Inhibition of Fibroblast Growth Factor 2 Expression by Antisense RNA Induced a Loss of the Transformed Phenotype in a Human Hepatoma Cell Line

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ABSTRACT

Fibroblast growth factor 2 (FGF-2 or basic FGF) is associated with the cell-transformed phenotype. To clarify the function of FGF-2 in the malignancy of tumor cells, we designed experiments to express antisense RNA in a hepatoma cell line. Using FGF-2 mRNA, alternative initiations of translation at one AUG and three CUG start codons led to the synthesis of four isoforms. SK-Hepl cells, which naturally produce the four FGF-2 proteins, were stably transfected with expression vectors that generate antisense RNAs targeted against different sites of human FGF-2 mRNA. A variable decrease of all of the isoforms of FGF-2 synthesis was observed compared with the control: the strongest inhibition was obtained with the smaller antisense targeted against AUG codon. Our results clearly demonstrated that inhibition of FGF-2 expression led to a loss of anchorage independence in soft agar. This effect was not reversed by adding exogenous FGF-2, indicating that an intracellular process of FGF-2 probably is involved in the phenotypic changes of SK-Hepl cells. Furthermore, the inhibition of FGF-2 synthesis was correlated with a loss of tumorigenicity in nude mice. These results clearly argue for a key role of endogenous FGF-2 in transformation and tumorigenesis of the hepatoma cell line used in this study.

INTRODUCTION

FGF-2, or basic FGF, a member of the heparin-binding growth factor family of proteins, is both a mitogen and differentiation factor for mesoderm- and neuroectoderm-derived cells and a potent angiogenic factor (1–4). Several forms of FGF-2 may be detected in most cell types that produce it (5). These different forms result from alternative initiations of translation at one AUG and three CUG start codons leading to synthesis of a small fragment of 18 kilodaltons and three forms of 21, 21.5, and 22.5 kilodaltons, respectively (6, 7). The relative amounts of these forms in different cell lines are substantially distinct, suggesting that this alternative initiation process is highly regulated. Subcellular cellular compartmentalization that depends on the presence or absence of a nuclear localization sequence and includes secretion has been described (8, 9). However, none of the four molecular forms includes a signal sequence for secretion, and the mechanism of this process remains unknown. Nonetheless, part of the biological activity of FGF-2 appears to be mediated through a family of high-affinity cell surface receptors and also requires low-affinity heparin-like molecules (10).

Although the role of FGF-2 in both normal and abnormal growth in vivo is unclear, several lines of evidence support the hypothesis that FGF-2 might play a role in neoplastic growth: (a) FGF-2 is produced by a variety of tumor cells (11–17); (b) Transfection of cells with FGF-2 cDNAs leads to transformation of transfected cells (18–23); (c) Neutralizing antibodies against FGF-2 inhibit cell growth and tumorigenesis of human glioblastoma cells (24) and reverse the transformed phenotype induced by transfection of FGF-2 cDNA in mouse BALB/c3T3 cells (23); and (d) Antisense oligonucleotides against FGF-2 inhibit proliferation of malignant melanomas (25) or a glioblastoma cell line (26, 27).

It seems more and more evident that FGF-2 is involved in autonomous cell growth and tumorigenesis as an autocrine growth factor in gliomas (3, 28, 29), as well as in human hepatocellular carcinomas (30). This study was designed to explore the involvement of endogenous FGF-2 in the control of cell proliferation, cell transformation, and tumorigenicity of the well-characterized human hepatoma cell line, SK-Hepl. For this purpose, we attempted to suppress FGF-2 synthesis in these cells using antisense RNAs targeted against different sequences of the FGF-2 mRNA.

The results of this study demonstrate that antisense RNAs against FGF-2 mRNA expressed in tumor cells strongly inhibit the endogenous production of FGF-2 with a concomitant loss of anchorage-independent growth in vitro and tumorigenicity in vivo.

