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All-*Trans*-Retinoic Acid Modulates Expression Levels of Thyroglobulin and Cytokines in a New Human Poorly Differentiated Papillary Thyroid Carcinoma Cell Line, KTC-1*

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ABSTRACT

A new human thyroid carcinoma cell line, KTC-1, was established from the malignant pleural effusion of a recurrent thyroid carcinoma patient. Cytogenetic analysis revealed a normal karyotype, and no p53 mutation in exons 5–9 was detected. This cell line is tumorigenic in athymic nude mice. Histological findings by light and electron microscopy, such as the absence of follicular structures and the existence of intranuclear cytoplasmic inclusions and psammoma bodies, indicated transplanted tumors to be a poorly differentiated papillary thyroid carcinoma. A low expression level of thyroglobulin was detected by immunocytochemistry and RT-PCR. Messenger ribonucleic acid (mRNA) expression of thyroid transcription factor-1 and PAX-8 was also detected. No mRNA expression of TSH receptors, thyroid peroxidase, or Na^+/I^- symporter was detected. Interleukin-6 and leukemia inhibitory factor were secreted into the medium. These findings suggest this cell line to be functionally poorly differentiated. Moreover, all-*trans*-retinoic acid increased the mRNA expression of thyroglobulin and decreased both the mRNA expression and secretion of interleukin-6 and leukemia inhibitory factor while significantly stimulating growth. RT-PCR analysis of retinoic acid receptors (RARs) revealed that KTC-1 cells express a moderate level of RARα and -γ, but a low level of RARβ. This cell line may be useful for studying redifferentiation therapy for thyroid carcinoma. (*J Clin Endocrinol Metab* **85:** 2889–2896, 2000)

ALTHOUGH well differentiated thyroid carcinoma is one of the most slow growing of malignancies, undifferentiated thyroid carcinoma grows rapidly, frequently metastasizes to distant organs, and is often fatal (1, 2). It is believed that undifferentiated thyroid carcinoma mostly originates from preexisting, well differentiated thyroid carcinoma. Several molecular mechanisms responsible for this transformation, such as mutations of p53 (3, 4) and β-catenin (5, 6) and Met activation (7), have been proposed. Recently, the clinicopathological importance of poorly differentiated thyroid carcinoma has been reported. Poorly differentiated thyroid carcinoma occasionally coexists with well differentiated thyroid carcinoma and increases the chance of recurrence and recurrence-related death (8, 9).

Several human thyroid carcinoma cell lines have been established and used for studying the cellular and molecular biology of thyroid carcinoma (10–30). Studies have indicated that cell lines originating from well differentiated thyroid carcinoma express thyroid differentiation markers, such as thyroglobulin (Tg), thyroid peroxidase (TPO), and TSH receptors (TSH-R). In contrast, cell lines originating from undifferentiated thyroid carcinoma do not express such markers, but express various cytokines, such as interleukin-6 (IL-6), colony-stimulating factors, and PTH-related protein (11, 15, 17, 18, 20, 24, 26, 28). No human thyroid carcinoma cell line originating from poorly differentiated thyroid carcinoma, which expresses both the differentiation markers and cytokines, has been reported to date.

We recently isolated a new human thyroid cancer cell line, KTC-1, derived from the malignant pleural effusion of a male patient with recurrent thyroid carcinoma. This cell line grows in a monolayer fashion *in vitro* and is tumorigenic in athymic nude mice. Cytogenetic analysis indicated that this cell line has a normal karyotype. Morphological analyses indicated KTC-1 transplanted tumors to be poorly differentiated papillary thyroid carcinoma. Immunocytochemistry and RT-PCR revealed a low expression level of Tg, but no expression of other differentiation markers, TPO, TSH-R, or Na^+/I^- symporter (NIS). Messenger ribonucleic acid (mRNA) expression of thyroid transcription factor-1 (TTF-1) and PAX-8 was also detected by RT-PCR. Biochemical analysis of culture medium revealed that this cell line secretes cytokines, IL-6, and leukemia inhibitory factor (LIF) (31). These findings suggest this cell line to be morphologically and functionally poorly differentiated.

Although disseminated, well differentiated thyroid carcinoma is effectively treated by radioiodine therapy, both poorly differentiated and undifferentiated thyroid carcinomas are resistant to various therapies, including cytotoxic chemotherapy and radiotherapy (2). Some researchers have

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indicated the usefulness of redifferentiation therapy using differentiation inducers, such as retinoids, for dedifferentiated thyroid carcinoma (32–34). To clarify the effect of all-*trans*-retinoic acid (ATRA), this newly developed thyroid carcinoma cell line was exposed to this compound, and its effects on differentiation and growth were investigated.

