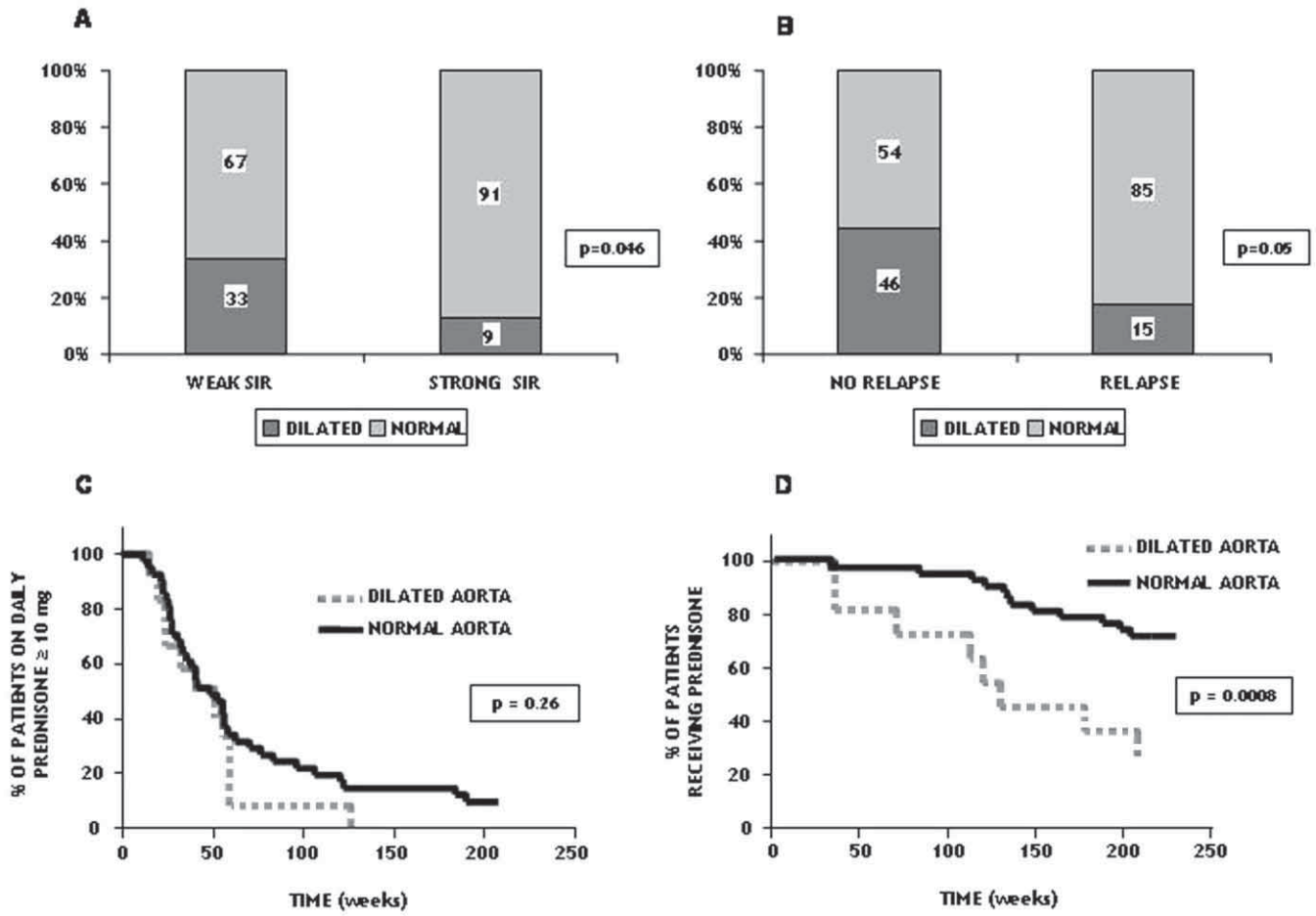

Figure 1. Systemic inflammatory response and clinical outcome in patients with giant cell arteritis with or without aortic structural damage. **A,** The proportion of patients with aortic structural damage was higher among patients with weak systemic inflammatory response (SIR; relative risk 1.7, 95% confidence interval 1.17–2.63, $P = 0.046$). Weak SIR was defined as the presence of ≤2 of the following: erythrocyte sedimentation rate ≥85 mm/hour, hemoglobin <110 gm/liter, fever >37°C, and weight loss >3 kg. Strong systemic inflammatory response was defined as the presence of 3–4 of the above items (20). **B,** Aortic structural damage did not preferentially occur in relapsing individuals. In contrast, aortic structural abnormalities tended to be more frequent among patients in sustained remission. **C,** Percentage of patients requiring ≥10 mg of daily prednisone over time. **D,** Percentage of patients requiring prednisone treatment over time.

significant aortic structural abnormalities (aneurysm or diffuse dilatation) after a median followup of 5.4 years. Thoracic aneurysms were much more frequent than abdominal aneurysms, as reported in retrospective studies.

Our screening method, chosen on the basis of its reasonable cost:benefit ratio and feasibility in general clinical practice, relied on a careful examination of a chest radiograph and an abdominal ultrasound, which may have reduced sensitivity. It is possible that performing echocardiography in patients with aortic murmurs, as recently suggested by Bongartz and Matteson (7), would increase sensitivity. Systematic screening with more sensitive imaging techniques such as CT scan would have probably revealed a higher prevalence of subtle aortic structural abnormalities, although perhaps not always clinically relevant. Despite the potential limitations of the screening method applied, the prevalence of aortic structural damage observed is higher than that reported in retrospective studies over a much more extended period. Based on the size of the aneurysm or the resulting aortic valve insufficiency, 5 patients (9.2% of the global series and 42% of those with aneurysm or dilatation) were considered candidates for surgery. The development of aortic structural damage is, therefore, a major health threat in the outcome of patients with GCA, with a potentially increasing impact given the growing life expectancy of elderly persons in developed countries.

A relevant question arising from the recognition of aortic aneurysm/dilatation as a major and frequent complication of GCA is whether aortic structural damage appears as a consequence of the initial injury or develops progressively due to persisting, low-grade inflammatory activity. Concerns about the potential development of aortic damage as a consequence of persisting low-grade inflammatory activity despite an apparently appropriate response to steroids arise from the repeatedly reported finding of inflammatory infiltrates in surgical or necropsy aortic specimens (4,19,20). However, a critical analysis of the reported cases reveals that the characteristics and extent of inflammatory infiltrates as well as the dose and duration of the corticosteroid treatment received until surgery or necropsy are not described in detail in most reports. Therefore it is not

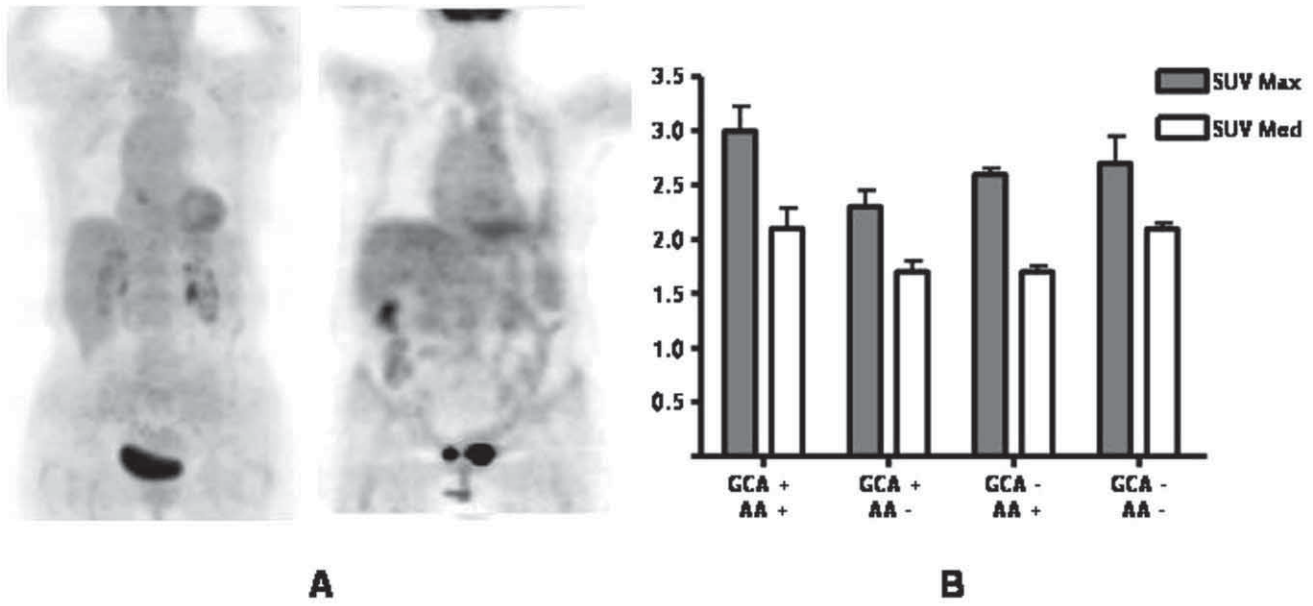

Figure 2. ^{18}F-fluorodeoxyglucose aortic uptake in patients with giant cell arteritis (GCA) according to the presence or absence of aortic aneurysm/dilatation. **A,** Positron emission tomography scan of a patient with GCA-related aneurysm (**left**) compared with a patient with noninflammatory aneurysm (**right**). **B,** Measurement of standard uptake value (SUV) maximal (Max) and median (Med) in patients with GCA and aortic aneurysm (GCA+, AA+), patients with GCA with a normal aorta (GCA+, AA−), patients with noninflammatory aneurysm (GCA−, AA+), and control individuals (GCA−, AA−).

clear whether active or residual inflammation is observed and whether specimens are obtained during active disease or in patients in remission under the current treatment strategy in terms of dose and duration of corticosteroids (13,20,23). Dense granulomatous lesions are usually described in specimens obtained from patients dying from aortic complications during active disease or in patients treated with low corticosteroid doses or treated for short periods (4,13). This important point was addressed by Lie who examined 35 aortic specimens from patients with GCA (19). Lie remarked that active granulomatous lesions were found in patients in whom the samples were obtained shortly after diagnosis, whereas the intensity and extent of inflammatory infiltrates were lower in treated patients. Our data, obtained from a cohort of prospectively treated patients according to the current standard of care, do not support that patients with smoldering or relapsing disease or patients with persistently elevated acute-phase reactants or proinflammatory cytokines are more prone to develop aneurysm/dilatation. The aortic specimens obtained during elective surgery from 2 of our patients showed scattered inflammatory foci in the media. Infiltrating leukocytes immunostained positive for MMP-2 and MMP-9. MMPs, particularly MMP-9, have been considered to be involved in elastin degradation and generation of aortic aneurysm in several models (24–27). However, gelatinolytic signal of MMP-9, which is mainly produced by activated inflammatory cells, was faint in aortic aneurysm compared with active GCA lesions in a temporal artery obtained at diagnosis. In contrast, active MMP-2, which can also be expressed by vascular smooth muscle cells and is involved in vascular reparative mechanisms (28,29), was detected equally in both active GCA lesions and aortic aneurysm. We cannot exclude that remaining infiltrates or MMP-2 produced during vascular remodeling increases vessel wall damage over the years. However, persisting inflammatory infiltrates were very scarce, whereas elastic lamellae disruption, which is an early finding in experimental aneurysm formation (24), was extensive, possibly as a consequence of the initial inflammatory injury.

Characteristics of the target tissue may play a significant role in the extent of the initial injury. Some patients may have unique substrate characteristics in their aortic tissue, making it more susceptible to aortic inflammation, whereas in others the aorta may remain relatively spared. Once inflammation and injury are established, characteristics of vascular remodeling may vary in different aortic segments. Necropsy studies have indeed demonstrated that inflammatory lesions in GCA equally target the thoracic and the abdominal aorta (4). This is in accordance with recent studies showing a similar proportion of thoracic and abdominal FDG uptake in individuals with active disease (11). However, in all series, thoracic aneurysms are much more frequent than abdominal aneurysms in patients with GCA (12–15). Thoracic and abdominal aortas differ greatly in lumen diameter, wall thickness, vasa vasorum density, content of elastic and collagen fibers, propensity to atherosclerosis, and susceptibility to infection-induced vasculitis (30,31). Thoracic and abdominal aortas may then respond differently to inflammatory injury. In addition, the thoracic aorta is subjected to a higher pressure, which might favor progressive dilatation of a weakened wall. Sex may also influence the development of aortic damage. In our series, aortic structural abnormalities were more frequently observed in men. Male predominance in susceptibility to experimental aortic aneurysms has also been demonstrated in experimental settings (32,33).

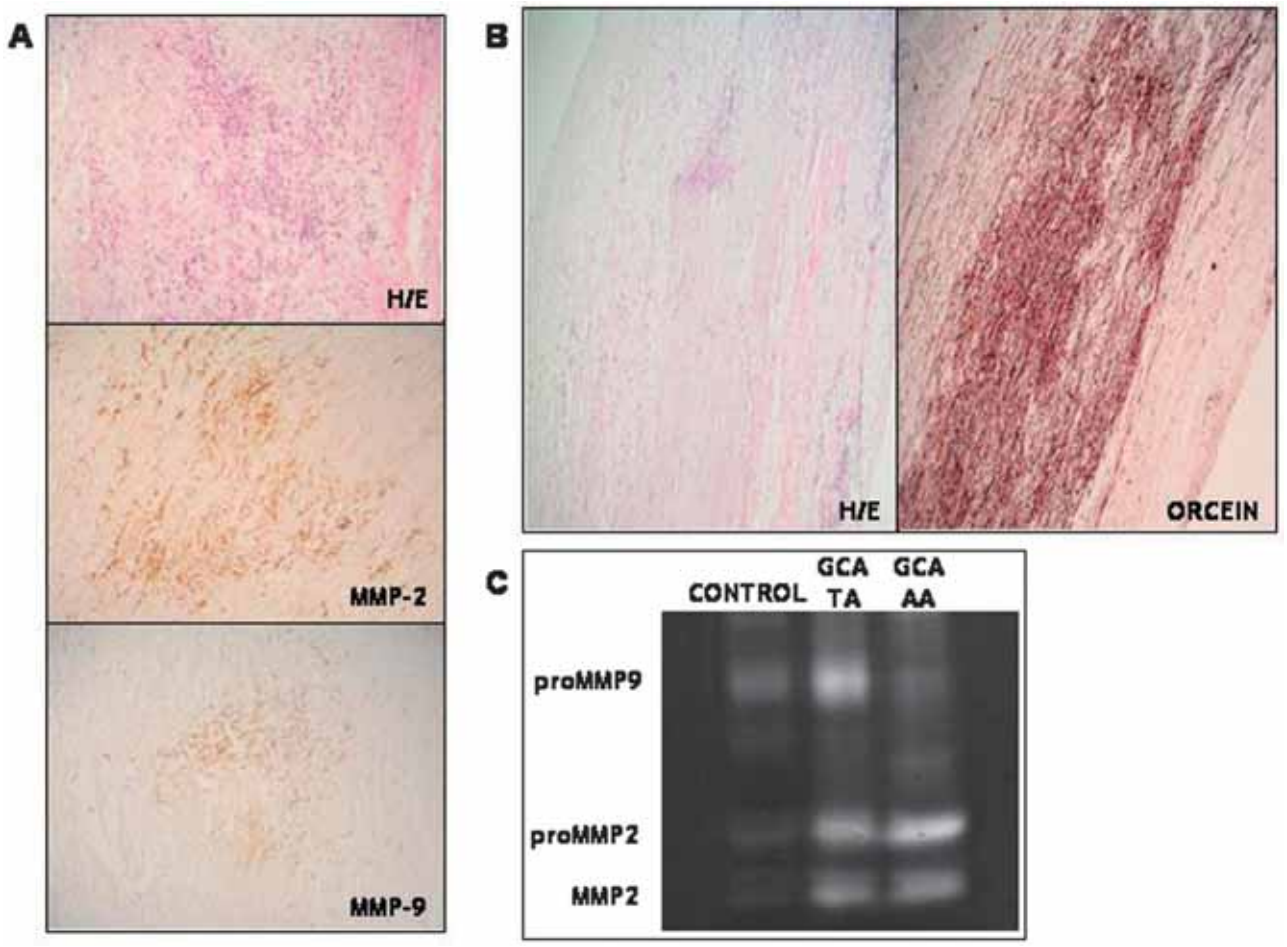

Figure 3. Histopathologic examination of the aortic wall in a patient with giant cell arteritis (GCA)–related aneurysm. **A,** Hematoxylin/eosin (H/E) staining showing scattered chronic inflammatory foci in the media and serial sections showing matrix metalloprotease 2 (MMP-2) and MMP-9 expression by inflammatory cells (magnification × 200). **B,** Lower magnification (magnification × 40) covering a wider area and showing a paucity of inflammatory infiltrates; orcein staining of elastic lamellae in a serial section displaying multiple foci of disruption in areas devoid of inflammatory infiltrates. **C,** Gelatin zymography of tissue extracts (80 μg/lane) from a normal temporal artery (TA); a TA with active, treatment-naive GCA lesions; and a GCA-related thoracic aortic aneurysm (AA). MMP-9 is only detected in active GCA lesions whereas active MMP-2 can be detected both in active lesions and in the GCA-related aneurysm. ProMMP = Promatrix metalloprotease.

Contrary to data gathered from retrospective studies (14,15), we did not observe a higher prevalence of aortic structural damage in patients with traditional cardiovascular risk factors. This may be due to the prospective nature of this study, in which tight control of vascular risk factors was part of the therapeutic approach. The higher prevalence of hypercholesterolemia, and consequently statin therapy, among patients with a preserved aortic wall raises the hypothesis of statins as protective agents against aortic wall structural damage.

In summary, prospective screening shows that a remarkable proportion of patients with GCA develop aneurysm/dilatation, in some instances severe enough to warrant surgical repair. The life-threatening nature of the potential complications derived from aortic structural damage indicates that patients with GCA should be subjected to a continuous surveillance by clinical examination and imaging. Our data do not support that in patients treated according to the current standard of care, aortic aneurysm formation results mainly from persistent activity; our data suggest interplay of heterogeneous factors. Investigating mechanisms involved in the development of aortic structural damage and its progression is of major relevance for patients with GCA.

AUTHOR CONTRIBUTIONS

Dr. Cid had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. García-Martínez, Arguis, Segarra, Lozano, Cid.

Acquisition of data. García-Martínez, Hernández-Rodríguez, Arguis, Paredes, Segarra, Lozano, Nicolau, Ramírez, Lomeña, Josa, Pons, Cid.

Analysis and interpretation of data. García-Martínez, Arguis, Paredes, Segarra, Lozano, Ramírez, Lomeña, Cid.

Manuscript preparation. García-Martínez, Cid.

Statistical analysis. García-Martínez, Cid.

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Bone marrow angiogenesis and angiogenic factors in multiple myeloma treated with novel agents

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Abstract

Introduction. An increased bone marrow (BM) angiogenesis is associated with poor outcome in multiple myeloma (MM). *Objective.* Angiogenesis study in MM treated with novel antimyeloma agents: thalidomide, lenalidomide, bortezomib, and with dexamethasone. *Patients and methods.* Forty-four patients with MM (14 newly diagnosed, 30 refractory/relapsed) were treated with novel agents at our institution. A BM biopsy was obtained before the initiation of therapy in 19. Angiogenesis was assessed by microvessel density (MVD) estimation in BM biopsies stained with the monoclonal anti-CD34 antibody, and by serum levels of angiogenic factors (VEGF, bFGF, and HGF) and cytokines (IL-6 and TNF-α). *Results*. A positive correlation was found between BM plasma cell involvement and MVD estimation ($p = 0.01$). However, MVD was not significantly correlated with either disease phase ($p = 0.065$) or response to therapy ($p = 0.79$). Neither baseline serum levels of angiogenic cytokines correlated to response to treatment. No significant correlation was found between BM MVD and serum levels of angiogenic cytokines. Serum levels of angiogenic cytokines before and after therapy showed a significant increase of bFGF ($p = 0.008$). *Conclusion.* There is no relationship between MVD estimation and baseline serum levels of angiogenic cytokines, neither between each of them and response to therapy.
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Keywords: Multiple myeloma; Angiogenesis; Microvessel density; Angiogenic cytokines

1. Introduction

Multiple myeloma (MM) is a prevalent disease, which accounts for about 1% of all neoplasias and for more than 10% of hematologic malignancies. It has a poor prognosis with a median survival of 3–5 years despite all treatment approaches including intensive chemotherapy followed by hematopoietic stem cell transplantation [1–8].

