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Autocrine Role of Insulin-like Growth Factor (IGF)-I in a Human Thyroid Cancer Cell Line

Noritaka Onoda, Eiji Ohmura, Toshio Tsushima, Yoshito Ohba, Naoya Emoto, Osamu Isozaki, Yasuko Sato, Kazuo Shizume and Hiroshi Demura

An established cell line (TC-cell, clone 78) derived from human thyroid papillary cancer cells was investigated for production of peptide growth factors. The cells had specific binding sites for insulin-like growth factor-I (IGF-I) and responded to this growth factor with increased proliferation. Culture medium conditioned by TC cells was found to contain insulin-like growth factor (IGF)-I and IGF-binding protein(s). Furthermore, reverse transcription-polymerase chain reaction revealed expression of IGF-I mRNA. When monoclonal antibody to IGF-I receptors (αIR3) was added, the growth of TC cells cultured in serum-free medium was significantly reduced. The growth rate of the cells was restored when the antibody was removed from the medium. These results strongly suggest that TC cells produce IGF-I, which is involved in the regulation of their own growth.
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INTRODUCTION

RECENT STUDIES have shown that neoplastic cells are able to synthesise a number of peptide growth factors which may be involved in their own growth [1, 2]. Insulin-like growth factors (IGF-I and II) are among the growing list of such growth factors. IGF-I is a growth hormone-dependent plasma factor with potent cell growth promoting activity in a variety of cultured cells [3]. Although serum IGF-I is largely derived from the liver, many extrahepatic tissues or cells synthesise IGF-I, suggesting an autocrine or paracrine role in growth regulation [4]. Moreover, the involvement of IGF-I in cell growth has been suggested by the expression of IGF-I mRNA in several neoplastic cells [5–8].

Although pituitary thyrotropin (TSH) is the major regulator of thyroid growth and function, it has been shown that a number of growth factors affect the growth and function of thyroid epithelial cells. For example, epidermal growth factor (EGF) is a potent mitogen and inhibitor of TSH-induced iodide metabolism in thyroid cells of several species [9, 10]. We and others have recently shown that IGF-I have a stimulatory effect on thyroid cell growth [11, 12]. It appears that IGF-I is involved in the growth of human thyroid neoplastic cells. Minuto *et al.* showed higher concentrations of immunoreactive IGF-I (irIGF-I) in human thyroid tumorous tissues [13], and Williams *et al.* reported inhibition of growth of human thyroid adenoma cells by antibody to IGF-I [14]. Furthermore, we have recently shown that the number of IGF-I receptors is much higher in human

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thyroid cancer tissues than in adjacent non-neoplastic tissues [15]. However, it has not yet been definitely determined whether IGF-I regulates the growth of human thyroid cancer cells through an autocrine mechanism. To address this question, we examined the secretion of IGF-I and its role in cell growth using a cell line established from human thyroid papillary carcinoma.

MATERIALS AND METHODS

Chemicals

The recombinant human IGF-I used was a product of Fujisawa Pharmaceuticals (Osaka). The human recombinant IGF-II and porcine monocomponent insulin used were products of Eli Lilly (Indianapolis). Monoclonal antibody to IGF-I receptors (αIR3) was kindly provided by Dr S Jacobs (Burroughs-Wellcome, Research Triangle Park). Fetal calf serum (FCS) was purchased from Filtron Ltd. (Victoria, Australia).

Cell culture

The human thyroid carcinoma cell line (TC cell) used in these studies was established from a patient with papillary carcinoma; the cell line was originally established and kindly provided by Dr Maeda (Tokyo Medical College). The cells possess tumorigenicity in nude mice and they produce a faint amount of thyroglobulin(Tg) but during the passage they lost the differentiated characters of thyroid cells, such as synthesis of thyroid hormones and thyroid peroxidase activity. However it possessed TSH receptor mRNA as described later. The cells were maintained in RPMI 1640 (Gibco) supplemented with 4% FCS, antibiotics (penicillin G 1000 U/ml, streptomycin 100 μg/ml, amphotericin B 2.5 μg/ml) in a humidified atmosphere of 5% CO_2 in air.

