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Transforming growth factor -α expression in rat experimental hepatocarcinogenesis

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Summary. Growth factors in general and transforming growth factor-α in particular have been related to cell proliferation and cell differentiation. This study was designed to clarify the distribution pattern of TGF-α in chemically-induced hepatocarcinogenesis. Sprague-Dawley rats were subjected to different non-intensive or intensive carcinogenic treatments using diethylnitrosamine (DEN) as carcinogen and ethinyl estradiol (EE) as promoter. The livers were fixed in 2% paraformaldehyde, dehydrated in a series of ethanol solutions, embedded in paraffin and sectioned. In the preneoplastic lesions no TGF-α immunoreactive cells were identified, but in some hepatic tumours cell immunostained with TGF-α antibody were observed. These results suggest that the cells capable of expressing TGF-α constitutively may be involved in neoplastic development *in vivo*.

Key words: Transforming growth factor-α, Tumour promotion, Hepatocarcinogenesis

Introduction

Transforming growth factor-α (TGF-α) was initially identified by its ability to induce proliferation of normal fibroblasts in a soft agar medium (De Larco and Todaro, 1978). TGF-α is a 50-amino acid polypeptide derived from a 160-amino acid precursor (Massagué, 1990). The amino acid sequence of TGF-α has 35% identity with the epidermal growth factor (EGF) sequence, which includes the conservation of all six cysteine residues (Derynck et al., 1984). Two-dimensional NMR studies provide evidence than in solution the two proteins have very similar polypeptide chain folds and both EGF and TGF-α compete with each other for binding to the EGF-receptor (Winkler et al., 1989).

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TGF-α is expressed in placenta (Lee et al., 1985) and embryonic tissues (Brown et al., 1990) as well as in normal skin, the adult brain and the gastrointestinal tract (Coffey et al., 1987; Wilcox et al., 1988; Malden et al., 1989). A number of human tumour cell lines, as well as biopsies from a range of human epithelium-derived tumours, have been found to co-express RNAs specific for TGF-α and EGF-R/c-erb B, renal cell carcinomas showing the highest levels of expression (Derynck et al., 1987). Similar observations have also been reported for hepatocellular carcinomas and papillary carcinomas of the thyroid (Yeh et al., 1987; Aasland et al., 1990). Over- expression of TGF-α has been involved in psoriatic epidermal hyperplasia (Elder et al. 1989). The production of TGF-α by various tumour cells suggests that it may play an important role in cellular transformation when expressed abnormally (Sandgren et al., 1990). It has been proposed that TGF-α or other growth factors may play a role in tumour formation via an autocrine mechanism whereby TGFs help to sustain the transformed character of some of the cell populations from which they are secreted (Sporn and Roberts, 1985).

The aim of the present study is to determine the distribution of immunohistochemically demonstrable TGF-α expression in altered populations of hepatocytes during hepatocarcinogenesis. A pattern of TGF-α expression among a range of hepatic lesions from preneoplastic foci to hepatocellular carcinoma is presented and results are discussed with reference to the role of this growth factor in tumourogenesis.

Materials and methods

Animals and treatments

Hepatic lesions were obtained from different carcinogenic protocols to Sprague-Dawley rats using Diethylnitrosamine (DEN) (Merck, Darmstadt, FRG) as carcinogen and Ethinyl estradiol (EE) (Sigma, St. Louis, Mo USA) as promoter. The animals were kept under alternate 12 - hours periods of light and darkness.

Preneoplastic tissues were obtained from the non-intensive carcinogenic treatments shown in Graph 1. DEN was administered intraperitoneally to 17-day-old males and females, diluted in 0.9% NaCl at a dose of 100 mg/kg body weight . EE was incorporated into the diet at a concentration of 10 ppm (Panlab. Spain). The estimated daily dose of EE was 0.5 mg/kg body weight. Two months after initiation, the NEE group was transferred to a «Usine d'Alimentation Rationnelle» (U.A.R) defined diet (Panlab, Spain) containing 10 ppm of EE, on which they were fed for 8 months. Groups NOO, NEO and NOE were used as controls, since in a previous study (Mayol et al., 1991) no effects were observed in the induction of the preneoplastic foci using short administration of the promoter. Three animals from the NEE group and two animals from the controls for each sex were used as a source of preneoplastic tissue for immunohistochemistry.