Formation of new blood vessels (angiogenesis) is a crucial pathogenetic mechanism for growth and dissemination in solid tumors, that has also been implicated in the pathogenesis of hematologic malignancies such as MM [9,10]. Vacca et al. [11] first recognized an increased angiogenesis in the bone marrow (BM) of patients with MM and its association with disease activity. Other studies have confirmed these findings, also supporting an increased vascularization as a poor prognostic feature [12–19], this leading to the introduction of antiangiogenic agents such as thalidomide in the treatment of this disease. However,

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the response to thalidomide has not always been associated with a decrease in BM vascularization, suggesting that the antimyeloma activity of this agent would possibly be mediated by other mechanisms [20–22].

Neovascularization is a complex process mediated by a balance of various positive and negative angiogenic molecules and growth factors released by tumor cells themselves as well as by the BM microenvironment [23–26]. Among these factors, some have been more clearly involved in angiogenesis in MM: vascular endothelial-growth factor (VEGF), basic fibroblastic-growth factor (bFGF or FGF-2), and hepatocyte-growth factor (HGF) [27–35]. On the other hand, some cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) have been implicated in the pathogenesis of monoclonal gammopathies due to its role on the proliferation of the myelomatous clone [36–39]. However, its contribution to disease progression has not been clearly established.

These confusing results led us to undertake this study to investigate angiogenesis in terms of BM vascularization and serum levels of angiogenic factors (VEGF, bFGF, and HGF) and cytokines (IL-6 and TNF-α) in patients with MM treated with novel agents (thalidomide, lenalidomide, and bortezomib) or dexamethasone. Furthermore, our purpose was to assess whether or not BM neovascularization estimated by microvessel density (MVD) correlates with serum levels of angiogenic cytokines as well as to study possible correlations of BM angiogenesis and angiogenic factors with response to therapy.

2. Patients and methods

2.1. Patients

From November 2002–September 2006, 44 patients (22 men and 22 women) diagnosed with MM were treated with novel agents or dexamethasone at our institution. Fourteen patients had untreated symptomatic MM while 30 had refractory or relapsed disease. Table 1 summarizes the characteristics of the two groups of patients before the initiation of antiangiogenic therapy. A serum sample was collected before the start of therapy in all cases, while a second sample at the time of maximum response or at the end of treatment was also available in 34 of them. A BM trephine biopsy was also obtained before the initiation of therapy in 19 of these patients.

Approval of the study by the Institutional Ethics Committee of our institution, as well as individual authorization and written informed consent for each patient to be included, were obtained.

2.2. Treatment schedule

The 14 untreated patients were included in the international, randomized, and double-blind clinical assay THAL-MM-003 [40] receiving: thalidomide plus high-dose dexamethasone (7 cases), or high-dose dexamethasone alone (7 cases).

Table 1
Characteristics of patients at the onset of therapy

Feature	Newly diagnosed	Refractory/ relapsed
No. patients	14	30
Male/female	9/5	13/17
Median age, yrs (range)	65 (59–79)	64 (52–82)
Immunological subtype, no. patients (%)		
IgG	7 (50)	18 (60)
IgA	5 (36)	7 (23)
Light chain (Bence Jones)	2 (14)	3 (10)
IgD	0 (0)	2 (7)
Light chain subtype, no. patients (%)		
Kappa	10 (71)	16 (53)
Lambda	4 (29)	14 (47)
Median M-protein size, g/L (range)	48 (25–71)	31 (6.8–62)
Median BM plasma cells, % (range)	33 (7–73)	26 (1–100)
Extramedullary involvement, no. patients (%)	2 (14)	4 (13)
Median time from diagnosis, yrs (range)	NA	47.8 (7.8–153.3)
Median no. of previous lines of therapy (range)	NA	1.5 (1–4)
Previous ASCT, no. patients (%)	NA	13 (43.3)

NA, not applicable.

Refractory and relapsed MM patients were treated with one of the following drugs: bortezomib, dexamethasone, thalidomide, lenalidomide plus dexamethasone, or with lenalidomide alone. Among the 10 patients who received bortezomib as rescue therapy, five were included in the APEX trial [41], while the other 5 were treated according to the clinical trial DOXIL-MMY-3001 [42] comparing bortezomib plus pegylated liposomal doxorubicin versus bortezomib alone. Eight patients were treated with high-dose dexamethasone alone, 4 included in the APEX trial and 4 in the context of another international clinical trial, CC-5013-MM-010 (Celgene) [43]. For the group of five patients treated with thalidomide, the drug was supplied by Grünenthal (Aachen, Germany) in tablets of 100 mg (Thalidomid 100). The last seven patients were treated with the immunomodulatory drug lenalidomide plus high-dose dexamethasone (6 patients) or lenalidomide alone (1 patient), in the context of clinical trials.

2.3. Methods

Angiogenesis in MM can be studied using two methods: the estimation of microvessel density (MVD) in BM trephine biopsies stained by immunohistochemistry and by measuring serum levels of angiogenic factors.

2.3.1. Grading of myeloma cell infiltration

Sections of paraffin-embedded BM specimens were stained by both hematoxylin-eosin (HE) and immunohisto-

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chemistry using the anti-CD138 antibody (Biotechnology, OBT0360H, dilution 1:600) to recognize plasma cells. According to the positivity observed independently by two examiners, four grades of BM infiltration by plasma cells were established: grade I (isolated plasma cells or clusters of no more than 10 cells in the BM intersticium), grade II (infiltration of $\leqslant$50% of the intertrabecular area), grade III (infiltration over 50% of the intertrabecular area), and grade IV (massive infiltration with isolated areas of normal haematopoesis and/or fat).

2.3.2. Estimation of BM angiogenesis

Bone marrow angiogenesis was evaluated by measurement of microvessels in sections of paraffin-embedded BM specimens previously stained by both HE and immunohistochemistry using an Automated immunostainer TechMate™ 500Plus (Dakocytomation, Denmark) and the two monoclonal antibodies that best recognize endothelial cells: Anti-CD34 (Novocastra, NCL-END, dilution 1:20) and Anti-vWF (Dako, F8186, dilution 1:400) antibodies. As the staining with the anti-vWF was much more faint than with the anti-CD34, we used only the staining with the second one for evaluation. Thereafter, angiogenesis was measured by light microscopy using the following described methods [23]: simple gradation and MVD semiquantitative estimation. Simple gradation led to the assignment of a low, intermediate, or high grade of angiogenesis. According to MVD estimation, three grades of angiogenesis were also established: low (MVD $\leqslant$ 10), intermediate (MVD 11–20), and high (MVD $>$ 20).

With the aim of increase consistence of our results, all stained sections were evaluated by two blinded reviewers using the same light microscopy and there was an excellent interobserver reproducibility of the two grading systems. With regard to MVD estimation, the mean of the values obtained by the two examiners was considered.

2.3.3. Measurement of angiogenic factors and cytokines

Peripheral venous blood samples were collected in sterile test tubes before initiation of therapy in all cases, and at the time of maximum response or at the end of treatment in 34 of them. These samples were centrifuged at 2500 rpm for 7 min and stored at −20 °C until they were used to measure the angiogenic factors and cytokines. Serum concentrations of VEGF, bFGF, and HGF were determined as serum immunoreactivity by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using commercially available kits from R&D Systems (Quantikine Human VEGF Immunoassay kit, Quantikine High-Sensitivity Human FGF Basic Immunoassay kit, and Quantikine Human HGF Immunoassay kit), while the ELISAs for TNF-α and IL-6 were performed using kits from BioSource (BioSource TNF-α EASIA kit and BioSource IL-6 EASIA kit). The limit of detection of each cytokine in serum was: 9.0 pg/mL for the VEGF, 0.05 pg/mL for the bFGF, 40 pg/mL for the HGF, 3 pg/mL for the TNF-α, and 2 pg/mL for the IL-6.

2.3.4. Statistical analysis

Descriptive statistics were used to summarize the patient's characteristics and to analyze the variables of the study. Comparison between groups was assessed by the Mann–Whitney U test for independent variables and the Wilcoxon test for matched-pair data, while box plots were used to show the median, minimum and maximum values and 25th–75th percentiles for each group. Correlation between quantitative variables was established according to the Spearman rank correlation coefficient. A value of $p < 0.05$ was required for statistical significance. The actuarial Kaplan and Meier method was used to estimate time to best response, as well as duration of response and time to progression [44]. At the time of this analysis, the median follow-up of the series has not been reached (range, 3.7 to 44.1+ months). All data were analyzed by using the software Statistica version 6.1. (StatSoft, Inc., Tulsa OK).

2.3.5. Evaluation of response

Effectiveness of the new antiangiogenic agents was evaluated according to the European Group for Blood and Marrow Transplant (EBMT), the International Bone Marrow Transplant Registry (IBMTR), and the Autologous Blood and Marrow Transplant Registry (ABMTR) criteria [45].

3. Results

3.1. Response to therapy

All the 14 previously untreated patients who received first-line therapy with thalidomide plus high-dose dexamethasone (7 patients) or high-dose dexamethasone alone (7 patients) responded, with 1 complete (CR: 7%), 11 partial (PR: 79%), and 2 minimal responses (MR: 14%). Among the 30 patients with refractory or relapsed MM, a response rate of 63% was observed (19/30). According to the specific rescue therapy, responses were as follows: 60% for bortezomib (3 PR and 3 MR), 62% for high-dose dexamethasone (4 PR and 1 MR), 60% for thalidomide (1 PR and 2 MR), 83% for lenalidomide plus high-dose dexamethasone (3 CR, 1 PR, and 1 MR), while the only patient who received monotherapy with lenalidomide did not respond.

With regard to the 14 newly diagnosed patients, median duration of treatment was 7.4 months (range, 5 to 32.5+). Among the responders (14/14), median time to best response was 0.9 months (range, 0.8–9.3) and median duration of this response was 9.7 months (range, 3.1 to 30.6+). At the time of this analysis, 7 of the 14 (50%) responding patients had progressed. Median time to progression in this group of patients was 12 months (range, 3.9 to 32.5+).

Median duration of the treatment administered to the group of 30 refractory/relapsed patients was 5.9 months (range, 0.3–25.8). Among the responders (19/30), median time to best response was 1.6 months (range, 0.7–9.3) and median duration of this response was 6.5 months (range, 1.8+ to 30.5+). At the time of this analysis, 12 of

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the 19 (63%) responding patients had progressed. Median time to progression for these patients was 10.8 months (range, 3.7 to 31.5+).

3.2. Estimation of BM angiogenesis

Among the 19 BM samples available before the initiation of therapy, a grade II–IV of plasma cell infiltration was observed in 84% of the biopsies, while only 3 cases showed grade I infiltration. With regard to the grade of BM angiogenesis, results obtained by simple gradation and MVD estimation showed a strong positive correlation (Spearman R correlation test, $R = 0.87$, $p = 0.000001$). According to MVD estimation, the grade of angiogenesis was high (MVD > 20 microvessels/×400 field) in 53% of cases, intermediate (DMV = 11–20) in 16%, and low (DMV ⩽ 10) in 31% of them. Moreover, there was a statistically significant correlation between the grade of infiltration by plasma cells and MVD estimation (Spearman R test, $R = 0.56$, $p = 0.01$). Microvessel density in patients with a II–IV grade of BM plasma cell infiltration was higher than in patients with a lower grade of BM involvement (Mann–Whitney U test, $p = 0.044$). A trend towards a higher angiogenic activity was also observed when comparing grades I–II vs III–IV ($p = 0.069$). Representative histological sections of BM with different grades of angiogenesis are shown in Fig. 1.

Table 2
Comparison of baseline serum levels of angiogenic factors and cytokines (median and range) depending on MVD estimation (Mann–Whitney U test)

Cytokine (pg/mL)	DMV > 20	DMV ⩽ 20	p
	$n = 10$	$n = 9$	
VEGF	171.5 (69.9–267.4)	467.4 (129.7–973.6)	0.0178
bFGF	6.7 (1.2–12.3)	7.7 (2.0–17.9)	NS
HGF	1161.1 (621.9–2823.6)	1083.9 (657.7–1796.7)	NS
	$n = 8$	$n = 4$	
TNF-α	23.0 (18.0–51.0)	10.5 (7.0–19.0)	0.0174
	$n = 8$	$n = 6$	
IL-6	6.0 (0–15.0)	2.5 (0–10.0)	NS

NS, non-significant ($p > 0.05$).

Table 3
Comparison of baseline/pre-treatment serum levels of angiogenic factors and cytokines (median and range) in responsive versus non-responsive patients (Mann–Whitney U test)

Cytokine (pg/mL)	CR, PR or MR	SD or progression	p
	$n = 33$	$n = 11$	
VEGF	223.6 (39.1–2351.8)	160.7 (63.9–1149.5)	NS
bFGF	5.7 (0–37.4)	4.4 (1.2–13.1)	NS
HGF	1046.4 (609.1–2823.6)	1240.5 (692.3–3210.3)	NS
	$n = 19$	$n = 5$	
TNF-α	23 (7–58)	23 (13–51)	NS
	$n = 21$	$n = 5$	
IL-6	10 (0–35)	4 (0–18)	NS

CR, complete response; PR, partial response; MR, minimal response; SD, stable disease; NS, non-significant ($p > 0.05$).

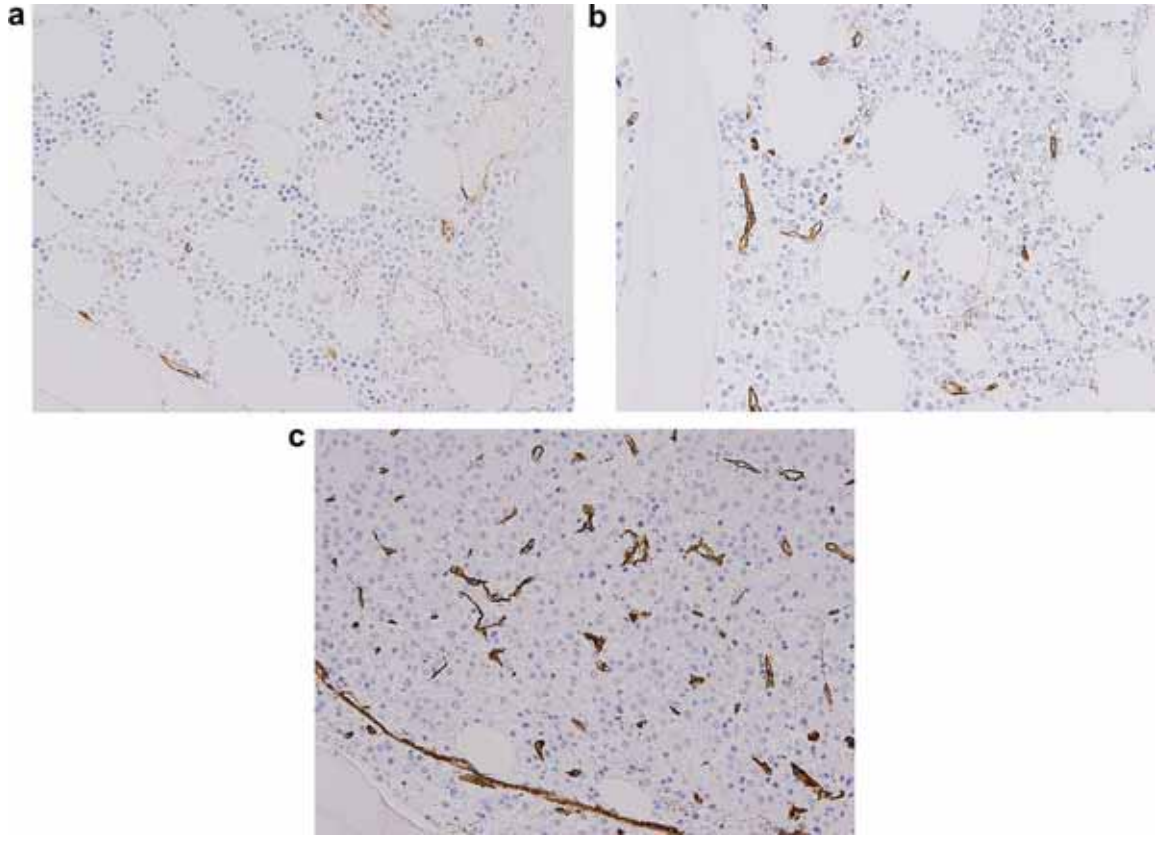

Fig. 1. BM biopsy specimens immuno-stained with anti-CD34 illustrating low-grade (a), intermediate-grade (b) and high-grade (c) angiogenesis in three patients diagnosed with MM (200× magnification).

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Median value for MVD at the onset of therapy was 20.2 microvessels/×400 field (range, 6.3–50.7) in the 19 patients with available biopsy. There was a trend towards a higher MVD in patients with untreated MM (median MVD, 30.7 [range, 9.3–47.8]) compared with those with refractory/relapsed disease (median MVD, 17.2 [range, 6.3–50.7]) (Mann–Whitney U test, $p = 0.065$). No significant difference was found in microvessel assessment between responsive- and non-responsive-to-therapy patients, with a median MVD of 22 (range, 6.3–50.6) and 18.6 (range, 8–35) microvessels/×400 field, respectively (Mann–Whitney U test, $p = 0.79$).

Table 4
Comparison of serum levels of angiogenic factors and cytokines before and after treatment (Wilcoxon test)

Cytokine (pg/mL)	Pre-therapy	Post-therapy	p	n
VEGF	219.1 (39.1–2351.8)	295.9 (33.7–1107.3)	NS	33
bFGF	4.9 (0–37.4)	8.2 (0–58)	0.008	34
HGF	1071.9 (621.9–2823.6)	987.2 (497.4–3623.4)	NS	34
TNF-α	22.5 (7–58)	32 (12–80)	NS	20
IL-6	9 (0–35)	7 (0–232)	NS	20

NS, non-significant ($p > 0.05$).

