k-FGF protooncogene expression is associated with murine testicular teratogenesis, but is not involved during mouse testicular development

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Summary. The k-FGF gene, which belongs to the family of the fibroblast growth factor genes, is implicated in tumoral and developmental processes. It is expressed in embryonal carcinoma cells, in embryonic stem cells, during limb and tooth formation and in some germ cell tumors. However, the expression of this protooncogene during testicular development as well its relationship to spontaneous teratogenesis have not been determined. Here we investigate k-FGF expression during testicular development in mice, as well as in a spontaneous testicular teratoma (STT) and in the OTT6050 teratocarcinoma (TC) by Northern blotting, RT-PCR and in situ hybridization. Several data indicate that k-FGF gene contains downstream regulatory sequences which bind octamer factors. One of these transcription factors which binds to k-FGF enhancer is Oct-4. Although the k-FGF gene is activated by Oct-4 in embryonal carcinoma and embryonic stem cells and Oct-4 is expressed in the germ cells of the embryo, our results indicate that there is no detectable k-FGF expression in mouse testicular germ cells at any stage of development. This indicates that Oct-4 does not activate transcription of the k-FGF gene in mouse germ cells, and that k-FGF is not implicated during testicular development. We also show that there is a high k-FGF expression in the experimental OTT6050 TC, but only very low levels in a murine differentiated STT, suggesting that k-FGF activation may be responsible for the genesis and development of STT, behaving as a marker of malignancy in these neoplasms.

Key words: k-FGF, Fgf-4, Spontaneous testicular teratoma, Mouse testicular development, OTT6050 teratocarcinoma, RT-PCR, In situ hybridization

Introduction

The k-FGF gene, also known as hst-1/HSTF1 (Sakamoto et al., 1986; Yoshida et al., 1988a-c), KS3 (Delli Bovi and Basilico, 1987) or FGF-4 (Hébert et al., 1990) is a member of the fibroblast growth factor (FGF) family of genes. This family includes nine members classified more or sequence comparisons than on functional equivalence (Burgess and Maclag, 1989; Tronick and Aaronson, 1995). FGFs are implicated in cell proliferation, cell differentiation, morphogenesis and neoplasia. They are mitogenic for cells derived from mesoderm and neuroectoderm lineages. The product of the k-FGF gene is a secreted glycoprotein of 22-kD that stimulates the proliferation of NIH 3T3 fibroblasts (Delli Bovi et al., 1987) as well as HUE vascular endothelial cells (Miyagawa et al., 1988; Yoshida et al., 1994), demonstrating a role for k-FGF in angiogenesis. In addition, the role of k-FGF in development has been demonstrated in Xenopus laevis by its mesoderm-inducing capacity of ectodermal tissue explants (Paterno et al., 1989) as well in the mouse. In mice, k-FGF is expressed in peri-implantation embryos (Rappolee et al., 1994), is essential for postimplantation mouse development (Feldman et al., 1995), and also participates in myogenesis and in specific epithelial-mesenchymal interactions during limb and tooth morphogenesis (Nisswander and Martin, 1992; Niswander et al., 1993, 1994; Vogel and Tickle, 1993; Jernvall et al., 1994). All these data suggest that FGFs in general, and particularly k-FGF, have different roles in normal development. On the other hand, k-FGF gene is implicated in the tumoral process. It was isolated after transfection of human stomach cancer DNA (Sakamoto et al., 1986) and from Kaposi sarcoma DNA (Delli Bovi et al., 1987) which contained DNA rearrangements upstream and downstream of the coding sequences.

Due to the fact that the proteins encoded in the oncogene and in the protooncogene are identical (Yoshida et al., 1987; Delli Bovi et al., 1988), the oncogenic activation of the k-FGF gene must result from unregulated expression and not from the genesis of a mutated form. Transfection of its eDNA induces cell motility, and malignancy (Delli Bovi et al., 1988; McLeskey et al., 1993; Taylor et al., 1993). It is expressed in human testicular germ cell tumors (Schofield et al., 1991; Strohmeyer et al., 1991) and in teratocarcinoma stem cells (Velcich et al., 1989;
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Schofield et al., 1991). In addition, it is found amplified (Chikuba et al., 1995) and coamplified with the int-2 gene (Yoshida et al., 1988a-d) and the bel-1 gene (Volling et al., 1993) in some human cancers, and there is an association between amplification, high k-FGF expression and metastatic phenotype in mouse mammary tumors (Murakami et al., 1990) and in an experimental system (Damen et al., 1991). Moreover, angiogenic properties of the k-FGF oncogene have also been determined, and its possible association with the metastatic phenotype (Brustle et al., 1992; Díaz-Flores et al., 1994).

