Mitogenic and Dedifferentiating Effect of the K-fgf/hst Oncogene on Rat Thyroid PC Clone 3 Epithelial Cells

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Abstract

Transfection of rat thyroid differentiated epithelial cells, PC clone 3, with the K-fgf/hst oncogene results in alleviation of thyrotropin dependency for growth and suppression of the differentiated phenotype without the acquisition of the fully transformed phenotype. An autocrine mechanism is responsible for these effects, since the proliferation of PC clone 3 cells, induced by K-FGF-conditioned medium, is blocked by anti-K-FGF-specific antibodies. Moreover, addition of K-FGF-conditioned medium inhibits the thyroid differentiated functions. Also, such other members of the fibroblast growth factor family as basic and acidic fibroblast growth factor are able to induce thyroid cell growth and to block expression of the differentiated functions.

Introduction

The K-fgf/hst oncogene has been isolated by transfection assay into NIH 3T3 cells using DNA extracted from a Kaposi sarcoma (1, 2) and from a gastric carcinoma (3). It encodes a protein whose structure and functions are analogous to those of b-FGF (4, 5), which is a potent mitogen for a variety of cell types (6–8) and can induce mesodermal differentiation (9, 10). A crucial difference between K-FGF and b-FGF is that the former is efficiently secreted, whereas the latter, which lacks a signal peptide, is inefficiently released (11, 12). b-FGF-related proteins have been identified in many normal and malignant tissues (13–19) and at different developmental stages (10, 20–22), implying that they may play a role in normal tissue function, embryonic development, and neoplastic progression.

Results

Transfection of PC CI 3 Cells with K-fgf and Analysis of Selected Clones

The K-fgf oncogene has the ability to transform mouse fibroblast cell lines (3, 4) and melanocytes (23) and to confer the fully malignant phenotype to the FGF-sensitive cell line SW-13 human adrenal cortex carcinoma (24). We thought that it would be interesting to investigate the transforming activity of the K-fgf oncogene in a different cell system, which might allow us to study its effects on differentiation. For this purpose, the epithelial cell system PC Cl 3, which has been well characterized in our laboratories, appeared to be a valid tool with which to investigate further K-fgf functions. In fact, PC Cl 3 cells are rat thyroid cells that retain in vitro the typical markers of thyroid differentiation, i.e., synthesis and secretion of TG, iodide uptake from the culture medium, and dependence on TSH for growth (25, 26).

Here, we show that transfection of PC Cl 3 cells with the K-fgf/hst oncogene alleviates the cells from TSH dependency and blocks TG expression and the ability to trap iodide, even though the fully neoplastic phenotype is not acquired. An autocrine mechanism is responsible for the K-fgf-induced effects, since analogous results have been obtained after treatment of PC Cl 3 cells with K-FGF-conditioned medium. It is worth noting that induction of cell proliferation in the absence of TSH and block of differentiated functions is not restricted to the K-FGF protein, because analogous results have been obtained with acidic and basic fibroblast growth factors.

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3 The abbreviations used are: b-, basic; FGF, fibroblast growth factor; a-, acidic; K-, Kaposi; CI, clone; TG, thyroglobulin; TSH, thyrotropin; cDNA, complementary DNA.
assay (27); no production of TG protein was observed in the culture medium (Table 1). Also, the ability to trap iodide, evaluated by a previously described technique (28), was not detected in the PC K-igf cell clones (Table 1).

Northern blot analysis was performed to ascertain whether the block of thyroglobulin secretion was dependent on lack of transcription of the specific gene. As shown in Fig. 5, no TG-specific mRNA was produced, which suggests a transcriptional block. Expression of the peroxidase-specific messenger was also abrogated in the K-igf cell clones (Fig. 6).

Since, in rat thyroid cells, independence from TSH for growth is correlated with loss of the TSH receptor (29), we analyzed the presence of the TSH receptor in PC K-igf cells by Northern blotting with a specific probe. Fig. 7 shows that the TSH receptor is not expressed in PC K-igf cell clones.

PC K-igf cell clones were also assayed for appearance of the neoplastic phenotype by soft agar growth assay and tumorigenicity in nude mice. No growth was detected in soft agar, and the injection of 2 × 10³ cells s.c. into athymic mice did not result in tumor formation at the inoculation site, even 4 months after injection.

Stimulation of Proliferation of PC Cl 3 Cells by K-FGF-Conditioned Medium and Basic and Acidic Fibroblast Growth Factors

An autocrine mechanism requires the secretion of the growth factor by the cells and the presence of specific receptors on the cell line. Therefore, to assess whether an autocrine cell mechanism was responsible for PC K-igf cell proliferation in the absence of its physiological growth stimulator to culture medium, we added the conditioned medium harvested from the K-igf-transfected COS-1 cells to the normal PC Cl 3 cells. Moreover, this kind of experiment allowed us to compare the possible effects on cell growth and differentiation induced by the K-FGF protein with those induced by other K-FGF-related proteins, such as basic and acidic fibroblast growth factors.