3.3. Correlation between MVD estimation and measurement of angiogenic factors and cytokines

We also studied the possible correlation between both ways of investigating angiogenesis in MM: bone marrow

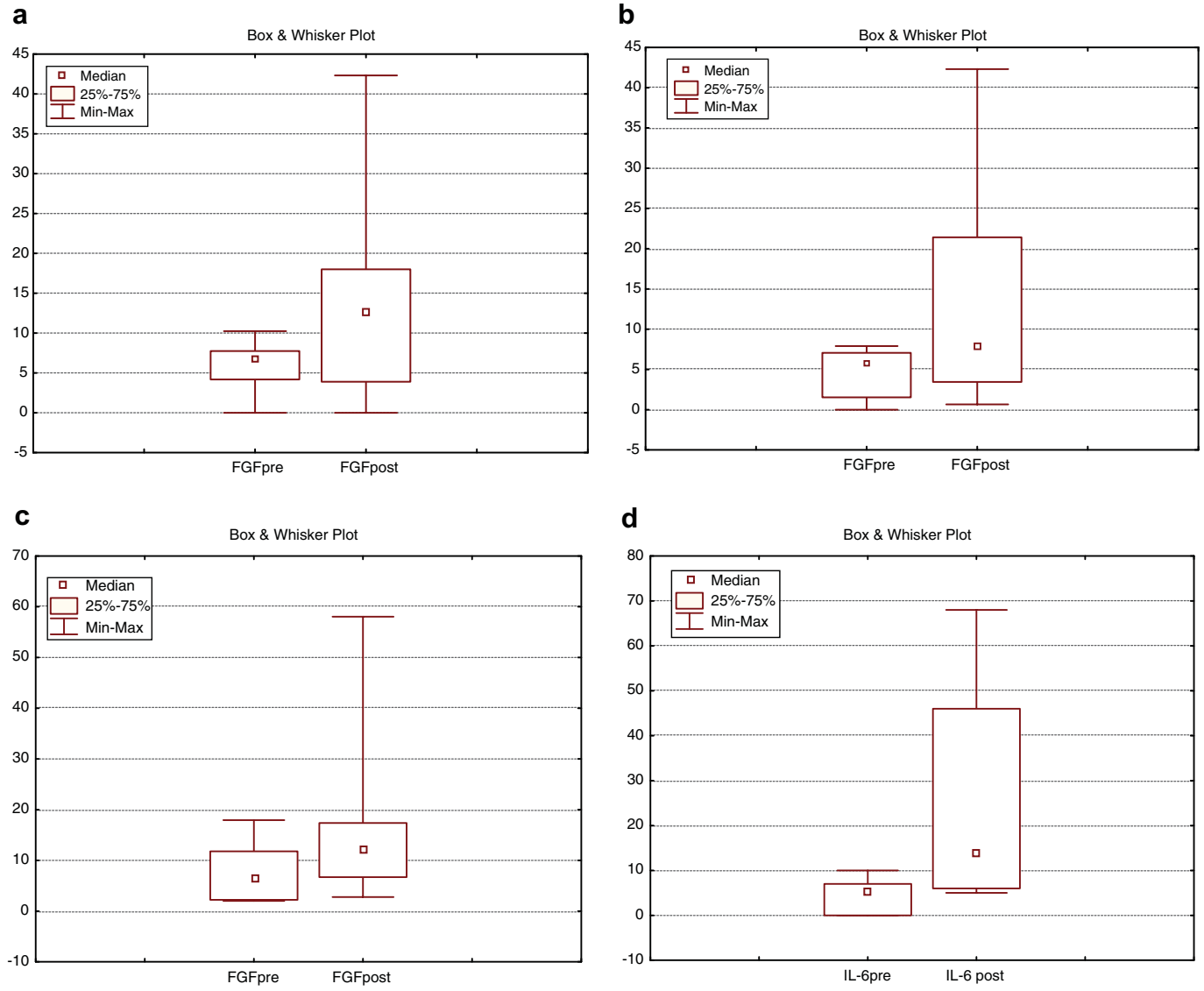

Fig. 2. These box plots show significant difference between levels of bFGF before and after treatment in patients with previously untreated MM ($p = 0.015$, Wilcoxon test; a), and in those who received dexamethasone as first-line therapy ($p = 0.046$, Wilcoxon test; b) or as rescue therapy ($p = 0.025$, Wilcoxon test; c). (d) Significant difference between levels of IL-6 before and after treatment in patients who received dexamethasone as rescue therapy ($p = 0.043$, Wilcoxon test).

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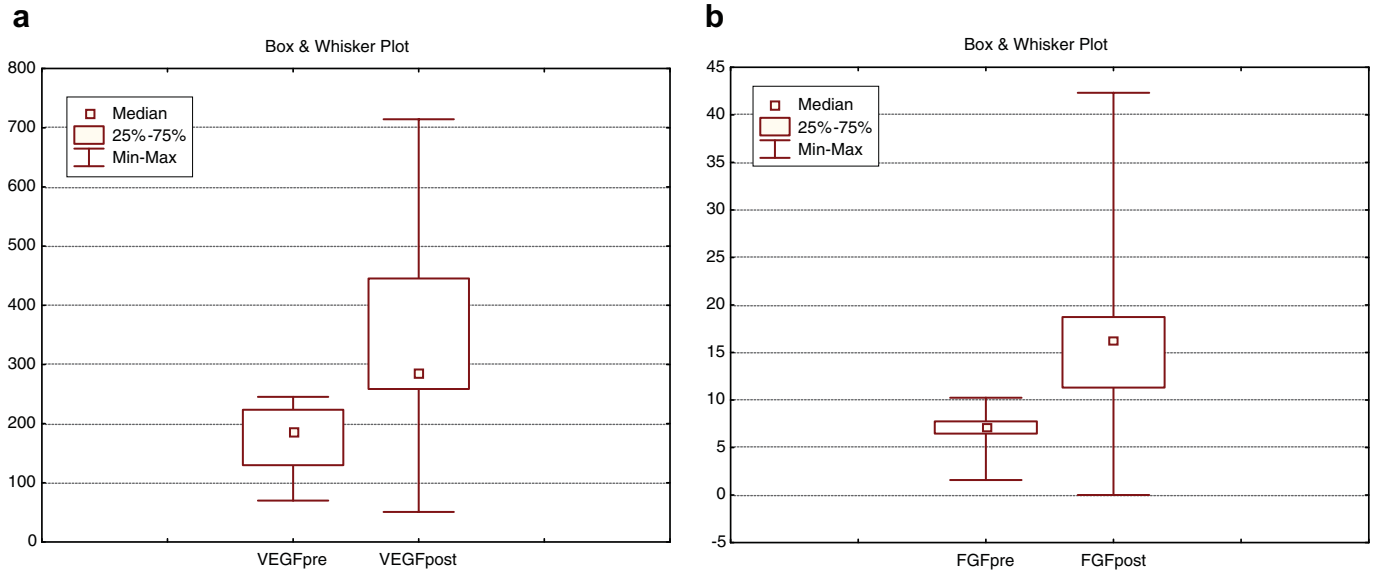

Fig. 3. These box plots show significant difference between levels of VEGF (a) and bFGF (b) before and after treatment in "responsive" patients to thalidomide/dexamethasone or dexamethasone alone as first-line therapy ($p = 0.020$ and $p = 0.038$, respectively; Wilcoxon test).

MVD estimation by immunohistochemistry and measurement of serum levels of angiogenic factors and cytokines (by ELISA) (Table 2). In our series, patients with high grade of angiogenesis (MVD > 20/×400 field) had lower serum levels of VEGF ($p = 0.01$) and higher serum levels of TNF-α ($p = 0.01$) pre-treatment than patients with intermediate or low grade of angiogenesis (Mann–Whitney U test).

3.4. Measurement of angiogenic factors and cytokines

A serum sample was collected before the onset of therapy in all 44 studied patients and a second sample was also available at the time of maximum response or at the end of treatment in 34 of them. No correlation between baseline serum levels of angiogenic factors and cytokines and response to therapy was found (Mann–Whitney U test) (Table 3). Comparison between serum levels of angiogenic factors and cytokines before and after treatment showed an statistically significant increase of bFGF from 4.9 pg/mL (range, 0–37.4) to a median of 8.2 pg/mL (range, 0–58) after therapy in the overall series (Wilcoxon test) (Table 4). This comparison was also made for every group of patients according to the phase of disease (initial vs refractory/relapsed) and type of therapy. An statistically significant increase of bFGF serum levels after therapy was found in the group of untreated MM patients and in those treated with dexamethasone alone independently of the phase of the disease (Wilcoxon test) (Fig. 2). Moreover, a statistically significant increase of IL-6 serum levels was also found in the group of patients who received rescue therapy with dexamethasone (Wilcoxon test) (Fig. 2). Among responsive patients to therapy whose second sample was collected at the time of maximum response, an statistically significant increase of VEGF and bFGF serum levels was found in the previously untreated MM patients (Fig. 3), while an increase of bFGF, HGF, TNF-α, and IL-6 was found in refractory/relapsed patients treated with dexamethasone monotherapy (Wilcoxon test) (Fig. 4).

4. Discussion

It is well established that angiogenesis plays a crucial role in the pathogenesis of solid tumors. It has also been shown to be important in the pathogenesis of hematologic malignancies [9,10]. Several authors have shown that an increased BM vascularization is associated with disease activity and poor prognosis in patients with MM. In fact, a higher degree of angiogenesis in patients with high grade of BM plasma cell involvement has also been one of our findings. The increased angiogenic activity was the rationale for the introduction of novel therapeutic agents with antiangiogenic effect in the treatment of MM, such as thalidomide, targeting not only the myeloma cells but also the interaction between MM cells and their microenvironment [46].

In this sense, Kumar et al. [18] investigated the effect of chemotherapy on the BM angiogenesis of MM by using immunohistochemistry techniques and MVD estimation. They found that the grade of vascularization before initiation of therapy was a predictive factor for survival but did not find significant changes in the grade of angiogenesis after chemotherapy, while other authors had found a significant decrease [47,48]. Also, the Mayo Clinic group has more recently reported the effect of thalidomide on the BM vascularization in 81 patients with MM treated with this agent alone or in association with dexamethasone [49]. They studied BM biopsies at baseline and after 4–6

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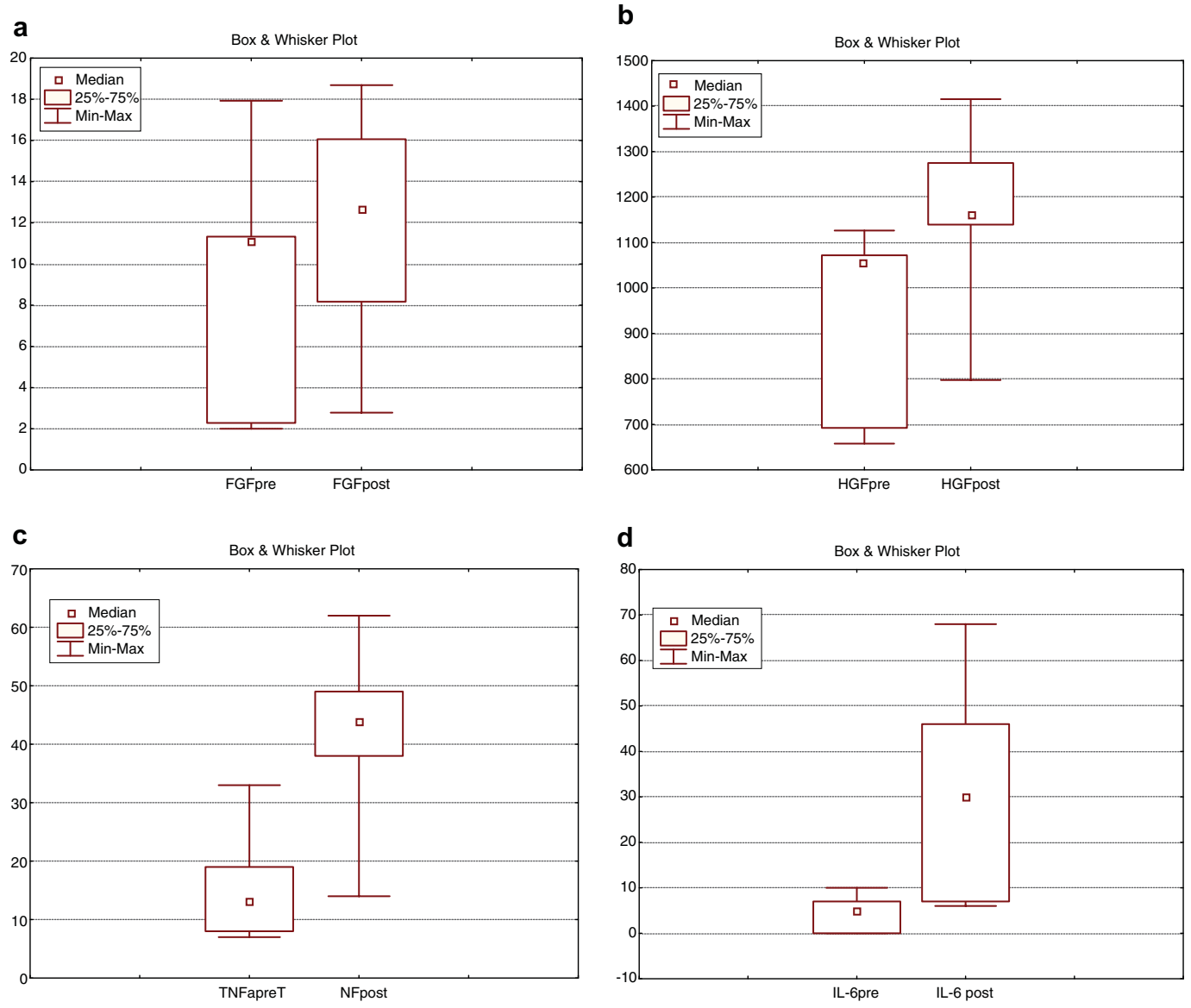

Fig. 4. These box plots show significant difference between levels of bFGF (a), HGF (b), TNF-α (c), and IL-6 (d), before and after treatment in "responsive" patients to dexamethasone as rescue therapy ($p = 0.043$, Wilcoxon test).

months of thalidomide therapy by using immunohistochemistry techniques with the anti-CD34 monoclonal antibody for MVD estimation, and found a significant decrease of MVD after thalidomide therapy in responding patients, these results supporting an antiangiogenic effect of thalidomide in MM.

The mechanism of the antiangiogenic effect of thalidomide is essentially unknown. It has been attributed partially to the inhibitory effect over VEGF and bFGF [50]. Although it has been considered crucial in the antimyeloma activity of this drug, the response to thalidomide has not always been associated with a decrease in BM vascularization or levels of angiogenic cytokines. Moreover, in our clinical experience, extramedullary plasmacytomas do not respond to thalidomide [51] despite that they are highly vascularized tissues [52]. On the other hand, the possible antiangiogenic effect of other agents such as bortezomib and lenalidomide [53,54], as well as the effect of dexamethasone, have been scarcely explored.

In the present study, we investigated the angiogenesis, assessed by BM MVD estimation and measurement of serum levels of angiogenic factors and cytokines, in patients with MM treated with novel agents or dexamethasone. We found a significant correlation between simple gradation and MVD estimation, and the degree of BM plasma cell involvement. We also investigated whether BM angiogenesis measured before initiation of therapy may be predictive for response. In contrast with other authors [55], we found no significant difference between BM MVD in responsive and non-responsive patients. This has been also reported by other groups [19,24,39]. On the other hand, a decrease in BM angiogenesis after response

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to thalidomide has been demonstrated by some authors [49,56], while others have not been able to confirm these "a priori" expected results [57,58]. It has been stated that these discrepancies might be explained by the assessment procedure for tumor angiogenesis in the BM [58]. Even measurement of BM microvessels could be not useful to assess the efficacy of antiangiogenic therapy due to influence on angiogenesis of shrinkage, necrosis or apoptosis of the tumor cells [59]. Of interest, the MVD in a regressing tumor under effective antiangiogenic therapy seems to be related to the ratio between tumor cell death and the endothelial cell apoptosis [9]. Ideally, pre- and post-therapy marrows should be examined but, unfortunately, we had no follow-up BM biopsies to sequentially study the BM angiogenesis in our patients.

We did not find predictive value of baseline serum levels of angiogenic factors and cytokines for response to therapy. This is in agreement with some authors [39] but not with the reports from others [28,33,60]. To study the effect of therapy on angiogenesis, follow-up serum samples were collected in 34 of the 44 patients at the time of maximum response or at the end of treatment. Of interest, the levels of bFGF significantly increased after treatment in the overall series. Levels of bFGF significantly increased in previously untreated patients as well as in those who received dexamethasone. This increase in bFGF levels was observed regardless of response to therapy. Moreover, we found that levels of VEGF and bFGF significantly increased at the time of maximum response in previously untreated patients, while all cytokines except VEGF significantly increased at the time of maximum response to rescue therapy with dexamethasone. We have no explanation for these unexpected results, given that growth factors and cytokines are supposed to be markers of increased angiogenesis and, in consequence, expected to decrease with response to antiangiogenic therapy. It has been postulated that the angiogenesis process in responding patients could be perpetuated through the secretion of angiogenic cytokines by residual cells [18,61]. Perhaps the antimyeloma activity of these agents is not caused by a specific inhibition of angiogenic cytokine secretion and, as suggested by Neben et al. [60], an effect on cell surface receptors or intracellular signalling events could be more likely in patients treated with thalidomide.

We also investigated the relationship between serum levels of angiogenic factors and MVD. Unexpectedly, we found that our patients with high grade of angiogenesis had lower serum levels of VEGF and higher serum levels of TNF-α pre-treatment. Otherwise, Andersen et al reported positive correlations between MVD and serum levels of syndecan-1 and HGF, but not with bFGF or IL-6 [62], while Thompson et al. found no significant association between baseline BM MVD and the pre-treatment levels of any cytokine [39]. In fact, there has never been any strict correlation between "angiogenic" cytokines and bone marrow MVD. In consequence, they can not be reliably used as a surrogate for bone marrow angiogenesis.

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Gelatinase expression and proteolytic activity in giant-cell arteritis

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EXTENDED REPORT

Gelatinase expression and proteolytic activity in giant-cell arteritis

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Objectives: Gelatinases (MMP2 and MMP9) are expressed in giant-cell arteritis (GCA) and are thought to play a role in vessel disruption. However, their activation status and enzymatic activity have not been evaluated. Our aim was to investigate the distribution and proteolytic activity of gelatinases in GCA lesions at different stages.

Methods: Expression of MMP2, MMP9, MMP2-activator MMP14 and their natural inhibitors TIMP1 and TIMP2 was determined by real-time PCR and immunohistochemistry in temporal artery sections from 46 patients and 12 controls. MMP activation status and enzymatic activity were assessed by gelatin and film in situ zymography.

Results: Vascular smooth muscle cells from normal specimens constitutively expressed pro-MMP2 and its inhibitor TIMP2 with no resulting proteolytic activity. In GCA MMP2, MMP9 and MMP14 were strongly expressed in their active form by infiltrating leucocytes. Inflamed arteries also expressed TIMP1 and TIMP2. However, the MMP9/TIMP1 and MMP2/TIMP2 ratios were higher in patients compared with controls, indicating an increased proteolytic balance in GCA which was confirmed by in situ zymography. Maximal gelatinase expression and activity occurred at the granulomatous areas surrounding the internal elastic lamina (IEL). Myointimal cells also expressed MMPs and exhibited proteolytic activity, suggesting a role for gelatinases in vascular remodelling and repair.

Conclusions: GCA lesions show intense expression of gelatinases. Activators and inhibitors are regulated to yield enhanced gelatinase activation and proteolytic activity. Distribution of expression and proteolytic activity suggests that gelatinases have a major role not only in the progression of inflammatory infiltrates and vessel destruction but also in vessel repair.

Giant-cell arteritis (GCA) is a granulomatous vasculitis involving large and medium-sized arteries.[1] Histopathological patterns observed in involved vessels suggest that leucocytes invade the vessel wall through the adventitial vasa vasorum and surrounding small vessels.[2 3] This interpretation is supported by immunopathological studies showing that adhesion molecules necessary for leucocyte recruitment are mainly expressed by vasa vasorum.[4] Inflammatory infiltrates subsequently extend towards the adventitia and the medial layer where they undergo granulomatous differentiation.[1 2] At this stage, inflammatory cells can be additionally recruited through inflammation-induced neovessels.[4 5]

To invade the vessel wall, infiltrating leucocytes need to break the basement membrane of the vasa vasorum, and to migrate through the interstitial matrix. As inflammatory cells proceed across the artery wall, the internal elastic lamina (IEL) is disrupted, allowing the progression of leucocytes, as well as myointimal cells towards the intima.[2 6] Among the proteolytic systems participating in this process, gelatinases (MMP2 and MMP9) may have an important role, given their elastinolytic activity and their unique ability to degrade basement membranes.[7–9] Rupture of elastic fibres may lead to deleterious consequences such as the development of aortic aneurysms, an increasingly recognised complication of GCA.[10 11] The relevance of gelatinases in vascular destruction has been demonstrated, indeed, in animal models of aortic aneurysms.[12]

As with other proteolytic systems, MMP activity is tightly regulated at several levels. Gelatinase production is transcriptionally regulated, but post-transcriptional control of enzymatic activity is even more crucial. Gelatinases are secreted as inactive zymogens and need to be activated by proteolytic cleavage.[7 8 13] MMP2 is activated at the cell surface through a unique multistep pathway requiring MMP14 and tissue inhibitor of metalloproteinase 2 (TIMP2).[13 14] Active MMP2 is, in turn, one of the most efficient activators of MMP9.[7 8 13] Gelatinase activity is subsequently modulated by interaction with their natural inhibitors, TIMPs, by forming noncovalent 1:1 stoichiometric complexes. TIMP2 preferentially inhibits MMP14 and MMP2, whereas TIMP1 is a potent inhibitor of MMP9.[8 13]

Gelatinases are known to be expressed in GCA.[15–19] However, molecules modulating gelatinase activity such as MMP14 or TIMPs have not been evaluated or have been detected in only a few cases. In order to gain a better understanding of the physiopathological role of gelatinases in GCA, the aims of our study were to investigate the expression and distribution of gelatinases, TIMPs and MMP2-activator MMP14 at the mRNA and protein level, and to determine gelatinase activation status and resulting proteolytic activity in GCA lesions.