Tumours were indistinctly obtained from three intensive carcinogenic treatments shown in Graph 2. In the ICT-A treatment the initial dose of DEN was 230 mg/Kg body weight. Two weeks later, a second dose of DEN (70 mg/kg) was given 24 hrs. after partial hepatectomy. One month after DEN initiation, EE was incorporated into the diet at a concentration of 10 ppm and retained in diet until 12 months after the first DEN initiation, when the animals were killed. In the ICT-B and ICT-C treatments the initial dose of DEN was 200 mg/kg body weight. Two weeks after initiation, EE was incorporated into the diet at a concentration of 10 ppm and retained until the animals were killed. The estimated daily dose of EE was 0.5 mg/kg body weight. Three weeks after initiation intraperitoneal injections of DEN were started. The doses of DEN were 40 mg/kg body weight each week for 8 weeks (group ICT-B) and 80 mg/kg body weight each week for 4 weeks (group ICT-C) (Graph 2). The animals were killed when they showed a marked decrease in body weight or other signs of serious illness. This normally occurred between 8 and 11 months after the first administration of DEN.

Tissue collection and preparation

Animals from the carcinogenic protocols (Graphs 1 and 2) were decapitated under ether anaesthesia. Livers were removed and examined for the presence of macroscopic tumours, which were dissected, fixed in 2% paraformaldehyde in PBS (pH : 7.2) for 24 h. The samples were processed routinely and 4 µm-thick paraffin serial sections were obtained.

TGF-α immunohistochemistry

After hydration, some paraffin sections were treated with 3% hydrogen peroxide-10% methanol for 5 min (to inhibit the endogenous peroxidase), rinsed in 0.01 M PBS (pH : 7.2) for 3 x 5 min. The slides were incubated at room temperature with normal rabbit serum (1 : 30, Dako Patts, ref. x 902) for 30 min. and overnight at 4°C in mouse monoclonal anti-TGF-α. It was diluted in 0.01 M PBS pH: 7.2, 0.1% bovine serum albumine (BSA) and 0.01% sodium azide (AzNa) to a 5 µgr/ml concentration. This antibody was acquired from Oncogene-Science (ref. GF-10). The anti-TGF-α recognizes human and rat TGF-α epitope (residues 34 - 50) and show no crossreactivity with EGF (Sorvillo et al., 1990).

The sections were then rinsed in PBS and incubated for 45 min at room temperature with rabbit anti-mouse IgG (1 : 80, Dako Patts, ref. Z259), washed with PBS and incubated in horseradish peroxidase and mouse anti-horseradish peroxidase complex (1 : 150, Dako Patts, ref. P650) for 45 min washed with PBS and incubated for 5 min. with 2.5 mg/100 ml 3,3'Diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Mo.) dissolved in PBS plus 0.03% hydrogen peroxide at room temperature (Sternberger, 1979). These slides were rinsed in distilled water, counterstained with Harris haematoxyline and dehydrated using graded ethanol solutions, cleared in xylene and mounted in DPX.

Some sections were not treated with hydrogen peroxide and after primary incubation with antibody against TGF-α they were incubated for 45 min. in fluorescein-conjugated rabbit IgG anti-mouse IgG, rinsed in PBS and mounted in glycerol: PBS (9:1) with p-pheyilendiamine. Negative controls included: 1º) Primary incubation with 0.01M PBS pH: 7.2, 0.1% BSA and 0.01% AzNa; 2º) Primary incubation with either anti-TGF-α at a 5µgr/ml concentration preabsorbed with both rat TGF-α (50µgr/ml, fragment 1-50. Bachem, ref. H-5545) and rat EGF (50µgr/ml. Janssen Biochemica, ref. 26.632.54) respectively.

Results

Livers from the non-intensive carcinogenic treatments displayed a high incidence of preneoplastic foci and nodules. Most of them were composed of clear cells, a

Fig. 1. Liver section from animal of the ICT-B protocol. The hepatocytes show heterogeneous size. Note that some vacuolate cells were dispersed through the hepatic parenchyma. H &E. x 450

