A Retrovirus Carrying the *K-fgf* Oncogene Induces Diffuse Meningeal Tumors and Soft-Tissue Fibrosarcomas

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The *K-fgf/hst* oncogene encodes a growth factor of the fibroblast growth factor (FGF) family and transforms cells through an autocrine mechanism which requires extracellular activation of its receptor(s). To identify the cell and tissue targets of *K-fgf* oncogenic potential in vivo, we constructed a recombinant retrovirus carrying the human *K-fgf* cDNA and injected it, together with helper Moloney murine leukemia virus, into immunocompetent as well as nude mice. The original construct was highly transforming in tissue culture but produced no detectable pathologies in vivo with the exception of a single fibrosarcoma which arose after a long latency. The virus produced by this tumor appears to have undergone a complex series of recombination events involving the helper Moloney murine leukemia virus. It encodes an Env/K-FGF fusion protein whose expression is under the control of a hybrid long terminal repeat. This virus (designated MFS, for meningeal fibrosarcoma) induces tumors in mice with high frequency and short latency. These neoplasms consist of aggressive fibrosarcomas of soft tissue as well as diffuse meningeal tumors originating from the dura mater that surround the whole central nervous system and cause severe hydrocephalus. The Env/K-FGF fusion protein expressed by the MFS virus has retained all of the biological properties of native K-FGF, including secretion, mitogenic activity, heparin binding, and neutralization by anti-K-FGF antibodies. These and other results indicate that the tumors induced by the MFS virus result from the oncogenic potential of K-FGF.

Oncogenes were originally identified as the genes responsible for the oncogenic activity of acutely transforming retroviruses. The analysis of the tumors induced by such viruses has provided important information on the cell types and tissues which are the target of these oncogenes in vivo. These studies have also given clues on the role that activation of the corresponding proto-oncogenes could play in naturally occurring tumors, as well as helping to elucidate the physiological function of the encoded proteins.

A number of dominant oncogenes which are not represented among viral oncogenes have been identified on the basis of their transforming potential in tissue culture or as sites of integration of slow transforming viruses. Among these is the *K-fgf/hst* oncogene, isolated after transfection of NIH 3T3 cells with DNA from human stomach cancer (26) and from a Kaposi sarcoma tumor (6). Expression of the *K-fgf/hst* proto-oncogene has been shown to be activated by mouse mammary tumor virus in a fraction of mammary carcinomas (23). It encodes a secreted growth factor (fibroblast growth factor 4 [FGF-4]) which belongs to the FGF family (2, 3) and transforms cells through an autocrine mechanism (8, 36). The mechanism of *K-fgf* activation consists of unregulated expression of a normal gene product (8, 46). In line with this finding, physiological expression of *K-fgf* appears to be limited to the early stages of mouse development (13).

The K-FGF protein is a powerful mitogen in vitro for a variety of cell types of mesodermal and neuroectodermal origin, including fibroblasts, endothelial cells, and melanocytes. The spectrum of cell types that are sensitive to the mitogenic action of K-FGF in vitro significantly overlaps

that of the earliest discovered members of the FGF family, acidic FGF and basic FGF (bFGF) (reviewed in reference 2). In line with its widespread mitogenic activity, K-FGF binds in vitro with high affinity to two FGF receptors (Flg and Bek) (18, 19), which appear to be expressed in many adult tissues (21). Fibroblast cell lines transfected to express K-FGF produce rapidly growing tumors in syngeneic and allogeneic immunocompetent mice, indicating that the *K-fgf* oncogene has a high transforming potential (36).

Although the *K-fgf/hst* oncogene was originally cloned from DNA from a human stomach carcinoma and Kaposi's sarcoma, evidence of its involvement in the etiology of such tumors is lacking (2). To identify the cells and tissues susceptible to *K-fgf* oncogenic potential in vivo, we constructed recombinant murine retroviruses containing the *K-fgf* cDNA and determined their tumorigenicity in mice. The results presented in this report show that a recombinant retrovirus expressing an Env/K-FGF fusion protein causes a high frequency of tumors in immunocompetent as well as in nude mice. These neoplasms consist of aggressive fibrosarcomas of soft tissues as well as multifocal meningeal tumors originating from the dura mater that envelop the whole central nervous system and cause severe hydrocephalus.

MATERIALS AND METHODS

Cell culture and transfection. ψ 2 (17), NIH 3T3, BALB/3T3, and COS-7 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum. ψ 2 and NIH 3T3 cells were transfected by the calcium phosphate technique (42). For selection of G418-resistant clones, geneticin (1 mg/ml; GIBCO) was added to the medium. Transfection of COS-7 cells was performed by the

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DEAE-dextran chloroquine procedure (16). The XC rat tumor cell line (25) was kindly provided by S. Goff.

Meningeal fibrosarcomas were isolated under a dissection microscope, digested with collagenase (Sigma), centrifuged, and resuspended in DMEM plus 20% fetal calf serum. Cultures of the in situ fibrosarcomas were started from the tumor tissue after digestion with collagenase. Cells were cultured in DMEM plus 10% fetal calf serum.

Plasmids. Plasmid pMDK-*fgf* was constructed by cloning the 1.1-kb *EcoRI* fragment encompassing the human K-*fgf* cDNA (7) into the unique *EcoRI* site of the MDRI retroviral vector (11).

Virus production and titration. To collect the virus, producer cells (2×10^6 /65-mm-diameter plate) were plated, and the next day the culture medium was replaced by 2 ml of DMEM plus 5% calf serum. Supernatants were collected 18 h later and filtered through a 45- μ m-pore-size filter. For virus titration, NIH 3T3 cells (10^5 /65-mm-diameter dish) were plated, and 1 day later cells were incubated for 2 h with dilutions of the supernatant in 1 ml of DMEM plus 5% calf serum in the presence of 8 μ g of Polybrene per ml. Complete medium was added, and 2 days later plates were split 1:3 into 100-mm-diameter dishes. Foci and G418-resistant colonies were scored 10 days later. For titration of helper virus, the XC plaque-forming assay was performed (25).

