Role of Insulin-Like Growth Factor-I in the Autocrine Regulation of Cell Growth in TT Human Medullary Thyroid Carcinoma Cells

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Since the TT human medullary thyroid carcinoma cell line required fewer exogenous growth factors (serum), we investigated whether this line has an autocrine mechanism by examining the effects of antibodies directed toward insulin-like growth factor I (IGF-I) and its receptor on TT cell growth in serum-free conditions. Treating cells with anti-IGF-I antibody for four days reduced the cell number by more than 50% compared with a nonimmune IgG control. Furthermore, a monoclonal antibody to the IGF-I receptor suppressed DNA synthesis when determined by a [^{3}H]thymidine incorporation assay. Exogenous IGF-I (20 ng/mL) stimulated [^{3}H]thymidine incorporation in serum-free medium; approximately 70% of the IGF-I-induced stimulation was blocked by the presence of the receptor antibody. Treating TT cells with IGF-I for 48 hours increased the cell population in the S phase by 62% when analyzed by flow cytometry. These data suggest that TT cells might respond to endogenously produced IGF-I and therefore provide an in vitro model for autocrine regulation of human tumor cell growth by IGF-I. (Henry Ford Hosp Med J 1992;40:293-5)

Many types of cancer cells produce polypeptide growth factors and often the same cells have functional receptors for the factors they release (1,2). This autocrine mechanism enables the cells to grow autonomously and may play a role in cellular transformation and in the continued proliferation of neoplastic cells.

Insulin-like growth factors (IGF-I and IGF-II)—mitogenic polypeptides with structural similarity to insulin (3,4)—have regulated in vitro cell growth for many human tumor types through autocrine and paracrine mechanisms (5). However, the importance of IGFs in the control of cell proliferation of human medullary thyroid carcinoma (MTC) cells remains unclear.

To examine the role of IGFs, we chose an established human MTC cell line, TT, which has maintained the morphological characteristics of MTC and the neuroendocrine properties of thyroid C-cells (6,7). The cell line has an abnormal karyotype with multiple rearranged chromosomes, including chromosomes 11 and 12 (8) where the IGF-I and IGF-II genes are located (9). Herein we examine the effects of a polyclonal antibody towards IGF-I and of a monoclonal antibody towards the IGF-I receptor on TT cell proliferation.

Materials and Methods

Cell culture and antibodies

The TT human MTC cell line was provided by Dr. Robert Gagel (M.D. Anderson Cancer Center, Houston, TX) and was initially established by Dr. Leong (Roswell Park Memorial Institute, Buffalo, NY). Cells were routinely passaged in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. Human recombinant IGF-I and the rabbit polyclonal antibody to IGF-I were obtained from Amgen Biologicals (Thousand Oaks, CA). αIR_3, a mouse monoclonal IgG$_1$ against the IGF-I receptor, was purchased from Oncogene Science, Inc. (Manhasset, NY). Nonimmune rabbit IgG was obtained from Zymed Laboratory (South San Francisco, CA). A mouse monoclonal IgG$_1$ against carcinoembryonic antigen (anti-CEA IgG$_1$), FP1-031490, was a gift from Dr. Chen (M.D. Anderson Cancer Center, Houston, TX).