Effects on Cell Growth. To study the effects of fibroblast growth factors on the growth of thyroid cells, the PC Cl 3 cells were plated at a density of 8 × 10⁴ cells, and FGF factors were added on the following day. After 72 h, the cells were counted. As shown in Fig. 8, the K-FGF-conditioned medium, b-FGF, and a-FGF were able to induce growth in PC Cl 3 cells. This growth stimulation was lower than that induced by the six growth factors added to the culture medium of the PC Cl 3 cells. The hypothesis that this effect could be due to other growth factors present in the conditioned medium of the K-igf-transfected COS-1 cells was excluded because the proliferative effect was suppressed by the addition of anti-K-FGF-specific antibodies, which were not able to suppress the growth of the PC Cl 3 cells stimulated only by serum plus six growth factors (data not shown). Moreover, the supernatant of untransfected PC Cl 3 cells or COS-1 cells transfected with a reporter gene did not have any effect on the growth of rat thyroid cells.

The confirmation that an autocrine loop is responsible for the proliferation of PC K-igf cells comes from the growth arrest induced by treatment of these cells with specific antibodies against the K-FGF protein (data not shown).

demonstrated by its capability to induce growth and morphological changes in NIH 3T3 cells (data not shown), as previously described (4). These effects on NIH 3T3 cells are blocked by anti-K-FGF-specific antibodies (data not shown).

Growth Properties of PC K-igf Cells

Photomicrographs (Fig. 3) of PC K-igf cells show changes in morphology, with a significant increase in refractility and the appearance of spindle-shaped cells, even though the epithelial morphology is maintained.

The growth rate in medium with or without six growth factors (TSH, insulin, hydrocortisone, human transferrin, somatostatin, and glycyl-L-histidyl-L-lysine acetate) or with only five growth factors (TSH excluded) was studied in three PC K-igf clones. No significant differences in the growth curve were observed among the clones. In Fig. 4, we show the growth curves of one representative PC K-igf clone under different growth conditions. The PC K-igf cells, in contrast to normal PC Cl 3 cells, were able to grow in the absence of the six growth factors. However, their addition seemed to increase the saturation density slightly and to reduce the doubling time. The effects of addition of the six growth factors are not dependent on TSH, since the addition of the remaining five growth factors had the same effect as the addition of all six.

Analysis of the Differentiation and Tumorigenicity of the PC K-igf Cell Lines

Three PC K-igf cell clones were analyzed for expression of the differentiation markers. Thyroglobulin synthesis and secretion were tested by a radioimmunological assay (27); no production of TG protein was observed in the culture medium (Table 1). Also, the ability to trap iodide, evaluated by a previously described technique (28), was not detected in the PC K-igf cell clones (Table 1).

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The confirmation that an autocrine loop is responsible for the proliferation of PC K-igf cells comes from the growth arrest induced by treatment of these cells with specific antibodies against the K-FGF protein (data not shown).
We also evaluated the time of the effects of K-FGF by analyzing the incorporation of thymidine at different time points after the addition of the growth factor alone or in association with TSH. Stimulation of DNA synthesis by K-FGF occurs at 18 and 24 h after treatment (data not shown).

Effects on Differentiation. Iodide uptake was measured on PC Cl 3 cells at various times after addition of the conditioned medium diluted 1:5 with normal medium. These experiments, like those with α-FGF and b-FGF, were performed in the presence of the six growth factors in the culture medium. As shown in Table 2, there was an inhibitory effect that started around the 17th hour and increased gradually. There was only a very low residual activity in iodide trapping after 3 days, and an almost complete loss of the capability to trap iodide was observed on the fourth day. However, this inhibitory phase was preceded by a stimulatory one. In fact, iodide trapping was enhanced 20% after 3 h, and it peaked at almost 50% around the seventh hour. Thereafter, there was a return to the normal level of activity; only after 17 h did we start to observe an inhibitory effect. The block of the dedifferentiating effect induced by anti-K-FGF antibodies excludes the possibility that the block of differentiation might be due to factors, other than K-FGF, that might be present in the conditioned medium (data not shown).

In addition, thyroglobulin expression was assayed by evaluating the level of TG-specific mRNA at several time points. The Northern blot in Fig. 9 shows that there was a decrease in expression at 24 h, with a maximal inhibitory effect at 72 h, at which point the expression of TG-specific mRNA was at least 10 times less than that of the untreated cells. The same experiments were performed with different amounts of acidic and basic fibroblast growth factors.