Mice and virus inoculation. BALB/c and ICR mice were obtained from Harlan Sprague Dawley and bred in our mouse colony at New York University School of Medicine. Newborn mice were injected intraperitoneally (i.p.) with 100 μ l of virus-containing supernatant. Four-week-old Swiss *nu/nu* mice were obtained from Taconic and injected with 250 μ l of supernatant subcutaneously (s.c.), i.p., or into the tail vein.

Immunoprecipitation. Cells were labeled with 200 μ Ci of [35 S]methionine for 6 to 12 h and then lysed in radioimmunoprecipitation assay buffer (7). The cell lysate was centrifuged at 12,500 rpm for 30 min at 4°C. The medium was mixed with 1/4 volume of 5 $\times$ radioimmunoprecipitation assay buffer. Cell lysates and media were immunoprecipitated with anti-K-FGF rabbit antiserum raised against human recombinant K-FGF. Immunoprecipitates were analyzed on 10 or 12% polyacrylamide gels. Following electrophoresis, the dried gels were fluorographed at -70°C.

In situ hybridization. Mice were anesthetized and perfused through the heart with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The head and the spine were removed and postfixed in the same fixative for 24 h at room temperature. Specimens were decalcified in 0.1 M EDTA-0.1 $\times$ PBS for 10 to 14 days (38) and embedded in Tissue-tek optimal cutting temperature (OCT) compound. Serial frozen sections of 4 μ m were collected onto poly-L-lysine-coated slides (1). Sections were dehydrated in increasing ethanol concentrations, air dried, and stored at -20°C in sealed boxes. Fibrosarcomas occurring in the soft tissues were embedded in OCT, and 4- μ m frozen sections were collected onto coated slides. Sections were fixed for 20 min at room temperature in 4% paraformaldehyde in PBS, rinsed in PBS, and dehydrated. For hybridization, slides were brought to room temperature, immersed for 15 min in 0.2 N HCl at room temperature, washed in distilled water for 5 min, and then treated with predigested proteinase K (2 μ g/ml) in 0.2 M Tris HCl (pH 7.4)-2 mM CaCl₂ at 37°C. Slides were then immersed for 1 min in PBS containing glycine (2 mg/ml) and subsequently washed in PBS. After dehydration, slides were immersed in 0.1 M triethanolamine buffer (pH 8.0), 0.25% acetic anhydride was added, and the slides were incubated

for 10 min at room temperature with constant stirring. After a rinse in 2 $\times$ SSPE (360 mM NaCl, 20 mM Na₂HPO₄, NaH₂PO₄, 2 mM EDTA) and dehydration, 40 μ l of hybridization solution (50% formamide, 0.6 M NaCl, 20 mM Tris HCl [pH 7.2], 5 $\times$ Denhardt's solution, 1 mM EDTA, 10% sodium dextran sulfate, 10 mM dithiothreitol, 150 μ g of salmon sperm DNA per ml, 1 mg of yeast tRNA per ml, 1×10^6 to 4×10^6 cpm of 35 S-labeled RNA probe) per slide was added. Siliconized coverslips were overlaid, and hybridization was carried out for 16 h at 50°C in a humid chamber. Slides were washed for 30 min in 50% formamide-0.6 M NaCl-20 mM β -mercaptoethanol, rinsed in 0.5 M NaCl-10 mM Tris HCl (pH 8.0), and then incubated for 30 min at 37°C in 0.5 M NaCl-10 mM Tris HCl (pH 8.0)-20 μ g of RNase per ml. After washes with 0.5 M NaCl-10 mM Tris HCl (pH 8.0), 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 0.1 $\times$ SSC at room temperature, slides were dehydrated in 70% ethanol-0.3 M ammonium acetate followed by 95% ethanol-0.3 M ammonium acetate and then air dried. Slides were dipped in Kodak NTB-2 nuclear emulsion and exposed at 4°C for 2 to 5 days. Slides were developed, stained, and mounted in Permount.

Plasmid pG3(B) *Ava*, containing a 263-bp *AvaI-EcoRI* fragment of the human K-*fgf* cDNA encompassing the 3' noncoding region (7), was used as a template for antisense and sense riboprobes. 35 S-labeled probes ([α - 35 S]UTP; >1,000 μ Ci/mmol) were synthesized with SP6 or T7 RNA polymerase, using an RNA transcription kit (Boehringer) in standard reactions. One microgram of template typically yielded $\approx 10^8$ cpm.

Genomic library construction and screening. High-molecular-weight DNA purified from KFS-1 cells was partially digested with restriction enzyme *Sau3A*, and 15- to 20-kb fragments were purified on a 10 to 40% sucrose gradient (28). The DNA was ligated to *Bam*HI-digested EMBL3 phage vector and packaged by using a Gigapak Gold kit (Stratagene). The library was screened with a 557-bp *SacI-EcoRI* fragment of the human K-*fgf* cDNA 32 P labeled by nick translation. Filters were hybridized overnight at 42°C in 50% formamide-5 $\times$ SSC-1 $\times$ Denhardt's solution-10 mM sodium phosphate buffer (pH 6.8)-10% sodium dextran sulfate-0.1% sodium dodecyl sulfate (SDS) and 100 μ g of denatured calf thymus DNA per ml. The filters were washed at 65°C with 2 $\times$ SSC-0.1% SDS for 30 min and with 1 $\times$ SSC-0.1% SDS for 45 min. The insert DNA was subcloned in pBluescript (Stratagene) and sequenced by the dideoxynucleotide termination method (30).