Cell proliferation assays

TT cells were plated in 96-well dishes at a density of 5×10^4 cells/well with RPMI medium containing 10% FCS and 2 mM glutamine. After 48 hours, cells were placed in serum-free medium (RPMI medium containing 0.3% bovine serum albumin and 2 mM glutamine) for another 24 hours. Triplicate cells were then fed with serum-free medium or media containing different amounts of FCS. The spent media were replaced with fresh media after two days. For proliferation-inhibition experiments, the antibody to IGF-I or control IgG was added to serum-free medium 24 hours after serum starvation. The antibody-containing media were refreshed after two days. Cell counts were determined by a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) after suspending the cells with trypsin-EDTA.
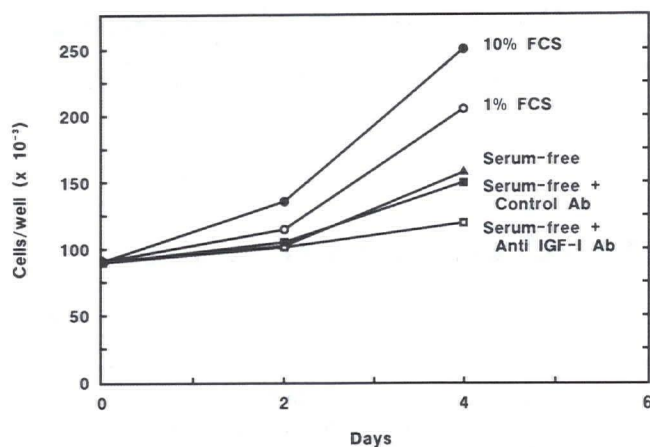
Fig 1—*Proliferation of TT cells in serum-containing, serum-free, and serum-free plus anti-IGF-I antibody media. Cells were grown in regular growth medium for two days and then placed into serum-free medium. After 24 hours (on day 0), the serum-free medium was replaced with fresh serum-free medium (indicated by solid triangles) or medium containing 1% (open circles) or 10% (solid circles) fetal calf serum. For proliferation-inhibition experiments, the antibody to IGF-I (open squares) or control IgG (solid squares) was added to serum-free medium on day 0. The spent media and antibody-containing media were refreshed on day 2. Cell counts were performed on days 0, 2, and 4. The figure shows the mean of triplicate determinations of a representative experiment.*

[³H]Thymidine incorporation studies

Cells were plated in the regular growth medium into 96-well dishes (5×10^4/well) and maintained for three days. After removing the medium, the monolayers were washed once with serum-free medium and incubated for 48 hours, with one additional change of the medium at 24 hours. The medium was replaced with fresh serum-free medium, and either the vehicle (control) or test agents were added to the wells. After incubation for 17 hours, [methyl-³H]thymidine (5 Ci/mmoL, final concentration 2 µCi/mL) was added, and the cells were incubated for an additional three hours. The labeling was terminated by removing the medium, washing once with ice-cold serum-free medium, and washing twice with 10% trichloroactic acid. The cell-associated radioactivity was recovered by solubilization with 0.5 N NaOH, neutralized with HCl, and counted in a scintillation spectrometer.

Flow-cytometric analysis

TT cells were first plated in the regular medium and maintained at 37 °C for 24 hours. After removing the medium, the cells were washed once with serum-free medium, incubated for an additional 24 hours, and treated with IGF-I (20 ng/mL) in refreshed serum-free medium for 48 hours. For flow-cytometric analysis, cells were harvested in single-cell suspensions, washed with saline, and fixed in 70% ice-cold ethanol. The fixed cells were stained in phosphate-buffered saline containing 0.05% Nonidet P-40, 0.2 mg/mL ribonuclease A, and 10 mg/mL

propidium iodine. About 5,000 cells were analyzed for the cell-cycle distribution on a FACSTAR-PLUS (Becton Dickinson, Mountain View, CA) cell sorter equipped with an argon laser operating at 488 nm. DNA profiles were analyzed by software provided by Becton Dickinson.

Results

Although TT cells are normally grown in RPMI medium containing 10% FCS, they also proliferated in medium containing a reduced amount of serum (1%) or even in serum-free medium, although with slower growth rates (Fig 1). Under serum-free conditions, the anti-IGF-I antibody inhibited cell growth more than 50% after four days in culture, suggesting that TT cells proliferated in serum-free conditions in response, at least partially, to endogenously secreted IGF-I or IGF-I-related substances.

Like other polypeptide hormones and growth factors, the biological activities of IGF-I are mediated by specific receptors on the cell surface. We therefore examined the effect of the IGF-I receptor blockade in serum-free medium using a monoclonal antibody, αIR_3, that specifically blocks IGF-I receptor binding (10,11). Fig 2 shows the αIR_3-induced inhibition of [³H]thymidine incorporation in TT cells. At a concentration of 15 µg/mL, αIR_3 inhibited [³H]thymidine incorporation by 45% compared with the anti-CEA IgG$_1$ control. The inhibition of αIR_3 was dose-dependent (data not shown).

To confirm the specificity of the inhibitory effect of αIR_3, we determined the stimulatory effect of exogenous IGF-I in serum-free medium in the presence or absence of the receptor antibody. IGF-I at a 20 ng/mL concentration stimulated DNA synthesis ([³H]thymidine incorporation) 1.6-fold more than the basal level (Fig 2). More than two-thirds of the IGF-I-induced mitogenic effect was suppressed by the monoclonal antibody against the IGF-I receptor, whereas the control IgG$_1$ had no significant effect.