Cell growth

TC cells were plated at a density of 0.5–3.0 × 10^4 cells/well in 12-well plates (3.8 cm^2 well, Costar). After 24 h culture, the medium was replaced by serum-free RPMI 1640 medium supplemented with 0.1% bovine serum albumin (BSA) (Tarui Chemicals, Kyoto). The cells were cultured in the presence or absence of desired concentrations of IGF-I for up to 8 days. Unless otherwise noted, the medium was replaced every 2 days. After dissociating the cells by treatment with 0.25% trypsin, the cell number was determined with a Coulter counter. The growth of TC cells were stimulated by FCS (0.5% to 4.0%) in a dose dependent manner. The cells also grew well in the absence of FCS, though the rate of growth was much slower. The doubling time of the cells was 24 h in the presence of 4% FCS, and 36 h in the serum-free medium supplemented with 0.3% BSA.

DNA synthesis

DNA synthesis was determined by the extent of [^{3}H]thymidine incorporation into the trichloroacetic acid (TCA)-insoluble cellular fraction. TC cells were allowed to grow into subconfluence in 12-well plates, and the cultures were shifted to serum-free RPMI 1640 medium containing 0.1% BSA for 24 h before the assay. The medium was then replaced by serum-free medium containing the test materials. After 12 h, the cells were pulsed with [^{3}H]thymidine (740 GBq/mmol; New England Nuclear) for 4 h. The cultures were washed twice with ice-cold phosphate buffered saline (PBS) and precipitated with ice-cold 10% TCA. The cultures were washed twice with ethanol–ether (1:3) solution and solubilised with 1 mol/l NaOH. An aliquot was neutralised and the radioactivity was measured with a scintillation counter.

Measurement of IGF-I and IGF binding protein

TC cells were grown to confluence in RPMI-1640 medium with 4% FCS in 80 cm^2 dishes. The cultures were then washed twice and replaced with 5 ml serum-free RPMI-1640 medium containing 0.1% BSA. After 48 h culture, the medium was collected and centrifuged at 200 *g* for 5 min. The supernatant was dialysed against distilled water and lyophilised. The lyophilised materials were treated with 1 mol/l acetic acid overnight. The aliquot was centrifuged at 10 000 *g* for 30 min and the supernatant was applied to a column (1.0 × 45 cm) of Sephadex G-50 (Pharmacia) equilibrated with 1 mol/l acetic acid. Fractions of 1 ml were collected, lyophilised, reconstituted with 500 μl of the assay buffer (50 mmol/l Tris–HCl buffer containing 0.3% BSA, 0.01% EDTA, and 0.02% NaN_3, pH 7.4), and assayed for IGF-I and IGF-binding protein. Radioimmunoassay (RIA) for IGF-I was performed using antibody prepared in this laboratory and IGF-I was radioiodinated by the Chloramine T method to a specific activity of approximately 3.7 TBq/mg. Antibody-bound ^{125}I-IGF-I was separated from the unbound fraction by polyethylene glycol 6000 (final concentration 12.5%). Sensitivity of the assay was 100 pg, and the coefficients of intra- and interassay variation were 5.2 and 12%, respectively. Crossreaction of human IGF-II in this assay system was less than 1% of that of IGF-I. The IGF-binding protein in the fractionated conditioned medium was identified according to the method described by Martin and Baxter [16]. Briefly, 100 μl of the fractionated aliquot was incubated with 20 000 cpm of ^{125}I-IGF-I for 20 h at 4°C in the presence or absence of 1 μg unlabeled human IGF-I in a final volume of 500 μl. Protein-bound ^{125}I-IGF-I was separated from the unbound fraction by adding 2% dextran-coated charcoal suspension in 50 mmol/l Tris–HCl buffer containing 2% BSA. Specific binding was calculated by the difference between the radioactivities bound in the absence and presence of an excess amount of unlabelled IGF-I.

