Effect of Testosterone on the Growth Properties and on Epidermal Growth Factor Receptor Expression in the Teratoma-derived Tumorigenic Cell Line 1246-3A

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ABSTRACT

1246-3A is an insulin-independent tumorigenic cell line isolated from the C3H mouse teratoma-derived adipogenic cell line 1246. In the present paper, we have demonstrated that testosterone inhibits the in vivo tumorigenic properties of the 1246-3A cells. Castrated male mice receiving injections of 1246-3A cells developed larger tumors at a higher frequency than sham-operated animals. Administration of testosterone to castrated male mice resulted in a dramatic decrease in tumor development. In vitro studies indicated that testosterone inhibited by 50% the proliferation of the 1246-3A cells in culture. However, growth inhibition was observed only if the cells had been cultured in the presence of testosterone for at least 4 days. In contrast, testosterone had little effect on the proliferation of the parent cell line 1246. Binding of several polypeptide growth factors was examined in cells cultivated in the absence and in the presence of testosterone. Testosterone increased 125I-EGF specific binding to 1246-3A cells. Scatchard analysis of EGF binding indicated that testosterone treatment induced a 2.4-fold increase in the number of cell surface EGF binding sites. This was accompanied by an increase in the intensity of cross-linked EGF receptors on the cells treated with testosterone. In addition, 1246-3A cells cultivated for 9 days in the presence of testosterone displayed a 10-fold increase in the level of EGF receptor mRNA when compared to 1246-3A cells maintained in its absence. Similar to its action on cell proliferation, the increase in EGF receptor number and mRNA expression was observed mainly if 1246-3A cells had been exposed to testosterone for 9 days. The data presented in this paper demonstrate that both in vivo and in vitro, testosterone induces in the teratoma-derived 1246-3A cell line phenotypic changes such as growth inhibition and modulation of EGF receptor expression.

INTRODUCTION

The 1246 cell line is a C3H mouse teratoma-derived adipogenic cell line which can proliferate and differentiate in defined medium and which stringently requires insulin for both processes (1). From 1246 cells maintained in the absence of insulin, several insulin-independent cell lines were isolated. One of them, called 1246-3A, was particularly studied. It was shown that 1246-3A cells could proliferate in the absence of insulin, have lost the ability to differentiate, and have become tumorigenic when injected into syngeneic hosts C3H mice (2). 1246-3A cells injected s.c. at a density of 10⁶ cells/animal gave rise to tumors within 30 days. These tumors have been analyzed as being similar to leiomyosarcomas.6 1246-3A-conditioned medium contained growth-promoting activity for the parent cell line 1246. Biochemical characterization of the growth factors produced by the 1246-3A cells was undertaken using 1246-3A-conditioned medium as starting material. It was found that the cells produced a factor similar to insulin which was purified to homogeneity (3, 4). This factor could bind to 1246-3A insulin receptors and stimulate the producer cell growth in an autocrine fashion (5, 6). In addition to producing insulin-related factor, 1246-3A cells could synthesize and secrete in their culture medium polypeptide growth factors belonging to the TGF-β family which were biologically active, since they stimulated anchorage-independent growth of normal rat kidney cells (7). Moreover, these factors could inhibit adipose differentiation (8, 9) via binding to TGF-β and EGF receptors (10). In order to examine the tumorigenic properties of the 1246-3A cells, we routinely used 6-week-old female C3H mice. However, when we compared the tumor growth of cells injected in age-matched female and male C3H mice, it was found that tumor growth was slower when cells were injected into male mice as compared to female mice, thus suggesting that testosterone may be acting as a negative regulator of the 1246-3A tumor growth in vivo. Sex steroids have been shown to influence the growth of tumors originating from tissues which are hormone responsive in vivo such as prostate for androgens and mammary epithelial cells for estrogens. In both cases, slow-growing prostastic tumors and mammary epithelial cell carcinomas require the presence of the corresponding steroid hormone for their sustained growth. The effect of both androgens and estrogens on the growth of normal and malignant prostate and mammary gland tissues has been studied (11, 12). However, in both cases it has not yet been possible to implicate androgen or estrogen directly in the regulation of tissue growth. This has led to the currently formulated hypothesis that polypeptide growth factors and/or their receptors may be the mediator of androgen or estrogen action on cell growth in vivo and possibly in vitro. Since we have found out that male mice receiving injections of 1246-3A cells develop tumors at a slower rate than female mice, we have investigated here whether testosterone inhibits the in vivo growth of the tumorigenic teratoma-derived 1246-3A cell line. Experiments were also carried out to determine the effect of testosterone on cell proliferation in vitro and on the binding of growth factors, particularly EGF, on the 1246-3A cells.

