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Insulin-like growth factor II mediates epidermal growth factor-induced mitogenesis in cervical cancer cells

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ABSTRACT **There is increasing evidence that activation of the insulin-like growth factor I (IGF-I) receptor plays a major role in the control of cellular proliferation of many cell types. We studied the mitogenic effects of IGF-I, IGF-II, and epidermal growth factor (EGF) on growth-arrested HT-3 cells, a human cervical cancer cell line. All three growth factors promoted dose-dependent increases in cell proliferation. In untransformed cells, EGF usually requires stimulation by a "progression" factor such as IGF-I, IGF-II, or insulin (in supraphysiologic concentrations) in order to exert a mitogenic effect. Accordingly, we investigated whether an autocrine pathway involving IGF-I or IGF-II participated in the EGF-induced mitogenesis of HT-3 cells. With the RNase protection assay, IGF-I mRNA was not detected. However, IGF-II mRNA increased in a time-dependent manner following EGF stimulation. The EGF-induced mitogenesis was abrogated in a dose-dependent manner by IGF-binding protein 5 (IGFBP-5), which binds to IGF-II and neutralizes it. An antisense oligonucleotide to IGF-II also inhibited the proliferative response to EGF. In addition, prolonged, but not short-term, stimulation with EGF resulted in autophosphorylation of the IGF-I receptor, and coincubations with both EGF and IGFBP-5 attenuated this effect. These data demonstrate that autocrine secretion of IGF-II in HT-3 cervical cancer cells can participate in EGF-induced mitogenesis and suggest that autocrine signals involving the IGF-I receptor occur "downstream" of competence growth factor receptors such as the EGF receptor.**

Progression through the cell cycle requires the orchestrated actions of two complementary classes of peptide growth factors. The notion that "competence" growth factors advance quiescent cells into the cell cycle and that those cells then become committed to DNA synthesis under the influence of "progression" growth factors was introduced in the late 1970s (1–3). In quiescent fibroblasts, the mitogenic effect of epidermal growth factor (EGF) requires activation of the insulin-like growth factor I (IGF-I) receptor by a progression factor such as IGF-I, IGF-II, or insulin (4). The importance of the IGF-I receptor in regulating cellular proliferation has been emphasized by *in vitro* studies which used antibodies, antisense oligonucleotides, or IGF-I peptide analogs to interfere with its activation; regardless of the method, cell proliferation could be inhibited in a wide variety of tumors (5–7).

The IGF-I receptor is virtually ubiquitous and its expression has been described in a vast array of tumors (8). The widespread physiologic significance of its activation was brought into focus by gene targeting experiments in mice (9, 10); mice with homozygous targeted mutations of the IGF-I receptor were severely growth retarded and died immediately after birth. Fibroblast cell lines generated from these mice grew more slowly than cell lines generated from wild-type littermates, and all phases of the cell cycle were prolonged (11); in addition, Coppola *et al.* (12) demonstrated that the EGF-mediated growth and transformation of these cells required the presence of a functional IGF-I receptor (12). Several investigators have suggested an important role for IGF-I and IGF-II in subverting the regulated growth of various gynecologic tumors (5, 13–15). In the present study, we characterized the mitogenic effects of IGF-I, IGF-II, and EGF on growth-arrested HT-3 cells, a human cervical cancer cell line, and investigated whether an autocrine pathway involving IGF-I or IGF-II participates in the EGF-induced mitogenesis of these cells.