Reverse transcription-polymerase chain reaction

TC cells were allowed to grow into confluence in 10 cm culture dishes and total RNA was isolated essentially as described by Chirgwin *et al.* [17]. Poly A+ RNA was selected by oligo(dT) cellulose chromatography [18]. Total or poly A+ RNA (0.5 μg) in 1 μl water was reverse transcribed. Contaminating DNA in RNA samples was digested with 2 U of RNase-free DNase (RQ DNase, Promega) in the presence of RNasin (Promega) for 30 min at 37°C. The RNA and 0.25 μg oligo-dT_{12-18} in 16 μl of reverse transcription (RT) buffer were heated at 95°C for 2 min, chilled on ice and incubated for 30 min to anneal. Then the mixture was incubated at 37°C for 60 min with 100 U (0.5 μl) cloned M-MLV reverse transcriptase (BRL, Gaithersburg). The reaction was stopped by heating at 90°C for 5 min and was kept on ice. Oligonucleotide primers were synthesised in the Bio Labo Inc. (Saitama) by the phosphoramidite method on an Applied Biosystem Model 380B DNA synthesiser (Foster City). The oligonucleotide in water was used after one step purification using Oligonucleotide Purification Cartridge (Applied Biosystems Inc). The proximal primer of IGF-I was designed to amplify segments starting at human IGF-I nucleotide position 436 (aminoacid 12), with the distal primer delimiting nucleotide position 584 (aminoacid 61). The proximal primer of TSH-R was designed to amplify segments starting at human TSH-R nucleotide position 622 (aminoacid 175), with the distal primer delimiting nucleotide position 1000 (aminoacid 301). The primer sequences are as follows.

IGF-I Proximal Primer:	5'-AT GCT CTT CAG TTC GTG TGT-3'
IGF-I Distal Primer:	5'-CA GTA CAT CTC CAG CCT CCT-3'
TSH-R Proximal Primer:	5'-CTA TGC AAT GAA ACC TTG AC-3'
TSH-R Distal Primer:	5'-A CAT CAA GGA CTC AAG GAT T-3'

These IGF-I sequences are based on the published data of Rotwein *et al.* [19] and the TSH-R sequences are based on the data of Nagayama *et al.* [20]. PCR was carried out according to the instructions provided with the enzyme. The following reagents were added: (1) 10 × reaction buffer, 1 μl; (2) dNTP mixture 2.5 mmol/l each dATP, dCTP, dGTP, or dTTP, BRL, 0.8 μl; (3) primer mixture (10 pmol each primer in water), 1 μl; (4) 0.5 U cloned *Thermus aquaticus (Taq)* DNA polymerase (BRL, Gaithersburg, Maryland) and (5) 1 μl of RT sample. The RT sample was always added last to avoid cross-contamination. Samples were incubated for 32 polymerase chain reaction (PCR) cycles. During each cycle, the samples were heated to 94°C to denature template complexes (120 s initially and 30 s during all subsequent cycles), cooled to 55°C to permit templates and primers to anneal (30 s) and heated to 72°C to allow extension to occur (60 s). The final 72°C incubation was extended for an additional 7 min to maximise strand completion. PCR products we run on 3% agarose gels. Standard size markers were phi × 174 RF DNA/*Hae* III fragments (1000 ng/lane; BRL). Gels were stained for 10 min in ethidium bromide (10 μg/ml), destained in water for 30 min, and examined on a 312 nm ultraviolet transilluminator.