MATERIALS AND METHODS

Materials

Calf serum and fetal bovine serum were purchased from HyClone (Logan, UT). Nutrient media DME and Ham's F12 were obtained from Gibco (Grand Island, NY). Human fibronectin, human recombinant basic FGF, and mouse submaxillary receptor grade EGF were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Porcine receptor grade 125I-insulin (374 μCi/μg) and [α-32P]UTP (1000 Ci/mm) were from New England Nuclear (Wilmington, DE). NaCl and chloroform form were from Baker (Pittsburgh, PA). Chemicals and molecular weight markers for polycrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). All other chemicals were from Sigma (St. Louis, MO).

The abbreviations used are: TGF, transforming growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; DME, Dulbecco's modified Eagle's medium; F12, Ham's F12 medium; Ch/XCS, charcoal-extracted calf serum.

Received 3/3/92; accepted 5/20/92.

1 Supported by Grant CA37589 from the NIH and Grant 2003 from the Council for Tobacco Research.

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3 G. Serrero and J. Hayashi, unpublished results.

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5 G. Serrero, unpublished results.

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7 G. Serrero, unpublished results.
Methods

In Vivo Tumorigenesis. Six- to seven-week-old C3H mice (Trudeau Institute, Saranac Lake, NY) were used in our experiments. Male mice were castrated and received s.c. injections in the flank of 10^6 1246-3A cells/animal, 1 day after castration. In some experiments, castrated males received a pellet containing 5 mg/pellet of testosterone (60-day release) (Innovative Research, Toledo, OH) or a placebo pellet which was placed under the skin in the back of the animals. Three to five animals were used per experimental condition. Experiments were repeated five times. Animals were examined daily for the appearance of tumors. After 30 days animals were sacrificed, and tumors were excised and weighed. We routinely checked that testicular tissue was absent and that testosterone and placebo pellets were still in place throughout the experiments.

Proliferation of 1246 and 1246-3A Cells in Vitro. 1246-3A stock cells were cultivated in DME-F12 medium supplemented with fibronectin (1 µg/ml), transferrin (10 µg/ml), and FGF (1 ng/ml). For experiments comparing the growth of 1246 and 1246-3A cells, 12,000 cells/well in a 12-well dish were inoculated in DME-F12 medium containing 2% ChXCS in the absence or in the presence of increasing concentrations of testosterone (10^-10 M to 10^-8 M). Cells were counted with a Coulter counter following trypsinization.

Insulin, FGF, and EGF Binding to 1246-3A Cells in the Presence and Absence of Testosterone. Cells were cultivated in 35-mm dishes in DME-F12 medium supplemented with 2% ChXCS in the absence or the presence of 10^-8 M testosterone for 8 days. Cells were then washed free of serum and hormone and incubated in 1 ml of DME containing 1 mg/ml bovine serum albumin for performing binding. Conditions for 125I-insulin binding have been described previously (5). 125I-EGF was kindly supplied by Dr. Mikio Kan (W. Alton Jones Cell Science Center). 125I-EGF binding was performed according to the method of Kan et al. (13). Iodination of EGF and 125I-EGF binding conditions were described elsewhere (8).