MATERIALS AND METHODS

Cell Culture. The HT-3 cell line, a poorly differentiated, epithelial-like cervical carcinoma which forms tumors in nude mice, was obtained from American Type Culture Collection and was certified to be free from contamination with *Mycoplasma.* Cells were maintained as exponentially growing, continuous monolayer cultures in medium consisting of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (penicillin, 100 units/ml; streptomycin sulfate, 100 μg/ml). Incubations were carried out at 37°C in a humidified 5% CO_2/95% air atmosphere. Pilot experiments were performed to determine the optimal cell plating conditions for the proliferation analyses. For individual experiments, 5×10^4 HT-3 cells were seeded into 96-well flat-bottom cell culture plates (Costar) and grown overnight in 0.2 ml of RPMI 1640 containing 10% fetal bovine serum. The cultures were then incubated for 48 hr in serum-free medium RPMI 1640 supplemented with bovine serum albumin (Sigma) at 1 mg/ml and transferrin at 10 μg/ml. The cells were then stimulated with defined serum-free medium: RPMI 1640 supplemented with bovine serum albumin at 1 mg/ml and transferrin at 10 μg/ml, with or without the addition of IGF-I, IGF-II, EGF, IGF-binding protein 5 (IGFBP-5), or both EGF and IGFBP-5. Proliferation assays were performed 24 hr later. For the RNase protection assay, cells were grown in 75-mm flasks and treated as described above.

Growth Factors and Binding Proteins. The growth factors IGF-I, IGF-II, and EGF were obtained from GIBCO/BRL. For dose–response experiments, EGF was added to the medium at 0.1, 1.0, and 10 ng/ml; IGF-I was added to the serum-free medium at 0.1, 1.0, 10, and 100 ng/ml; and IGF-II was added to the defined serum-free medium at 10, 100, and 1000 ng/ml. For all other experiments, EGF and IGF-I were used at 10 ng/ml. For proliferation assays, IGFBP-5 (Austral Biologicals, San Ramon, CA) was added to defined serum-free

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Abbreviations: EGF, epidermal growth factor; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDGF, platelet-derived growth factor.

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medium at 1, 10, and 100 ng/ml in the presence or absence of EGF at 10 ng/ml.

Oligodeoxynucleotides. The antisense oligodeoxynucleotide corresponding to the IGF-II mRNA initiation site, 5′-TTC-CCC-ATT-GGG-ATT-CCC-AT-3′, and the sense oligodeoxynucleotide, 5′-ATG-GGA-ATC-CCA-ATG-GGG-AA-3′, were purchased from Research Genetics, Inc. (Huntsville, AL) and were synthesized with phosphothionate modification. Cultures of HT-3 cells were treated with oligodeoxynucleotides (40 μg/ml) 24 hr after plating in defined serum-free medium. The next day, the medium was replaced with defined serum-free medium containing EGF (10 ng/ml) and oligodeoxynucleotides (20 μg/ml). Proliferative effects were assessed 24 hr later.

Proliferation Assay. The assay used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) and was based on that previously described by Mosmann (16). MTT was dissolved at 5 mg/ml in phosphate-buffered saline, filter sterilized, and stored at 4°C in a darkened bottle. Fifty microliters of stock MTT solution was added to each well and the plates were incubated at 37°C for 4 hr. All medium was then removed from each well, and the plates were air dried overnight. To dissolve the dark blue formazan crystal precipitate, 150 μl of mineral oil was added to each well and the plates were once again incubated overnight at 37°C. The plates were read on a scanning multiwell spectrophotometer (ELISA reader; Titertek Multiscan model MCC/340 MK II; Flow Laboratories) at a test wavelength of 570 nm. Cell number was estimated by extrapolating the optical density readings from a standard curve.

IGF-I Binding Assay. The IGF-I binding assay was performed as described (17), but with modifications. In brief, 5×10^4 cells per 0.2 ml of tissue culture medium were seeded in 96-well flat-bottom Remove-a-Cell plates (Dynatech). Twenty-four hours later, the cells were exposed to ^{125}I-labeled IGF-I (100 pM; 2 mCi/pmol; Amersham; 1 Ci = 37 GBq) along with various concentrations of unlabeled IGF-I (0–1000 ng/ml). The plates were incubated for 4 hr at 4°C and rapidly washed, then each well was individually separated and placed in a vial for measurement of radioactivity in a γ counter. Nonspecific binding was assessed in the presence of unlabeled IGF-I at 1000 ng/ml. Cell numbers were determined by MTT assay from six replicate wells which were manipulated in the same way as those wells used for the binding measurements. Scatchard analysis was performed with the LIGAND computer program to determine the apparent equilibrium dissociation constant (K_d) and the number of binding sites per cell (18).