IGF-I Binding study

TC cells were grown in confluence in 12-well plates were washed twice and then incubated with ^{125}I-IGF-I (50 000 cpm), in the presence or absence of 1 μg of unlabeled IGF-I for 20 h at 4°C in 1 ml Hepes binding buffer (100 mmol/l Hepes, 50 mmol/l Tris, 10 mmol/l $MgCl_2$, 2 mmol/l EDTA, 10 mmol/l glucose, 10 mmol/l $CaCl_2$, 50 mmol/l NaCl, 5 mmol/l KCl, and 0.2% BSA). At the end of incubation, the medium was removed and the cells were washed twice with ice-cold Hepes binding buffer and solubilized with 1 ml 1 mol/l NaOH. Radioactivity of the aliquots was measured using an automated gamma-counter.

Statistical analysis

The data were subjected to analysis of variance. Then, statistical analysis of differences between the groups was carried out using Student's *t*-test if the variation in the data was uniform. When the variation in the data was not uniform, Cochran–Cox's test was employed. *P* values of less than 0.05 were considered significant.

RESULTS

Effect of IGF-I

We examined whether exogenous IGF-I affects the growth of cells. Figure 1 shows that human recombinant IGF-I stimulates, in a dose-dependent manner, DNA synthesis of the cells placed in serum-free medium. The effect was detected at 1.25 ng/ml and plateaued at concentrations greater than 12.5 ng/ml. To demonstrate that DNA synthesis represents the mitogenic effect of IGF-I, TC cells were cultured for up to 4 days in the presence or absence of IGF-I, and the cells were counted. It can be seen that IGF-I actually stimulates cell proliferation. The effect

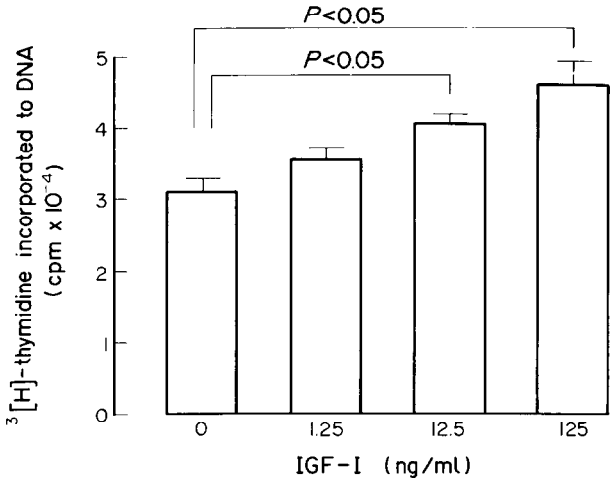

Fig. 1. Effect of IGF-I on [^{3}H]thymidine incorporation of TC cells. TC cells in subconfluent condition were preincubated for 24 h. Then, the cells were exposed to the indicated concentrations of IGF-I for 12 h and the [^{3}H]thymidine incorporation was determined as described in Materials and Methods. The values are means (S.E.) (bars) of three determinations.

was dose-dependent and maximal at 12.5 ng/ml (Fig. 2). The number of cells cultured in the presence of 125 ng/ml IGF-I amounted to 123% and 220% compared with the number of untreated cells at 2 and 4 days of culture, respectively.

IGF-I receptors

The mitogenic effect of IGF-I suggests the presence of IGF-I receptors on TC cells, which was confirmed by the ^{125}I-IGF-I