Northern (RNA) analysis. RNA was purified from cultured cells by the guanidine thiocyanate-cesium chloride method (5). RNA was fractionated on a 1% formaldehyde gel and transferred to nitrocellulose. Probes were a 1.4-kb *PvuII-EcoRI* fragment of the murine *bek* cDNA (18) and a 0.9-kb *EcoRI-PvuII* fragment of the murine *flg* cDNA (19), labeled with 32 P by nick translation (28). The amount of RNA in each lane was normalized upon hybridization with a 1.4-kb rat glyceraldehyde 3-phosphate dehydrogenase cDNA probe (24).

Binding of 125 I-labeled bFGF or K-FGF to cells. Human recombinant bFGF was iodinated by using chloramine T (15). The specific activity was usually 1,000 cpm/fmol. Human recombinant K-FGF was iodinated with 125 I-labeled Bolton-Hunter reagent and had a specific activity of about 200 cpm/fmol. Tumor cells were plated on gelatin-coated dishes and incubated for 48 h with or without polyclonal anti-K-FGF-neutralizing antiserum. Cells were then washed and incubated for 2 h at 4°C in DMEM containing 0.15%

FIG. 1. Structure of the proviral DNA of the recombinant retrovirus pMDK-*fgf*. pMDK-*fgf* was constructed by inserting the 1.1-kb *Eco*RI human K-*fgf* cDNA into the MDRI retroviral vector under the control of the SV40 promoter/enhancer.

gelatin, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), and 5 ng of 125 I-labeled bFGF or K-FGF per ml. At the end of the incubation period, the medium was removed, and the cells were washed with PBS and then with 2 M NaCl in 20 mM HEPES (pH 7.4) to remove the growth factor bound to the extracellular matrix (20). 125 I-labeled bFGF or K-FGF bound to receptors was released by washing cells with 2 M NaCl in 20 mM sodium acetate (pH 4.0). The amount of radioactivity released in the washes was determined in a gamma scintillation counter.

RESULTS

Low tumorigenicity of recombinant retroviruses constructed in vitro. To study the tumorigenicity of the K-*fgf* oncogene in vivo, we constructed a recombinant retrovirus containing the K-*fgf* cDNA. We chose the MDRI retroviral vector, which has the long terminal repeats (LTRs) of the myeloproliferative sarcoma virus (MPSV). This vector had been shown to be more efficient than Moloney murine leukemia virus (MoMLV)-based retroviral vectors in promoting gene expression in nonhematopoietic cells both in vitro and in vivo (11). A 1.1-kb *Eco*RI fragment encompassing the coding region of the human K-*fgf* cDNA (7) was inserted into the unique *Eco*RI site of the MDRI plasmid (Fig. 1) under the control of the simian virus 40 (SV40) promoter/enhancer region. The recombinant plasmid was transfected into the ψ 2 helper-free retrovirus packaging cell line (17), G418-resistant colonies were isolated, and the supernatants from 40 independent clones were used to infect NIH 3T3 cells. To evaluate the retrovirus titer, both the number of foci (focus-forming units [FFU]) and the number of G418-resistant colonies (CFU) were determined. The cell line ψ MPSVq, producing the pMDK-*fgf* retrovirus with a titer of 10^5 FFU/ml, was chosen for the in vivo studies. The clone was found to also produce a helper virus, presumably resulting from recombination and reconstitution of a packaging signal in the ϕ genome following transfection. The helper virus had a titer of 10^6 PFU/ml.

Newborn ICR mice were injected (10^4 FFU per animal), and only leukemia was observed in most of the animals starting at about 16 weeks after injection. Seven months after infection, however, a tumor was observed adjacent to the spine in one of the mice. Examination of the tumor histologically and by electron microscopy revealed a highly undifferentiated fibrosarcoma. A culture that was readily established as a continuous cell line was started from a fragment of the tumor tissue.

A K-*fgf* recombinant retrovirus generated in vivo is highly tumorigenic. The cell line established from the fibrosarcoma (designated KFS-1) produced a highly transforming retrovirus that readily induced the formation of foci in NIH 3T3 cells. The titer of the transforming retrovirus was estimated to be 10^6 FFU/ml. Titration of the helper virus in the XC plaque-forming assay yielded 10^7 PFU/ml.

We reasoned that this virus could have been selected for high tumorigenicity in vivo. We therefore used the supernatant from the KFS-1 cell line to infect 4-week-old Swiss

nu/nu mice (2.5×10^5 FFU per animal) and newborn ICR mice (10^5 FFU per animal). Swiss *nu/nu* mice were injected i.p., s.c., or intravenously (i.v.). All of the nude mice injected i.p. developed fibrosarcomas at the site of virus inoculation (Table 1) within 6 weeks after injection. Mice injected i.v. developed fibrosarcomas after 8 weeks of latency; the tumors were usually localized on the back of the animal, along the base of the spine. Mice injected s.c. developed fibrosarcomas with longer latency, and the tumors grew at a slower rate than did those in the animals that had been injected i.p. or i.v. In the fibrosarcomas, interlacing fascicles of pleomorphic, atypical spindle cells were observed. Mitotic figures were present. The tumors invaded surrounding tissues, but no evidence of metastasis was found. Electron microscopy showed typical fibroblastic differentiation, and retroviral particles were present (data not shown).