MATERIALS AND METHODS

Plasmid Construction. Eukaryotic bicistronic expression vectors were constructed as described previously (31). In brief, FGF-2 cDNA fragments (indicated below and in Fig. 1A) were placed in an antisense orientation downstream of the CMV promoter element as the first open reading frame, and the neomycin gene was placed directly downstream of the internal ribosome entry site of the encephalomyocarditis virus as the second open reading frame (Fig. 1B). Plasmid constructions were as follows: pRFAS1, antisense FGF-2 cDNA corresponding to a 171-bp fragment covering the AUG start codon; pRFAS2, antisense FGF-2 cDNA corresponding to a 312-bp fragment covering the 5′ UTR; pRFAS3, antisense FGF-2 cDNA corresponding to a 538-bp fragment covering the 5′ UTR plus the alternatively translated region containing the four initiation codons; pRFAS4, antisense FGF-2 cDNA corresponding to a 958-bp fragment covering the full coding sequence; and pEN, control vector without insert. These constructions are shown in Fig. 1. All plasmids were prepared using cesium chloride purification.

Cell Culture. SK-Hepl cells were grown in DME-M supplemented with 10% FCS serum, 27 mm glutamine, 0.1 mg/ml amphotericin, and 0.1 mg/ml gentamycin. Cells were incubated at 37°C in a 10% CO2 atmosphere. To measure proliferation, cells were plated in six-well plates at 5 X 10^4 cells/well and counted in triplicate in a Coulter counter (Coulter Electronics, Coultronics S.A., France).

Stable Transfection. SK-Hepl cells (25 X 10^5/mm plate) were transfected by the calcium phosphate method with 5 μg recombinant plasmid. Two days later, cells were split 1:4 and cultured thereafter in DME/M containing 0.7 mg/ml of geneticin (G418 Sigma). The surviving clones were tetrasyzed and expanded individually. Mock cells were similarly transfected with a control vector from which the FGF-2 insert was absent.

Preparation of Cell Extracts and Western Blot Analysis. Frozen cell pellets were rapidly resuspended in PBS-SDS 0.2% containing PMSF (175 mg/ml) and aprotinin (1 mg/ml) and then sonicated. Total proteins were quantified by BCA assay (BioProbe). For Western blots, lysates were heated for 2 min at 95°C in SDS, dithiothreitol, and β-mercaptoethanol containing sample buffer, separated by 12.5% SDS-PAGE, and transferred to a nitrocellulose membrane. FGF-2 proteins were immunodecorated using anti-FGF-2 antibodies (Oncogene Science Inc., NY) and an enhanced chemiluminescence kit (Amersham).

Soft Agar Assay. Assays of cell growth in soft agar were performed as described previously (19). After 3 weeks at 37°C, 1 ml of a 1 mg/ml solution of dimethylthiazol diphenylditerazolium bromide (Sigma) was added to each
RESULTS

Antisense FGF-2 mRNAs Induced a Decrease of FGF-2 Synthesis. Expression vectors used in this study were constructed from different fragments of FGF-2 cDNA placed in antisense orientation as indicated in "Materials and Methods" and in Fig. 1. After transfection of SK-Hepl cells with these different constructions and G418 selection, we observed equivalent numbers of clones with all plasmids transfected, except for pRFAS4, coding for the longer mRNA, which produced fewer clones. For each construction, a pool of the clones obtained, and at least 10 separated clones were analysed by Western-blot. Of the constructions studied, pRFAS1 showed the strongest reduction of FGF-2 expression, as indicated in Table 1 and Fig. 2: the wild type cells (Fig. 2, A, compare Lanes 2, 3, and 4 with Lane 1, and B). A marked reduction in FGF-2 expression also was produced by using pRFAS3 (Fig. 2A, Lanes 7, 8, and 9), but the extent of the reduction varied. pRFAS2 and pRFAS4 constructions did not induce a significant decrease of FGF-2 expression (Fig. 2A, Lanes 5 and 6, and 10 and 11, respectively). The pool of pEN clones did not differ significantly from the wild-type cells (Fig. 2A, Lane 13).

Effect of FGF-2 Endogenous Inhibition on Cell Proliferation and Morphology of SK-Hepl Cells. No significant difference in the growth of the various populations of transfected cells compared with the wild-type or mock-transfected cells was observed. The addition of recombinant FGF-2 to the culture medium did not influence cell proliferation of wild-type cells or of transfected cell lines, even when the cells were grown in medium containing only 0.5% FCS (data not shown).