PATIENTS AND METHODS

Patients

We studied 46 patients with biopsy-proven GCA. Thirty-three patients had received no treatment before the temporal artery excision, whereas the remaining 13 had received 1 mg/kg/day of prednisone for 9±2.5 days (mean ± SEM). Unless otherwise indicated, only treatment-naive patients were considered in quantitative measurements. Twelve normal temporal arteries from patients in whom GCA was initially considered but

Abbreviations: GCA, giant-cell arteritis; IEL, internal elastic lamina; VSMC, vascular smooth muscle cells

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subsequently excluded served as controls. In all of them, symptoms were related to other conditions, and in none of them was the clinical suspicion strong enough to prescribe treatment in spite of a negative biopsy. The study was approved by the Ethics Committee of our institution, and all patients signed informed consent.

Specimens were embedded in OCT, snap-frozen in isopenthane prechilled in liquid nitrogen and stored at −80°C. Additional fragments from 6 patients and 2 controls were directly frozen in liquid nitrogen and stored at −80°C in order to perform gelatin zymography and western-blot analysis.

Histopathological evaluation

Temporal artery biopsies were classified according to the extension of inflammatory infiltrates. Sixteen specimens had inflammatory infiltrates limited to the vasa vasorum and adventitial layer. The remaining 30 had fully developed lesions with inflammatory infiltrates extending through the entire artery wall. The topographic distribution of MMPs was separately evaluated in both groups. Additional aspects evaluated were IEL integrity and extent of intimal hyperplasia. Elastic lamina was stained with 1% Shikata's orcein (Scharlau Chemie S.A., Barcelona, Spain) in 70% ethanol. IEL disruption was scored as follows: 1, IEL preserved in >80% of the circumference; 2, IEL preserved in 50–80%; 3, IEL preserved in 30–50%; and 4, IEL remaining in <30%. Intimal hyperplasia was scored from 0 to 4 as described.[5]

Immunostaining

Serial 4–6-μm temporal artery sections from the 46 patients and 12 controls were incubated with the following primary antibodies: monoclonal mouse antihuman MMP9 (clone GE-213) (Chemicon International, Inc., Temecula, CA) at 1/1000 dilution, polyclonal rabbit antihuman MMP2 (Chemicon) at 1/500 dilution, polyclonal rabbit antihuman MMP14 (Chemicon) at 1/250 dilution, monoclonal mouse antihuman TIMP1 (clone Ab-2) (Calbiochem, Cambridge, MA) at 1/40 dilution and monoclonal mouse antihuman TIMP2 (clone Ab-1) (Calbiochem) at 1/40 dilution. Immunoglobulins obtained from the same species were used as negative controls. Immunodetection was carried out with an HRP-labelled polymer conjugated to secondary antibodies (EnVision kit from Dako, Carpinteria, CA), as reported.[20 21]

Quantification of the immunostaining at the granulomatous area was performed in the 30 specimens with fully developed lesions according to a semiquantitative 0–4 score, as described.[20 21]

mRNA quantification

Total RNA was obtained from 150 serial sections (5 μm thick) per biopsy using TRIzol (Invitrogen, Carlsbad, CA). RNA could be obtained from 35 patients (27 untreated and 8 treated) and from the 12 controls. One microgram of total RNA was reverse-transcribed to cDNA using the Archive kit (Applied Biosystems, Foster City, CA). Samples were stored at −20°C until use.

MMP2, MMP9, MMP14, TIMP1 and TIMP2 expression was measured by real-time quantitative PCR using specific Assay-on-Demand Taqman Gene expression probes from Applied Biosystems.[20 21]

Film in situ zymography (FIZ)

Topographic distribution of gelatinase activity was assessed by FIZ (Fuji Photo Film Co., Ltd, Tokyo, Japan). Five-micrometre-thick cryostat sections from the same biopsy samples used for immunostochemistry were applied to 7-μm polyester membranes cross-linked with gelatin or with gelatin containing the gelatinase inhibitor (1,10)-phenanthroline as a control for specificity. Films were incubated for 20 h in a moist chamber at 37°C and stained with 0.5% Amido Black 10B (Sigma) in 70% methanol, 10% acetic acid for 10 min and distained by washing in 70% methanol, 10% acetic acid. Gelatinase activity was visualised as destained areas on a dark-blue background.

Gelatin zymography

Frozen temporal artery samples (0.5 cm long) from 6 patients and 2 controls, were homogenised in 1 ml of TRIzol. Given the substantial amount of tissue required, this study could not be extended to more specimens. One hundred micrograms of total protein per sample, extracted according to the manufacturer's instructions, was subjected to gelatin zymography as described.[22 23]

Western-blot

Twenty micrograms of protein obtained from the homogenised arteries was subjected to SDS-PAGE and transferred onto nitrocellulose. Membranes were incubated overnight at 4°C with a polyclonal rabbit antihuman MMP14 (Chemicon) at 1:1000 dilution. Immunodetection was performed as published.[23]

Statistics

The Mann–Whitney U test was used to compare quantitative variables and Spearman test for correlations, and the Fisher exact test was used for contingency tables.

RESULTS

MMP expression in temporal artery biopsies from patients with GCA and controls

No MMP9 or MMP14 expression was detected by immunohistochemistry in normal arteries. In contrast, MMP2 was expressed by vascular smooth muscle cells (VSMC) in the medial layer, as previously observed[15–17 19] (fig 1). MMP9 and MMP14 mRNA were detected at low concentrations in normal biopsies but were significantly more abundant in samples from patients with GCA (fig 2). No differences were found in MMP2 mRNA between patients and controls, further supporting constitutive MMP2 expression in non-inflamed arteries.

MMP9 and MMP14 were detected by immunohistochemistry in all specimens with GCA lesions and were mainly expressed by inflammatory cells. Although, at the mRNA level, no differences were found in MMP2 expression between patients and controls, immunostaining revealed important differences in MMP2 distribution. In normal arteries, MMP2 was mainly expressed by VSMC, whereas in GCA specimens MMP2 was expressed not only by remaining VSMC but also, and more intensively, by infiltrating leucocytes (fig 1). The pattern of MMP expression varied according to the extent of inflammatory involvement (fig 1). In specimens with inflammatory infiltrates restricted to vasa vasorum and adventitial layer, MMPs were expressed by adventitial inflammatory cells, and MMP2 was also expressed by preserved VSMC at the media, similarly to normal arteries. In GCA arteries with fully developed lesions, VSMC were destroyed, and MMPs were intensively expressed by the granulomatous infiltrates at the media and intima/media junction. MMP2, MMP9 and MMP14 were also expressed by myointimal cells at the hyperplasic intima (fig 1).

Immunostaining scores for all 3 MMPs were significantly correlated (MMP2 vs MMP9, $r = 0.80$, $p<0.0001$; MMP2 vs MMP14, $r = 0.78$, $p<0.0001$; MMP9 vs MMP14, $r = 0.78$, $p<0.0001$). In accordance with previous studies showing coordinated expression of MMP2 and MMP14,[22 23] a significant correlation between MMP2 and MMP14 mRNA was observed ($r = 0.39$, $p = 0.048$). Intriguingly, there was a negative

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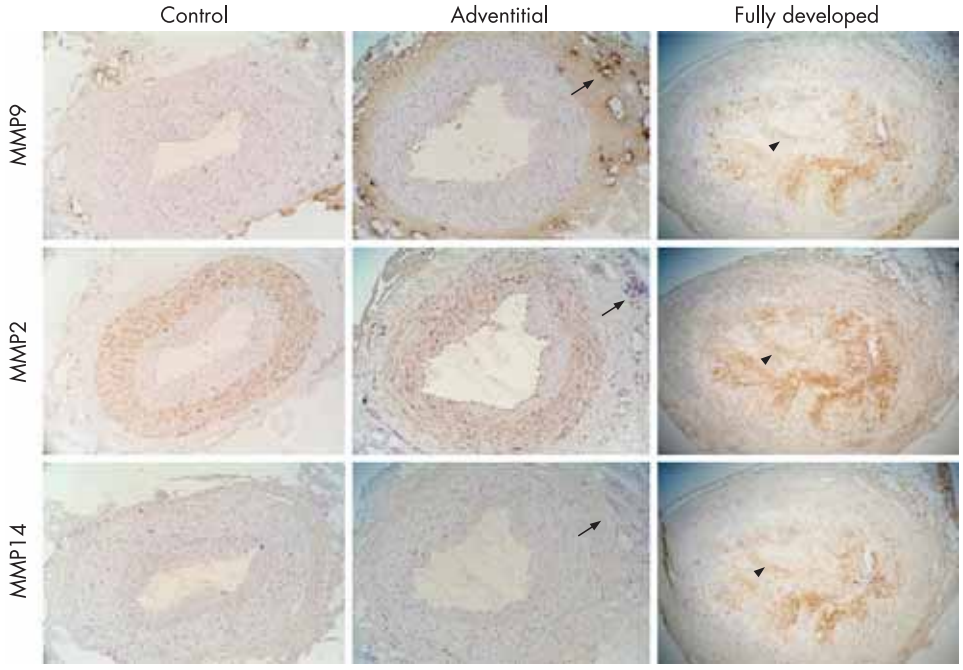

Figure 1 Expression of MMP2, MMP9 and MMP14 in serial sections of temporal arteries from controls and from patients with GCA, according to the extent of inflammatory infiltrates. In specimens with inflammatory infiltrates involving vasa vasorum and adventitia only, MMP expression was similar to controls, with some MMP2 and MMP9 expression by inflammatory cells (arrows). In fully developed lesions, expression predominates at the granulomatous areas. MMP expression by myointimal cells surrounding the lumen can also be observed (arrowheads).

correlation between MMP2 and MMP9 mRNA concentration ($r = -0.45$, $p = 0.019$).

Activation status of MMPs in GCA

In order to assess the activation status of gelatinases in GCA lesions, temporal artery protein extracts were subjected to gelatin zymography (fig 3A). Interestingly, in control arteries, both MMP9 and MMP2 pro-enzymes could be detected by this sensitive method, even though immunohistochemistry was only able to detect MMP2. No activated forms of gelatinases were detected in normal samples. In contrast, in fully inflamed arteries, activated forms of both gelatinases were present. Interestingly, in the artery with inflammatory infiltrates restricted to the adventitia with a preserved media, the activated form of MMP9 was apparent, but MMP2 was mostly present in its latent form, as in control arteries (fig 3A).

MMP14 detection by western blot in temporal artery biopsy extracts disclosed a 45-kDa form in addition to the 57-kDa active species (fig 3B). The 45-kDa form is inactive and results from proteolytic removal of the catalytic domain of MMP14 by active MMP2 and by active MMP14 itself.[24 25] This constitutes a counter-regulatory mechanism limiting MMP14 activity. The generation of the 45-kDa form requires, thus, full activation of the MMP14/MMP2 system, and its detection in lesions further indicates that the MMP2/MMP14 system is functionally operative. As shown in fig 3B, active MMP14 and particularly its 45-kDa form were more abundant in GCA patients than in controls.

TIMP counterbalance

Like MMP2, its inhibitor TIMP2 was constitutively expressed by VSMC in normal biopsies, at both the mRNA and protein level (fig 4 and on-line figure). As mentioned, TIMP2 is necessary for MMP2 activation by MMP14.[13 14] Constitutive TIMP2 expression guarantees that MMP2 can be activated when required but, at the same time, maintains constitutive MMP2 functionally inactive in quiescent arteries.

In GCA samples, TIMP1 mRNA was significantly upregulated, whereas TIMP2 mRNA significantly decreased compared with controls (fig 4). Immunohistochemical analysis showed that, in GCA biopsies, TIMP1 was expressed by inflammatory cells, whereas TIMP2 was expressed by both VSMC and infiltrating leucocytes (on-line figure). Distribution of gelatinases was, thus, coincident with that of their respective inhibitors and may constitute a counter-regulatory mechanism restricting the destructive potential of gelatinases, However, the MMP9/TIMP1 and MMP2/TIMP2 mRNA ratios were significantly higher in GCA specimens compared with controls (fig 4). In agreement with this observation, the intensity and extent of immunostaining were lower for TIMPs than for MMPs. MMP9 median score was 3 (range 1–4), whereas the TIMP1 median score was 2 (range 1–3); $p = 0.015$. The MMP2 median score was 3 (range 1–4), whereas the TIMP2 median score was 2 (1–3); $p = 0.03$. (on-line figure). These findings indicate that, in inflammed arteries, MMP/TIMP balance favours proteolytic activity.

Gelatinolytic activity of MMP2 and MMP9 in GCA

As shown in fig 5, in accordance with the inactive status of MMP2 expressed by VSMC, normal temporal arteries did not exhibit proteolytic activity. In arteries with inflammatory infiltrates limited to the adventitia, a weak gelatinolytic activity could be observed in areas with inflammatory infiltrates. No gelatinolytic activity could be observed in the media, as in control specimens. In fully developed lesions, a strong gelatinolytic activity was observed in inflamed zones, particularly in granulomatous areas at the media and at the intima/media junction. Myointimal cells also exhibited proteolytic activity (fig 5). Gelatinase activity, thus, required co-expression of MMP-9, MMP2 activator MMP14 and activated MMP2, which in turn may activate MMP9. According to the elevated MMP/TIMP ratio observed, colocalisation of MMPs with their respective inhibitors did not prevent gelatinolytic activity (on-line figure). Unfortunately, this system could not discern how much MMP9 or MMP2, respectively, contributed to the resulting proteolytic activity.

Effect of corticosteroids on MMP expression

Samples from patients who had received corticosteroids had significantly lower immunohistochemical scores for MMPs than samples from untreated patients (fig 6). However, no significant differences in MMP2 and MMP14 mRNA levels

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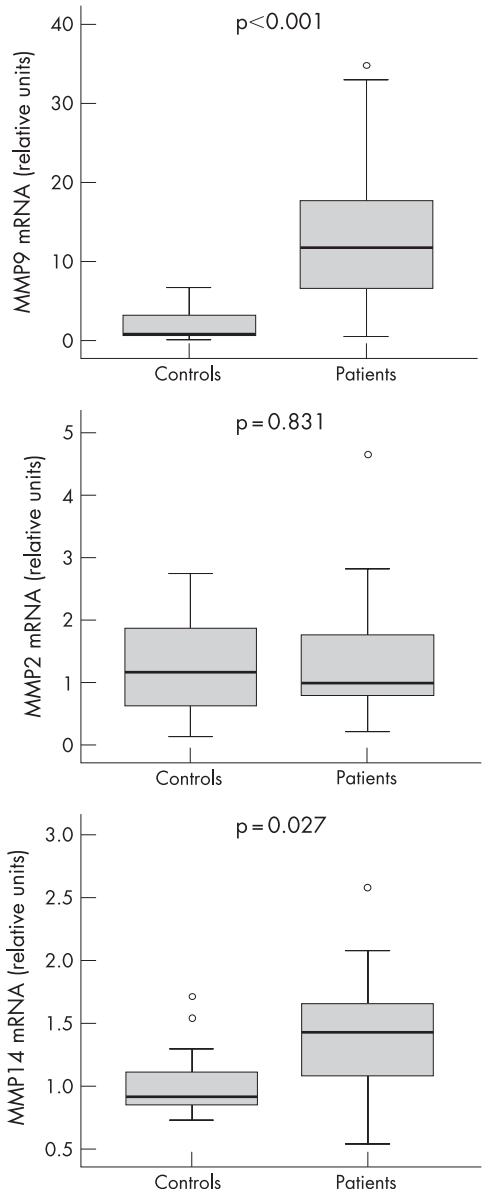

Figure 2 MMP9, MMP2 and MMP14 mRNA concentrations in temporal arteries from 27 untreated GCA patients and 12 controls.

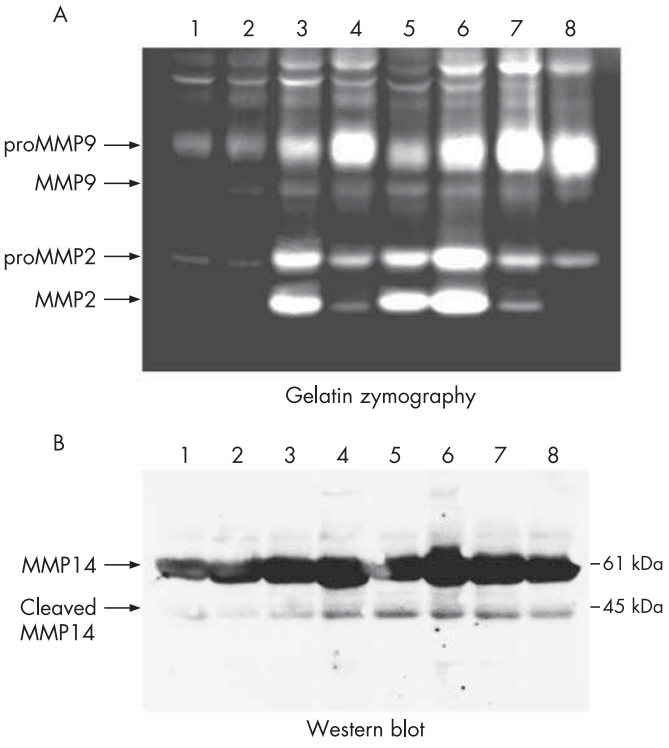

Figure 3 Activation status of MMPs. (A) Gelatin zymography of temporal artery extracts disclosing gelatinolytic bands corresponding to zymogens and activated forms of MMP2 and MMP9. Lanes 1–2 are controls, lanes 3–7 samples with fully developed lesions, and lane 8 corresponds to a specimen with adventitial inflammation only. (B) Detection of MMP14 in the same samples by western blot.

could be found between untreated patients and those who had received treatment (MMP2: 1.02 (0.23–4.67) vs 1.43 (0.58–6.93), p = 0.43; MMP14: 1.44 (0.55–2.54) vs 1.32 (0.83–2.48), p = 0.81). MMP9 mRNA clearly tended to decrease, but the difference did not reach statistical significance (11.74 (0.59 – 34.78) vs 5.08 (2.29–23.55), p = 0.11).

Potential role of gelatinases both in vascular destruction and vascular remodelling

High (3–4) IEL disruption scores were significantly more frequent in temporal arteries with fully developed lesions than in those with adventitial inflammation only (OR 31, 95% CI 3.02–329.1, p = 0.0011), supporting a relevant elastinolytic activity of macrophages at the granulomatous area. The participation of MMP2 and MMP9 in this process is supported by the localisation of maximal gelatinolytic activity in the granulomatous area. IEL rupture positively correlated with the extent of intimal hyperplasia (r = 0.5, p = 0.036), suggesting that, through MMP expression, myointimal cells may contribute and, at the same time, take advantage of the breakdown of IEL to migrate towards the intima.

However, in samples with fully developed lesions, no significant correlation was observed between MMP immunohistochemical scores and IEL disruption scores (MMP9 r = 0.22, p = 0.38; MMP2 r = 0.34, p = 0.17; MMP14 r = 0.22, p = 0.38) or intimal hyperplasia scores.