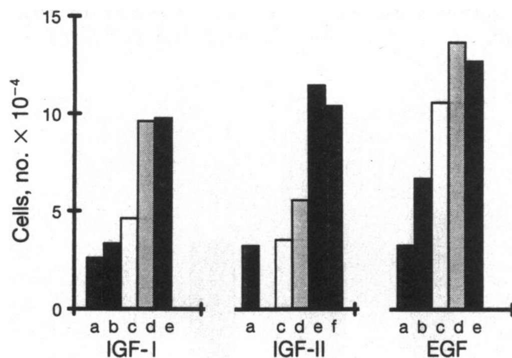

FIG. 1. Dose–response for the HT-3 cell line treated with various concentrations of IGF-I, IGF-II, or EGF. Cells were seeded in 96-well plates in 10% serum for 12 hr and then maintained in serum-free medium for 48 hr. The medium was then replaced with serum-free medium with or without the addition of IGF-I (0.1–100 ng/ml), IGF-II (1–1000 ng/ml), or EGF (0.1–100 ng/ml). Twenty-four hours later, proliferation was assessed by the MTT assay. Bars: a, 0 ng/ml; b, 0.1 ng/ml; c, 1 ng/ml; d, 10 ng/ml; e, 100 ng/ml; f, 1000 ng/ml. Data are representative results from four independent experiments.

RNA Extraction. Total RNA from HT-3 cells was prepared by the guanidinium isothiocyanate/cesium chloride method of Chirgwin *et al.* (19). Absorbance of each RNA sample was measured at 260 nm in a Beckman DU-64 spectrophotometer (Beckman).

Solution Hybridization/RNase Protection Assay. The probes used for solution hybridization were derived from human IGF-I and human IGF-II cDNAs and details of their construction have been described (20, 21). Both probes were labeled with [α-^{32}P]CTP (Amersham) by use of T7 RNA polymerase and the MAXIscript *in vitro* transcription kit (Ambion, Austin, TX). To monitor the amounts of RNA in each sample, an RNA probe complementary to human β-actin mRNA (Ambion) was also labeled. The probes were purified over a Quick Spin Sephadex G-50 column (Boehringer Mannheim). The RNase protection assay with the RPA II kit (Ambion) included some optimizing changes. In brief, 10 μg of each sample total RNA was hybridized overnight at 45°C with 10^5 cpm of RNA probe (specific activity, 5×10^6 cpm/μg). Single-stranded RNA was digested with RNase ONE (Promega). The samples were then precipitated and run on a 6% polyacrylamide/8 M urea gel. The gel was dried and exposed to x-ray film.

Phosphorylation of the IGF-I Receptor. Cells were stimulated either with IGF-I (10 ng/ml), with EGF (10 ng/ml), or with EGF (10 ng/ml) plus IGFBP-5 (150 ng/ml) for various times at 37°C. The cells were placed on ice and rinsed with cold phosphate-buffered saline. Cells were kept in lysis buffer [50 mM Tris, pH 8.0/150 mM NaCl/1% (vol/vol) Nonidet P-40] with 100 μM sodium orthovanadate and protease inhibitors (phenylmethylsulfonyl fluoride, 100 μg/ml; leupeptin, 2 μg/ml; and aprotinin, 2 μg/ml) for 20 min at 4°C, and the lysate was centrifuged for 10 min at 4°C to remove nuclei. The cleared lysate was transferred to a fresh tube and immunoprecipitation was carried out overnight at 4°C with monoclonal antibody to the IGF-I receptor, α-IR-3 (2 μg/ml; Oncogene Science). The immunocomplexes were washed four times with cold lysis buffer and then collected on protein A/G beads (Pierce) and eluted with 2× SDS sample buffer (100 mM Tris Cl, pH 6.8/4% SDS/20% glycerol/100 mM dithiothreitol/0.2% bromophenol blue). Samples were boiled for 5 min, and proteins were separated in a 7.5% polyacrylamide gel and then electroblotted onto a nitrocellulose filter. Phosphorylated proteins were detected by Western immunoblot analysis with an anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). Bound antibody was detected with the ECL system (Amersham).