Materials and Methods

Patient and cell culture

Reductive thyroidectomy followed by radioiodine and TSH suppression therapies were performed in February 1990 for a 60-yr-old male patient with advanced thyroid cancer with mediastinal lymph node metastases. Pathological examination revealed a well differentiated papillary thyroid carcinoma. Radiotherapy to refractory lymph node metastases was given in October 1994. Right pleural effusion appeared in October 1998. Thoracic drainage was performed to remove the pleural effusion. Cytological analysis showed atypical epithelial cells. Approximately 50 mL of the heparinized effusion were collected for cell culture. After centrifugation, the cell pellet was resuspended with DMEM supplemented with 5% FBS and cultured. Atypical epithelial cells rapidly grew in a monolayer fashion and have been passed at a 1:10 split once or twice a week more than 70 times for over a year. No additional supplement, such as TSH, was needed to maintain these cells.

Morphological analysis

Cultured cells were observed, and phase contrast microphotographs were taken with an inverted Nikon Diaphot-TMD microscope (Nikon, Tokyo, Japan). Hematoxylin-eosin staining of paraffin-embedded specimens, including cell pellets and transplanted tumors, was performed using the conventional method. Microphotographs were obtained with an Olympus Corp. AH-2 microscope (Olympus Corp., Tokyo, Japan). For transmission electron microscopy, transplanted tumors were resected, minced into blocks 1 mm in size, and fixed with 2.5% glutaraldehyde in PBS for 2 h at 4 C. After being washed with PBS, the blocks were postfixed with 1% osmium tetroxide in 0.1 mol/L cacodylate buffer and embedded in epoxy resin. These blocks were cut into thin sections with a Supernova ultracutter (Reichert-Jung, Vienna, Austria) with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Hitachi H-7100 electron microscope (Hitachi, Tokyo, Japan). For immunocytochemical staining, paraffin sections of tumor samples or cell pellets were dewaxed with xylene, hydrated with phosphate-buffered saline, treated with hydrogen peroxide for elimination of endogenous peroxidase, and then processed by the immunoperoxidase procedure. Rabbit anti-Tg polyclonal antibody (DAKO Corp. Japan, Tokyo, Japan), mouse monoclonal anticytokeratin antibody (Immunotech, Marseilles, France), and mouse monoclonal anti-carcinoembryonic antigen (Takara Biochemicals, Kyoto, Japan) were used as the first antibody. Control experiments were performed by substituting normal rabbit or mouse serum for the first antibody. The reaction was visualized with a Histofine kit (Nichirei, Tokyo, Japan). The sections were also counterstained with methyl green.

Chromosomal analysis

Cytogenetic analysis was performed at the 5th and 64th passages. Semiconfluent cells were exposed to 0.1 μg/mL colcemid for 4 h and detached with a trypsin solution. After the addition of a hypotonic solution of 0.075 mol/L potassium chloride, the cells were fixed with 3:1 methanol-acetic acid and stained with Giemsa.

RT-PCR

Total cellular RNA from KTC-1 cells was extracted with a TRIzol RNA extraction kit (Life Technologies, Inc., Gaithersburg, MD). One microgram of total RNA and 1 μmol/L oligo(deoxythymidine)$_{18}$ primer in 12.5 μL diethyl pirocarbonate-treated water were heated to 70 C for 2 min, followed by cooling on ice for 1 min. Complementary DNA (cDNA) synthesis was initiated with 200 U recombinant Moloney murine leukemia virus reverse transcriptase (CLONTECH Laboratories, Inc., Palo Alto, CA), and the reaction was allowed to proceed at 42 C for 1 h. The reaction was terminated by heating at 94 C for 5 min. cDNA was dissolved to a final volume of 100 μL by adding 80 μL diethyl pirocarbonate-treated water and then was frozen at −20 C until use. Oligonucleotide primers for the RT-PCR were designed using a published sequence of each target gene and were synthesized by the solid phase triester method. The primers and conditions used and the expected sizes from the reported cDNA sequence are shown in Table 1.