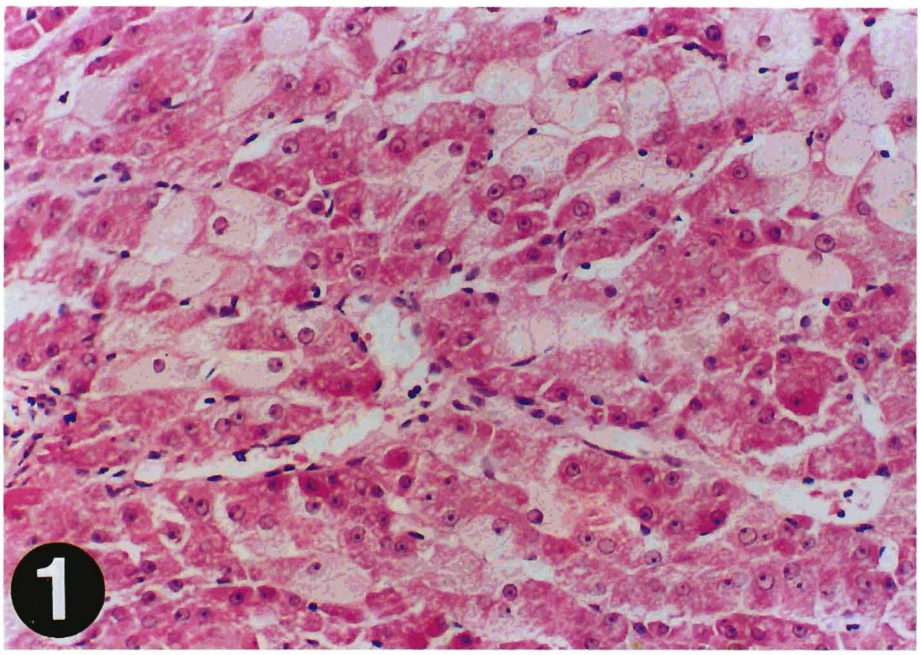
Fig. 2. Neoplastic cells from the protocol ICT-B stained with TGF-α antibody. Some negative cells were shown dispersed throughout a large area with strong immunoreactive TGF-α cells. PAP method. x 800

Fig. 3. Liver section from animal of the protocol ICT-C. Vacuolate cells were observed. Occasionally some of them were binucleate (arrow). H&E. x 450

Fig. 4. TGF-α immunoreactive cells from the protocol ICT-B. These cells show an atypical shape (arrows) and someone display two nuclei (large arrow). Indirect immunofluorescence method. x 800

Fig. 5. TGF-α immunoreactive cells from the protocol ICT-A. Note the binucleate cell (big arrow). Indirect immunofluorescence method. x 800