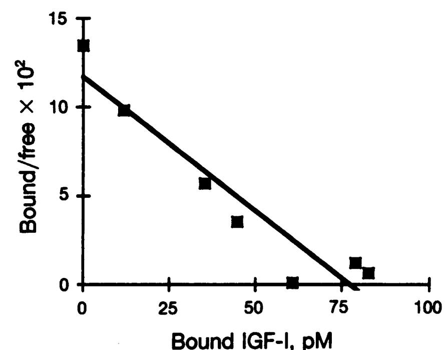

FIG. 2. Scatchard plot of IGF-I binding sites in HT-3 cervical cancer cells. The plot yields K_d = 0.4 nM and B_{max} = 99,037 sites per cell; coefficient of determination, r^2 = 0.8987. The method for determining the number of IGF-I receptors per cell is given in *Materials and Methods.*

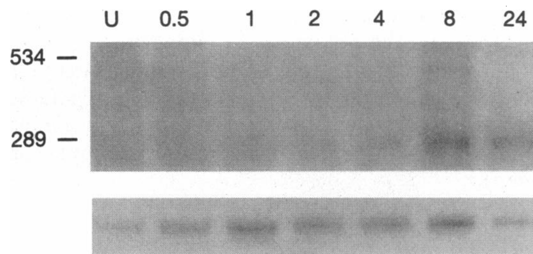

FIG. 3. Expression of IGF-II mRNA in HT-3 cells after stimulation with EGF. Cells were made quiescent in defined serum-free medium. RNA was extracted from unstimulated cells (U) or from cells stimulated with EGF (10 ng/ml) for 0.5, 1, 2, 4, 8, and 24 hr. (*Upper*) IGF-II mRNA. The bands at 289 bases represent IGF-II mRNA transcribed from the fetal promoter. No mRNA is transcribed from the adult promoter (540 bases). Autoradiographic exposure was for 48 hr. (*Lower*) β-Actin mRNA. Exposure was for 15 min. The experiment was repeated three times with similar results.

RESULTS

Effects of IGF-I, IGF-II, and EGF on the Growth of HT-3 Cells. The mitogenic actions of IGF-I, IGF-II, and EGF on HT-3 cells were measured with the MTT assay (Fig. 1). The dose–response curves for cell growth after 24 hr of exposure to either IGF-I or EGF indicate that maximal proliferation occurred at a dose of 10 ng/ml, with higher doses inducing equivalent proliferation. Maximal proliferation after exposure to IGF-II occurred at a dose of 100 ng/ml. No appreciable mitogenic effect was observed with IGF-II at 1 ng/ml; at the intermediate dose of 10 ng/ml, submaximal proliferation occurred.

^{125}I-IGF-I Binding. Scatchard analysis of ^{125}I-IGF-I binding to HT-3 cells that had been incubated overnight in 10% serum conforms to a linear model (Fig. 2), indicating a single type of high-affinity IGF-I receptor on the HT-3 cells. Extrapolation toward the abscissa of the values at low IGF-I concentrations gave a value of 9.9×10^4 IGF-I receptors per cell with a dissociation constant of 0.4 nM.