An aspect that has not been studied is its expression during testicular development in mice. Testicular development involves cell migration, proliferation and differentiation processes. During embryogenesis, small amounts of primordial germ cells (PGC) are clustered around the hind gut of Embryonic stem (ES) cells (Scholer et al., 1989; Scholer, 1991) and that k-FGF is expressed in EC cells (Scholer et al., 1989; Scholer, 1991) and in germ cell testicular tumors, in a spontaneous testicular teratoma (STT) by Northern blotting, RT-PCR and in situ hybridization.

Materials and methods

Testis, tumor and embryo obtention

Postnatal testis at different stages of development were obtained from 129/Sv male mice. 129/sSv isogenic mice were obtained from Prof. Jacob of the Pasteur Institute (Paris, France). Day of partum was designed as T0, T2, T5, T10, T18, T25 and T72 testis were dissected and extracted from the intraperitoneal cavity of male 129/Sv mice.

OTT6050 mouse TC used in these experiments was obtained by injecting subcutaneously 10^5 embryoid bodies (EB) in PBS into 129/Sv isogenic mice, and dissecting the tumor after 2 weeks. OTT6050 TC was derived from 129/Sv mouse E6.5 grafted to a histo-compatible host testis. The ascitic form of the tumor (EB) was obtained after several intraperitoneal passages of solid TC (Stevens, 1970). EB were kindly supplied by Prof. Jacob. A STT was obtained from an adult 129/Sv mouse left testis.

Embryos were obtained from pregnant 129/Sv isogenic mice. 129/Sv mice have been maintained in our laboratory since 1984. Noon of the day on which the copulating plug was observed was considered to be 0.5 days of gestation (E0.5). Embryos at early post-implantation stages (E7.5 to E9.5) were processed including decidua, E10.5 to E11.5 were dissected from the implantation site and processed including extra-embryonic annexes and E12.5 to E14.5 were dissected from decidua and annexes. For Northern blot and RT-PCR analysis, the head and the body were dissected (below the liver) in E13.5 to E14.5.

Cell culture

F9 embryonal carcinoma cell line was cultured in gelatin-coated petri dishes in Dulbecco’s Modification of Eagle’s Medium (DMEM) (Flow Lab.) containing 10% fetal calf serum (FCS) (Serva) and 1% L-Glutamine. Cells were cultured at 37 °C in a 5% CO_2 atmosphere to confluence. They were disrupted in a solution containing 0.25% trypsin and 0.04% EDTA and washed in PBS.

RNA and DNA isolation

Total cellular RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). DNA from F9 cells was isolated by the method described by Blin and Stafford (1976).

Primer design

For specific priming in RT-PCR analysis, two 20nt oligonucleotide primers spanning the second intron of the...
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mouse k-FGF gene (Brookes et al., 1989), RTKF2 5'TGGTGAGACATTTGAGGTG 5' and RTKF1 5' TTATGGTAGGGCGACACTCG 3' were designed and produced in a DNA synthesizer (Applied Biosystems).

RT-PCR reaction

For the reverse transcriptase reaction, 100 ng of RNA samples were heated at 95 °C for 10 min, cooled on ice and cDNA synthesis was performed at 42 °C for 30 min in 50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% Triton X-100, 1.5mM MgCl₂ using 1mM dNTPs (Pharmacia), 20 U RNasin (Promega), 100 pmol sodium downstream primer (RTKF1) and 10 U AMV reverse transcriptase (Promega). Then the reaction mixture was denatured at 95 °C for 1 min and extension of the primers at 72 °C for 1 min. This cycle was repeated 22 times.