In contrast to the nude mice, ICR mice (which had been injected only i.p.) developed more distinctive abnormalities. Fibrosarcomas arising in the abdominal region, presumably at the site of injection, were observed in about 10% of the ICR mice, but the most prominent pathology was a severe hydrocephalus, which developed by 8 to 16 weeks after infection (Table 2). The affected mice were small, their weight being about 50% of that of the unaffected siblings and control mice of the same age, and they showed an enlarged and bulging head, often accompanied by a curved spine. Histological analysis of cranial sections showed a massive proliferation of the dural portion of the meninges (Fig. 2A to D). The neoplasms had the appearance of fibrosarcomas, but they grew as an unusual plaque-like tumor along the inner surface of the dura mater. At times the tumor also tracked into the arachnoid or invaded the bone. The tumor cells themselves were spindle cells of variable atypicality. The tumors did not show typical features of human meningiomas, which are generally thought to originate from the arachnoid (4), and on electron microscopy appeared to be fibroblasts. For this reason, we classified these tumors as meningeal fibrosarcomas. Mast cells were also noted among the spindle cells. In some of the affected animals, histological analysis was also performed on the spinal cord, and a massive proliferation of the dura, similar to that observed in the cranium, was detected (Fig. 2E and F). In a few cases, both hydrocephalus associated with meningeal tumors and ab-

TABLE 1. Tumorigenicity of the MFS virus in 4-week-old Swiss *nu/nu* mice^a

Injection	Incidence (%)	Latency (wk)
i.p.	100	5
i.v.	100	7–14
s.c.	50	10–18

^a Mice were injected with 250 μ l of supernatant collected from KFS-1 cells (5×10^6 cells per 100-mm-diameter plate). Animals were monitored twice a week for appearance of solid tumors. Tumors (fibrosarcomas) were scored when their diameter was ≥ 1 mm. Each group of inoculated animals consisted of 12 mice.

TABLE 2. Tumorigenicity of the MFS virus in newborn ICR mice^a

Pathologic effect(s)	Incidence (%)	Latency (wk)
Hydrocephalus/meningeal fibrosarcoma	61.0	8–16
Soft-tissue fibrosarcoma	11.8	15–21
Hydrocephalus/meningeal fibrosarcoma and soft-tissue fibrosarcoma	8.5	12–16
Leukemia	8.5	16–20
Hydrocephalus/meningeal fibrosarcoma and leukemia	6.8	18–20
None	3.4	

^a Mice were injected i.p. with 100 μ l of supernatant from KFS-1 cells. Meningeal fibrosarcomas were detected by the bulging head and reduced size of the animals. Animals were sacrificed, and histological analysis of the skull was performed. Soft-tissue fibrosarcomas were scored when their diameter was ≥ 1 mm. Results are pooled from two independent experiments. The total number of inoculated mice was 59.

dominal fibrosarcomas were observed. Leukemia was observed in a small number of injected animals, and some of the mice developed hydrocephalus and leukemia (Table 2). The possible development of meningeal tumors in nude mice was not systematically determined, since these animals were generally sacrificed early after infection because of the rapid growth of their fibrosarcomas. However, it should be noted that a meningeal fibrosarcoma was also observed in one of the nude mice.

As a control, the helper virus produced by the KFS-1 cell line was purified from the transforming virus by infection of NIH 3T3 cells at limiting dilution and was injected into newborn ICR mice. The animals developed leukemia after ~20 weeks of latency and no detectable solid tumors.

We also infected nude mice with an inoculum of KFS-1-produced virus about 20 times lower (1.2×10^4 FFU per animal) than that used in the previous experiments. These animals still developed fibrosarcomas with high efficiency and short latency, while 3×10^4 FFU of the original pMDK-*fgf* virus per animal did not produce any tumors 3 months after injection.

The highly tumorigenic retrovirus results from recombination in vivo. To elucidate the molecular basis for the oncogenic potential of this novel retrovirus (designated MFS, for meningeal fibrosarcoma), we analyzed the properties of the KFS-1 cell line as well as the MFS virus itself. When immunoprecipitation was performed on the KFS-1 cells after metabolic labeling, a surprising result was observed. Anti-K-FGF antibodies immunoprecipitated from both the cell lysate and the culture medium a polypeptide of about 60 kDa, much larger in size than the native K-FGF protein, which has an apparent molecular size of 22 to 24 kDa (Fig. 3). The 60-kDa protein was recognized by all anti-K-FGF antibodies used, which had been raised against the entire K-FGF protein or against peptides corresponding to amino acids (aa) 100 to 114 and 182 to 198 of the K-FGF sequence. The 60-kDa protein appeared to be efficiently secreted in the medium of the tumor cells.

Southern blot analysis of DNA extracted from KFS-1 cells after digestion with a panel of informative restriction enzymes (data not shown) showed that the pMDK-*fgf* provirus had undergone a rearrangement in the 5' region of the *K-fgf* sequences, which could explain the finding that KFS-1 cells were not G418 resistant (Fig. 1). The observation that the 60-kDa polypeptide was secreted by the tumor cells suggested that a rearrangement had joined the 5' region of the

K-fgf cDNA to a sequence encoding a secretory protein. Since a helper virus was present in the virus preparation used for infection, and it is known that coinfecting retroviral genomes undergo a high frequency of illegitimate recombination, the rearrangement could have involved the helper MoMLV.

To verify this hypothesis and define the sequences encoding the 60-kDa K-FGF protein, we constructed a genomic library from KFS-1 cells in the EMBL3 phage vector. The library was screened with a ³²P-labeled *K-fgf* cDNA as a probe, and one of the positive phages identified after secondary screening was found to encompass human *K-fgf* cDNA sequences. A 2.2-kb *KpnI-EcoRI* fragment, containing all of *K-fgf* and about 1.3 kb of upstream sequence, was subcloned and sequenced. Sequence analysis revealed that a rearrangement had joined nucleotide 279 in the *K-fgf* cDNA to nucleotide 6694 of the MoMLV *env* gene (41). The fused gene would encode for 306 aa of the MoMLV gp70 followed by 194 aa of K-FGF (Fig. 4A and B). This region corresponds to all of the mature K-FGF protein plus an additional 19 aa that are part of the signal sequence normally cleaved during processing (8). The fusion protein would contain two of the seven N-linked glycosylation sites present in MoMLV gp70. The phage isolated appeared to contain the entire provirus. Fine restriction mapping analysis with 16 different enzymes was performed, and selected subclones of the proviral genome were sequenced. The results obtained indicated that the MFS virus contained the *gag* and *pol* genes of MoMLV. While the *gag* gene appeared intact, the *pol* sequences had suffered a 431-nucleotide deletion. Sequencing of the LTR region and comparison with the sequences reported for MoMLV and MPSV LTRs revealed that the U3 region was derived from MoMLV, while R and U5 sequences were from MPSV (data not shown). We analyzed by polymerase chain reaction the structure of the LTR of the helper virus, which had been purified from the transforming MFS virus as described above. The MoMLV/MPSV LTR rearrangement was also present in the helper virus. While the precise site of rearrangement between MPSV and MoMLV LTRs cannot be determined because of the high degree of homology between these two sequences (34), it is clear that the MFS virus arose as a result of multiple recombination events. The structure of the defective transforming retrovirus is shown in Fig. 4C.