Expression of the IGF-I and IGF-II Genes in HT-3 Cells. The levels of IGF-I and IGF-II mRNA in HT-3 cells after stimulation with EGF for various lengths of time were measured by solution hybridization/RNase protection assay. IGF-I gene expression was not observed in any of the samples (data not shown). The antisense RNA used to detect IGF-II mRNA contained the 5′ untranslated region generated from the adult promoter and spanned the divergence in the 5′ untranslated regions, so its use in the RNase protection assay should have resulted in two protected bands, 534 and 289 bases in length; the 289-base band represents IGF-II mRNAs with 5′ untranslated regions generated from either the fetal or the fetal–neonatal promoter, whereas the 534-base band corresponds to IGF-II mRNAs with 5′ untranslated regions generated from the adult promoter (21). Minimal IGF-II gene expression was observed in HT-3 cells maintained for 24 hr in serum-free medium, even after prolonged autoradiographic exposure. When the cells were stimulated with EGF, IGF-II mRNA transcribed from the fetal, but not the adult, promoter increased in a time-dependent manner (Fig. 3 *Upper*). Greater amounts of IGF-II mRNA were initially apparent after 2 hr of EGF exposure and increased progressively at 4, 8, and 24 hr. The amount of RNA in each sample was monitored with a β-actin probe (Fig. 3 *Lower*). Expression of the β-actin gene, which is not growth-regulated, was evident in each sample and was not appreciably influenced by EGF. IGF-II mRNA increased by 6-fold (as determined by densitometry) during the 24-hr time course, and when the IGF-II signal was normalized for β-actin, there was a 10-fold increase in IGF-II mRNA expression.

Inhibition of EGF-Induced Mitogenesis with IGFBP-5. The mitogenic actions of EGF in the presence of various amounts of IGFBP-5 were assessed by the MTT assay (Fig. 4). The affinity of IGFBP-5 for IGF-II is higher than that demonstrated by the IGF-I receptor, effectively neutralizing IGF-II (22, 23). When added to defined serum-free medium, IGFBP-5 at a wide range of doses had a negligible effect on the cells' growth. However, when the cells were stimulated with EGF in the presence of various concentrations of IGFBP-5, dose-dependent inhibition of cell proliferation was observed. Similar inhibition of EGF-induced mitogenesis also occurred when IGFBP-4 or a polyclonal antibody to IGF-II was added to the EGF-containing medium (data not shown).

An Antisense Oligomer to IGF-II Inhibits EGF-Mediated Cell Growth. To further assess whether IGF-II synthesis is a consequence of EGF stimulation, the mitogenic actions of EGF were assessed in the presence of an antisense or sense oligodeoxynucleotide to IGF-II RNA (Fig. 5). The antisense oligodeoxynucleotide substantially inhibited EGF-induced proliferation, indicating that translation of IGF-II mRNA is involved in the proliferative EGF stimulus. The sense oligomer had only a minimal effect. There was no appreciable evidence of toxicity in response to the oligodeoxynucleotides as determined by loss of cells into the medium (data not shown); in addition, cells maintained in defined serum-free medium were essentially unaffected by the addition of either oligodeoxynucleotide to the medium.

Stimulation with EGF Causes Activation of the IGF-I Receptor. Since IGF-II-mediated mitogenesis results from

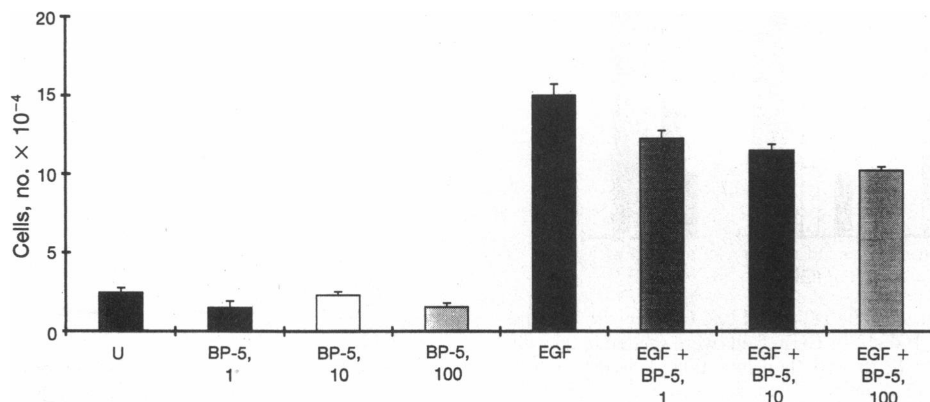

FIG. 4. Inhibition of EGF-induced mitogenesis by IGFBP-5 (BP-5). Quiescent cells were stimulated for 24 hr under the listed conditions and then assessed for proliferation by using the MTT assay. IGFBP-5 (ng/ml) concentrations are shown on the abscissa. U, unstimulated (cells in serum-free medium without addition). Data are expressed as the mean ± SD of four replicate wells and are representative results from three independent experiments.