DISCUSSION

Gelatinase expression has been previously reported in GCA,[15–19] but this is the first attempt to investigate gelatinase functional regulation and activity. Indeed, gelatinase expression was not always associated with enzymatic activity. Normal specimens and samples with infiltrates restricted to the adventitia did not disclose gelatinolytic activity in the media in spite of significant MMP2 expression by VSMC. These findings indicate that constitutively expressed MMP2 is not functionally active and that additional stimuli (ie, inflammation or injury) are required for its activation. As shown by gelatine zymography, MMP2 was produced, indeed, as a pro-enzyme in normal arteries whereas, in GCA lesions, MMP2 was activated, likely as a consequence of concomitant induction of MMP14. Moreover, MMP2 inhibitor TIMP2, intensively expressed by VSMC in normal specimens, decreased in GCA, favouring MMP-2 proteolytic balance in inflamed arteries.

MMP9 was strongly upregulated and activated in GCA. MMP9 inhibitor TIMP1, which is induced by inflammatory mediators,[26] was also upregulated in GCA. However, MMP9/TIMP1 ratio favoured proteolysis in lesions. Maximal gelatinolytic activity occurred in areas where coexpression of the three MMPs investigated was observed in inflammatory or in myointimal cells. These findings suggest that gelatinolytic activity in GCA requires coordinated regulation of the whole

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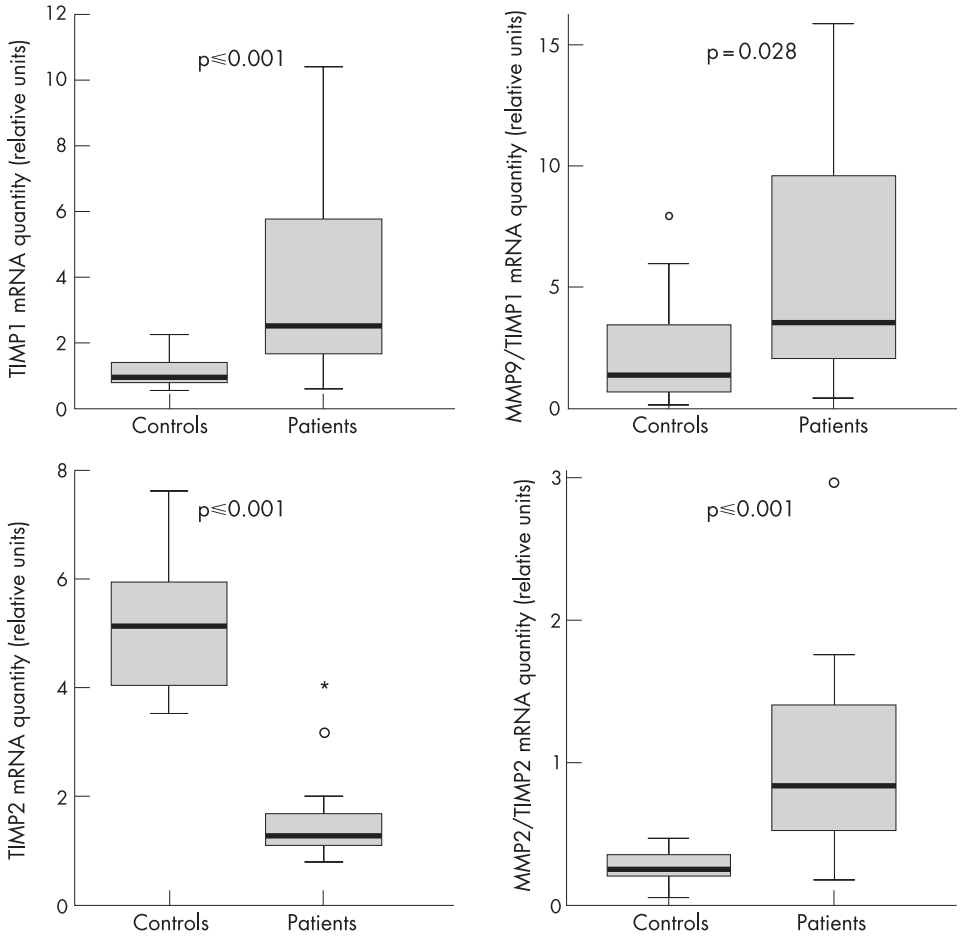

Figure 4 TIMP1 and TIMP2 mRNA concentrations in temporal arteries from 27 untreated GCA patients and 12 controls. MMP9/TIMP1 and MMP2/TIMP2 ratios in the same specimens.

system: increased expression of MMP9 and MMP14, activation of MMP2 by MMP14, subsequent activation of MMP9 by MMP2, and absolute or relative downregulation of TIMPs.

Although, at the protein level, there was a positive correlation between MMP2, MMP9 and MMP14 expression scores in granulomatous areas, a negative correlation between MMP2 and MMP9 transcripts was observed. Since MMP9 is mainly expressed by infiltrating leucocytes, increased MMP9 transcripts may indicate intense inflammatory activity, resulting in destruction of the medial layer and decreasing the contribution

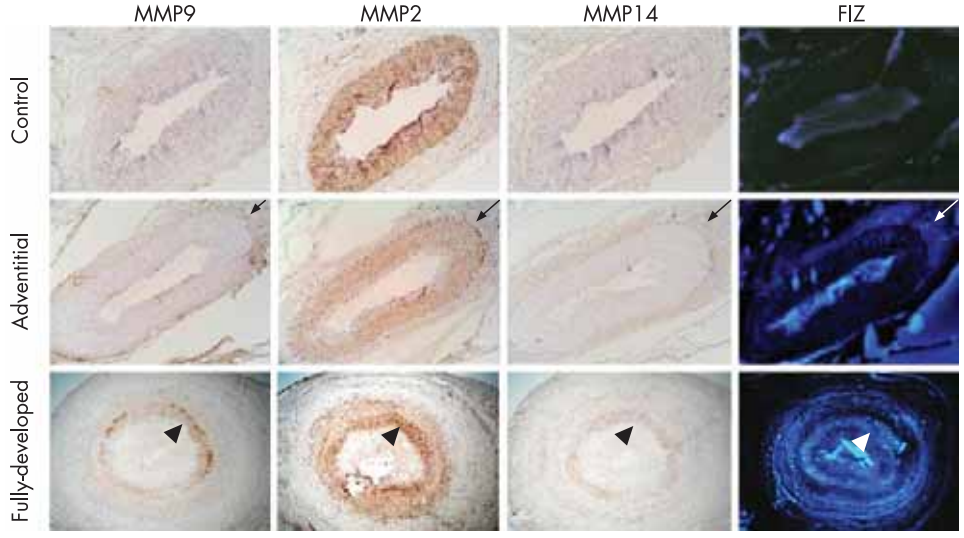

Figure 5 Gelatinolytic activity of MMP2 and MMP9 according to the extension of inflammatory infiltrates. In spite of strong MMP2 expression in the media of normal temporal arteries and of specimens with adventitial inflammation only, gelatinolytic activity appears only in the areas with inflammatory infiltrates (arrows). Activity is maximal in fully developed lesions, particularly at the granulomatous areas (arrowheads). Gelatinolytic activity can also be observed in the hyperplastic intima of fully developed lesions.

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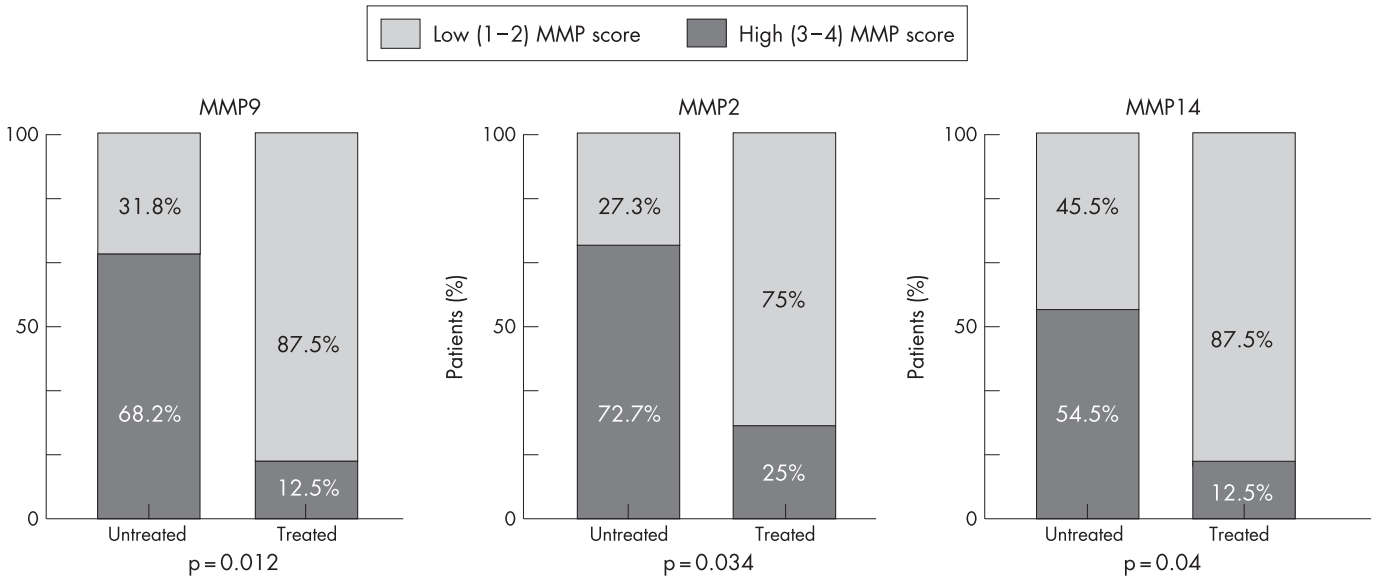

Figure 6 Effect of corticosteroid treatment on MMP expression in the granulomatous areas. Graphs show the percentage of specimens disclosing high (3–4) versus low (0–2) immunohistochemical scores, evaluated in the 30 samples with fully developed granulomatous lesions (22 from treatment-naive patients and 8 from patients treated with corticosteroids).

of VSMC to MMP2 expression. Moreover MMP2-deficient mice have increased production of MMP9, and a negative regulation of MMP2 on MMP9 expression has been demonstrated.[27 28]

Production of active MMPs by infiltrating leucocytes may be crucial in the development of vascular inflammation by allowing leucocyte progression across the vessel wall.[29 30] Moreover, gelatinases modulate bioavalability and cleavage of many cytokines, chemokines and growth factors, resulting in additional regulatory functions in inflammatory diseases. The recent observation that MMP2-null mice develop more severe lesions in various models of chronic inflammatory diseases[27 28] indicates that the roles of MMPs in inflammation are far more complex than anticipated.

Gelatinases may contribute to vessel-wall disruption in GCA, which may convey severe complications such as the development of aneurysms. Maximal gelatinolytic activity occurred in the granulomatous areas where activated macrophages have been demonstrated to have additional destructive activities, such as production of reactive oxygen species and nitric oxide.[31] This location suggests, indeed, a role for gelatinases in the destruction of IEL. In fact, specimens with adventitial inflammation only, which had no gelatinolytic activity at the media, had significantly more preserved IELs than arteries with fully developed lesions, which showed significant enzymatic activity at the intima/media junction. However, in specimens with fully developed lesions, no correlation was found between the intensity of gelatinase expression and the extent of IEL disruption. This observation indicates that regulation of MMP enzymatic activity may have a stronger functional impact than regulation of MMP expression. Moreover, elastin-degrading enzymes produced in the granulomatous area may include enzymes other than MMP2 or MMP9.

In specimens with fully developed inflammatory lesions, myointimal cells also expressed MMPs. The development of intimal hyperplasia is a significant source of morbidity in patients with vasculitis.[1 5] However, it provides a mechanism reinforcing the vessel wall when IEL is destroyed. Intimal hyperplasia correlated, indeed, with the extent of IEL disruption, as observed by others.[32] IEL degradation may promote intimal hyperplasia by facilitating myointimal cell activation and migration towards the intima.[1 2 33] However, in arteries with fully developed lesions, no significant correlation was found between the extent of intimal hyperplasia and the intensity of MMP expression. MMP may have a dual function in vascular remodelling: by disrupting IEL may promote and allow myointimal cell migration but, at the same time, increased MMP expression and activity may prevent excessive matrix deposition and lumen occlusion.

Immunostaining scores at the granulomatous area were significantly lower in samples from patients who had received corticosteroids. Interestingly, although MMP9 mRNA tended to decrease, no significant differences were found in MMP mRNA concentrations. Corticosteroids exert post-transcriptional and post-translational regulatory activities, which may regulate protein and mRNA levels differently,[34–36] and the treatment period may not have been long enough to downregulate the whole system efficiently. Moreover, although MMP expression in granulomatous areas may decrease upon corticosteroid treatment, MMP, and particularly MMP2, mRNA may be upregulated in regenerating VSMC during the process of vascular repair.

In summary, increased MMP expression and activity are prominent in GCA. The pattern of MMP expression and activity supports complex roles not only in vessel-wall inflammation and disruption, but also in vascular remodelling and repair. A better understanding of the specific roles of various MMPs at different disease stages is necessary before MMPs can be considered therapeutic targets to limit vessel inflammation and destruction in GCA.

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Dual function of focal adhesion kinase in regulating integrin-induced MMP-2 and MMP-9 release by human T lymphoid cells

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ABSTRACT

Integrin engagement induces matrix metalloproteinase (MMP) production by lymphocytes, allowing their progression into tissues. Focal adhesion kinase (FAK) is a key component of integrin-mediated signaling pathways regulating cell migration. We explored the role of FAK in integrin-induced gelatinase production by Jurkat T cells. Elevation of FAK expression by transient transfection increased cell invasiveness and gelatinase production and release driven by fibronectin. FAK point mutants revealed that gelatinase release was not dependent on FAK kinase activity but did require Y397, a binding site for Src-type tyrosine kinases. Requirement of Src kinases was further demonstrated by transfection with Src kinase-deficient mutants and treatment with a Src inhibitor. Transfection of truncated forms demonstrated dual functional elements in the FAK molecule. The FRNK fragment decreased gelatinase release, whereas the FAT subfragment enhanced it. FRNK inhibitory signals were transduced through Src-dependent pCAS phosphorylation and subsequent ERK1/2 activation. In contrast, FAT stimulated gelatinase secretion, which was also dependent on Src-kinase activity, was associated with a decreased ERK1/2 phosphorylation. This dual function of FAK in gelatinase secretion is then associated with changes in ERK1/2 activation status, a pathway coordinating cycles of adhesion/release required for cell migration and defines a novel regulatory step in the complex control of MMP function.

Key words: matrix metalloproteinases • lymphocytes • fibronectin

Lymphocyte transmigration into tissues is essential for many physiological and pathological conditions, including immune surveillance, graft rejection, lymphoma dissemination, host defense against tumors, and the development of inflammatory infiltrates underlying chronic inflammatory diseases (1–4). Tissue infiltration by lymphocytes requires focal degradation of the basement membrane and interstitial matrix (5, 6). To accomplish this process, lymphocytes produce small amounts of proteases among which matrix metalloproteinases (MMP) have a significant role based on their ability to degrade virtually any component of the extracellular matrix.

Cells from the lymphoid lineage are able to produce several MMPs (7–10). Among them, gelatinases appear to have a major role in lymphocyte transmigration due to their ability to degrade type IV collagen, a major component of basement membranes. In addition to extracellular matrix breakdown, gelatinases and other MMPs have important additional functions by cleaving a wide array of proteins such as adhesion molecules, cytokines, chemokines and growth factors, by exposing functionally active cryptic sites of large extracellular matrix proteins and by allowing access of cells to growth factors sequestered in the surrounding matrix (4, 11–15). This newly expanded range of biological functions may have unexpected impact on the regulation of inflammatory disorders and in early stages of tumor development (16, 17).

The mechanisms regulating gelatinase production and secretion by lymphocytes are largely unknown. Several cytokines, chemokines, and growth factors have been demonstrated to induce pro-MMP9 and to a lesser extent pro-MMP2 in lymphocytes (18–20). However, the most effective mechanism not only in inducing but also in activating both gelatinases appears to be integrin-dependent lymphocyte adhesion (7, 21, 22). Integrin engagement by cell membrane counter receptors (VCAM-1) or by extracellular matrix proteins, particularly fibronectin (FN), can elicit not only gelatinase production but also MMP-2 activation by inducing coordinated expression of MMP-14 (7). Among integrins, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ have been demonstrated to mediate adhesion-dependent gelatinase production and activation by T cells (7, 21, 22).

The signaling pathways involved in integrin-mediated gelatinase production by lymphocytes are unknown. On the basis of the results obtained from studies performed in other cell types, particularly fibroblasts, integrin engagement is followed by the recruitment of adaptor molecules connecting with cytoplasmic kinases and cytoskeletal proteins, leading to the formation of multimolecular complexes named focal adhesions (23, 24). These structures appear to be crucial for mediating the cycles of adhesion/release necessary for cell motility (25). Equivalent structures develop upon lymphocyte adhesion and migration (26).

A crucial molecule in focal contacts is focal adhesion kinase (FAK) (27). FAK and its paralog proline-rich tyrosine kinase-2 (Pyk-2) are expressed in cells of the lymphoid lineage (26). FAK is involved in the regulation of integrin-dependent signals regulating cell motility and survival (28–30). FAK has been shown to promote an aggressive phenotype in human tumors and is overexpressed in some lymphoproliferative disorders (31–35).

Upon integrin binding to the extracellular matrix, focal adhesion assembly occurs through incompletely characterized mechanisms (36, 37). FAK interacts with integrins at focal adhesions through direct binding of its N-terminal domain to the cytoplasmic tail of integrin β chains, or indirectly by binding of its C-terminal region to cytoskeletal proteins such as paxillin and talin

that bind to integrin chains (38, 39). Subsequently, FAK is activated by phosphorylation at residue Y397, providing a binding site for Src-type tyrosine kinases (40, 41), which, in turn, phosphorylate additional tyrosine residues, including Y925, located within the focal adhesion targeting (FAT) sequence of the FAK C-terminal domain. Phosphorylated Y925 has been suggested to recruit the adaptor protein Grb2, leading to the activation of the GTP binding protein Ras, which can trigger a signaling cascade, leading to ERK1/2 activation (42). Increasing evidence indicates that ERK1/2 can be also activated by alternative mechanisms and sites on FAK (43–45).

Interestingly, high activation of the Ras/Raf-1/MEK1/ERK1/2 pathway leads to a decrease in integrin avidity, facilitating focal adhesion turnover and promoting cell migration (46, 47). Moreover, FAK has two proline-rich domains that allow the assembly of additional signaling complexes, among which pCAS (Crk-associated substrate) and GRAF (GTPase-activating protein for Rho associated with FAK) are the best characterized (48, 49). The role of FAK in focal adhesion turnover is underscored by the increases in focal adhesion formation and cell adherence associated with reduced cell motility displayed by FAK–/– cells (50, 51).

Given the seminal role of FAK in integrin-mediated cell migration, the aim of our study was to assess the role of FAK in integrin-mediated gelatinase production by T lymphoid cells. By transfection of FAK wild-type and FAK-truncated forms and point mutants, we found that FAK transmits both stimulatory and inhibitory signals for gelatinase production and release. This dual role of FAK is achieved through its scaffolding function rather than through its intrinsic kinase activity. Stimulatory and inhibitory signals for gelatinase expression coordinated by FAK are transmitted through pathways known to control focal adhesion formation and disassembly required for cell migration.

MATERIALS AND METHODS

Cell culture

Human T lymphoblastoid cell lines (Jurkat and J.CaM1.6) were obtained from the European Collection of Cell Cultures (Salisbury, UK). Peripheral blood mononuclear cells were obtained from a healthy donor and depleted from monocytes and B cells by nylon wool adherence. After this procedure, primary T cell purity was 97% as assessed by flow cytometry. T lymphoblasts were generated by culturing primary T cells with phytohemagglutinin (Gibco Life Technologies, Grand Island, NY) at 2.5 μg/ml during the first 48 h and then stimulated with interleukin-2 at 25 U/ml until 2 days before use. Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (Biological Industries, Kibbutz, Beit Haemec, Israel), 2 mM L-glutamine and 50 μg/ml gentamycin at 37°C in 5% CO_2, and adjusted to a concentration of 0.3×10^6 cells/ml the day before performing the experiments.