Southern blotting

One tenth of the RT-PCR reaction was fractioned in a 1.8% agarose TBE gel, stained with ethidium bromide, photographed, treated with 0.25N HCl, 1M NaCl/0.5M NaOH, equilibrated in 0.5M Tris-HCl pH 7.4/1.5M NaCl and blotted to Genescreen membranes (Dupont) in 10xSSC. DNA was immobilized by baking the membrane at 80 °C. Hybridization was performed in 50% formamide, 6xSSPE, 1% SDS, 50 μg/ml denatured salmon sperm DNA at 42 °C for 16 to 24 h. A mouse 417 bp pb mouse genomic k-FGF fragment including 3rd probe. Both were alkaline hydrolyzed to reduce probes to an average size of 400 nt. For Northern blotting experiments, 25 μg of total RNA were denatured in 50% formamide, 2.2M formaldehyde, 20mM MOPS pH 7.0, 5mM sodium acetate, and 1mM EDTA. RNA samples were diluted in loading buffer containing 0.1% bromophenol blue and 10% glycerol and electrophoresed in 1% agarose-formaldehyde-MOPS gel. The gel was transferred onto Genescreen membranes (Dupont) in 10xSSC. Hybridization was performed in 50% formamide, 5x Denhardt's solution, 5xSSPE, 1% SDS, 200 μg/ml denatured, and fragmented salmon sperm DNA at 42 °C for 16 to 24 h. Riboprobes were used at a specific activity of 2x10⁶ cpm/ml. Washes were made at high stringency conditions and autoradiography was performed as Southern blots. For β-actin hybridization, k-FGF riboprobe was removed from the filter by washing in 50% formamide, 6xSSPE for 40 min at 65 °C.

In situ hybridization

Testis, tumors and embryos were fixed in 4% paraformaldehyde, 5mM MgCl₂ in PBS, dehydrated, embedded in paraffin wax and sectioned at 6 μm. In situ hybridization conditions were performed at high stringency conditions according to the method of Frohman et al. (1990). k-FGF and β-actin riboprobes were used at a specific activity of 10³ cpm/μl. Slides were exposed to an autoradiographic emulsion (NTB2, Kodak) 1:1 diluted in 0.6M ammonium acetate at 4 °C in the dark between three to four weeks (k-FGF) or one week (human β-actin) before being developed and stained with hematoxylin-eosin.

Results

Northern blot analysis of kFGF mRNA in postnatal testis

It is known that murine gonocytes and testicular germ cells prepared from neonatal mice proliferation in vitro. Due to the fact that k-FGF mRNA is expressed in human and murine embryonal carcinoma cells, mouse embryonic stem cells and human testicular germ cell tumors, we investigated the expression of the k-FGF gene in postnatal testis at different days postpartum (T2, T5, T10, T14, T25 and T72) by Northern blotting. As shown in Fig. 1 there was no detectable expression in mouse testis at different ages of development.

We also analyzed k-FGF expression in a STT, in the OTT6050 TC and in F9 cells. We were unable to detect any transcript (Fig. 1), even after exposing the X-ray film for several days in STT. In OTT6050 TC and in F9 cells we detected a transcript of 3.5 kb, although the level of expression was several fold higher in F9 cells. Finally, we could not detect any k-FGF
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Fig. 1. Northern blot analysis of RNA samples from F9 cells (lane 1), OTT6050 TC (lane 2), 129/Sv STT (lane 3), testis of 2, 5, 10, 14, 25 and 72 days p.c. (lanes 4 to 9) and mouse embryos of 7.5 to 12 days (lanes 10 to 15) and head of 13.5-day and 14.5-day embryos (lanes 16 and 18) and body of 13.5 days and 14.5-day embryos (lanes 17 and 19). 25 µg of total RNA are electrophoresed in each lane. A α32P-UTP-labelled antisense k-FGF riboprobe is used, as described in materials and methods. Film was exposed overnight. The same membrane was reprobed with the antisense human β-actin riboprobe.

Fig. 2. RT-PCR analysis of k-FGF transcripts. Ethidium bromide-stained agarose gel of reverse transcriptase-polymerase chain reaction (RT-PCR) products (A). 100bp ladder (lane 1), F9 DNA (lane 2), RNA from F9 cells (lane 3), from OTT6050 teratocarcinoma (lane 4), from a spontaneous testicular teratoma (lane 5), from 2, 5, 10, 14, 25 and 72 days p.p. testis (lanes 6 to 11), from 7.5-, 8.5-, 9.5-, 10.5-, 11.5-, 12.5-day mouse embryos (lanes 12 to 17), from heads of 13.5- and 14.5-day embryos (lanes 18 and 20) and from bodies of 13.5- and 14.5-day embryos (lanes 19 and 21). Southern blot of the same gel, using as a probe a α32P-dCTP-labelled 55 bp Avall fragment (see material and methods) (B). The positions of molecular weight markers are indicated.
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transcript in postimplantation mouse embryos (E7.5 to E12.5 and from head and body of E13.5 to E14.5) on Northern blots under our experimental conditions (Fig. 1).

These results showed that k-FGF mRNA expression was apparently restricted to undifferentiated F9 cells and OTT6050 TC, being undetectable in testis, STT and embryos under our experimental conditions. However, the possibility that expression was restricted to few clumps of cells of the testis and of the STT, which could not be detected by this technique, was not ruled out.