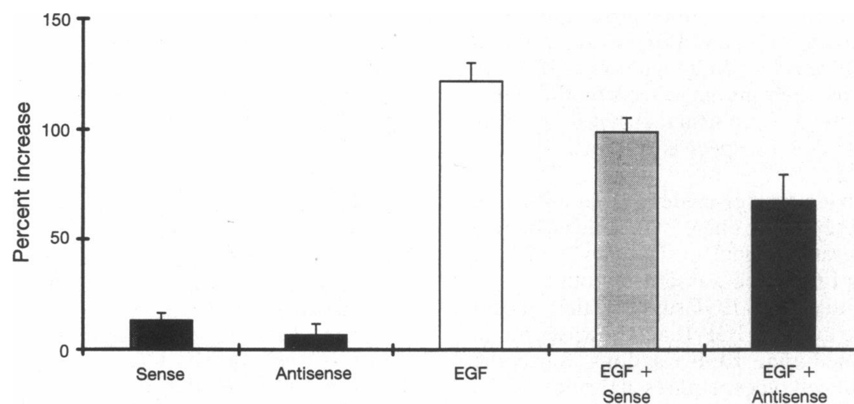

FIG. 5. Inhibition of EGF-induced mitogenesis by an antisense oligodeoxynucleotide to IGF-II. Quiescent HT-3 cells were stimulated for 24 hr under the listed conditions and then assessed for proliferation. The ordinate gives the percent increase over the value for unstimulated (serum-free medium) cells. Data are expressed as the mean ± SD of four replicate wells. The experiment was repeated three times with similar results.

activation of IGF-I receptor, autophosphorylation of the receptor was assessed after the cells were stimulated in various conditions by immunoprecipitation and staining with an anti-phosphotyrosine antibody (Fig. 6). Clear evidence of IGF-I receptor autophosphorylation was detected when the cells were stimulated for 1 hr with IGF-I, but no band was apparent after stimulation with EGF for 1 hr or 4 hr, indicating that EGF cannot directly activate the IGF-I receptor. However, when the cells were stimulated with EGF for 7 hr, evidence of IGF-I receptor autophosphorylation was readily apparent. In contrast, when the cells were exposed to both EGF and IGFBP-5 for 7 hr, considerably less activated IGF-I receptor was detected. This result suggests that EGF can indirectly activate the IGF-I receptor by inducing the production of an IGF which, in turn, can cause IGF-I receptor autophosphorylation.

DISCUSSION

In this study, we have demonstrated that EGF induces the autocrine production of IGF-II, which, in turn, mediates the mitogenic effects of EGF on HT-3 cervical cancer cells. These experiments suggest that autocrine signals involving the IGF-I receptor occur "downstream" of competence growth factor receptors such as the EGF receptor. Several previous reports support this notion. For example, using antisense oligonucleotides to the IGF-I receptor, Pietrzkowski *et al.* (24) showed that activation of an IGF-I/IGF-I receptor autocrine loop was required for EGF-induced mitogenesis of BALB/c 3T3 cells transfected to overexpress the IGF-I receptor (24). In addition, mouse fibroblasts harboring a null mutation for the IGF-I receptor gene are unable to grow or to be transformed after transfection and overexpression of the EGF receptor, but reintroduction into these cells of the IGF-I receptor gene restores EGF-mediated growth and transformation (12).

In mouse fibroblasts, IGF-I-mediated mitogenesis requires costimulation with platelet-derived growth factor (PDGF). It appears that PDGF enhances IGF-I binding sites (25), and Rubini *et al.* (26) have confirmed this by showing that PDGF induces transcription of the IGF-I receptor gene promoter. It is hypothesized that progression through the cell cycle may be regulated by quantitative differences in IGF-I receptor expression (27, 28). For example, nontransformed cells, such as murine fibroblasts, typically express about 10,000 IGF-I receptors per cell, whereas malignant cell lines often express 10 times this quantity (6, 29). Furthermore, various investigators have demonstrated that ligand-dependent neoplastic transformation and proliferation are promoted by cells which overexpress the IGF-I receptor (30–32). This finding suggests that transformed cells which overexpress the IGF-I receptor may subvert growth regulation by minimizing their dependency on additional growth factors. Since the cervical cancer cells used in this study constitutively overexpress the IGF-I receptor, it is not surprising that these cells proliferate when stimulated solely with IGF-I or IGF-II.