Expression of the *K-fgf* oncogene in the tumors. Northern blot analysis of RNA expression in the sarcomas arising in situ revealed high levels of expression of *K-fgf* RNA. These tumors appeared to be mono- or oligoclonal, as shown by Southern blot analysis (data not shown).

On the other hand, expression of *K-fgf* in the meningeal tumors could not be analyzed on purified RNA because of the difficulty of isolating the meninges from the skull. We therefore analyzed the expression of *K-fgf* on cranial sections by in situ hybridization. Also, K-FGF being a secreted growth factor, direct analysis of its expression at the single-cell level would have allowed us to determine whether cell recruitment was occurring in the tumors. In situ hybridization was also performed on the fibrosarcomas at the site of injection. After fixation in paraformaldehyde and demineralization of the skull and the spine, frozen sections were hybridized with [³⁵S]UTP-labeled RNA probes corresponding to a 263-bp *AvaI-EcoRI* fragment encompassing the 3' region of the *K-fgf* cDNA (7). Upon autoradiography, intense labeling was observed in the areas of meningeal proliferation (Fig. 5). Areas of intense labeling were observed together with practically negative areas, suggesting

FIG. 2. Histopathology of meningeal fibrosarcomas. (A) Medium-power view of meningeal fibrosarcoma in dura of skull, indicated by arrows (hematoxylin-eosin; magnification, $\times 93$); (B) medium-power view of dura of a control animal (hematoxylin-eosin; magnification, $\times 186$); (C) high-power view of meningeal fibrosarcoma (hematoxylin-eosin; magnification, $\times 372$); (D) brain of an affected animal showing severe hydrocephalus (hematoxylin-eosin; magnification, $\times 9$); (E) low-power view of spine showing diffuse meningeal fibrosarcoma, indicated by arrows (hematoxylin-phloxin-safronin; magnification, $\times 19$); (F) medium-power view of meningeal fibrosarcoma in spinal cord (hematoxylin-phloxin-safronin; magnification, $\times 149$).

FIG. 3. Immunoprecipitation analysis of metabolically labeled KFS-1 fibrosarcoma cells. Cells (2×10^6 /65-mm-diameter plate) were labeled for 8 h with 200 μ Ci of [35 S]methionine per ml. Cell lysates (L) and media (M) were immunoprecipitated with an anti-K-FGF serum; immunoprecipitates were fractionated on a 12% polyacrylamide gel. NIH3T3, NIH 3T3 cells; ψ MPSVq, ψ 2 cells producing the pMDK-*fgf* retrovirus and expressing native human K-FGF; KFS-1, cells from the KFS-1 fibrosarcoma line.

recruitment of uninfected cells by the secreted growth factor. Within the positive areas, the labeling was not homogeneous, with a few cells showing a much higher number of grains than the average in the same area. A more diffuse pattern was observed in the fibrosarcomas arising in situ (data not shown), which showed no clearly negative areas.

Properties of the 60-kDa Env/K-FGF fusion protein. The recombination event that resulted in the production of a fused K-FGF protein created a highly oncogenic retrovirus that clearly differs in this property from the original pMDK-*fgf* construct. In principle, the 60-kDa protein could have properties different from those of the native K-FGF growth

factor. To analyze the biological activity of the Env/K-FGF polypeptide, the 2.2-kb *KpnI-EcoRI* fragment encoding the fused protein was cloned into the *EcoRI* site of the 91023B mammalian expression vector (44) under the control of the adenovirus major late promoter. The resulting plasmid, p9env/K-*fgf*, was transfected in COS cells, and immunoprecipitation after metabolic labeling was performed. As shown in Fig. 6, a 60-kDa polypeptide was detected in the cell lysate and medium of p9env/K-*fgf*-transfected cells. Like native K-FGF, the fusion protein was stabilized in the medium by the glycosaminoglycan heparin (8). The COS cell conditioned medium was used to test its ability to stimulate [3 H]thymidine incorporation in serum-starved BALB/3T3 cells as well as for its capacity to produce morphological transformation of NIH 3T3 cells. The same tests were performed by using conditioned medium from COS cells transfected with plasmid p9KS3A (7), which carries the wild-type K-*fgf* cDNA. These experiments showed that the fusion protein was able to stimulate DNA synthesis and to induce morphological changes similarly to native K-FGF but with a somewhat lower potency (Fig. 7). This activity was blocked by anti-K-FGF neutralizing antibodies.