Since it has been reported in fibroblasts that IGF-I is required for cells to progress through G$_1$ to the S phase (12), we also determined whether the mitogenic effect of IGF-I was associated with IGF-I-induced alterations of the cell cycle distribution in the TT cell line. The cells were grown in serum-free conditions in the presence or absence of IGF-I; DNA histograms of the treated and untreated controls were then analyzed by flow cytometry. The data revealed that treatment with 20 ng/mL IGF-I for 48 hours increased the cell population in the S phase by 62% compared with the untreated control (8.8 ± 0.7% versus 5.4 ± 0.4%).

Discussion

Our initial observations that the TT cell line needed little or no serum in culture prompted us to investigate whether these cells possessed an autonomous growth system. An anti-IGF-I antibody alone suppressed cell proliferation more than 50% in serum-free conditions, leading us to believe that TT cells may produce IGF-I, which in turn stimulated cell growth. The role of endogenously secreted IGF-I in autocrine regulation was again demonstrated by the inhibition of basal DNA synthesis by the

antibody-mediated IGF-I receptor blockade. This inhibitory effect was specifically associated with IGF-I receptor inhibition and was not due to nonspecific toxic effects of the antibody because simultaneous addition of IGF-I under serum-free conditions reversed the αIR_3 antibody-induced suppression of [^{3}H]thymidine incorporation. The presence of functional IGF-I receptor in TT cells was supported by the fact that exogenous IGF-I, at physiological concentrations, stimulated [^{3}H]thymidine incorporation, and the apparent IGF-I-induced mitogenic effect could be suppressed by simultaneous addition of the receptor antibody. IGF-I increased the cell population in the S phase, further demonstrating IGF-I's growth-promoting effect. Taken together, these data suggest that activated pathways for production of IGF-I and expression of the IGF-I receptor may have been turned on in TT cells. Identification of quantities of IGF-I in the conditioned medium and the IGF-I receptor on TT cell surfaces that are high enough to suggest gene activation is required to confirm this notion. These studies are currently under way.

Since the IGF-I antibody alone could suppress more than 50% of the basal growth of this cell line, IGF-I must be one of the major growth factors involved in the regulation of TT cell proliferation. Whether structurally related IGF-II and other unrelated growth factors act in concert with or opposition to IGF-I in the autocrine regulation of TT proliferation is also being investigated in our laboratory.

In conclusion, this study suggests that TT cells might respond to endogenously produced IGF-I and therefore provide an in vitro model for autocrine regulation of human tumor cell growth by IGF-I.

Acknowledgments

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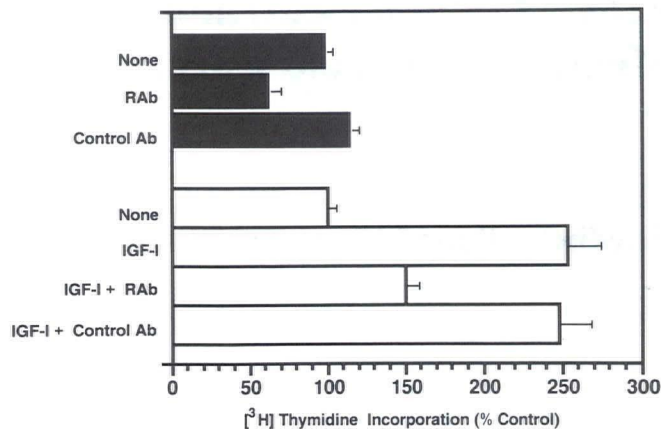4. Hall K, Sara VR. Growth and somatomedins. Vitam Horm 1983;40:175-233.

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Fig 2—Effect of the anti-IGF-I receptor antibody (αIR_3) on [^{3}H]thymidine incorporation in TT cells under serum-free conditions in the presence or absence of exogenous IGF-I. After cells were seeded with regular growth medium and grown for three days, the medium was changed to serum-free medium. Forty-eight hours later, αIR_3 (RAb) or control IgG$_1$ (control Ab) was added. In some wells where the effect of exogenous IGF-I was determined, IGF-I was added. [^{3}H]thymidine incorporation assays were performed after 17 hours by procedures described in the text. Data are the mean of triplicate determinations from a representative experiment; bars = SD.