RT-PCR analysis of postnatal testis, a 129/Sv STT, OTT6050 TC, F9 cells and embryos

Amplification of RNA-specific sequences via cDNA (RT-PCR) is a powerful and very sensitive method to analyze RNA (Kawasaki et al., 1988). Due to the fact that we could not detect k-FGF mRNA in postnatal testis, STT or embryos, we decided to analyze the presence of k-FGF transcripts by RT-PCR. The analysis of the PCR products revealed (Fig. 2) a 249 bp visible band on the agarose gel and a strong signal onto the blot corresponding to the specific primed RNAs in F9 cells and OTT6050 TC. We also detected a clearly visible fragment in postimplantation mouse embryos and only a faint band in the STT on the blot. However, we did not observe appreciable expression in T2 testis. As a control of k-FGF-specific priming 500 ng of F9 DNA was used, detecting a band of 649 bp as expected (Fig. 2).

In situ hybridization in testis, tumors and embryo sections

Using testis sections of different postnatal ages (T2,

Fig. 3. Bright field (A) and dark field photograph (B) of OTT6050 TC hybridized with a α32P-UTP-labelled antisense k-FGF riboprobe. Embryonal carcinoma (EC) cells express high amounts of k-FGF transcripts. x 250

Fig. 4. Bright field (A) and dark field photograph (B) of a 7.5-day mouse embryo hybridized with a α32P-UTP-labelled antisense k-FGF riboprobe. Arrows indicate k-FGF transcription in the primitive streak. x 250
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T5, T10, T25, T72) and hybridizing with the antisense k-FGF riboprobe, we could not detect any expression, even after exposing the slides for one month, as expected from the results obtained in RT-PCR experiments (data not shown). On the other hand, in STT sections, where we could not observe the presence of undifferentiated EC cells, we did not detect remarkable k-FGF expression in any differentiated tissue (data not shown). However, and in agreement with the results obtained by RT-PCR, we detected k-FGF expression in EC cells within the OTT6050 TC (Fig. 3), differentiated cells being completely negative. On the other hand, in mouse embryo sections we detected expression in the primitive streak of E7.5 (Fig. 4), but we were unable to detect any specific expression in the developing mouse gonad (Fig. 5).

Sections were hybridized with an antisense human β-actin riboprobe, giving a high signal in all the cells (data not shown). All the sections were hybridized with sense k-FGF and β-actin riboprobes using the same conditions as antisense probes, and as expected, we did not observe any signal.

**Discussion**

The k-FGF gene, as previously reported by Paterno et al. (1989), is a secreted growth factor (GF), member of the fibroblast growth factor family that has been involved in cell proliferation and differentiation during early development. In addition to its role in the normal development, it has been implicated in the tumoral, as mentioned above. Therefore, K-FGF expression has been studied mainly in many tumors and during embryogenesis. However, k-FGF expression has not been studied during testicular development. Testicular germ cells prepared from neonatal (48 h after birth) mice proliferate in vitro (Maekawa and Nishimune, 1991) and there is a high proliferative activity of gonocytes in the pre- (day 14 postcoitum) and early postnatal period (day 2 postpartum) of murine testicular development (Vergouwen et al., 1991), effects with presumably are the consequence of the action of a different GFs. Several GFs play different roles during testicular development. Insulin or IGF-1 is required during the initial phase of testicular organization (Taketo et al., 1991) and nerve growth factor-like proteins with mitogenic activity have been isolated from round spermatids and pachytene spermatocytes (Onoda et al., 1991). In particular, a basic fibroblast growth factor has been detected in immature and adult testis (Mayerhofer et al., 1991; Lahr et al., 1992).