Plasmid p9env/K-*fgf* induced foci of stable transformation in NIH 3T3 cells with about the same efficiency as did p9KS3A. In a representative experiment using 2.5 μ g of plasmid DNA per 10^6 cells, this activity corresponded to 800 foci per μ g of DNA for p9env/K-*fgf* and 670 foci per mg of DNA for p9KS3A. We previously showed (36) that K-*fgf*-transformed 3T3 cells revert to a normal phenotype, as shown by morphological changes and loss of the ability to grow in serum-free medium, when exposed to anti-K-FGF neutralizing antibodies. We thus tested the effect of the same neutralizing antibodies on 3T3 cells which had been transformed by p9env/K-*fgf*. Growth in serum-free medium of

FIG. 4. Schematic representation of the Env/K-FGF fusion protein and of the MFS provirus. (A) Structure of the MoMLV *env* gene, encoding gp70 and p15, and of the human K-FGF cDNA (7) are indicated. The nucleotide numbers of the MoMLV genome (41) and of the human K-*fgf* cDNA (7) are indicated. Arrows indicate the breakpoint. The stippled area of K-FGF represents the signal peptide; the dark area indicates the mature protein. (B) Env/K-FGF fusion protein. (C) Structure of the MFS proviral DNA. The U3 region is derived from MoMLV, while R and U5 are from MPSV; the *pol* gene (Δ *pol*) contains a deletion spanning nucleotides 3430 to 3860.

FIG. 5. In situ hybridization analysis of K-*fgf* expression in meningeal tumors. (A) Bright-field micrograph of section from skull of an ICR mouse with meningeal fibrosarcoma after in situ hybridization with an ^{35}S -labeled antisense RNA probe transcribed from the 263-bp *AvaI-EcoRI* fragment of the human K-*fgf* cDNA; (B) dark-field image of panel A; (C) bright-field micrograph of section from spinal cord of an ICR mouse with meningeal fibrosarcoma after in situ hybridization with the human K-*fgf* RNA probe; (D) dark-field image of panel C.

FIG. 6. Immunoprecipitation analysis of the Env/K-FGF fusion protein expressed in COS cells. COS-7 cells were transfected by the DEAE-dextran chloroquine method; 40 h later, cells were labeled for 6 h with 200 μ Ci of [35 S]methionine per ml in the presence (+) or absence (-) of heparin (90 μ g/ml). Cell lysates (L) and media (M) were immunoprecipitated with rabbit anti-K-FGF serum. Immunoprecipitates were fractionated on a 12% polyacrylamide gel. —, untransfected cells; p9KS3A, cells transfected with a plasmid containing the *K-fgf* cDNA; p9env/*K-fgf*, cells transfected with a plasmid encoding the Env/K-FGF fusion protein.

cells transformed by p9env/*K-fgf* was inhibited to the same extent as was growth of cells transformed by p9KS3A (Fig. 8).

One of the consequences of the recombination event that fused the *K-fgf* cDNA to the viral *env* gene has been shifting the control of expression of the oncogene from the SV40 promoter to a novel hybrid LTR (Fig. 4). To assay whether the expression of K-FGF from the MFS retrovirus was more efficient than in the original pMDK-*fgf* construct, we infected NIH 3T3 cells with equal titers of virus produced by the Ψ MPSVq and KFS-1 cell lines. Three days after infection, the cells were metabolically labeled, and immunoprecipitation of lysates and media with anti-K-FGF antibodies was performed. As shown in Fig. 9, we did not detect higher levels of protein in cells infected with the MFS virus. Taken together, these results indicate that the high tumorigenic ability of the MFS retrovirus is not due to the production of a protein with higher mitogenic activity or to very elevated levels of growth factor expression.

In vitro properties of cells from MFS virus-induced tumors. To analyze the properties of the tumors induced by the MFS virus and to gain clues on the specificity exhibited by this oncogenic agent, we established cultures from several MFS-induced fibrosarcomas and meningeal tumors (see Materials and Methods).

Cultures were started from several independent meningeal fibrosarcomas as well as from in situ tumors. As expected, they all produced infectious virus. Immunoprecipitation analysis with anti-K-FGF antibodies showed that the Env/K-FGF growth factor was expressed at similar levels in the cell lines deriving from the different tumors and comparably stabilized by heparin (data not shown).

We performed binding assays on the tumor cultures by using 125 I-labeled bFGF or K-FGF. Binding to the high-affinity cell surface receptors was quite low, probably because the receptors are downregulated as a result of the sustained production of the growth factor by the tumor cells. Support for this hypothesis came from experiments in which cells were treated with anti-K-FGF neutralizing antibodies (37). After 48 h of antibody treatment, the receptor number had increased to about 10^4 per cell (data not shown). Anti-K-FGF antibodies had no effect on the binding to NIH 3T3 cells. The values measured are within the range obtained

FIG. 7. Stimulation of DNA synthesis by K-FGF and Env/K-FGF proteins in BALB/3T3 cells. Cells (3×10^4 /16-mm-diameter well) were plated in DMEM plus 10% calf serum and the next day washed and incubated with DMEM plus 0.5% calf serum (1 ml per well). Two days later, different volumes of conditioned medium (ranging from 0.25 to 40 μ l) from COS-1 cells transfected with plasmid p9KS3A or p9env/*K-fgf* were added; 18 h later, the cells were labeled with 1 μ Ci of [3 H]thymidine per ml for 6 h. Cells were then washed twice with PBS, and the incorporation into trichloroacetic acid-precipitable material was determined. The conditioned media had been collected after 6 h of incubation with or without 50 μ g of heparin (hep) per ml in DMEM plus 0.1% serum. To quantitate the amounts of K-FGF or Env/K-FGF present in these media, aliquots were fractionated on a 12% polyacrylamide gel, transferred to a nitrocellulose filter, and incubated with an anti-K-FGF polyclonal antibody followed by 125 I-labeled protein A. The relative amounts of K-FGF or Env/K-FGF protein in each conditioned medium were estimated by scanning the filter with Ambis radioactive analyzer. Values corresponding to dilutions of conditioned media containing equal amounts of K-FGF or Env/K-FGF proteins are shown for each point. The data are averages for two wells minus background incorporation ($\sim 2,500$ cpm per well). Conditioned medium from mock-infected COS cells did not produce any incorporation above background.

for other tissues or cell lines (2, 3), but we cannot determine whether receptor number was completely restored. Binding to the low-affinity sites, the heparan sulfates present on the cell surface, did not appear to differ, in terms of number or affinity, from that observed in murine fibroblasts.