When the effects of ATRA on mRNA expression levels of Tg, IL-6, LIF, and retinoic acid receptors (RARs) and the basal expression levels of TTF-1 and PAX-8 were investigated to amplify both the internal control gene (β-actin) and one of the target genes in a single reaction, multiplex PCR was carried out. The ratios of primer sets between the target gene and the control gene are shown in Table 1. These ratios and the numbers of PCR cycles were determined to amplify both products logarithmically. Because the PCR product size of RARβ was close to that of β-actin, glyceraldehyde-3-phosphate dehydrogenase gene (human glyceraldehyde-3-phosphate dehydrogenase Control Amplimer Set, CLONTECH Laboratories, Inc.) was used as the internal control gene for RARβ. When mRNA expression levels of thyroid differentiation markers were studied, the single PCR was carried out using samples from normal thyroid gland and well differentiated papillary thyroid carcinoma as the positive controls (35). These samples were also used as the positive controls for the expression study of TTF-1 and PAX-8. In addition, to compare the relative expression levels of RARs, a human breast cancer cell line, KPL-4 (36), was used as a positive control. This cell line was also used as a negative control for the expression study of TTF-1 and PAX-8.

Each PCR reaction contained 1:100 cDNA, the indicated concentrations of primers of each target gene and/or the control gene, 200 μmol/L deoxynucleotide triphosphates, 10 mmol/L Tris-HCl (pH 8.8), 1.5–2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.08% Nonidet P-40, and 1 U recombinant *Thermus aquatics* DNA polymerase (MBI Fermentas, Vilnius, Lithuania) in a final volume of 20 μL. After an initial denaturation at 94 C for 4 min, various cycles of denaturation (at 94 C for 15 s), annealing (at various temperatures, as shown in Table 1 for 15 s), and extension (at 72 C for 30 s) were performed on a DNA Thermal Cycler 2400 (PC-960G Microplate Gradient Thermal Cycler, Mortlake, Australia). The final extension was performed for 5 min.

After visualization of the PCR products on 1.2% agarose gel stained with ethidium bromide, gel images were obtained using the FAS-II UV image analyzer (TOYOBO Co. Ltd., Tokyo, Japan), and the densities of the products were quantified using Quantity One (version 2.5, PDI, Inc., Huntington Station, NY). The relative expression levels were calculated as the density of the product of the respective target genes divided by that of the control gene.

Cell growth in vitro

KTC-1 cells (2×10^5 cells/well) were seeded into 12-well plates (SB Medical, Tokyo, Japan) and grown in DMEM supplemented with 5% FBS at 37 C in a 5% CO_2 atmosphere. Triplicate wells were trypsinized every other day, and the cells were enumerated with a Coulter counter (Coulter Electronics, Harpenden, UK).

ATRA was purchased from Sigma (St. Louis, MO) and was dissolved in absolute ethanol at a concentration of 10 mmol/L, stored at −20 C, and protected from light. The final concentration of ethanol in medium was 0.1%.

To investigate the effects of ATRA on cell growth and the secretion of cytokines, KTC-1 cells (2×10^5 cells/well) were seeded in 12-well plates (SB Medical) and grown in DMEM supplemented with 5% FBS at 37 C in a 5% CO_2 atmosphere for 2 days. After being washed with PBS, the cells were incubated with phenol red-free RPMI 1640 supplemented with 2% dextran-coated charcoal-stripped FBS (37) plus 0.1–10 μmol/L ATRA in the dark for 2 or 4 days to reduce the influences of impurities of phenol red and unknown factors in FBS on the activity of ATRA. After the incubation, the culture medium was collected and stored at −80 C until use, and the cells were enumerated with a Coulter counter.

To investigate the effects of ATRA on mRNA expression levels of thyroid differentiation markers, cytokines, and RARs, after a wash with PBS, semiconfluent KTC-1 cells in six-well plates (SB Medical) were incubated with phenol red-free RPMI 1640 supplemented with 2% dextran-coated charcoal-stripped FBS plus 1 μmol/L ATRA for 24 h. After the incubation, KTC-1 cells were collected and stored at −80 C until use.

TABLE 1. Primer sequences, conditions, and product sizes for the multiplex RT-PCR