Affinity Labeling of EGF Receptors. For affinity labeling studies, monolayers of 1246-3A cells which had been in the presence or the absence of testosterone (10^-8 M) were washed twice with DME medium containing 1 mg/ml bovine serum albumin. Binding was performed for 2 h at 25°C with 300,000 cpm/dish of 125I-EGF (375 µCi/µg) alone or in the presence of 1 µg/ml unlabeled EGF. The solution was removed by aspiration, and the cells were washed three times with ice-cold phosphate-buffered saline. Cells were then treated with disuccinimidyl suberate at a final concentration of 1 mM for 30 min at 4°C. The reaction was quenched by the addition of 2 volumes of cold 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. The washed cells were resuspended in 1 ml of DME containing 2% ChXCS. EGF receptors were affinity labeled by the method of Bringman et al. (14). Affinity labeled EGF receptors were precipitated with twenty µg of tRNA, resuspended in 4 µl of loading buffer, heated to 92°C for 5 min, and electrophoresed in a 7.5% slab gel. Gels were stained with Coomassie blue stain and destained in 25% acetic acid and 25% methanol, destained, dried, and subjected to autoradiography using Kodak XAR film and intensifying screen.

EGF Receptor mRNA Expression in 1246-3A Cells. Expression of EGF receptor transcript in 1246-3A cells was measured by RNase protection assay. The plasmid pMEGFr, which is a 444-base pair polymerase chain reaction fragment of the intracellular domain of the mouse EGF receptor subcloned into SK plasmid,6 was linearized by restriction enzyme Acyl, digested with proteinase K, phenol/chloroform extracted, ethanol precipitated, and resuspended in water at a concentration of 0.5 µg/µl. An antisense [α-32P]UTP-labeled RNA probe 230 nucleotides in length was generated using T3 RNA polymerase from a RNA transcription kit (Stratagene). Total RNA was extracted from 1246-3A cells by the method of Chomczynski and Sacchi (15). Twenty µg of total RNA and 5 x 10^6 cpm of probe were hybridized overnight at 50°C in 50% formamide and 1× 1.4-piperazinediethanesulfonic acid in a total volume of 40 µl. The next day samples were digested for 30 min at room temperature with 300 µl of an RNase digestion buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3 M NaCl, 12 µg RNase A, and 150 units RNase T1. The samples were treated with proteinase K, phenol/chloroform extracted, precipitated with twenty µg of tRNA, resuspended in 4 µl loading buffer, heated to 92°C for 5 min, and electrophoresed on a 6% denaturing polyacrylamide gel. Dried gels were analyzed by autoradiography. Protected EGF receptor transcripts are 160 nucleotides in length.

EGF Radioceptor Assay for Measuring EGF Receptor Competing Material. The presence of EGF/FGF-α-like polypeptide in 1246-3A culture medium was determined by its ability to compete with 125I-EGF for binding to CCL64 cells (16). 1246-3A cells were cultivated in 2% ChXCS in the presence or the absence of testosterone (10^-8 M) for 8 days. Medium was then changed to factor-free, serum-free DME medium in the presence or absence of testosterone and collected every 3 days. Medium was concentrated 10- to 20-fold by ultrafiltration through a YM 2 membrane (Amicon, Danvers, MA) and used directly in the EGF radioceptor assay using CCL64 cells as described by Bringman et al. (16).

RESULTS

Tumorigenicity of 3A Cells Injected into Syngeneic Host C3H Mice. We have shown previously that 1246-3A cells form tumors when injected s.c. at a cell density of 10^6 cells into adult female C3H mouse (2) and that tumors developed in male mice with a slower rate and a decreased frequency than in age-matched female mice. Here, we compared the rate of tumor growth in male mice which had been castrated or sham operated. Castrated mice received a pellet containing either testosterone or a placebo compound which was implanted s.c. 1 day prior to injecting the 1246-3A cells. The data of Table 1 show that all castrated mice developed tumors with an average weight of 1.68-2.16 g/tumor after receiving injections of 10^5-10^6 3A cells. If the castrated mice received testosterone, only 10 of 14 mice (60%) developed tumors with a lower weight (Table 1) and size (Fig. 1). In sham-operated animals, 10 of 16 animals developed tumors with an average weight of 0.34 g/tumor. These experiments indicate that testosterone inhibits the in vivo growth of 1246-3A cells.