Expression of two receptors for K-FGF, FGFR-1 (*fgf*) and FGFR-2 (*bek*), was analyzed by Northern blots in the tumor-derived cell cultures. The *bek* mRNA is expressed at high levels in the brain and at low levels in other tissues and most cell lines, while *fgf* expression is much higher and quite widespread (2, 21). Figure 10 shows representative results of our analysis of the tumor-derived cell lines. High levels of expression of FGFR-2 were detected in the meningeal tumors but not in the in situ fibrosarcomas. In the meningeal tumor lines, FGFR-1 expression was lower than in NIH 3T3 cells or brain tissue. FGFR-2 binds the K-FGF growth factor with about 10 times higher affinity than does FGFR-1 (18). It is possible to speculate that the relatively high levels of expression of this high-affinity receptor for K-FGF in the meningeal fibrosarcomas may contribute to tumorigenesis by the MFS virus in this tissue.

FIG. 8. Inhibition of serum-free growth of NIH 3T3 clones transformed by *p9env/K-fgf* or wild-type *K-fgf* by anti-K-FGF immunoglobulin G (IgG). A total of 5×10^4 cells per 35-mm-diameter dish were plated in DMEM containing 10% calf serum. The next day (time 0), cells were washed twice with DMEM without serum and refed with DMEM-F12 (GIBCO) containing transferrin (5 μ g/ml) and insulin (10 μ g/ml). A 50- μ g/ml concentration of IgG purified from a rabbit polyclonal anti-K-FGF antiserum by adsorption to protein A-Sepharose CL-4B or control IgG was added. Duplicate plates were trypsinized and counted at days 2 and 4. 6A is an *env/K-fgf*-transformed line; 8bG is a wild-type *K-fgf*-transformed line.

DISCUSSION

To identify the cell and tissue targets of the transforming activity of the *K-fgf* oncogene in vivo, we constructed a recombinant retrovirus carrying the human *K-fgf* cDNA and injected it into newborn ICR immunocompetent mice as well as nude mice. The retrovirus was constructed by using the MDRI plasmid vector, an MPSV-based retroviral vector in which expression of *K-fgf* was driven by the SV40 promoter/enhancer region. Helper virus was present in the virus preparations injected. No pathology that could be ascribed to the expression of *K-fgf* was observed, with the exception of a single fibrosarcoma which arose in one of the ICR mice

FIG. 9. Immunoprecipitation analysis of NIH 3T3 cells infected with recombinant retroviruses. NIH 3T3 cells (5×10^5 /100-mm-diameter plate) were infected with 10^5 FFU of pMDK-*fgf* or MFS recombinant retrovirus in the presence of 8 μ g of Polybrene per ml for 2 h at 37°C. Three days after infection, cells were labeled for 10 h with 100 μ Ci of [³⁵S]methionine per ml in medium containing 50 μ g of heparin per ml. Cell lysates (L) and media (M) were immunoprecipitated with polyclonal anti-K-FGF antiserum. Immunoprecipitates were fractionated on 12% polyacrylamide gel. —, uninfected cells; pMDK-*fgf* cells infected with supernatant from the ψ MPSV cell line; MFS, cells infected with supernatant from the KFS-1 cell line.

after about 7 months of latency. The retrovirus produced by this tumor appeared to have undergone a complex series of recombination events involving the pMDK-*fgf* retroviral genome and the helper virus. It now encoded an Env/K-FGF fusion protein composed of 306 aa of the MoMLV gp70 protein followed by the entire K-FGF polypeptide, and expression of the oncogene sequences was placed under the control of a novel hybrid MoMLV/MPSV LTR. This novel retrovirus induces tumors at very high frequency and with relatively short latency and is therefore quite comparable to many natural acutely transforming retroviruses.

The tumors induced by the MFS virus are of two types: malignant fibrosarcomas, which arise near or at the site of injection and are prevalent in nude mice, and unusual tumors of the meninges, which are best classified as meningeal fibrosarcomas. These tumors, which are mainly seen in ICR mice, induce a severe form of hydrocephalus that is associated with locomotor problems and stunted growth. The tumors are likely to be multifocal, as they appear in distinct areas of the meninges around the brain and the spinal cord and eventually envelop the whole central nervous system.

Fibrosarcomas developing at the site of injection have frequently been observed in animals infected by acutely transforming retroviruses (40). The process of tissue repair occurring upon injection is believed to play a role in fostering tumor progression (10). The observation that fibroblasts are a primary target for the *K-fgf* oncogene in vivo is not surprising. Fibroblasts express a high number of high-affinity receptors for K-FGF, and they are extremely sensitive in vitro to the mitogenic and transforming activity of the growth factor (8). On the other hand, meningeal tumors have rarely been observed among experimental brain tumors in mice (39). In humans, tumors of dural fibroblasts represent a small fraction of meningeal tumors, while the majority are meningiomas, benign tumors which derive from the arachnoid (4). The tumors induced by the MFS virus are reminiscent of a rare human pathology, designated meningeal sarcomatosis, of which very little is known and which could be multicentric in origin (47). The MFS virus could therefore be

FIG. 10. Northern analysis of FGFR-2 and FGFR-1 expression in cells derived from MFS-induced tumors. Total RNA (15 μ g) was fractionated on a 1% formaldehyde gel, transferred to nitrocellulose, and hybridized with a 32 P-labeled 1.4-kb *PvuII-EcoRI* fragment of FGFR-2. The hybridized probe was stripped, and the filter was hybridized with a 0.9-kb *EcoRI-PvuII* fragment of FGFR-1. To quantitate the amount of RNA in each lane, the filter was then hybridized with a 1.4-kb *PstI* fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). MEN B, MEN H, and MEN A, cells derived from MFS-induced meningeal fibrosarcomas; FIBR 6, cells from soft-tissue fibrosarcoma.

useful in establishing an experimental mouse model for meningeal tumors.