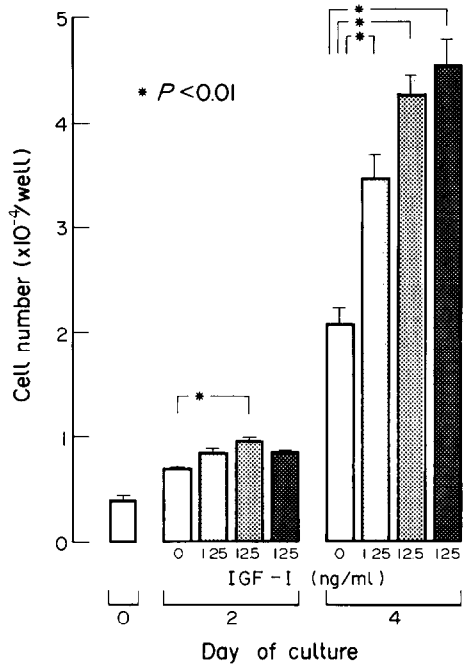

Fig. 2. Effect of IGF-I on TC cell growth. TC cells were grown in RPMI 1640 medium with 4% FCS. Then, the cells were cultured in RPMI 1640 containing 0.1% BSA with the indicated concentrations of IGF-I. The cells received fresh medium every 2 days. The cell number was determined as described in Materials and Methods. The values are means (S.E.) (bars) of three determinations.

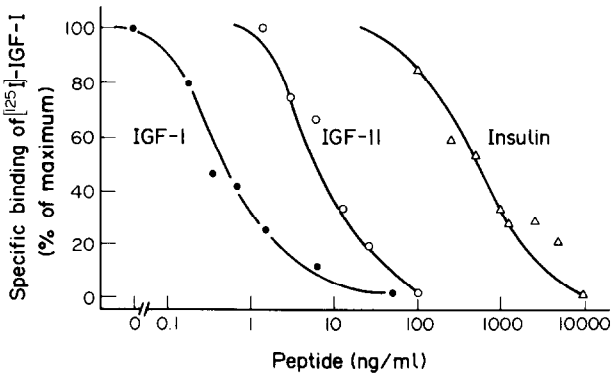

Fig. 3. Binding study of IGF-I to TC cells. Radioreceptor assay of ^{125}I-IGF-I was performed using TC cells. The cells were incubated with ^{125}I-IGF-I in the presence of the indicated concentrations of unlabeled IGF-I (●), IGF-II (○) or insulin (△) at 4°C for 20 h. The bindings of ^{125}I-IGF-I were determined as described in the Materials and Methods. The values are the means of triplicated determinations.

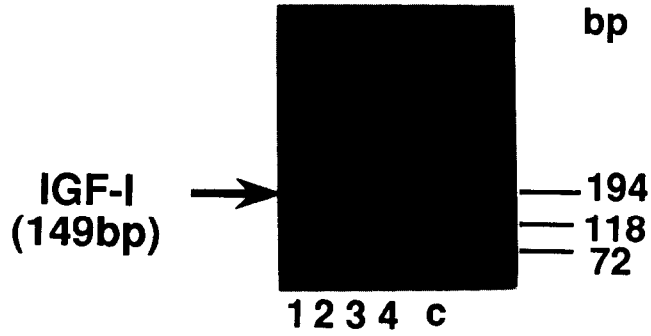

Fig. 5. Detection of IGF-I mRNA by RT-PCR. PCR was carried out for 52 cycles with an annealing temperature of 55°C using IGF-I primers. The product was electrophoresed in 3% agarose gel and stained with ethidium bromide. cDNA of total RNA from TC cells (lane 1), cDNA of poly A+ RNA from TC cells (lane 2), cDNA of total RNA from rat liver (lane 3), cDNA of total RNA from rat kidney (lane 4) and cDNA without RNA (lane C). The expected size of IGF-I PCR product was 149 bp as indicated.

binding study (Fig. 3). Binding was inhibited by unlabelled IGF-I in a concentration dependent manner; half maximal inhibition was attained with 0.5 ng/ml. Scatchard analysis of the binding data from three separate experiments revealed a single class of IGF-I receptors with high affinity. (K_d = 0.07 nmol/l). As shown in Fig. 3, IGF-II and porcine insulin were able to displace ^{125}I-IGF-I. However, their potencies were only 1/20 and 1/1000, respectively, as compared with IGF-I. Thus, specificity of ^{125}I-IGF-I was characteristic of that of type I-IGF receptors [21].