Fig. 6. TGF-α immunoreactive cells from the protocol ICT-A. These cells exhibit a heterogeneous size and some cells displayed a vacuolated cytoplasm (arrows). Indirect immunofluorescence method. x 800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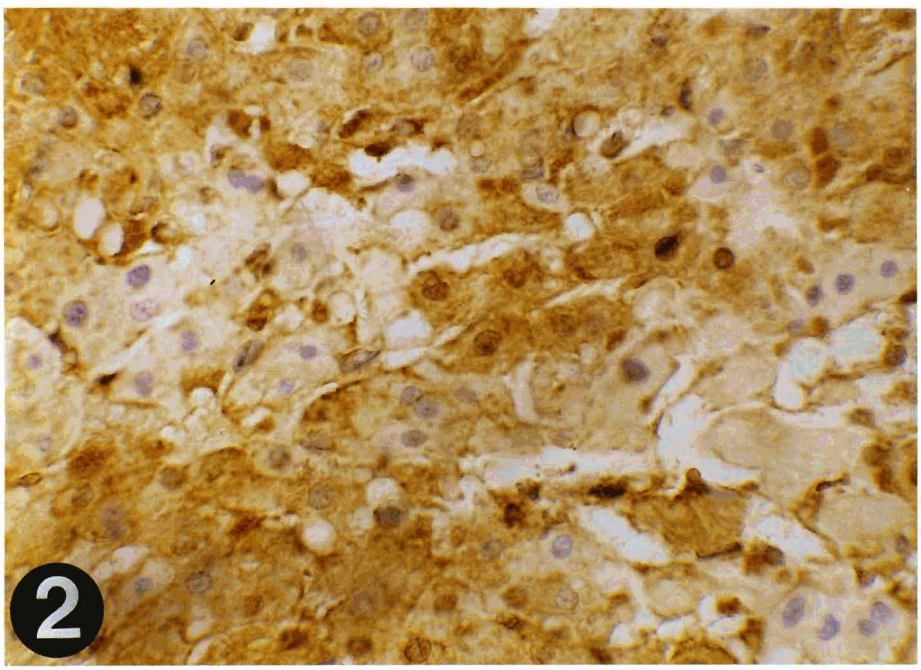
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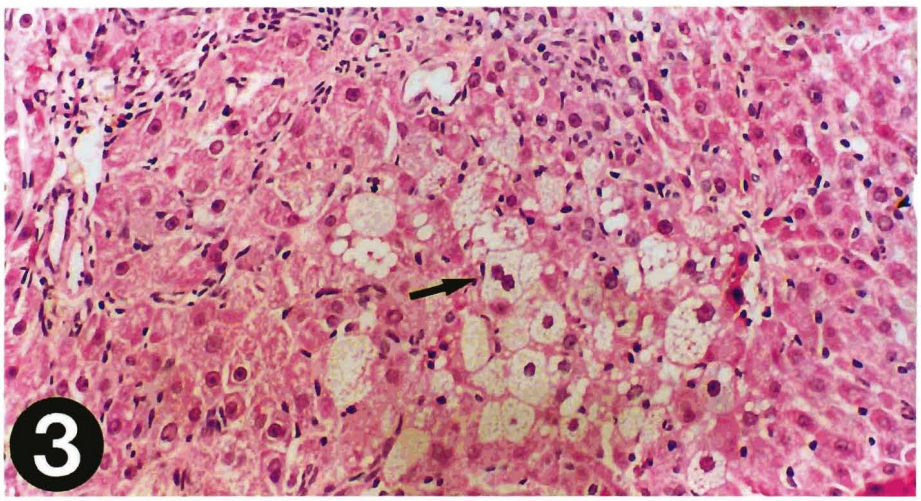
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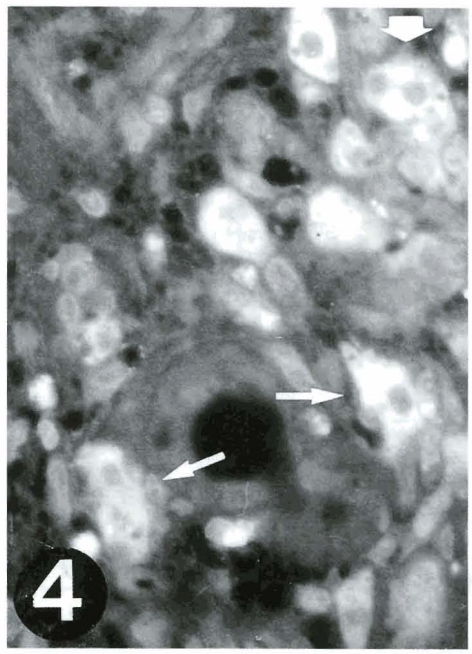
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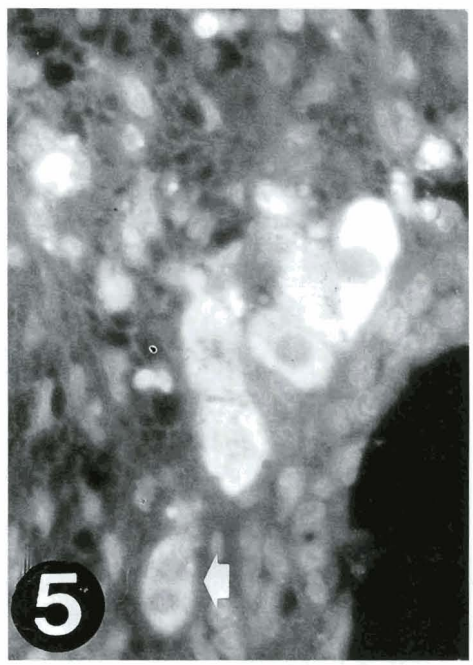
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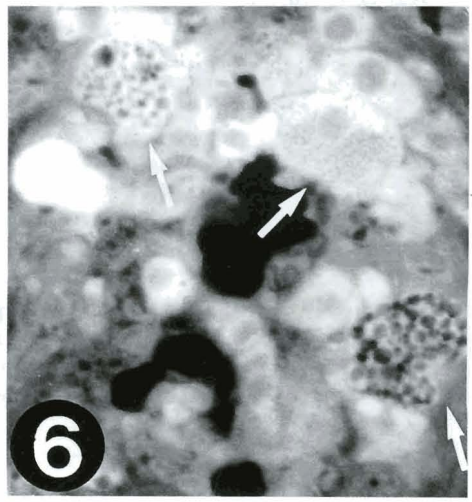
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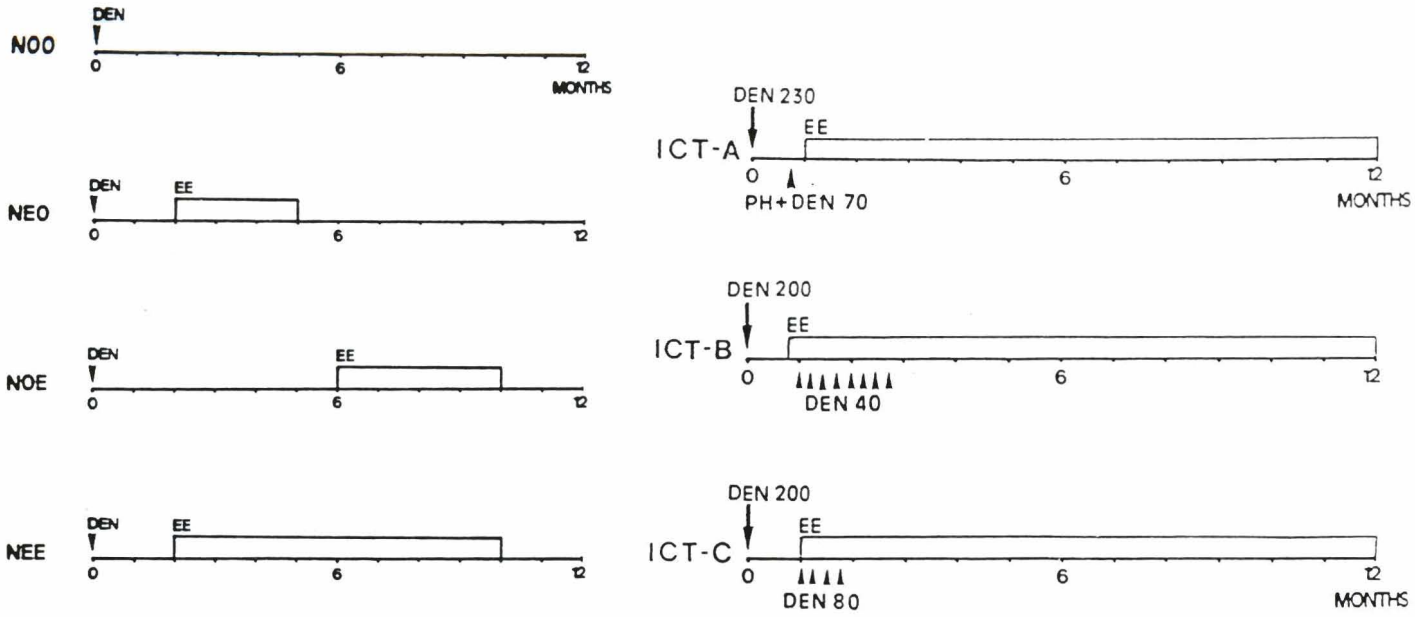