In contrast, decreased FGF-2 expression in SK-Hepl cells caused a substantial change in cell morphology (Fig. 3). Scattered colony structure, characteristic of the transformed (wild-type) phenotype, also was observed for pEN (control) and AS2 and AS4 cell lines.
INHIBITION OF FGF-2 BY ANTISENSE RNA

Fig. 3. Morphology of the studied SK-Hep1 lines. The morphology of the parental cells (WT) and the transfected cells (AS1-7, AS2-2, AS3-1, AS4-3, and pEN-5) was examined by phase-contrast microscopy (×100 magnification). One typical clone/transfection is represented.

which exhibited a normal level of FGF-2 expression. In contrast, AS1 and AS3 cells, which exhibited a significant decrease of FGF-2 expression, remained cohesive and formed clusters (Fig. 3).

Because AS2 and AS4 clones appeared to behave similarly, only two clones of AS1-, AS3-, AS4-, and PEN-transfected cells were further characterized in comparison with the parental cells.

Antisense FGF-2 mRNA Induced Anchorage-dependent Growth. The parental and transfected cells were examined for their ability to form colonies in soft agar medium, an accepted criterion for transformation (Fig. 4). As expected, wild-type cells formed numerous large colonies in soft agar. pEN- and AS4-transfected clones presented similar results. On the other hand, as shown in Fig. 4, AS4- and AS3-transfected cells lost their ability for anchorage-independent growth.

When recombinant FGF-2 was added in soft agar (1 and 10 ng/ml), the number of clones for wild-type cells or pEN cells was not affected and the loss of anchorage-independent growth observed with AS1 cells was not reversed (data not shown).

Decrease of FGF-2 Synthesis Induced Inhibition of Tumorigenesis in Nude Mice. The tumorigenicity of the parental and transfected cells was tested in nude mice by s.c. injection. As shown in Table 2, all mice injected with wild-type SK-Hep1 cells and more than 80% of mice inoculated with AS4 or pEN cells rapidly developed tumors at the site of inoculation. No tumor growth was observed in tested nude mice after injection of AS1 cells, even after 10 weeks. Three of six nude mice developed tumors after inoculation of AS3 cells. Variability was observed in tumor volume measured at day 50 regardless of whether 5 or 10 × 10⁶ cells were injected.

To ensure that no modification of FGF-2 expression had arisen during tumor development in mice, the tumors were removed and dissociated, and the cells were regrown in vitro. Cells were still resistant to G418 and synthesized similar amounts of FGF-2 as did the originally injected cells (data not shown).

DISCUSSION

It has been suggested that FGF-2 may be one of the autocrine growth factors in several tumor cells, and various strategies have been used to determine its role in the tumoral process, including transfection of FGF-2 cDNA in normal cells with or without signal peptide (9, 18, 19, 22), antibodies against FGF-2 (24, 32), and antisense oligodeoxynucleotides against FGF-2 (25, 27). An effect of exogenous FGF-2 on growth of the antisense FGF-2 RNA transfected melanoma cell line has been reported (33).