Effect of Testosterone on the Proliferation of 1246-3A Cells in Culture in Vitro. We then investigated whether testosterone added to the culture medium of the 1246-3A cells would affect their proliferation in vitro. As shown in Fig. 2a, testosterone inhibited the proliferation of the 1246-3A cells in a dose-dependent fashion. At a testosterone concentration of 10^-8 M, proliferation was inhibited by 50%. A higher concentration of testosterone did not have a higher inhibitory effect. In contrast,

<table>
<thead>
<tr>
<th>Mice</th>
<th>No. of mice developing tumors</th>
<th>Average weight of tumors (g)</th>
</tr>
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<tbody>
<tr>
<td>I Sham</td>
<td>10/16</td>
<td>0.34 ± 0.08</td>
</tr>
<tr>
<td>II Castrated</td>
<td>6/6</td>
<td>2.16 ± 0.36</td>
</tr>
<tr>
<td>III Castrated placebo</td>
<td>10/10</td>
<td>1.68 ± 0.32</td>
</tr>
<tr>
<td>IV Castrated testosterone</td>
<td>10/14</td>
<td>0.28 ± 0.06</td>
</tr>
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* D. P. Eisinger and G. Serrero, manuscript in preparation.
long-term exposure of 1246-3A cells to testosterone in conditions that inhibited their proliferation resulted in changes in specific cell surface binding for the polypeptide growth factors known to stimulate 1246-3A cell growth (i.e., insulin, FGF, and EGF). As shown in Table 2, specific binding of radiolabeled insulin and FGF remained the same whether or not the cells were treated with testosterone. In contrast, EGF binding increased by 3-fold when the 1246-3A cells were treated with $10^{-8}$ M testosterone when compared to the EGF binding on 1246-3A cells cultivated in the absence of the hormone. However, if the cells were treated with testosterone only for 4 h prior to performing EGF binding, EGF binding remained the same. These results would suggest that the increase in cell surface binding testosterone did not inhibit the proliferation of the nontumorigenic adipogenic 1246 cell line (Fig. 2B). The growth curve of 1246-3A cells in the absence and the presence of $10^{-8}$ M testosterone showed that the inhibitory effect of testosterone on cell growth was not immediate and became apparent after the cells had been in culture for several days (Fig. 3).

**Effect of Testosterone on Polypeptide Growth Factor Binding on 1246-3A Cells.** We have shown previously that 1246-3A cell proliferation is stimulated by several growth factors (7) and that 1246-3A cells present specific cell surface binding sites for several polypeptide growth factors, some of which are being produced by the cells (3–7, 10). One possible explanation for the delayed effect of testosterone on 1246-3A cell proliferation (Fig. 3) is that testosterone acts indirectly via modulation of the production or response to one or several of these growth factors. To investigate this possibility, we determined whether...
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collected and analyzed for the presence of EGF receptor competing activity as described in “Materials and Methods.” Media collected from three separate experiments were assayed. The results indicate that the amount of EGF receptor competing activity when normalized to the cell number was about the same whether the cells had been cultivated in the presence or in the absence of testosterone [0.22 ± 0.01 (SE) ng/10^6 cells in the absence of testosterone and 0.37 ± 0.05 ng/10^6 cells in its presence]. These results would suggest that the increase in EGF binding sites cannot be explained by a decrease in the amount of EGF receptor competing activity produced by the treatment of the 1246-3A cells with testosterone.

Scatchard Analysis of EGF Binding to 1246-3A Cells Treated or Not Treated with Testosterone. Scatchard analysis of ^125I-EGF binding to 1246-3A cells cultivated in either the presence or the absence of 10^-8 M testosterone for 8 days was performed. Typically, Scatchard representation of EGF binding was curvilinear for 1246-3A cells cultivated in the absence of testosterone and linear for 1246-3A cells cultivated in its presence (Fig. 4). In both cases, linear portions of the Scatchard plots were used to calculate binding parameters (Table 3). The results indicate a 2.4-fold increase in the number of EGF receptors in the testosterone-treated cells when compared to the untreated 1246-3A cells without a change in the value of the dissociation constant.