Several lines of evidence indicate that activation of the IGF-I receptor is a convergence point for the transduction of mitogenic signals initiated by other tyrosine kinase growth factor receptors. For instance, PDGF and fibroblast growth factor stimulate production of IGF-I in human fibroblasts (33); EGF can also stimulate IGF-I production in fibroblasts under low-density culture conditions (34), and mouse fibroblasts with targeted disruption of the IGF-I receptor gene are unable to grow in defined medium containing EGF, PDGF, and IGF-I, whereas their parental cell counterparts, which possess a functional IGF-I receptor gene, grow appropriately under these conditions (11). Under physiologic conditions, IGF-I and IGF-II are the principal ligands which activate the IGF-I receptor and stimulate phosphorylation of its tyrosine kinase domains. This study demonstrates that EGF can also stimulate production of IGF-II by HT-3 cervical cancer cells and that the maximum mitogenic effect of EGF on these cells requires the autocrine participation of IGF-II.

Activation of the IGF-I receptor is controlled by several regulatory mechanisms. Apart from autocrine, paracrine, or endocrine pathways, the bioavailability of the ligands to bind to the receptor is modulated by one of six IGFBPs. The affinity of the IGFBPs for the IGFs is higher than that demonstrated by IGF-I receptor (23). The molecular mechanisms involved in this process are complex, with factors such as cell surface association, extracellular membrane association, phosphorylation, or proteolysis altering the ligand (IGF-I or IGF-II)/receptor interaction. Consistent with our results, IGFBP-5 has been shown to have inhibitory effects on DNA and glycogen synthesis (22) as well as steroidogenesis (35).

In rodents, IGF-II is widely expressed in the developing embryo, but its expression is progressively extinguished in virtually all tissues after birth (36, 37). On the basis of our data, increased expression of fetal, but not adult, IGF-II mRNA indicates that subversion of growth factor regulation may occur through inappropriate activation of the fetal promoter of the IGF-II gene and suggests that the malignant phenotype may be a reversion toward growth pathways important in developing tissues. IGF-II also appears to play a rate-limiting role in

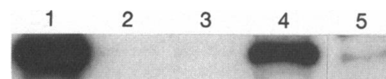

FIG. 6. Immunoprecipitation of autophosphorylated IGF-I receptor after stimulation with IGF-I for 1 hr (lane 1), with EGF for 1, 4, or 7 hr (lanes 2–4, respectively), or with EGF plus IGFBP-5 for 7 hr.

multistage oncogenesis by promoting tumor growth and malignancy (38), consistent with IGF-I and IGF-II expression in a variety of tumor types *in vitro* (8). Although both IGF-I and IGF-II are potent mitogens, their mutual effects on the IGF-I receptor may also activate an important survival pathway which subverts apoptosis. This prospect is the focus of considerable investigation (38, 39).

These experiments provide further evidence that autocrine activation of the IGF-I receptor by one of its ligands can be a consequence of a proliferative signal initiated by a distinct growth stimulus, such as EGF. The autocrine production of IGF-II in response to stimulation by EGF supports the concept of a growth factor cascade in which IGF-II acts downstream of EGF to participate in mediating EGF's actions. Since the growth of several different cell types requires activation of the IGF-I receptor by one of its ligands (40), it is likely that subversion of regulated growth involves contributions from various signaling pathways which converge as cells progress into the S phase of the cell cycle. The inappropriate autocrine production of IGF-II, a neonatal/fetal progression factor, in response to a mitogenic stimulus, represents an important signaling pathway that malignant cells may exploit to escape growth regulation.

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