Inhibition of FGF-2 Synthesis by Different Antisense RNAs. Our studies indicate that an inhibition of the endogenous expression of FGF-2 in hepatoma cells can be obtained using antisense RNAs, leading to a modification of the cell phenotype associated with a loss...
of tumorigenicity. We have established cell lines in which antisense RNA is generated by a sequence that is stably incorporated into the genome and is able to constitutively express a transcript. The SK-Hep1 human cell line naturally expresses the four isoforms of FGF-2. Because the mechanism of translational regulation of the mRNA involves a choice of initiation codon (6), the inhibition of gene expression must present quantitative and qualitative aspects; these requirements compelled us to investigate the effect of antisense mRNAs directly at the protein expression level. We have reported distinct functions for these CUG- and AUG-initiated forms in another cell system previously (19). In this study, we investigated the possibility of a selective inhibition of initiation codon usage using different antisense RNA sequences. How antisense RNA functions to reduce expression in eucaryotic cells is not known as yet (34). In keeping with the hypothesis that the initiation of translation could be inhibited by a double-stranded RNA, only initiation at the AUG codon should have been affected by AS1 construction. AS2 was expected to inhibit the CUG-initiated forms but not the AUG-initiated one, because of the presence of an internal ribosome entry site between the CUG3 and AUG codons (35). AS3 and AS4 were expected to inhibit all FGF-2 forms. Our results showed a variable efficiency of the four antisense RNAs tested. The shortest one (pRFAS1) appeared to be the most efficient, whereas the longest one (pRFAS4) was unable to induce a significant decrease of FGF-2 expression. It is striking that the inhibition of FGF-2 expression obtained with AS1 and AS3 corresponded to a decrease in all isoforms. This result suggests a destabilization of mRNA instead of specific blocking of a particular translation initiation codon. It may be of interest to note that the 5' UTR sequence is present in AS3 but not in AS1, suggesting that the difference in efficacy between the two constructions could be caused by this G+C-rich sequence (36) easily making secondary structures.

Biological Effects of FGF-2 Synthesis: Evidence for Intracrine Regulation. It is widely known that tumor cells in culture exhibit reduced requirements for serum or growth factors. Indeed, wild-type SK-Hep1 cells show similar proliferation with or without exogenous FGF-2, using either 10 or 0.5% serum. It has been reported previously that SK-Hep1 cells expressed high-affinity receptors but respond poorly to exogenous FGF-2 (37, 38). In our experiments, cell proliferation appears to be independent of the level of endogenous FGF-2 and of the addition of exogenous FGF-2. This finding is in contrast with reports about melanoma cells for which neutralizing antibodies to FGF-2 as well as antisense oligonucleotides targeted against FGF-2 mRNA were shown to inhibit cell proliferation (25, 39). Like the study by Becker et al. (25), our study also provides evidence that loss of FGF-2 expression induces morphological and behavioral changes. Furthermore, tumorigenicity appears to be associated directly with FGF-2 expression in SK-Hep1 derived cell lines, as reported in other tumor cells (3). However, our results show that the loss of tumorigenicity is not necessarily associated with a modification of proliferation parameters when a growth factor expression is modulated. These results support the hypothesis that an intracrine process of FGF-2 may be involved in the transformed phenotype of SK-Hep1 cells. This hypothesis is also sustained by previous work demonstrating that overexpression of the nuclear localized forms of FGF-2 leads to a transformed phenotype (9, 19) that cannot be reversed by neutralizing antibodies or by the dominant negative mutant of the receptor (40).

In conclusion, regardless of what mechanisms are responsible for the inhibition of FGF-2 synthesis, this effect has definite biological consequences. Our findings provide evidence that a strong decrease of tumorigenicity is not necessarily associated with a modification of proliferation parameters when a growth factor expression is modulated.

Table 2 Tumorigenicity of wild-type and transfected SK-Hep1 cell lines in nude mice

<table>
<thead>
<tr>
<th>SK-Hep1 cell line</th>
<th>Tumor formation^b</th>
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<tbody>
<tr>
<td>AS1-7</td>
<td>0/6</td>
</tr>
<tr>
<td>AS3-1</td>
<td>3/6</td>
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<tr>
<td>AS4-3</td>
<td>5/6</td>
</tr>
<tr>
<td>pEN-5</td>
<td>4/5</td>
</tr>
<tr>
<td>WT</td>
<td>6/6</td>
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</tbody>
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^a Each mouse was inoculated s.c. with 5 × 10^6 cells. Tumor formation was taken into account when tumor volume was at least 30 mm^3.

^b Mice with tumors/mice that received injections; tumor size was monitored and measured twice weekly, as indicated in “Materials and Methods.”
FGF-2 synthesis in SK-Hep1 cells is associated with a change of morphology and the loss of anchorage-independent growth and tumorigenicity. Of the possible FGF-2 mechanisms of action implicated in tumorigenicity, our results strongly suggest that an additional intracellular process is involved in this tumor cell model.

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