Production of IGF-I and IGF binding protein by TC cells

TC cells were cultured in serum-free medium for 48 h and the conditioned medium was analysed for the content of immunoreactive IGF-I. It has been reported that a number of cultured cells secrete both IGF-I and IGF binding proteins with different molecular weights. Since the binding proteins probably interferes with IGF-I RIA by interacting with ^{125}I-IGF-I [22], the conditioned medium was fractionated by gel-chromatography under acidic conditions to separate IGF-I from IGF binding proteins. Figure 4 illustrates that IGF-I like immunoreactivity

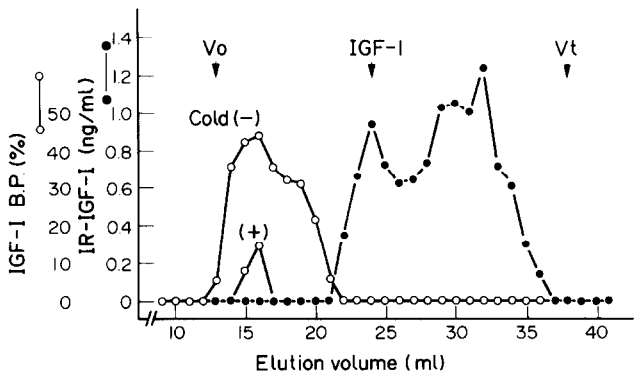

Fig. 4. Elution profile of immunoreactive IGF-I and IGF-I binding activity of TC cells. The medium of TC cells cultured with 0.1% BSA for 2 days was extracted with 1 mol/l acetic acid and processed as described in Materials and Methods. The immunoreactive IGF-I (●), IGF-I binding activity (○) in the absence and presence of excess IGF-I of each of the fractions are shown. Vo and Vt indicate the void volume and total volume of the column, respectively. The elution position of authentic IGF-I is shown as IGF-I.

was largely eluted in the position corresponding to authentic IGF-I or slightly retarded fractions. The dilutions of the active fractions reacted to IGF-I antibody in a manner parallel to that of standard IGF-I. The absolute amount secreted by TC cells is approximately 0.1 to 0.2 ng/ml/24 h/10^6 cells. The irIGF-I values in the void volume fractions are not shown in figure because RIA gave negative values; when an aliquot was measured for irIGF-I, the counts of ^{125}I-IGF-I precipitated by polyethylene glycol (PEG) were much greater than those obtained in the absence of unlabeled IGF-I. This suggests that ^{125}I-IGF-I complexes to protein(s) other than antibody to IGF-I and the complex were precipitated by polyethylene glycol. This was indeed the case. When the void volume fractions were incubated with ^{125}I-IGF-I in the presence or absence of unlabeled IGF-I, binding protein specific for IGF-I was detected (Fig. 4).

RT-PCR

To further characterise ir-IGF-I produced by TC cells, we examined the expression of mRNA for IGF-I by RT-PCR. Since liver and kidney are rich sources of IGF-I, RNA samples were also prepared from rat kidney and liver in the validation of the RT-PCR for IGF-I mRNA. As shown in Fig. 5, single PCR product of expected size was obtained in RNA from liver and kidney. In case of TC cells, IGF-I transcript was evident in poly A+ RNA but is very faint in total RNA. These data indicate the amount of IGF-I gene transcript in TC cells is not as abundant as in liver or kidney. Next the TSH receptor message expression in TC cell was examined to confirm the origin of TC cells. As shown in Fig. 6, TSH receptor mRNA was present in the poly A+ RNA preparation from TC cells as well as RNA preparations from normal thyroid tissues and papillary cancer tissues of thyroid. The fact that thyroid cancer tissue expresses TSH receptor mRNA is compatible with the previous report [23] and the fact that TC cell expresses TSH receptor mRNA confirmed that TC cell is originated from thyroid follicular cell.