Although k-FGF is not expressed in any adult tissue, including adult testis (Yoshida et al., 1988a-c), previous data have shown that a great number of human testicular germ cell tumors express the k-FGF protooncogene (Strohmeyer et al., 1991). However, we have not detected k-FGF expression by Northern blotting (Fig. 1). In situ hybridization (data not shown) or even by the very sensitive RT-PCR method, at any stage of testicular development (Fig. 2). There is not very much information about factors that regulate K-FGF gene during embryogenesis. However, recent evidence suggests that k-FGF expression is regulated by a NK-Y transcription factor that binds to k-FGF promoter (Hasan et al., 1994; Bryans et al., 1995) and several ubiquitous as well as cell-specific octamer factors that bind to k-FGF enhancer (Koda et al., 1994). One of the factors that can control its expression is the product of Oct-4 gene, also known as Oct-3 or NF-A3. Although it is known that Oct-4 is expressed in PGC of embryo tests, but not in the sperm of adult tests (Scholer, 1991) and that this factor activates k-FGF transcription in undifferentiated EC and ES cells (Schoorlemmer and Kruijer, 1991), it clearly does not do so in immature male germ cells. The cause of this lack of expression is not clear; one possibility might be that in these cells Oct-4 is neither necessary nor sufficient for the regulation of k-FGF expression. The other possibility could be that...
after birth, with the appearance of the A spermatogonia and the onset of spermatogenesis, Oct-4 was downregulated, k-FGF transcription not being activated. It should be noted that Oct-4 is a target for negative regulation by retinoic acid (RA) (Okamoto et al., 1990), which in turn mediates its action through RA receptors. Vanbeek and Meistrich (1992) reported the importance of retinoic acid in the maintenance of spermatogenesis. Therefore, it should be of interest to analyze RA and RA receptor expression in mouse testis after birth and in early spermatogenesis (prepubertal stage).

In a spontaneous, 129/Sv mouse testicular teratoma (STT), we did not detect appreciable k-FGF expression by Northern blotting, although we detected a faint band after blotting the products of the RT-PCR reaction and hybridizing with a specific probe (Fig. 2). This fact indicates that probably only some small groups of undifferentiated cells, which were not included in the analyzed STT sections, express k-FGF, reflecting the conversion to benignancy of the tumor. This clearly associates k-FGF with the degree of undifferentiation of this tumor. Supporting this idea, k-FGF is expressed in primary human testicular tumors (Yoshida et al., 1988a-d; Schofield et al., 1991; Strohmeyer et al., 1991). We used the experimental OTT6050 TC as a positive control for k-FGF expression. We detected expression in the stem cells of the tumor (EC cells), but not in the differentiated tissue by in situ hybridization. The fact that k-FGF is expressed in the stem cells of the tumor and that it is known that this GF acts via an autocrine mechanism (Delli Bovi et al., 1988; Talarico and Basilico, 1991; Moscatelli, 1994), suggest a role of k-FGF in OTT6050 TC in vivo growth.

By Northern blot analysis, we also detected a different level of expression between the nullipotent F9 cell line and the multipotent OTT6050 TC. This result is not surprising and may be explained because OTT6050 TC contained some differentiated cells which are known not to express k-FGF, and also because there are differences in the level of k-FGF expression between several EC cell lines, as previously demonstrated by Schoorlemmer and Kruijer (1991).

In agreement with the results previously reported by Niswander and Martin (1992) indicating k-FGF expression during gastrulation, myogenesis and limb and tooth development, we detected k-FGF transcripts in mouse embryos from E7.5 to E12.5 (Figs. 2, 4) and in the head and body of E13.5 and E14.5 (Fig. 2) by RT-PCR. Using an antisense riboprobe for the k-FGF gene, we confirmed some of these results by detecting k-FGF expression in the distal part of the primitive streak of E7.5 (Fig. 4). This zone of the primitive streak contains cells that are fated to become embryonic, but not extraembryonic mesoderm. As discussed by Niswander and Martin (1992), this finding suggests that k-FGF is involved in the specification of some cells within the streak as well as in mesoderm induction in mammals, as demonstrated in amphibia by Paterno et al. (1989). In E7.5, the base of the allantosis, the area where PGC are located, is completely negative, indicating that PGC do not express the k-FGF gene. This result is surprising, because k-FGF is directly transactivated by Oct-4, which is known to be expressed in PGC and in the mouse gonad (Schöler, 1991). However, some evidence suggests that there may be an interaction between Oct-4 and an ElA-like factor. One could speculate that Oct-4 could be complexed by an ElA-like factor, avoiding it binding to the k-FGF enhancer. Indeed, ElA acts as a bridging factor for transcriptional regulation by Oct-4 (Schöler et al., 1991). Another possibility for this lack of k-FGF expression in PGC could be addressed to the presence of a transcriptional repressor. This lack of k-FGF expression in the developing gonad was also maintained in later stages (E12.5, E13.5 and E14.5 mouse embryo sections).

These results indicate that although k-FGF gene is not involved in mouse testicular development, it is involved in mesodermal induction and in the growth of the experimental OTT6050 TC. At the same time, the detection of very low levels of k-FGF transcription in STT, its absence in differentiated cells of STT, as well as its expression in undifferentiated OTT6050 TC cells suggest that the activation of this gene is probably related to the genesis and progression of STT.

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