Characterization of EGF Receptor Species in 1246-3A Cells Exposed or Not Exposed to Testosterone. Cross-linking experiments of ^125I-labeled EGF to 1246-3A cells cultivated with or without 10^-8 M testosterone were performed after 9 days in culture. An autoradiograph of the results (Fig. 5) showed in both cell lines a cross-linked doublet band (Fig. 5, Lanes 1 and 3) which was competed by adding excess cold EGF (Fig. 5, Lanes 2 and 4). This band had the expected molecular weight of 4245

\[ \frac{B}{F} \]

\[0.2 \]

\[0.1 \]

\[0.4 \]

\[0.3 \]

\[0.2 \]

\[0.1 \]

Fig. 4. Scatchard analysis of ^125I-EGF binding to 1246-3A cells cultivated in the absence (A) or in the presence (B) of 10^-8 M testosterone. ^125I-EGF binding conditions are described in “Materials and Methods.”

may not have been due to a direct effect of testosterone on EGF binding (data not shown). Moreover, long-term exposure of 1246 cells to testosterone did not have any effect on cell surface EGF binding (Table 2).

Testosterone Does Not Change the Production of EGF Receptor Competing Activity by the 1246-3A Cells. We have previously shown that medium conditioned by the 1246-3A cells contains a TGF-α/EGF-like activity which can bind and occupy EGF receptors on the producer cells (7). Based on this information, it is possible to assume that the increase in the number of cell surface binding sites observed when the 1246-3A cells are treated with testosterone is due to the fact that testosterone inhibits the production of EGF receptor competing activity by the 1246-3A cells, resulting in a reduced occupancy of EGF cell surface binding cells. Medium conditioned by 1246-3A cells cultivated in the absence and the presence of testosterone was collected and assayed for EGF receptor competing activity as described in “Materials and Methods.”

Table 3 EGF binding parameters for 1246-3A cells cultivated in the absence or the presence of testosterone

<table>
<thead>
<tr>
<th>Cells/dish</th>
<th>Kd (nM)</th>
<th>Sites/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>With testosterone</td>
<td>1.9 ± 0.3 × 10^6</td>
<td>5.0 ± 1.1 × 10^-11</td>
</tr>
<tr>
<td>Without testosterone</td>
<td>0.97 ± 0.19 × 10^6</td>
<td>3.4 ± 0.8 × 10^-11</td>
</tr>
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G. Serrero and D. Mills, unpublished results.