Effect of αIR3 on growth of TC cells

The results described above strongly suggest that TC cells secrete IGF-I. To demonstrate the autocrine role of IGF-I on TC cells, the effect of monoclonal antibody to IGF-I receptors (αIR3) on cell growth was examined. Since preliminary studies showed that the antibody (final dilution 1:200; 25 μg IgG/ml)

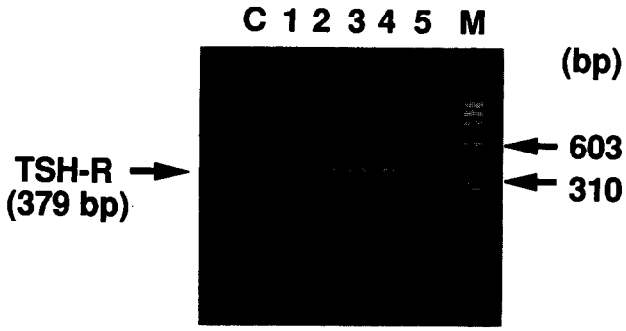

Fig. 6. Detection of TSH receptor (TSH-R) mRNA by RT-PCR. PCR was carried out for 52 cycles with an annealing temperature of 55°C using TSH receptor primers. PCR product was processed in the same manner as described in Fig. 5. cDNA without RNA (lane C), cDNA of poly A+ RNA from TC cells (lane 1), cDNA of total RNA from normal thyroid tissue (lane 2 and 4), and cDNA from RNA from papillary carcinoma of thyroid (lane 3 and 5). The expected size of TSH receptor PCR product was 379 bp as indicated.

was able to inhibit ^{125}I-IGF-I binding by more than 90%, this concentration of antibody was employed throughout the experiments. The antibody did not affect ^{125}I-IGF-II binding at this concentration. The same concentration of monoclonal mouse antibody to pork insulin prepared in our laboratory served as a control. TC cells were cultured in serum-free RPMI 1640 medium supplemented with 0.3% BSA in the presence of αIR3 or control IgG. Figure 7 illustrates that the presence of αIR3 significantly ($P < 0.01$) inhibits the growth of TC cells. The number of cells was 74% on day 2 and 47% on day 4 compared with control cells not exposed to αIR3. When the antibody was removed on day 4, the subsequent growth rate was identical to that of control cells.

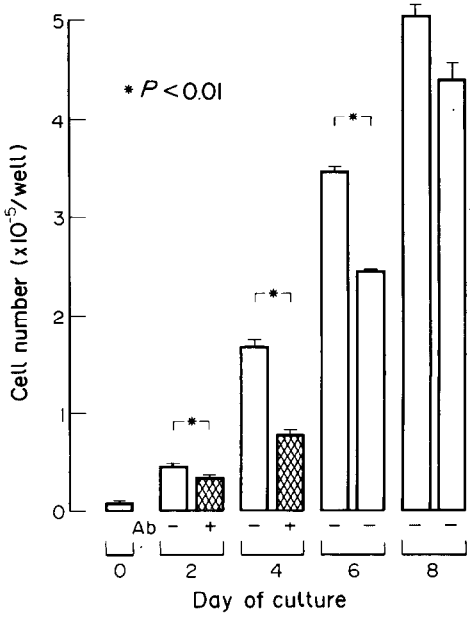

Fig. 7. Effect of anti-IGF-I receptor antibody (αIR3) on growth of TC cells. The medium of the TC cells was changed to PRMI1640 with 0.1% BSA and the cells were further cultured for in the presence of the antibody(+) or control immunoglobulin(−). The cells received fresh medium every 2 days. After the indicated periods, the cell number was determined. The values are means (S.E.) (bars) of three determinations.

DISCUSSION

Several lines of evidence indicate that peptide growth factors are important in cell proliferation and transformation. Autocrine stimulation of cell growth including IGF-I has been suggested for several neoplastic cell lines [5, 24]. The data presented here demonstrates that TC cells, a human thyroid cancer cell line, have specific binding sites for IGF-I. The specificity, and the affinity of the receptors were similar to those reported for human thyroid tissues [15]. The specificity was compatible to that characteristic of type 1 receptors, which interact with IGF-I with high affinity, and interact with insulin with lower affinity [21]. The binding of ^{125}I-IGF-I to the IGF-I receptors on TC cells may be affected by IGF-BP, if present in the incubation medium. Although the culture medium conditioned by TC cells produced IGF-BP as described later, we were not able to detect IGF-BP in the incubation medium under the conditions employed in the IGF-I binding study (incubation for 18 h at 4°C).