Inhibitors

PP2, GM6001, curcumin, and PD98059 were purchased from Calbiochem (La Jolla, CA). Actinomycin D, brefeldin A, monensin, and wortmannin were obtained from Sigma (St. Louis, MO). SP600125 was a generous gift from Dr. B. Bennett (Celgene, San Diego, CA). Inhibitors were added to the cells 30 min before addition of fibronectin (Calbiochem). Except when

otherwise indicated, cells were exposed to drugs during experiments for 4 h. Cell viability was confirmed by trypan blue exclusion.

Transient transfection

FAK wild-type cDNA and FAK point mutants K454R, Y397F and Y925F, were expressed using the pcDNA3 vector containing the hemagglutinin (HA) epitope generated, as described previously (45). The FAK truncation form termed FRNK and the point mutants FAK P712/715A and FRNK P712/715A were expressed using the pRK-VSV vector containing the vesicular stomatitis virus (VSV) epitope (43, 52). A shorter FAK construct containing FAT domain was constructed in pRK-VSV as follows: the fragment of FAK encoded by the DNA sequence 2630-3268 of GenBank accession number M95408 was PCR amplified from the HA-FAK plasmid using the following forward and reverse primers: 5′-GGATCCGGATCCCGAGGCAGCATCGACAGGGAAGA-3′ and 5′-TCTAGATCTAGATCAGTGTGGCCGTGTCTGCCCTA-3′. The PCR product was digested with BamHI and XbaI and cloned into pRK-VSV. The Src-type kinase-deficient mutants Fyn KD and Lck KD were inserted into pSRα-puro (a kind gift from Dr. O. Acuto, Molecular Immunology Unit, Institut Pasteur, France) and wild-type Lck was subcloned into pSRα (prepared by Dr. C. Serra, Immunology Department, Hospital Clinic, Barcelona, Spain).

Superfect Transfection Reagent (Qiagen, Valencia, CA) was used for transient transfection of Jurkat cells following the protocol advised by the manufacturer, using 2 μg of cDNA for 2.5 × 10^6 cells in a 6-well plate.

Primary T cells were transiently transfected with the Nucleofector kit (Amaxa, Cologne, Germany), following the protocol recommended for stimulated human primary T cells. Transfection efficiency was estimated by cotransfecting the cells with pEGFP-C3 or pSRα-HA-GFP. Cells were used for experimental procedures 48 h post-transfection. Equivalent levels of transfection were verified by Western blot analysis of cell lysates. Experiments were repeated at least 4 times with consistent results.

Gelatin zymography

Cultured Jurkat cells were washed twice with plain RPMI 1640 and resuspended at 0.5 × 10^6 cells/ml. FN was added at 10 μg/ml, and 5 × 10^6 cells per condition were incubated at 37°C in 5% CO_2. Unless otherwise specified, the supernatant fluid was collected 4 h later and concentrated 200-fold with Urifil-10 concentrator devices (Millipore, Molsheim, France). Concentrated samples were analyzed as described previously (7).

RT-PCR

RNA was extracted from 5 × 10^6 Jurkat cells using TRIzol Reagent (Gibco) following the manufacturer's instructions. One microgram of RNA was reverse-transcribed into cDNA using Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA).

Primers for MMP-2 amplification were 5′-GGCACCCATTTACACCTACACCAA-3′ (position 1552-1575) (sense) and 5′-GCTTCCAAACTTCACGCTCTTCAG-3′ (position 2245-2222)

(antisense); for MMP-9, 5′-CTCCTGCTCCCCCTGCTCACG-3′ (position 376-395) (sense) and 5′-CGGGTGTAGAGTCTCTCGCT-3′ (position 855-836) (antisense) and for MMP-14, 5′-CAACATCACCTATTGGATCC-3′ (position 265-285) (sense) and 5′-CTCACCCCCATAAAGTTGCTG-3′ (position 1092-1072) (antisense). The expected sizes for the amplification products were 670 bp (MMP-2), 480 bp (MMP-9) and 828 bp (MMP-14). TIMP-1 and TIMP-2 were detected as described previously (7).

Thirty-five reaction cycles were run, each consisting of three steps of 45 s at 94°C, 57°C (for MMP-2 and MMP-9) or 60°C (MMP-14), respectively, followed by an elongation period of 10 min at 72°C. PCR products were analyzed in 1.2% agarose gels (Invitrogen). Multiplex amplification of β2-microglobulin was used as an internal control.

Adhesion assay

Ninety-six well plates were coated with FN (50 μg/well) overnight at 4°C, and the remaining fluid was aspirated. Cells were suspended in serum-free medium, plated on FN-coated wells at 0.15×10^6 cells/well and incubated at 37°C for 1 h. Nonadherent cells were aspirated and the remaining cells were fixed and stained with 0.2% crystal violet (Sigma) in 20% methanol. Wells were washed with distilled H_2O and air-dried. Dye was solubilized with 1% SDS, and optical density was read with a spectrophotometer at 600-nm wavelength. Conditions were tested in quadruplicate wells.

Invasion assay

Ten micrometer-pore polycarbonate filters (Nucleopore, Toronto, Canada) were coated with Matrigel (kindly provided by Dr. Hynda K. Kleinman, National Institutes of Health, Bethesda, MD) diluted in RPMI 1640 at 1:8, and placed between the lower and the upper compartment of 48-well Boyden chambers (Neuro Probe Inc., Gaithersburg, MD). The lower compartments were filled with 25 μl RPMI 1640 with 10% FCS and 0.1×10^6 cells in serum-free medium and were loaded onto the upper chambers. After 6 h-incubation at 37°C, the filter was removed, fixed with methanol, and stained with hematoxylin. Cells on the upper side were swept, and cell number/field in the lower side was counted in 6 randomly selected fields/well. Experiments were performed in quadruplicate wells.

Western blotting

5×10^6 cells per condition were incubated with FN at 10 μg/ml in serum-free RPMI 1640 medium and were lysed with 0.5 ml of modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton-X-100, 2 mM EDTA, 1% sodium deoxycholate, 0.1% SDS) supplemented with freshly added protease inhibitors (Complete, Boehringer Mannheim, Mannheim, Germany) and Na_3VO_4 at 200 μM. Protein content of lysates was measured with the BCA protein assay (Pierce, Rockford, IL).

Twenty micrograms of lysate per condition were subjected to SDS-PAGE and transferred onto nitrocellulose. Membranes were incubated overnight at 4°C with the appropriate primary antibody: FAK variants were detected with a rabbit polyclonal antibody against the carboxy-terminal domain (anti FAK C-20 from Santa Cruz Biotechnology, Santa Cruz, CA) at 1:400

dilution. A mouse monoclonal antibody anti-Lck (3A5) (Santa Cruz Biotechnology) was used to assess Lck content at 1:500 dilution. ERK activation state was evaluated using anti-phospho-p44/42 MAP kinase and then reprobed with p44/42 MAP kinase rabbit polyclonal IgG antibodies (Cell Signaling, Beverly, MA) at 1:1000 dilution. JNK MAP kinase required a preceding enrichment procedure. Cell lysates were incubated overnight at 4°C with SAPK/JNK rabbit polyclonal IgG antibody (Cell Signaling) at 1:100 dilution and immunoprecipitated with GammaBindPlus Sepharose beads (Amersham Biosciences, Uppsala, Sweden). Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose. The phosphorylation state of JNK was detected using phospho-JNK/SAPK rabbit polyclonal IgG antibody (Cell Signaling) at 1:1000 dilution. In parallel experiments, a mouse monoclonal anti-phosphotyrosine (4G10) from Upstate Biotechnology at 1 μg/ml was used as a primary antibody and, after immunodetection, blots were stripped and reprobed with a rabbit polyclonal antibody against the N-terminal fragment of pCAS (N-17) (Santa Cruz Biotechnology) at 1:300 dilution.

Immunodetection was performed by incubating membranes with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit HRP-conjugated, Cell Signaling or anti-mouse HRP-conjugated, Transduction Laboratories, Lexington, KY) at 1:2000 dilution. Chemiluminescence signals were detected with LUMIGLO chemiluminescent reagent (Cell Signaling). Membranes were exposed to *X*-ray film Hyperfilm ECL (Amersham Life Science, Buckinghamshire, England) and developed with a Kodak RP-X-OMAT processor.

RESULTS

Focal adhesion kinase regulates integrin-dependent gelatinase (MMP-2 and MMP-9) expression and release by T lymphoid cells

Transient transfection of FAK wild-type cDNA into Jurkat T cells and into primary T cells increased MMP-2 and MMP-9 production in response to FN, as assessed by gelatin zymography of the conditioned medium, consistent with a role for FAK in the regulation of integrin-mediated gelatinase production (Fig. 1*A*). The increase in gelatinase production was followed by an increase in invasiveness, which was inhibited by the MMP inhibitor GM6001 (Fig. 1*B*). To assess whether FAK-stimulated MMP secretion was associated with higher rates of MMP expression, semiquantitative RT-PCR was performed with total RNA samples obtained from mock- and FAK- transfected cells. As shown in Fig. 1*C*, FAK transfected cells showed enhanced MMP-2, MMP-9, and TIMP-1 mRNA in response to FN with no significant changes in MMP-14 and TIMP-2 expression. FAK transfection alone was unable to increase gelatinase production by T cell lines without exposure to FN (data not shown), confirming that the increase in MMP production achieved by FAK transfection is dependent on additional signals driven by integrin engagement.

Given the important role of FAK in the focal adhesion turnover required for cell migration through the extracellular matrix, we developed the hypothesis that besides inducing MMP gene expression, FAK activation by integrin engagement might have an even more prominent role in regulating gelatinase release. As shown in Fig. 2*A* and *B*, upon FN exposure, both MMP-2 and MMP-9 appeared in the supernatant fluid long before an increase in gelatinase mRNA could be detected by RT-PCR. Furthermore, blocking transcription with actinomycin D, which was effective in abrogating gelatinase mRNA induction by FN (Fig. 2*C*), did not prevent gelatinase

detection in the cell culture supernatant medium and did not inhibit the increase in gelatinase secretion triggered by FAK transfection (Fig. 2*D*). This early release was markedly reduced by the protein secretion inhibitors monensin and brefeldin A, further supporting that FN stimulates secretion of prestored gelatinases (Fig. 2*E*). These findings indicate a prominent role for FAK in mediating post-transcriptional release of gelatinases by T lymphoid cells.

We next explored potential differences in integrin-driven signaling pathways leading to gelatinase expression and to gelatinase release. As shown in Fig. 2*F*, inhibition of Src-type tyrosine kinases with PP2, blocking ERK1/2 activation with PD98509, and inhibition of JNK activity by SP600125, all inhibited the FN-induced increase in gelatinase mRNA. However, although Src-tyrosine kinase and JNK inhibition also resulted in decreased gelatinases in the supernatant medium, inhibition of ERK activation and inhibition of PI 3-kinase with wortmannin produced a significantly increased release of gelatinases (Fig. 2*G*). In agreement with these findings, curcumin, which at low concentrations inhibits JNK and at higher concentrations also prevents ERK activation (53), displayed bimodal effects (Fig. 2*G*).

To better understand the mechanisms through which FAK regulates gelatinase production, point mutants of FAK were transiently transfected into Jurkat cells in order to determine which docking or signaling FAK domains are involved in MMP-2 and MMP-9 production and release induced by FN. Point mutation of tyrosine 397 (FAK Y397F) has been shown to prevent recruitment of tyrosine kinases of the Src family, while mutation of lysine 454 (FAK K454R) abolishes FAK intrinsic kinase activity and mutation of tyrosine 925 (FAK Y925F) has been suggested to prevent Grb2 binding and the subsequent activation of the Ras/Raf-1/ERK signaling pathway (42). As shown in Fig. 3*A*, mutation at the catalytic site did not decrease gelatinases in the conditioned medium, indicating that intrinsic FAK kinase activity is not required for gelatinase release. Mutation of Y925 also did not reduce gelatinase release compared with FAK wild-type transfected cells. In contrast, mutation at Y397 remarkably decreased MMP gelatinolytic signal, suggesting that recruitment of Src and possibly other kinases by FAK into focal adhesions is necessary for gelatinase production.

Src and Src-FAK interaction are crucial for integrin-mediated gelatinase release by T lymphoid cell lines

These results led us to focus on the role of Src-type protein kinases in gelatinase production induced by FN. As shown in Fig. 2*F* and 2*G*, PP2, a specific inhibitor of Src-type tyrosine kinases, strongly reduced gelatinase production induced by FN.

Given that Lck is a major Src-type tyrosine kinase in cells of lymphoid lineage, we explored gelatinase induction by FN in J.CaM1.6 cells, a Jurkat-derived Lck-deficient T cell line. JCaM1.6 cells displayed dramatically reduced production of gelatinases in response to FN compared with their Jurkat parental cell line (Fig. 3*C*). Gelatinase production by J.CaM1.6 cells in response to FN was restored by transfection with wild-type Lck. Although the levels were markedly decreased, J.CaM1.6 exhibited some gelatinase production, indicating that Lck function may be partially compensated by other Src-type tyrosine kinases with redundant functions (Fig. 3*C*). Subsequently, Jurkat cells were transiently transfected with Lck and Fyn kinase-deficient mutants. As shown in Fig. 3*D*, both mutants resulted in decreased gelatinase production elicited by FN, indicating that Src-family kinase activity contributes to gelatinase

induction by FN and providing further evidence for functional redundancy among Src-type tyrosine kinases.

The reduced gelatinase production in Y397F-transfected cells, combined with the role of Src tyrosine kinases in gelatinase induction, suggests that FAK/Src complex formation is crucial for integrin-mediated induction of gelatinases. In accord with this hypothesis, cotransfection of Lck and FAK wild-type into the Lck-deficient cell line J.CaM1.6 resulted in more increased MMP-2 and MMP-9 production than transfection with either Lck or FAK individually (Fig. 3*E*).

Integrin-mediated stimulatory and inhibitory signals for gelatinase release are orchestrated by distinct domains of FAK: functional divergence between FRNK and FAT fragments

We have previously shown that exposure to FN transduces stimulatory and inhibitory signals for MMP production by T lymphoid cells (7). We next explored whether these dual signals were coordinated by FAK by inducing overexpression of truncated forms. These expressed constructs consisted of the naturally occurring C-terminal domain FRNK, which lacks the kinase domain but retains binding sites for adaptor proteins (54) and FAT, a shorter C-terminal truncated form that retains binding sites for paxillin and talin but lacks the proline-rich domains present in FRNK (Fig. 4*A*).

As shown in Fig. 4*B*, FRNK and FAT fragments had intriguingly opposite effects on gelatinase release. FRNK overexpression elicited a much lower production of released gelatinases than FAK wild-type in response to FN. In contrast, FAT transfection produced a remarkable increase in both MMPs that was considerably higher than even FAK wild-type. Similar results were obtained when cells were exposed to soluble or to solid-phase FN (Fig. 4*B*).

The effects of FRNK and FAT were more prominent on gelatinase release than on gelatinase mRNA expression (Fig. 4*D*). Interestingly, FRNK and FAT also had an opposite effect on cell adhesion to FN: while FRNK decreased cell attachment, FAT increased it (Fig. 4*E*). These findings suggest that these mutants may decrease cell motility through different mechanisms: by decreasing cell attachment or by inducing cell arrest. Disturbed cell motility and altered gelatinase release resulted in a decrease in cell invasiveness in cells transfected with either truncated form (Fig. 4*E*).

We next tried to elucidate the molecular basis for the divergent effects of FRNK and FAT on gelatinase production. Both FRNK and FAT are able to displace native FAK from its physiological location at focal adhesions and compete with FAK for binding proteins resulting in inhibition of FAK-mediated effects on cell motility (50, 55, 56). Both lack the catalytic domain, indicating that, in accordance with the results obtained from FAK K454R-transfected cells (Fig. 3*A*), the absence of the catalytic domain in FRNK, and in FAT, is not a major determinant of their divergent effects. The key determinants for the opposing effects of FRNK and FAT fragments should instead reside in FRNK domains absent in FAT.

Compared with FRNK, FAT lacks two proline-rich domains with important functions (Fig. 4*A*). The proline-rich 1 domain is able to interact with SH3-containing pCAS (Crk-associated substrate) and the proline-rich 2 region is a binding site for GRAF (GTPase-activating protein for Rho associated with FAK) (57, 58). Activated GRAF, as well as phosphorylated pCAS, lead to

Rho inhibition, favoring focal adhesion disassembly and facilitating cell motility (59). In other cell types, FAK-recruited pCAS is phosphorylated by FAK itself and by locally recruited Src-type tyrosine kinases (60). pCAS interacts with Crk and activates Rac and, subsequently, JNK (61, 62). Both activated Rac and JNK regulate gene expression and have been shown to increase gelatinase production in other cell systems (63, 64). Crk is also able to activate PI 3-kinase and Ras/Raf-1/ERK (65, 66), which, according to our results, are inhibitory pathways for gelatinase secretion (7) (Fig. 2*G*).

pCAS-mediated signals could be, then, stimulatory (through JNK) or predominantly inhibitory (through Ras/ERK or PI 3-kinase activation) for gelatinase secretion. Since FRNK is able to bind pCAS but unable to phosphorylate it, we considered that FRNK might compete with pCAS binding to native FAK thereby preventing pCAS downstream signaling events leading to JNK activation. However, even though JNK is necessary for FN-induced MMP expression, as indicated by the abrogation of gelatinase expression and release obtained with JNK inhibitor SP600125 (Fig. 2*F* and *G*), the reduced integrin-mediated gelatinase production by FRNK does not appear to result from interference with JNK activation (Fig. 5).

FRNK is able to mediate pCAS downstream signaling events regulating gelatinase release through mechanisms requiring Src-kinase activity

The absence of FRNK inhibition on JNK phosphorylation may indicate that FRNK-recruited pCAS can be phosphorylated by other kinases and effectively transmit downstream-signaling events. In fact, it has been recently shown that pCAS is able to directly recruit Src-type tyrosine kinases (67). This hypothesis might explain, at least in part, the reduced JNK2 phosphorylation achieved by FAT, which lacks proline-rich regions and is unable to recruit pCAS (Fig. 5).

As a test of this hypothesis, Jurkat cells were transfected with FRNK mutated at prolines 712/715 (FRNK P712/715A), which is unable to recruit pCAS. As displayed in Fig. 6*A*, this mutation prevented the inhibitory effect of FRNK on gelatinase release. Supporting the role of Src-type tyrosine kinases in phosphorylating FRNK-bound pCAS, FRNK transfection into the Lck-deficient J.CaM1.6 cell line not only failed to reduce MMP production but, in fact, resulted in an enhancement of MMP release, even stronger than that achieved by FAT (Fig. 6*B*). Furthermore, the suppressing effect of FRNK was reversed by inhibiting Src-type tyrosine kinase activity with PP2 (Fig. 6*C*). The FRNK inhibitory effect is, consequently, dependent on Src-type tyrosine kinases, particularly Lck.

Further supporting the ability of FRNK to recruit pCAS and the ability of Src-type tyrosine kinases to phosphorylate it, FRNK overexpression did not interfere with phosphorylation of two pCAS family members present in lymphoid cells, CasL and Sin (Fig. 6*D*). However, both CasL and Sin phosphorylation were reduced by PP2 only in FRNK-transfected cells and not in the mock-transfected controls, supporting that FRNK cooperates with Src-type tyrosine kinases to transmit downstream CasL/Sin signaling (Fig. 6*D*). Consistent with this idea, FRNK-induced ERK phosphorylation was abrogated by the Src inhibitor PP2 (Fig. 6*E*). Importantly, as shown in Fig. 6*C*, the FRNK inhibitory effect on gelatinase release was reversed with the MEK1 inhibitor PD98059 indicating that FRNK transmits inhibitory signals for gelatinase release by a process requiring ERK activation.