Irrespective of the differences from or similarities to human pathologies, the high frequency and the diffuse nature of the meningeal sarcomas in infected mice indicate that the fibroblasts of the dura are exquisitely susceptible to the oncogenicity of the MFS virus. We have considered several possibilities to explain this finding. It could result from a specific tropism of the MFS virus for viral receptors present on meningeal fibroblasts. However, a variety of studies conducted with MoMLV, Moloney sarcoma virus, and MoMLV-based vectors, which all share a common coat and therefore should infect the same cell types, have not indicated a preferential targeting to meningeal cells (14, 29, 31, 40, 41). Rather, MoMLV receptors appear to be widespread. Thus, since the MFS virus coat should derive from MoMLV, a preferential virus uptake by meningeal cells seems unlikely. It is also possible that the hybrid LTR, which governs the expression of the MFS virus oncogene, is particularly active in meningeal cells. We have, however, obtained evidence that this LTR is quite efficient in 3T3 fibroblasts and therefore is unlikely to be specific for meningeal cells.

A plausible explanation is that meningeal fibroblasts of the dura express receptors highly specific for K-FGF or are located in an environment which is particularly favorable to the presentation of this growth factor to its high-affinity receptors. To study this possibility, we established cell cultures from several sarcomas and meningeal tumors and studied their expression of FGF receptors. All of these cells produced the Env/K-FGF growth factor in the culture medium. Surface expression of FGF receptors in these cells was, not surprisingly, downregulated because of the constitutive growth factor expression. Following upregulation, which could be obtained by using neutralizing anti-K-FGF serum, the number of binding sites for K-FGF or bFGF was

restored to values similar to those observed in many other fibroblast cultures. The only distinctive feature of the cells derived from the meningeal tumors was the relatively high expression (at the RNA level) of FGFR-2 (*bek*), which in other fibroblast cell lines is generally expressed at much lower levels of FGFR-1 (*flg*). Bek has about 10 times higher affinity for K-FGF than does Flg (18), which may have contributed to the high susceptibility of these cells to transformation by the MFS virus.

K-FGF, like other members of the FGF family of growth factors, binds to heparan sulfate proteoglycans, which are a major component of the extracellular matrix (2). Heparin or heparan sulfates appear to protect FGFs from degradation, and they may play a role in providing a cell surface-bound reservoir of the growth factors (12, 27, 45). Yayon et al. (45) have recently shown that binding of bFGF to the Flg receptor requires heparan sulfates, suggesting that FGFs binding to high-affinity receptors may require a conformational change that is induced upon interaction with these glycosaminoglycans. It is possible that the extracellular matrix in the dura mater is quantitatively or qualitatively different from that in other tissues, leading to a higher stability or more efficient interaction of K-FGF with its receptors. However, we could not detect an unusually high expression of low-affinity FGF receptors in these cells, although it is possible that they are qualitatively unusual. In addition, it is possible that some factor which might potentiate K-FGF action is present in the cerebrospinal fluid.

Only a few examples are known of acutely transforming retroviruses constructed in the laboratory by inserting oncogene or proto-oncogene coding sequences into retroviral vectors similar to the ones that we used (22, 33, 35, 43). Souyri et al. (32, 33) showed that the choice of retroviral vector greatly influenced the oncogenicity of their *N-ras* recombinant retroviruses. It was therefore not totally surprising that our initial retrovirus constructs were not tumorigenic. The MFS retrovirus which spontaneously arose in the animal is, in contrast, highly tumorigenic, but the precise reason why this novel virus is oncogenic whereas the original constructs were not is still unclear. The Env/K-FGF fusion protein produced by the MFS virus appears to possess the same biological properties as does native K-FGF, at least in vitro. Furthermore, there is evidence that unearranged K-*fgf* has oncogenic potential in mice. Some mouse mammary tumors express the K-*fgf* oncogene that has been activated by mouse mammary tumor virus insertion (23), and we have obtained a fibrosarcoma in a mouse made transgenic for human K-*fgf* (37a). This tumor clearly expressed a wild-type K-FGF protein. The Env/K-FGF protein does not appear to bind to MoMLV receptors, since NIH 3T3 cells transfected to express Env/K-FGF show no interference with MoMLV infection (data not shown). Thus, the results available indicate that the high tumorigenicity of the MFS virus does not specifically result from the expression of K-FGF as a fusion protein, although the possibility that such a fusion protein acquired some novel in vivo properties cannot be formally ruled out.

The only consistent and reproducible difference that we have found so far between the MFS virus and the original K-*fgf* retrovirus is that the MFS virus grows to higher titers (about 10-fold) in 3T3 cells than does the pMDK-*fgf* virus with its helper. Thus, at present the most likely explanation of the difference in oncogenicity is that selection has led to the isolation of a virus which is much more efficient than the original constructs in multiplying and spreading in the infected animals. While this obviously highlights the impor-

tance of natural selection in producing a virus which is viable in an animal environment, the molecular reasons for this difference remain to be elucidated and could involve a number of steps in the virus life cycle.

In closing, it is worth mentioning that only one acutely transforming retrovirus carrying a growth factor oncogene was known previously. The simian sarcoma virus *sis* oncogene encodes a fusion protein linking sequences of the viral *env* gene to the B chain of platelet-derived growth factor (9, 41). The isolation of the MFS virus, which carries the K-*fgf* oncogene, provides a new tool with which to probe the effects of alterations of growth factor expression on growth regulation and development. The MFS virus induces a high frequency of malignant fibrosarcomas and multifocal meningeal tumors. Further work should reveal the molecular basis for the extremely high susceptibility of meningeal fibroblasts to K-*fgf* oncogenic potential and should be useful in understanding the mode of action of K-*fgf* as well as its involvement in human malignancies.

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