4245

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The data presented in this paper demonstrate that the in vivo tumorigenic property of the 1246-3A cells is inhibited by testosterone. Castrated male C3H mice developed larger tumors with a higher frequency than sham-operated animals. When castrated animals received testosterone, the frequency, rate of appearance, and size of the tumors decreased. Testosterone supplementation also inhibited tumor growth in female C3H mice that received injections of 1246-3A cells (data not shown). Examination of the effect of testosterone on the proliferation of the 1246-3A cells in vitro indicates that only long-term exposure of the cells to testosterone leads to growth inhibition. In contrast, testosterone did not inhibit the proliferation of the nontumorigenic parent cell line 1246 even after 16 days in culture. Estradiol and progesterone added in the same range of concentrations had no effect on 1246-3A and 1246 cell proliferation (data not shown). As shown in Fig. 3, the effect of testosterone on cell growth in vitro was not immediate but became apparent only after the cells had been exposed to the hormone for at least 4-5 days. Since 1246-3A cells produce several polypeptide growth factors which act as autocrine growth stimulators, these results suggest that the inhibitory effect of testosterone might not be direct but rather could be via changes in the response to growth factors that the cells produce or to which they respond. The inhibition of proliferation caused by testosterone could be due to the fact that either the production or the response to the polypeptide growth factors found in 1246-3A-conditioned medium is being altered by long-term cultivation with the hormone. In this paper, we focused on studying the effect that testosterone would have on the expression of EGF-competing activity and EGF receptor in the 1246-3A cells. Several observations support this study: TGF-α or EGF-like polypeptides and EGF receptors are coexpressed in many tumors and tumor cell lines (18, 19); another sex steroid, estrogen, which stimulates the growth of mammary epithelial cells, has been found to modulate TGF-α and EGF receptor expression in normal and malignant mammary epithelial cells (20, 21); we have shown previously that the insulin-independent 1246-3A cells produce insulin and EGF-like competing activities which stimulate their growth in an autocrine fashion and which occupy cell surface receptors for insulin and EGF, respectively (4-7). In this paper we examined the binding of insulin, EGF, and FGF, which are growth stimulators of 1246 and 1246-3A cell proliferation. Long-term cultivation of the 1246-3A cells with testosterone resulted in a 3-fold increase in EGF specific binding without changes in insulin and FGF specific binding. This increase in EGF binding corresponded to a 2.4-fold increase in the number of high-affinity EGF receptors without a change in the dissociation constant. In addition, long-term exposure and not short-term exposure to testosterone resulted in a 10-fold increase in EGF receptor mRNA expression in 1246-3A cells. It is not yet determined at this point why the increase in the level of EGF receptor mRNA caused by the exposure to testosterone is higher than the increase in EGF receptor number on the cell surface. Binding studies have shown that testosterone stimulates EGF receptor number in the prostate (22, 23). The data presented in this paper indicate that testosterone increases the number of EGF receptors in a cell type that is other than prostate and that is of mesodermal origin. Moreover, the data in this paper also show that testosterone stimulates EGF receptor mRNA expression in the 1246-3A cells. In contrast to its stimulatory effect on EGF receptor expression, testosterone did not affect the level of production of EGF receptor competing activity by the 1246-3A cells. Thus, it is unlikely that the increase in the number of EGF
binding sites is also due to a decrease in the occupancy of cell surface EGF receptors by the secreted factor. Moreover, the addition of EGF to the culture medium of 1246-3A cells maintained in the presence of testosterone for 9 days when EGF receptor number had been up-regulated did not stimulate proliferation and did not overcome the inhibition of proliferation caused by the hormone (data not shown). However, one cannot exclude the possibility that testosterone induces changes either in the production of TGF-α/EGF-like polypeptides or in the response to these factors in the microenvironment of the cells which could lead to growth inhibition but which are too small to be detected in our experiments. In fact such minute changes at the level of the microenvironment of cells have been hypothesized to play an important role in the activation of TGF-β latent precursor and in its action (24). Moreover, it has been shown in human breast cancer cells that although estrogen modulates TGF-α and EGF receptor expression, blockade of EGF receptor inhibitors TGF-α-induced but not estrogen-induced growth (25). Based on these results, it is possible to assume that the modulation of EGF receptor expression by testosterone is not necessarily the cause of its growth-inhibitory effect on the 1246-3A cells either in vivo or in vitro. Since modulation of EGF receptor number and mRNA expression only occurred after long-term exposure of the 1246-3A cells to testosterone, one can assume that this change in EGF receptor is one of the phenotypic changes that the hormone has induced in the cells. It has been shown in the prostate that programmed cell death induced by castration was accompanied by an increase in the expression of TGF-β which is a growth inhibitor for the prostatic epithelial cells (11, 26). In the case of the 1246-3A cells, long-term exposure to testosterone did not cause any change in the level of secretion of TGF-β (data not shown). We have shown previously that the totality of TGF-β produced by the 1246-3A cells is secreted as a latent precursor which is known to be biologically inactive (27) and that TGF-β added exogenously is a growth simulator for the cells (10). Therefore, it is unlikely that modulation in TGF-β level or response could explain the growth-inhibitory effect of testosterone for the tumorigenic 1246-3A cells in vivo and in vitro. However, the possibility is not excluded that testosterone affects the level of response or production by the cells of yet unidentified growth stimulators or growth inhibitors in vivo and in vitro. In addition, it is possible that testosterone inhibits tumor growth in vivo not only via changes in the response to local and/or circulating growth regulators but also by modifying other biological phenomena, such as vascularization, which are related and necessary for tumor growth in vivo (28). These various possibilities are currently under investigation in our laboratory.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Mikio Kan for the gift of 121I-FGF, Marina LaDuke and Carol Baine for the illustrations, and Valerie Oliver for preparation of the manuscript.

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