FAT-increased gelatinase release requires Src activity and is associated with reduced ERK activation

FAT subfragment retains binding sites for important signaling and cytoskeleton proteins such as paxillin and talin and may compete with native FAK for focal adhesion location and for Y925 phosphorylation. As shown in Fig. 7*A*, FAT enhanced gelatinase release was inhibited by the Src-family kinase inhibitor PP2 and was virtually unmodified by curcumin and the more specific JNK inhibitor SP600125, indicating that Src kinase activity but not JNK activity are necessary for FAT-enhanced gelatinase release. As shown in Fig. 7*B*, FAT was, indeed, less efficient than FAK wild-type in inducing tyrosine phosphorylation of ERK1/2 in response to FN, suggesting that interference with ERK1/2 activation may be a mechanism through which FAT increases gelatinase release. In accord with this mechanism, further inhibition of ERK 1/2 activation by PD98509 elicited only a weak additional increase in gelatinase release induced by FAT (Fig. 7*A*). However, given the lack of increase in gelatinase release achieved by the point mutant Y925F, it appears likely that FAT decreases ERK activation and gelatinase release through additional mechanisms besides the Y925 site. Alternatively, FAT effects would not be mediated by competing with endogenous FAK and FAT itself with its bound molecules and devoid of inhibitory proline-rich regions would directly mediate gelatinase release through mechanisms requiring Src-kinase activity and ERK inhibition. Further experiments are needed to better identify the mechanisms involved in FAT-induced gelatinase release.

DISCUSSION

Gelatinase production by lymphocytes permits their migration through tissues where they fulfill crucial biological functions in health and disease. In this study, we show that FAK has an important role in regulating integrin-induced gelatinase production and, particularly, release by T lymphoid cells.

Transient transfection of Jurkat cells with full-length FAK increased MMP-2 and MMP-9 expression and release in response to FN, resulting in an enhanced invasiveness of transfected cells. It has been recently shown that, in melanoma cells, MMP-2 and MMP-9 are stored in vesicles, closely associated with microtubules (68). Reorganization of the cytoskeleton during FAK coordinated focal adhesion turnover, might then, promote gelatinase release.

Point mutation of FAK at Y397, which upon phosphorylation, becomes a binding site for SH2 domains of Src-type tyrosine kinases and other signaling molecules such as PLCγ, PI 3-kinase, and Grb7 (28, 29), decreased gelatinase production, suggesting that FAK interaction with Src-type tyrosine kinases and possibly other SH2-bearing signaling molecules is required for FAK-increased gelatinase production. In support of this mechanism, Lck-deficient Jurkat cells displayed reduced gelatinase expression in response to FN. Transfection with kinase-deficient mutants showed that, among Src-type tyrosine kinases, both Lck and Fyn participate in integrin-dependent gelatinase production by T lymphoid cell lines. This finding is consistent with previous studies demonstrating that constitutive Src activation or viral Src transformation is associated with cell invasiveness in malignant cells (69). Recently, Hsia et al. have demonstrated that, while viral Src infection rescues impaired motility in FAK–/– cells, interaction with FAK is necessary for viral Src-mediated cell invasiveness and MMP production (64). Our results indicate that interaction of FAK with endogenous Src-type tyrosine kinases is relevant not only

for invasiveness of v-Src-transformed cells but is also crucial for physiologic responses such as integrin-mediated induction of gelatinases in T lymphoid cells. Even though Src tyrosine kinase activity is essential for FN-induced MMP production and release, as indicated by the abrogation of gelatinase production achieved by the Src-type tyrosine kinase inhibitor PP2, intrinsic FAK kinase activity was not required for integrin-mediated gelatinase production, as demonstrated by the lack of any inhibitory effect of the FAK K454R kinase-dead point mutant.

Interestingly, as revealed by transient transfection of truncated forms, FAK is able to transduce both stimulatory and inhibitory signals for gelatinase production by T lymphoid cells through pathways known to regulate cell motility and survival in other cell types (70). Transient transfection of Jurkat cells with FRNK elicited a reduced release of gelatinases compared with full-length FAK, while transfection with FAT substantially increased gelatinase release. Although FAK truncated forms exhibited opposite effects on cell adhesion and gelatinase release, both resulted in decreased cell invasiveness, indicating that coordination of gelatinase release with focal adhesion turnover is essential for cell invasion.

FAT-increased gelatinase release required Src tyrosine kinase activity, as it was abrogated by the Src inhibitor PP2. The FAT-stimulated gelatinase release may occur, at least in part, by interfering with ERK activation. This is supported both by the fact that FAT elicited reduced ERK phosphorylation compared with FAK wild-type and that inhibition of ERK1/2 phosphorylation in nontransfected cells actually resulted in an increased gelatinase release in response to FN. However, transfection of FAK mutated at Y925 did not result in a substantial increase in gelatinase release, suggesting that FAT may inhibit ERK activation through pathways other than interfering with Y925-mediated Grb2 recruitment by endogenous FAK.

JNK activation has been implicated in MMP-9 production in FAK–/– cells infected with viral v-Src (64), as well as for MMP-3 and MMP-13 production in in vivo models of arthritis (63). JNK was, indeed, necessary for gelatinase production in response to FN, as demonstrated by the fact that SP600125 prevented the increase in both gelatinase mRNA and protein induced by FN. However, JNK was not involved in FAT-stimulated gelatinase release, as demonstrated by the decrease in JNK2 phosphorylation obtained with FAT overexpression and by the absence of any significant inhibitory effect of curcumin or SP600125 on FAT-mediated gelatinase release by T lymphoid cells. Moreover, interference with JNK, also does not appear to be a major mechanism underlying the reduced effect of FRNK on gelatinase production by T lymphoid cells: JNK2 was highly phosphorylated in Jurkat cells before exposure to FN (data not shown), and FRNK transfection did not result in a major decrease in JNK phosphorylation, contrary to the effects of FAT. Consequently, even though JNK2 activation is necessary for gelatinase production, other mechanisms may activate it more efficiently than integrin engagement. Serum factors, rather than integrin engagement, have been shown to induce JNK activation and localization, along with FAK, in lamellipodia and invadopodia in FAK restored and viral-Src infected FAK–/– cells (64).

We show that FRNK is able to transmit some stimulatory signals for gelatinase release through activation of JNK and perhaps other pathways. In our system, FRNK was inefficient in increasing FN-induced gelatinase production compared with the smaller FAT truncation, probably due to the parallel transduction of more powerful inhibitory signals from FRNK via ERK1/2 activation and possibly other pathways.

The ability of FRNK to transduce downstream predominantly inhibitory signals is dependent on pCAS binding to FRNK, as a FRNK mutant unable to recruit pCAS (FRNK P712/715A) reversed FRNK inhibitory effect. Moreover, FRNK inhibition is dependent on Src-type tyrosine kinases, which can be recruited by pCAS (71), given that Src inhibition substantially enhanced gelatinase release in FRNK-overexpressing cells, and FRNK expression in Lck-deficient J.CaM1.6 cells resulted in a strong gelatinase release. Src-dependent FRNK inhibitory signals were likely transduced through CasL/Sin phosphorylation and subsequent Crk-derived ERK activation, because in FRNK-overexpressing cells, Src inhibitors decreased CasL/Sin phosphorylation and inhibitors of ERK phosphorylation increased gelatinase release.

Interestingly, our findings indicate that FRNK is not a dominant-negative for all FAK functions. In support of our findings, it has been recently demonstrated that, as opposed to FAT, which induces apoptosis, FRNK can efficiently transduce survival signals for serum-deprived fibroblasts through Src-dependent pCAS phosphorylation and subsequent JNK activation (72). In our setting, FRNK dominant-negative function for gelatinase release also depended on Src-tyrosine kinase activity. FRNK has been recently demonstrated to inhibit invasiveness and MMP-2 secretion in viral v-Src-transformed NIH3T3 fibroblasts (55). In v-Src-transformed cells, where Src is constitutively active, Src-dependent FRNK inhibition of gelatinase release might be constantly activated, and FRNK could always function as a dominant-negative for gelatinase production. In contrast, during physiological migration on FN, Src-tyrosine kinases are only transiently activated and, through their interaction with FAK, may transduce stimulatory and inhibitory signals for gelatinase release.

Although T lymphocytes are not major agents in proteolytic tissue remodeling, they are able to produce tiny amounts of gelatinases that are crucial in allowing their progression into tissues in physiological and pathological conditions. Our findings indicate that, in T lymphoid cells, FAK regulates not only gelatinase production but also post-transcriptional gelatinase release in response to FN, and the scaffolding function of FAK—rather than its kinase activity—is the most relevant feature in this process. FAK emerges, then, as an important mediator in coordinating stimulatory and inhibitory signals for gelatinase release using pathways controlling cell migration. This fine control may adapt pulse release of gelatinases to focal adhesion turnover.

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Fig. 1

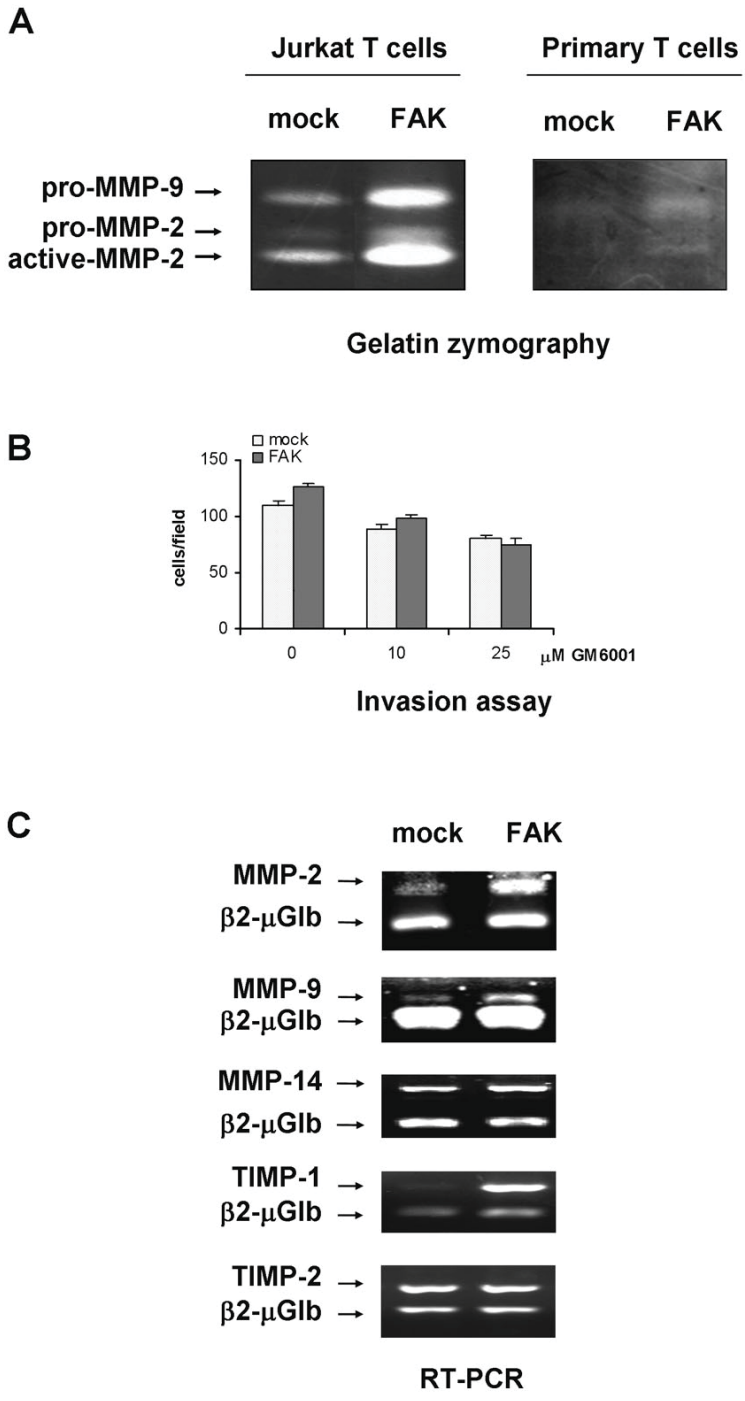

Figure 1. Transient transfection of FAK wild-type increases gelatinase production in response to fibronectin. ***A*)** Gelatin zymography of concentrated conditioned medium obtained from mock and FAK-transfected Jurkat and primary T cells exposed to 10 μg/ml of fibronectin (FN) for 4 h. ***B*)** Invasion of FAK and mock-transfected Jurkat cells through Matrigel-coated filters in the absence and in the presence of the MMP inhibitor GM6001. FAK vs. mock $P = 0.0134$; baseline mock vs. mock with GM6001 (10 μM), $P = 0.0039$; baseline mock vs. mock with GM6001 (25 μM) $P = 0.0018$; baseline FAK vs. FAK with GM6001 (10 μM and 25 μM) $P < 0.0001$ (Mann Whitney U test). ***C*)** RT-PCR amplification of MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2, and β2-microglobulin (β2-μGlb), using RNA obtained from mock and FAK wild-type transfected Jurkat cells after exposure to FN for 4 h.

Fig. 2

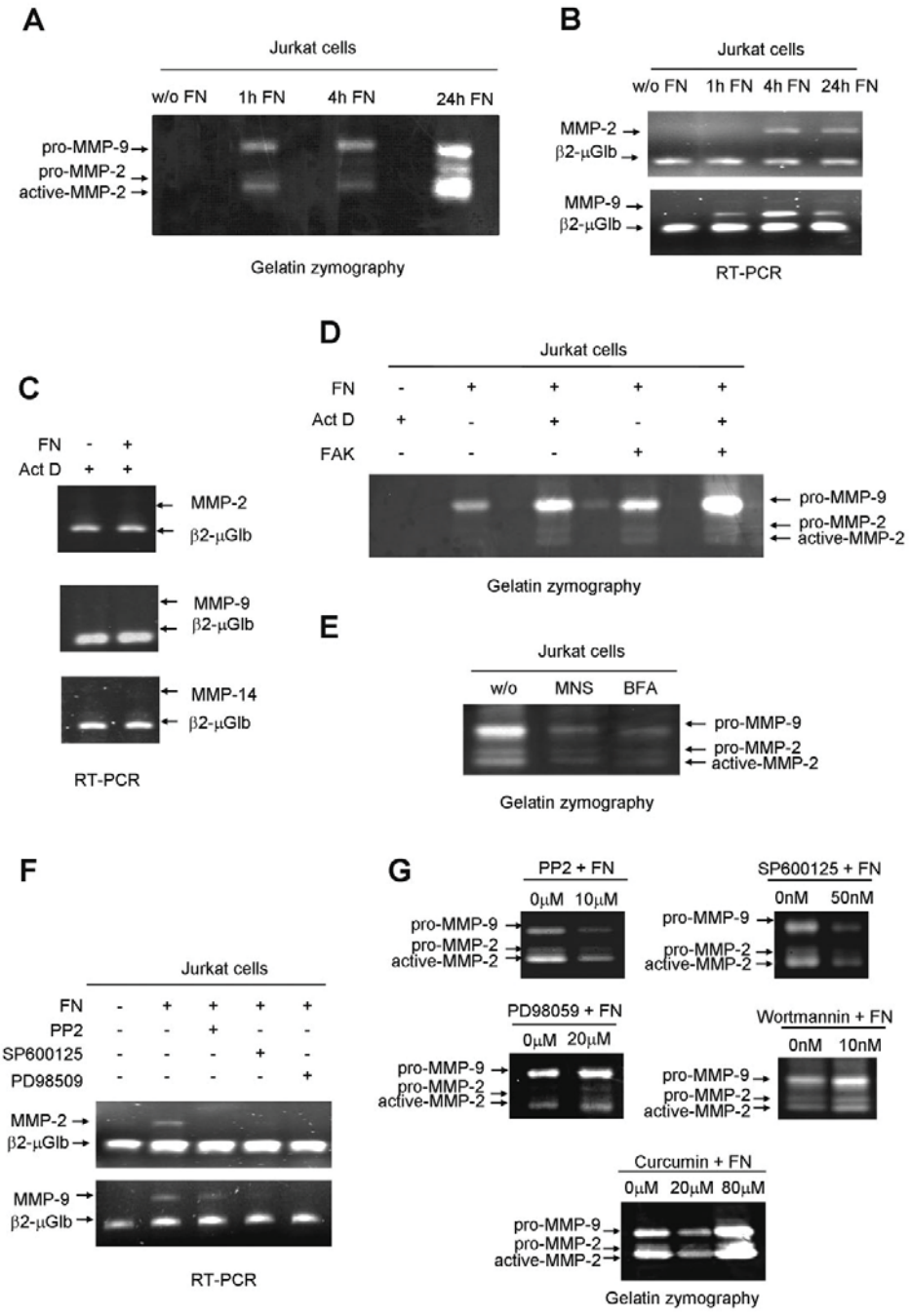

Figure 2. FAK mediates fibronectin-induced gelatinase release. ***A*)** Gelatin zymography of concentrated conditioned medium of Jurkat cells collected at the indicated time points after exposure to FN (10 μg/ml). ***B*)** RT-PCR detection of MMP-2, MMP-9, and β2-μGlb using RNA obtained from the same cells as in (***A***). ***C*)** RT-PCR amplification of MMP-2, MMP-9, MMP-14, and β2-μGlb of RNA obtained from Jurkat cells exposed to FN for 4 h with (+) or without (–) pretreatment with actinomycin D (Act D) at 2 μg/ml. ***D*)** Gelatin zymography of concentrated conditioned medium obtained from mock and FAK-transfected Jurkat cells exposed to FN for 4 h with or without pretreatment with actinomycin D. ***E*)** Gelatin zymography of Jurkat cells exposed to FN in the absence (w/o) and in the presence of monensin (MNS) or brefeldin A (BFA). ***F*)** RT-PCR amplification of MMP-2 and MMP-9 using RNA obtained from Jurkat cells under basal conditions compared with exposure to FN for 4 h, with or without pretreatment with the Src-tyrosine kinase inhibitor PP2 at 10 μM, JNK inhibitor SP600125 at 50 nM, or MEK1 inhibitor PD98509 at 20 μM. ***G*)** Gelatin zymography of the conditioned medium of Jurkat cells exposed to FN for 4 h and pretreated with PP2, SP600125, PD98059, wortmannin or curcumin at the indicated concentrations.