Graph 1. Non-intensive carcinogenic treatment. Protocols, NOO, NEO, NOE and NEE. DEN was administered as a single injection of 100 mg/Kg body weight. EE was incorporated into the diet at 10 p.p.m. and the periods of administration are shown by white bars..

Graph 2. Intensive carcinogenic treatment. Protocols ICT-A, ICT-B and ICT-C. In the protocol ICT-A the dose of initiation with DEN was 230 mg/Kg body weight. Two weeks later a second dose of DEN (70 mg/Kg) was administered 24 hrs. after performing a partial hepatectomy (PH). In the protocols ICT-B and ICT-C the dose of initiation with DEN was 200 mg/Kg body weight. In the protocol ICT-B the intensive doses of DEN were 40 mg/Kg body weight each week during 8 weeks (arrowheads). In the protocol ICT-C the intensive doses of DEN were 80 mg/kg body weight each week during 4 weeks (arrowheads). EE was incorporated into the diet at 10 p.p.m. and the periods of administration are shown by white bars.

Multinodular carcinomas	4(7)
Undifferentiated carcinomas	4(7)
Well-differentiated carcinomas	1(3)
Neoplastic nodules	1(5)

Table 1. Tumours from the intensive carcinogenic treatments which showed immunostaining to monoclonal antibody to TGF-α. In parenthesis, total number of tumours analyzed.

smaller number were eosinophil and only a minority were basophil. No TGF-α immunoreactivity cells were observed in any of the these preneoplastic lesions.