Corresponding to the presence of high affinity IGF-I receptors, TC cells responded to physiological concentrations (1.25–125 ng/ml) of IGF-I with a dose-dependent increase in DNA synthesis and cell proliferation. We also showed that TC cells secrete immunoreactive(ir) IGF-I into the medium. IrIGF-I was eluted in the position corresponding to authentic IGF-I and also in the more retarded fractions on Sephadex G-50 column equilibrated with 1 mol/l acetic acid. The property of irIGF-I in the retarded fractions is unknown at present. This may be degradation products of IGF-I. Alternatively, it may be a variant form of IGF-I. Further evidence that TC cells synthesise IGF-I was provided by RT-PCR which revealed IGF-I mRNA transcripts as reported for another types of transformed cells [5–8]. Secretion of IGF-I has also been described in normal human and sheep thyroid [25, 26].

To evaluate the role of endogenously produced IGF-I in growth of TC cells, we studied the effect of IGF-I receptor antibody. The result strongly suggested that the endogenous IGF-I involved in their own growth of TC cells. It is not likely that the inhibition of the growth by the antibody is non-specific cytotoxic effect because the effect was reversible. In FRTL-5 cells and sheep thyroid cells, production of IGF-II has been demonstrated [27, 28]. We have not determined yet whether TC cells produce IGF-II, which may be involved in growth of the cells as well. However, binding of ^{125}IGF-II to TC cells was not inhibited by anti-IGF-I receptors (αIR3) at the concentration capable of inhibiting ^{125}I-IGF-I by more than 90%. Thus, it is unlikely that IGF-II, if produced by TC cells, stimulates cell proliferation through binding to type I receptors.

Autocrine role in thyroid cell growth has been recently reported by Williams *et al.* [14]. They showed that antibody to IGF-I inhibits the growth of human thyroid adenoma cells. Although the present study strongly suggests an autocrine role of IGF-I in growth of TC cells, it is to be noted that the concentration of IGF-I in the medium conditioned by TC cells was much lower as compared with that of exogenous IGF-I required to stimulate DNA synthesis or cell proliferation. Several explanations would be possible. The majority of secreted IGF-I may immediately interact with IGF-I receptor and become internalised, with only a part of the IGF-I being detected in the medium. Alternatively, TC cells might produce other growth factors which potentiate the mitogenic activity. Actually we have found EGF-like peptide (possibly TGF-α) in the conditioned medium (data not shown).

TC cells was found to secrete IGF-I binding proteins as well,

as reported for other types of transformed cells [5, 29, 30, 31]. At least six types of IGF-BP have been identified and sequenced. Preliminary studies with ligand blot have shown that TC cells produce at least two species of IGF-BP with mol. wt. 24K and 33K. (data not shown). Although IGF-BP have been reported to modify IGF actions [32–35], the role of IGF-BP is still unknown. When the conditioned medium of TC cells was fractionated on a Sephadex G-50 column, irIGF-I was recovered in the void volume fractions. Thus, it appears that the majority of irIGF-I in the medium exist as a complexed form with IGF-BP. Further studies are required to determine whether these IGF-BP modify IGF-I action on growth of TC cells.

In conclusion, our results indicate that a human thyroid cancer cell line produces IGF-I, which regulates its own growth. Together with previous findings that the number of IGF-I receptors in thyroid cancer tissues is greater than in the adjacent normal tissues [15], the present study strongly suggests that IGF-I is one of the factors responsible for the growth of human thyroid cancer.

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