Fig. 3

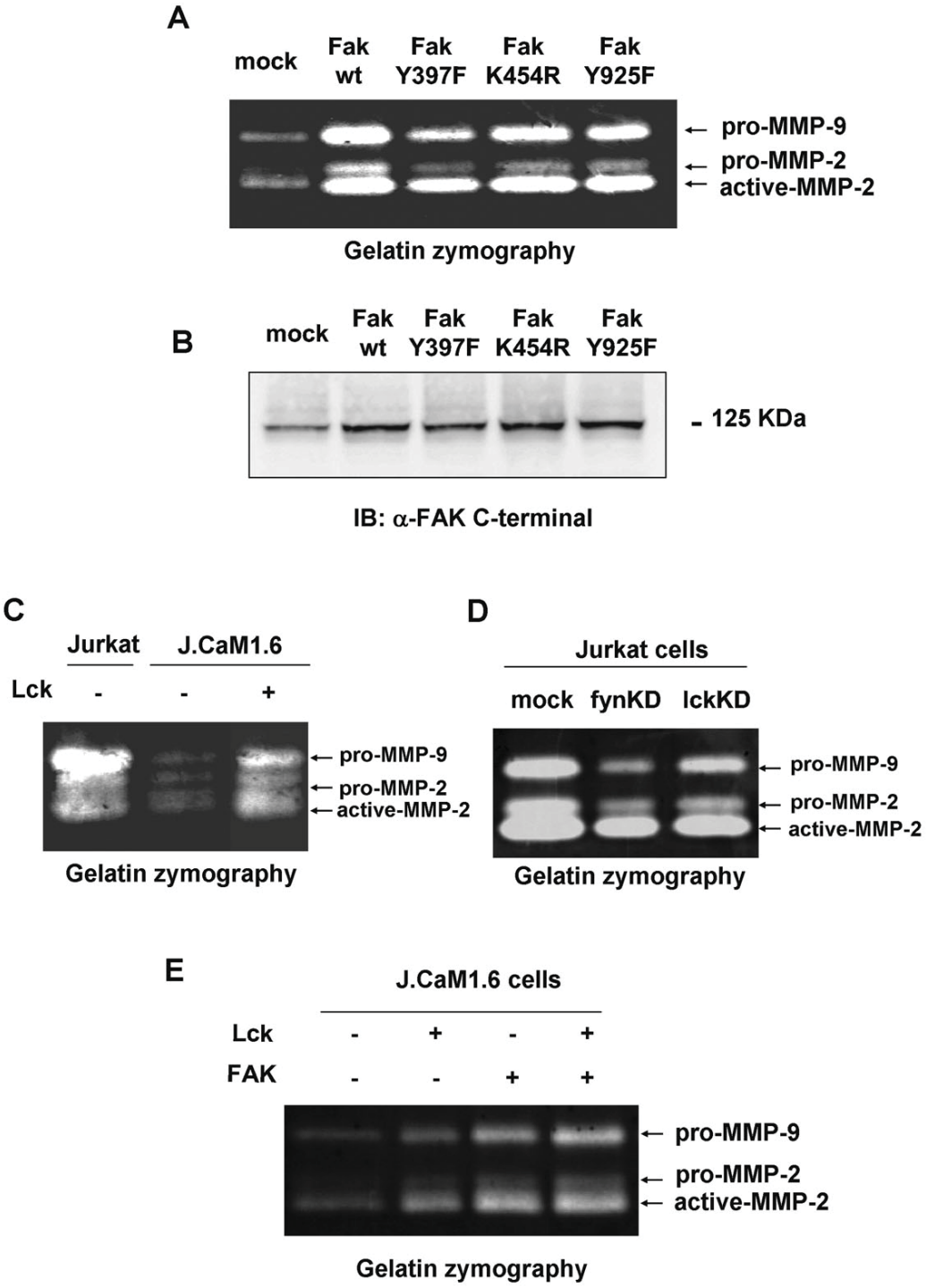

Figure 3. Functional participation of distinct FAK domains in gelatinase expression. Role of Src kinases Lck and Fyn. ***A***) FAK point mutants Y397F, K454R, and Y925F were transiently transfected into Jurkat cells and incubated with FN (10 μg/ml) for 4 h. Conditioned medium was concentrated and subjected to gelatin zymography. ***B***) Western blot assessing FAK protein content in lysates of transfected cells. ***C***) Gelatin zymography of conditioned medium from Jurkat cells, Lck-deficient JCaM1.6 cells, and J.CaM1.6 cells transiently transfected with wild-type Lck exposed to FN for 4 h. ***D***) Gelatin zymography of concentrated conditioned medium from mock-transfected Jurkat cells and transfected with kinase-deficient Fyn and Lck mutants exposed to FN for 4 h. ***E***) Gelatin zymography of conditioned medium of JCaM1.6 cells mock transfected (–) or transfected with wild-type Lck (+) or FAK (+), cultured with FN (10 μg/ml) for 4 h.

Fig. 4

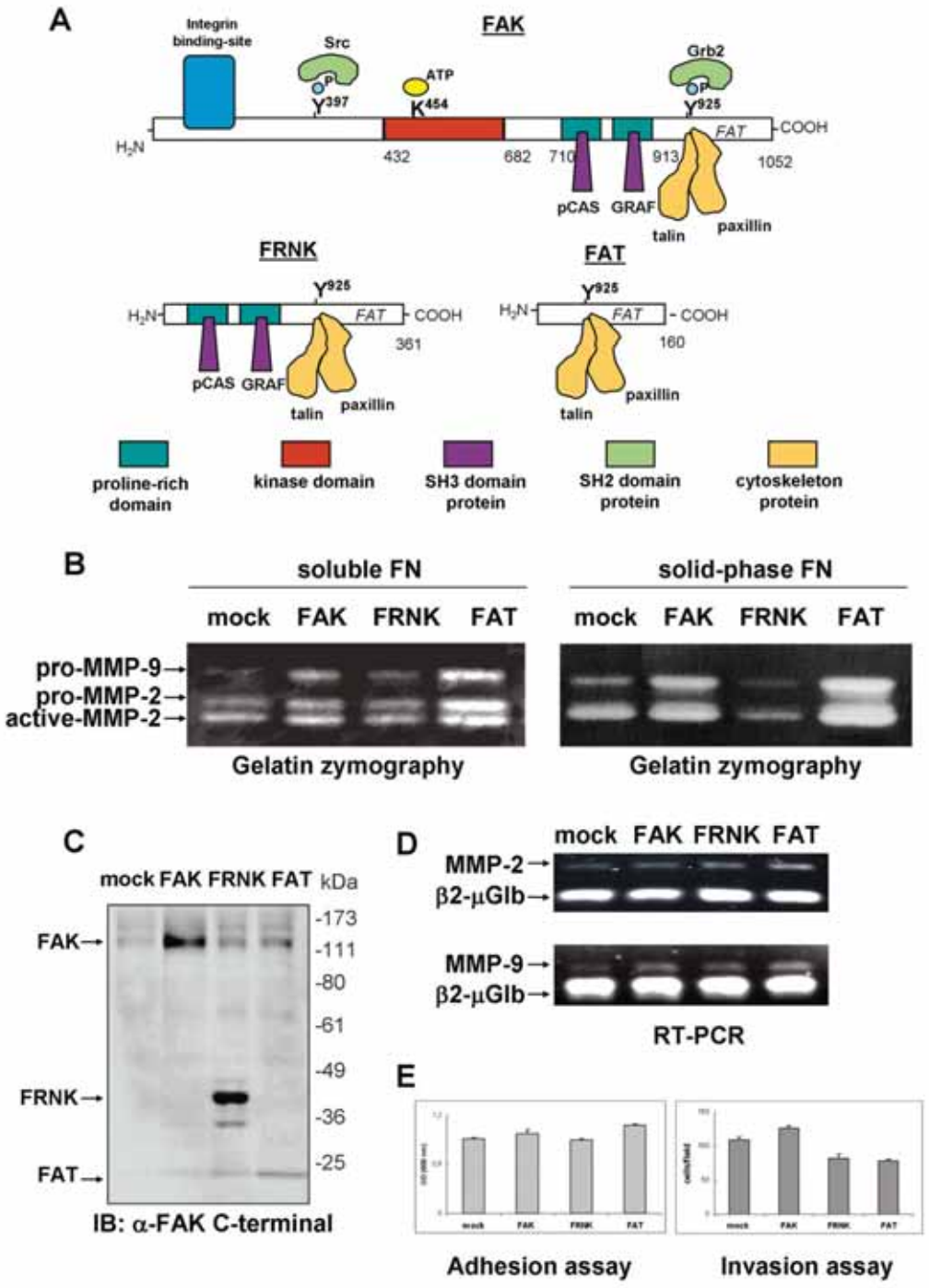

Figure 4. Different effects of FAK truncated forms on gelatinase expression and release. ***A*)** Schematic representation of FAK wild type and truncated forms FRNK and FAT used in this study. ***B*)** Gelatin zymography of concentrated conditioned medium from Jurkat cells mock-transfected or transfected with FAK wild-type or FAK truncated forms FRNK and FAT, after 4 h exposure to soluble FN (10 μg/ml) (***left***) or solid-phase FN (100 μg/ml) (***right***). ***C*)** Western blot performed with lysates of transfected cells indicating the relative expression of FAK wild-type, FRNK fragment, and FAT subfragment. ***D*)** RT PCR amplification of MMP-2, MMP-9 and β2-μGlb using RNA obtained from the same cells as in (***B***). ***E*)** Adhesion of transfected Jurkat cells to solid-phase FN and invasion through Matrigel-coated filters. For adhesion: FAK vs. mock, $P = 0.0571$; FAK vs. FRNK, FAK vs. FAT, and FRNK vs. FAT, $P = 0.0286$. For invasion: FAK vs. mock, $P = 0.0134$; FAK vs. FRNK and FAT, $P < 0.0001$ (Mann-Whitney *U* test).

Fig. 5

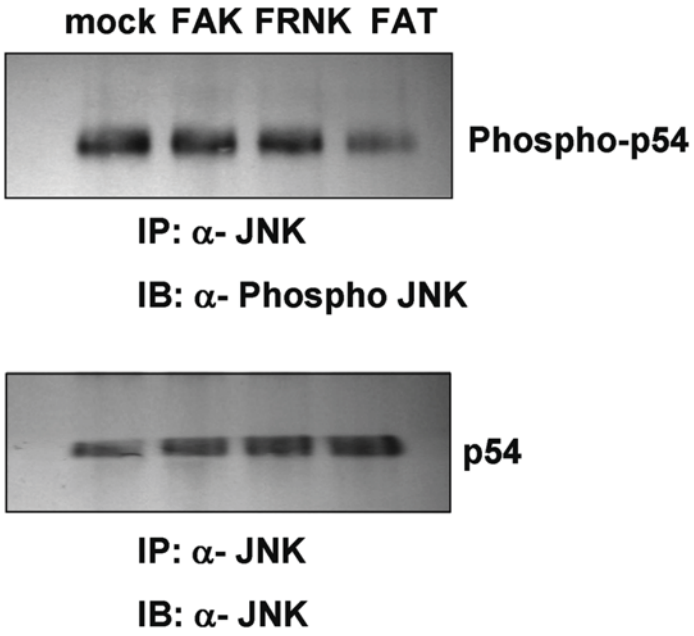

Figure 5. FRNK overexpression does not interfere with JNK activation. Cell lysates obtained from Jurkat cells mock transfected or transfected with FAK wild-type or FAK truncated forms FRNK or FAT and exposed to FN for 1 h were immunoprecipitated with anti-JNK antibody, subjected to SDS-PAGE and blotted onto nitrocellulose. Western blot analysis was performed with anti-phosphorylated JNK.

Fig. 6

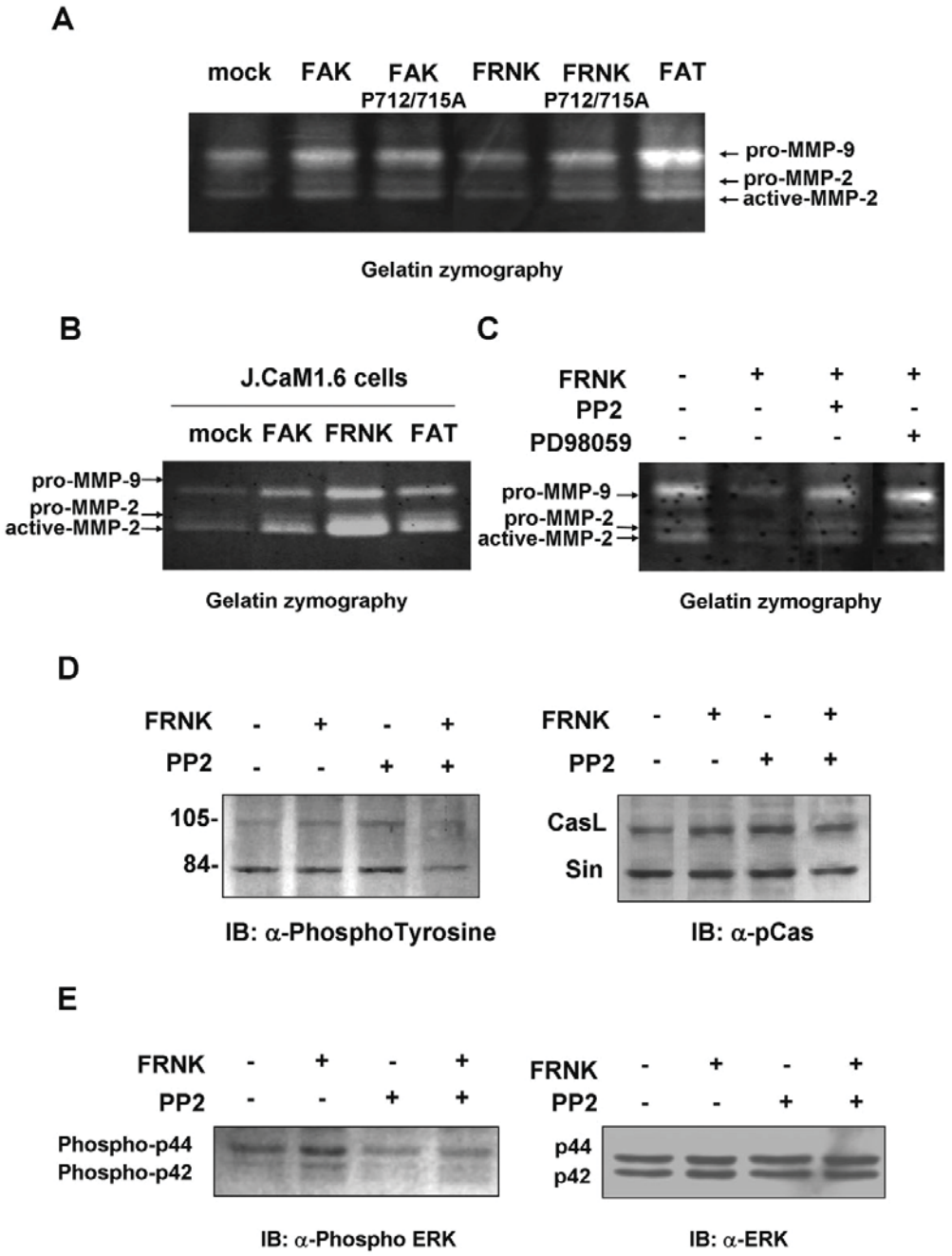

Figure 6. FRNK inhibitory effect on gelatinase release requires Src activity and is associated with CasL phosphorylation and ERK activation. ***A*)** Gelatin zymography of conditioned medium obtained from Jurkat cells transiently transfected with the indicated FAK mutants and truncated forms after 4 h exposure to FN (10 μg/ml). ***B*)** Gelatin zymography of concentrated conditioned medium obtained from baseline JCaM1.6 cells, and J.CaM1.6 cells transiently transfected with FAK wild-type or truncated forms FRNK and FAT. Gelatin zymography of concentrated conditioned medium obtained from Jurkat cells mock-transfected (–) and Jurkat cells transiently transfected with FRNK (+), untreated (–), or treated with Src-inhibitor PP2 (+) or MEK1 inhibitor PD98059 (+). All cells were exposed to FN for 4 h. ***C*)** Cell lysates obtained from Jurkat cells mock transfected (–) or transfected with FRNK (+), untreated (–), or treated with Src-inhibitor PP2 (+) and incubated with FN for 1 h were subjected to SDS-PAGE and blotted onto nitrocellulose. Western blot analysis was performed with a phosphotyrosine antibody; then the blot was stripped and reprobed with anti-pCAS antibody. ***D*)** Western blot analysis of cell lysates obtained from mock (–) or FRNK (+) transfected Jurkat cells, untreated (–), or treated with PP2, and exposed to FN for 1 h. ***E*)** Blots were incubated with anti-phosphorylated ERK antibody, stripped, and reprobed with anti-ERK antibody.

Fig. 7

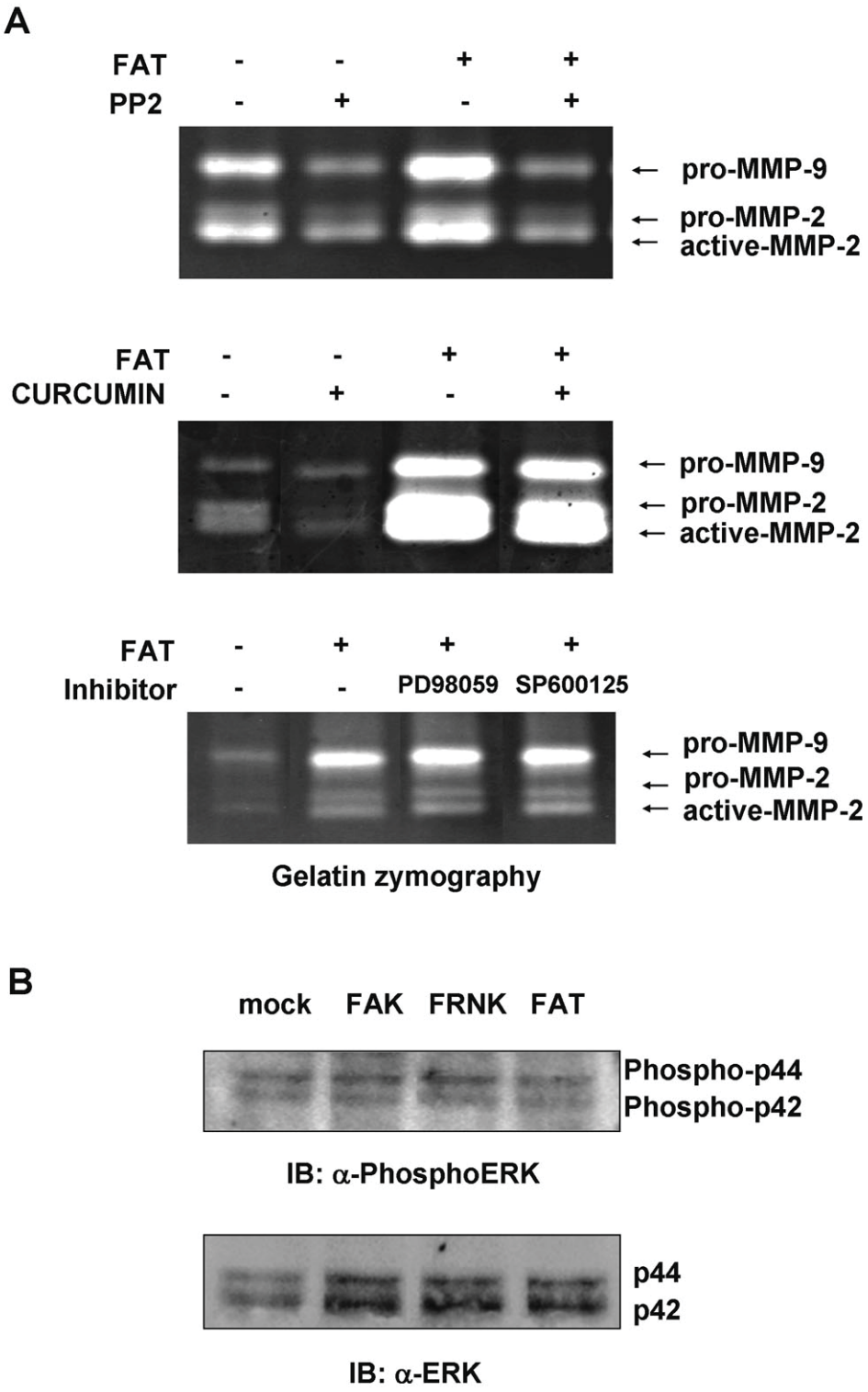

Figure 7. FAT enhanced gelatinase release requires Src activity and is associated with a reduction in ERK activation. ***A*)** Gelatin zymography of concentrated conditioned medium obtained from Jurkat cells mock-transfected (–) or transfected with FAT (+), untreated (–), or treated with PP2 at 10 μM (+), curcumin at 20 μM (+), PD98509 at 20 μM (+), or SP600125 at 50 nM (+), as indicated in the figure. All cells were cultured with FN at 10 μg/ml for 4 h. ***B*)** Western blot analysis of cell lysates from Jurkat cells mock-transfected or transfected with FAK wild-type or truncated forms FRNK and FAT, exposed to FN (10 μg/ml) for 1 h, and analyzed using anti-phosphorylated ERK antibody. The blot was subsequently stripped and reprobed using anti-ERK.

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