Macroscopic tumours, defined as those > 0.5 cm in diameter were counted in the livers of the animals under intensive carcinogen treatment. Bile duct hyperplasia and hypertrophy, small collangiomas and oval cells forming expansive trabeculae were found to a greater or lesser extent inside all the tumours of hepatocellular origin. All the animals from the ICT-A protocol developed hepatic tumours. Histologically they were neoplastic nodules and well-differentiated carcinomas. In the intensive carcinogenic treatments from the ICT-B and ICT-C protocols all the animals also had tumours and, in addition to neoplastic nodules and well differentiated carcinomas, undifferentiated and multinodular carcinomas were identified. In all the tumour classes there were cases of TGF-α antibody immunoreactivity. Furthermore, it appeared that in the more advanced neoplastic lesions (multinodular and poorly differentiated carcinomas) TGF-α immunostaining was more frequent than in the less advanced ones (neoplastic nodules) (Table 1).

The cells immunoreactive to TGF-α antibody, displayed an atypical shape with a heterogeneous size (Figs. 1 - 3). Polynucleate (Figs. 3 - 5) or vacuolate (Figs. 3, 6) cells and occasionally, abnormal mitotic figures were seen. Numerous cystic and haemorrhagic lesions were also observed. Necrosis areas with degenerating or pyknotic cells were dispersed throughout the hepatic tumoral tissue. The neoplastic-immunoreactive-TGF-α cells were spread over large areas and also formed nodules or small groups of up to twenty cells (Fig. 6). None of the tumours was enterirely stained by the antibody; only subpopulations of them showed TGF-α immunoreactivity (Fig. 2).

Discussion

Roden hepatocarcinogenesis is a multi-step process (Farber and Sarma, 1987), in which altered populations of hepatocytes arise during the carcinogenic treatments. Preneoplastic foci and nodules are thought to be the precursors of neoplastic tumour formation. High levels of the TGF-α mRNA in hepatocytes has been observed during liver regeneration, suggesting that a TGF-α autocrine circuit is transiently activated (Mead and Fausto, 1989). We were not able to detect immunohistochemically reactive TGF-α expression in

preneoplastic lesions of the liver. However, we found that TGF-α was detectable in some hepatic tumours. It appeared that the incidence of TGF-α positivie tumours was higher in carcinomas than in neoplastic benign nodules. No differences in this incidence were observed among the different types of carcinomas that we considered: well-differentiated homogeneous, well differentiated multinodular; and poorly differentiated hepatocellular carcinomas. These results suggest that TGF-α expression in hepatic lesions during hepatocar-cinogenesis may be involved in the later steps of tumour formation, associated with the stage of progression. However, it does not seem to be an essential factor in the process of tumour formation, since there were hepatocellular carcinomas that did not show TGF-α immunoreactivity. Given the limited resolution of the immunohistochemical method used, it cannot be ruled out that low levels of TGF-α expression in those immunohistochemically-negative tumours could also be involved in their neoplastic development process. In some of the animals with hepatocellular carcinomas, pulmonary micrometastasis from the liver arose (data not shown) and immunohistochemistry for TGF-α showed that they were all negative for the factor. Furthermore, there was no preferential staining in the more advanced tumours such as multinodular or poorly differentiated ones. Therefore, although TGF-α may play a role in liver tumour progression, presumably by binding to and activating EGF-receptors, which are abundant in the rodent liver (Gladhaug and Christoffersen, 1988), it does not seem to be involved in the processes leading to the acquisition of malignancy.

Previous studies have also indicated that TGF-α is a potent inducer of angiogenesis in a model *in vivo* (Schreiber et al., 1986). Haematopoietic tumours which do not require neovascularization for their development do not contain TGF-α mRNA. However, the endogenous synthesis of TGF-α in various types of solid tumours may not only contribute to an autocrine growth stimulation of these tumour cells, but may also play a paracrine role in the tumour-induced angiogenesis, probably in conjunction with other angiogenic factors similar to TGF-ß (Evarts et al., 1990). Massive proliferation of endothelial cell was not characteristic in our tumours, thus further study is required to elucidate the involvement of TGF-α as an angiogenic growth factor in hepato-carcinogenesis.

Jhappan et al. (1990) affirm that TGF-α specific lesions may arise predominantly in tissues containing cells that are highly responsive to TGF-α/EGF, owing to the possession of either excessive endogenous EGF-receptors, as in the liver, or a more productive signal transduction pathway. Thus, in our rats, liver carcinogenesis may involve, at least in part, the selection of cells capable of expressing constitutive TGF-α, which may be of significance for the development of malignancies *in vivo*.

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