The results of iodide uptake are shown in Table 3; the effects were comparable to those observed with the conditioned medium obtained from K-1gl-transfected COS-1 cells. There was, in fact, a similar surprising increase in activity during the first 5–10 h and then an inhibitory effect which at 72 h resulted in a 70–80% reduction in the ability to trap iodide. The effects on TG expression with α-FGF and b-FGF were also evaluated. The results were analogous to those obtained with the K-FGF-conditioned medium (data not shown).

The results concerning iodide uptake derive from an average of at least two experiments with a slight variability (less than 5%) among them, indicating clear reproducibility of the results obtained.

Discussion

We have studied the effect of the K-1gl/hst oncogene on a differentiated cell system, the rat thyroid epithelial cell line PC Cl 3. The data demonstrate that the K-1gl onco-
gene can alleviate PC CI 3 cells from dependency on TSH for growth. This effect on cell growth is associated with a loss of thyroid differentiated markers, but not with appearance of the neoplastic phenotype. Experiments are in progress to investigate whether the transformed phenotype is obtained by cooperation with other oncogenes, as occurs in the same cell line by cooperation between v-ras and myc (25) and between E1A and v-ral (30). Preliminary results do not indicate any form of cooperation between K-igf and other transforming and immortalizing oncogenes.

Our data show that an autocrine mechanism is involved in the block of differentiation by the K-igf gene since the use of conditioned medium induced the same effects observed in the PC K-igf cell line, and treatment with antibodies against the K-FGF protein inhibited this effect. Other members of the FGF family, such as basic and acidic fibroblast growth factors, showed the same properties as those of the K-FGF protein. The ability of members of the FGF family to replace TSH in the stimulation of mitogenic activity of thyroid cells is particularly

**Fig. 3.** Phase-contrast photomicrographs of PC CI 3 and PC CI 3 transfected with the K-igf oncogene. A, normal PC CI 3 cells, × 150. B, PC K-igf cells, × 150.

**Fig. 4.** Growth curves of uninfected PC CI 3 and PC K-igf CI 1 grown in the presence of the six growth factors, in the presence of five growth factors (TSH excluded), or in the absence of the six growth factors. PC CI 3 cells grown in the presence (○) or absence (●) of the six growth factors; PC K-igf grown in the presence (●) or absence (○) of the six growth factors; PC K-igf/ grown in the presence of five growth factors, without TSH (△).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Iodide uptake*</th>
<th>TG production*</th>
<th>Colony-forming efficiency (%)</th>
<th>Tumorigenicity†</th>
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<tr>
<td>PC CI 3</td>
<td>31</td>
<td>680</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td>PC K-igf CI 1</td>
<td>0.5</td>
<td>ND*</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td>PC K-igf CI 2</td>
<td>0.7</td>
<td>ND</td>
<td>0</td>
<td>0/5</td>
</tr>
<tr>
<td>PC K-igf CI 3</td>
<td>0.4</td>
<td>ND</td>
<td>0</td>
<td>0/5</td>
</tr>
<tr>
<td>NIH K-igf</td>
<td>0.5</td>
<td>ND</td>
<td>65</td>
<td>5/5</td>
</tr>
<tr>
<td>PC MPSV</td>
<td>0.5</td>
<td>ND</td>
<td>75</td>
<td>6/6</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>0.6</td>
<td>ND</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td>RAT-2</td>
<td>0.7</td>
<td>ND</td>
<td>0</td>
<td>0/5</td>
</tr>
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</table>

*Counts per min of 125I incorporated × 10⁷ cells, expressed as percentage of the total 125I added to the medium.

*Expressed as ng of TG produced/ml × 10⁶ cells as determined by radiomunnoassay.

† Assayed by injection of 2 × 10⁷ cells into athymic mice.

‡Not detected.
interesting since it suggests an alternative pathway in thyroid growth regulation. This pathway might play a role in some thyroid neoplasms. The recent finding of an altered expression of b-FGF protein in a follicular carcinoma cell line seems to support this suggestion. Also, the ability of rat thyroid cells to respond to mitogenic and dedifferentiating effects of the fibroblast growth factors indicates the presence of a receptor for these factors in thyroid cells. Consequently, alterations of this receptor might also have a potential role in thyroid carcinogenesis.

The results obtained on differentiation are similar to those described with other transforming oncogenes (26, 31, 32). However, it is noteworthy that the capability to trap iodide increased immediately after treatment with the fibroblast growth factors. A rapid stimulation of adenylate cyclase activity by FGF probably accounts for this surprising effect, as it has been described previously (33). The effects of FGF proteins on growth and iodide uptake of rat PC 3 cells are consistent with those obtained by treating porcine thyroid cells with b-FGF (34).

Thyroglobulin transcription was blocked by addition of FGF-related proteins to the culture medium of PC 3 cells. It has been demonstrated that two nuclear specific factors, TTF-1 and TTF-2, are responsible for the specific expression of TG (35) and that these factors are involved in the dedifferentiation of rat thyroid cells induced by Ha-ras, Ki-ras, and the middle T gene of Polyoma virus. Moreover, it is known that lack of phos-
K-fgf cells was prepared from confluent cells in culture without serum. After 48 h, the medium was harvested, and heparin was added at a concentration of 45 μg/ml. The conditioned medium was aliquoted and stored at −20°C until treatment.

Transfections and Plasmids. All transfections were performed with the calcium phosphate procedure as described (37). The pPy K-fgf plasmid, a eukaryotic expression vector carrying the K-fgf oncogene under the transcriptional control of an ORI-Py immediate early promoter enhancer, was used as transformant plasmid. The pSVneo carrying the gene for Genetin resistance was used as a selectable marker (38). Cotransfections were carried out with the selectable marker and the specific gene at a ratio of 1:5.

Assay of the Transformed State. Tumorigenicity of the cell lines was tested by s.c. injections of 2 × 10⁶ cells into athymic mice. The animals were monitored at regular intervals for the appearance of tumors. Soft agar colony assays were performed as described (39).

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* P. Delli Bovi, unpublished results.
(a) a 680-base pair EcoRI-PstI fragment corresponding to the 3' end of the rat thyroglobulin gene (41); b) the purified insert from clone TBABF, which represents residues -54 to 2780 of the nucleotide sequence of the rat thyrotropin receptor gene, a generous gift of Dr. Leonard D. Kohn (42); c) a 0.7-kilobase EcoRI-SalI fragment corresponding to the 5' coding region of the rat thyroid peroxidase gene, a generous gift of Dr. R. Di Lauro (43); d) a 1152-base pair EcoRI insert of the plasmid pPy K-lfg, representing the cDNA of the K-lfg oncogene (2). Iodide uptake was assayed according to a previously published procedure (28).

Labeling of the probes was performed with a random oligonucleotide primer kit (Amersham Corp.) as described elsewhere (40).

**Immunofluorescence Staining.** Immunofluorescence staining was performed on PC K-lfg cells fixed with absolute methanol at -20°C as previously described (4).

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**Table 1: Effects of a-FGF and b-FGF on iodide trapping in rat thyroid PC Cl 3 cells**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Treatment (ng)</th>
<th>Iodide uptake*</th>
<th>Inhibition or stimulation effect†</th>
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<tr>
<td>0</td>
<td>30.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>a-FGF</td>
<td>44.2</td>
<td>+42.6</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>47.1</td>
<td>+51.2</td>
</tr>
<tr>
<td></td>
<td>b-FGF</td>
<td>42.2</td>
<td>+36.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>47.0</td>
<td>+51.0</td>
</tr>
<tr>
<td>8</td>
<td>a-FGF</td>
<td>44.0</td>
<td>+42.0</td>
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<td>20</td>
<td>49.0</td>
<td>+66.1</td>
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<tr>
<td></td>
<td>b-FGF</td>
<td>56.3</td>
<td>+78.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>54.0</td>
<td>+72.0</td>
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<tr>
<td>12</td>
<td>a-FGF</td>
<td>19.7</td>
<td>-40.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>16.1</td>
<td>-52.1</td>
</tr>
<tr>
<td></td>
<td>b-FGF</td>
<td>9.8</td>
<td>-70.3</td>
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<td>20</td>
<td>15.2</td>
<td>-55.1</td>
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<tr>
<td>24</td>
<td>a-FGF</td>
<td>14.6</td>
<td>-57.0</td>
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<td>14.6</td>
<td>-57.0</td>
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<td>b-FGF</td>
<td>8.4</td>
<td>-74.8</td>
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<td>20</td>
<td>8.6</td>
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<td>48</td>
<td>a-FGF</td>
<td>14.6</td>
<td>-57.0</td>
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<td>b-FGF</td>
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<tr>
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<td>20</td>
<td>4.5</td>
<td>-86.5</td>
</tr>
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</table>

*Counts per min (cpm) incorporated × 10⁶ cells, expressed as percentage of the total cpm added to the medium.
†Increased or decreased iodide trapping activity expressed as percentage of that shown by untreated cells.

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**Fig. 9.** Northern blot hybridization of total RNA from PC Cl 3 cells treated, for different times, with conditioned medium from COS-1 cells transfected with a K-FGF SV40 expression vector and expressing the K-FGF protein (COS-KFGF-CM) at 1:4 dilution plus heparin at 45 μg/ml. 5 μg of total RNA were run for each lane. The Northern blot was hybridized with the p57.5 cDNA TG probe. Sources of RNA were as follows: Lane 1, untreated PC Cl 3 cells; Lane 2, PC Cl 3, 24 h of treatment; Lane 3, PC Cl 3, 48 h of treatment; Lane 4, PC Cl 3, 72 h of treatment.

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