Target genes	Primer sequences		Annealing temperature (C)	Primer ratio	No. of PCR cycles[a]	Expected size (bp)
	Forward	Reverse				
Tg	5′-ggctaatgctacatgtcctg-3′	5′-gcttctgttggagatgctgg-3′	57	2:1	35	231
TSH-R	5′-tgaagctgtacaacaacggc-3′	5′-tcagttccttcaggtgctcc-3′	56	2:0.5	35	213
TPO	5′-gtctgtcaggctggttatgg-3′	5′-caatcactccgcttgttggc-3′	57	2:1	35	242
NIS	5′-tccatgtatggcgtgaacc-3′	5′-cttcgaagatgtccagcacc-3′	60	Single PCR used	40	234
TTF-1	5′-acgtgagcaagaacatggc-3′	5′-ggtggttctggaaccagatc-3′	61	2:1	35	195
PAX-8	5′-aaggtggtggagaagattgg-3′	5′-gctgctctgtgagtcaatgc-3′	60	2:0.4	35	387
IL-6	5′-gaactccttctccacaagcg-3′	5′-gaatccagattggaagcatcc-3′	58	2:0.2	40	316
LIF	5′-atgaaccagatcaggagcc-3′	5′-acagcacgttgctaaggagg-3′	60	2:0.5	40	325
RAR-α	5′-gcatcatcaacaaggtgacc-3′	5′-gttgttctgagctgttgttcg-3′	59	2:0.4	38	261
RAR-β	5′-attccagtgctgaccatcg-3′	5′-ggtaattacacgctctgcacc-3′	58	2:0.5	40	560
RAR-γ	5′-agctcatcaccaaggtcagc-3′	5′-tctcggtgtcagccatctcc-3′	60	2:0.5	33	420
β-actin	5′-tgacgggtcacccacactgtgcccatcta-3′	5′-ctagaagcatttgcggtggacgatggaggg-3′	NA[b]	NA	NA	661
G3PDH	NK[c]	NK	58	2:0.5	40	983

[a] Numbers of cycles were determined to amplify both products logarithmically and in relatively similar amounts.
[b] Not assessible.
[c] Not known (the Human G3PDH Control Amplimer Set, CLONTECH Laboratories, was used).

Cell growth in vivo

Semiconfluent KTC-1 cells were trypsinized and harvested, and viable cells were counted in a hemocytometer using trypan blue exclusion. Approximately 5×10^6 viable KTC-1 cells were sc inoculated into the dorsal region of 5-week-old female or male nude mice (CLEA Japan, Tokyo, Japan). The three-dimensional tumor size was measured once a week. Tumor volume was calculated as the product of the largest diameter, the orthogonal measurement, and the tumor depth. After the mice had been sacrificed by cervical dislocation, transplanted tumors were resected. After measurement of tumor weight, the resected samples were fixed with 5% buffered formalin and embedded in paraffin for the immunocytochemical and morphological analyses. A part of the sample was fixed with 2.5% glutaraldehyde in PBS for the electron microscopic study.

The animal protocols for these experiments were approved by the animal care and use committee of Kawasaki Medical School.

Measurement of Tg, IL-6, and LIF in culture medium

Tg was measured with an immunoradiometric assay kit (Sanofi Pharmaceuticals, Inc., Diagnostics Pasteur, Marnes La Coquette, France; sensitivity, 1.5 ng/mL). IL-6 was measured with the Quantikine HS immunoassay kit (R&D Systems, Minneapolis, MN; sensitivity, 0.16 pg/mL). LIF was measured with an enzyme-linked immunosorbent assay kit (Amersham Pharmacia Biotech, Aylesbury, UK; sensitivity, 15.6 pg/mL). The secretion of each factor per cell was calculated as the product of each concentration $\times$ volume of medium divided by mean cell number.

Single strand conformation polymorphism (SSCP) analysis for p53 mutation

DNA was purified by digestion with proteinase K, extraction with phenol/chloroform, and precipitation by ethanol. PCR amplification of p53 exons 5–9 (hot spots) and SSCP analysis were performed according to the method of Gaidano *et al.* (38). The oligonucleotide primers for exons 5–9 were synthesized by the solid phase triester method and were identical to those used by Gaidano *et al.* (38). PCRs were performed using 100 ng genomic DNA. The products were loaded onto a 6% acrylamide/Tris-borate-EDTA gel containing 10% (vol/vol) glycerol. Autoradiography was performed with an intensifying screen overnight.

Statistical analysis

All values are expressed as the mean $\pm$ SE. ANOVA using StatView computer software (ATMS Co., Tokyo, Japan) was used to compare the differences in cell number and secretion of IL-6 and LIF between two groups. A two-sided P value less than 0.05 was considered statistically significant.

Results

Morphological analysis

KTC-1 cells grew in a monolayer fashion like cobble stones with a large nucleus and prominent nucleoli (Fig. 1A). Subcutaneous injections of KTC-1 cells into nude mice produced cystic tumors. Tumor cells associated with diffuse squamous metaplasia lined the cyst wall and occasionally exhibited papillary projections inside the cyst wall. No typical follicular structure was observed (Fig. 1B). Intranuclear cytoplasmic inclusions and psammoma bodies were observed (Fig. 1C). Electron microscopic study revealed large ground glass-like nuclei with cytoplasmic invaginations and intranuclear cytoplasmic inclusions (Fig. 2, A and B). Well developed endoplasmic reticulum, desmosome structures and microvilli were observed (Fig. 2C). These findings were stable during the passages and indicate that the morphological

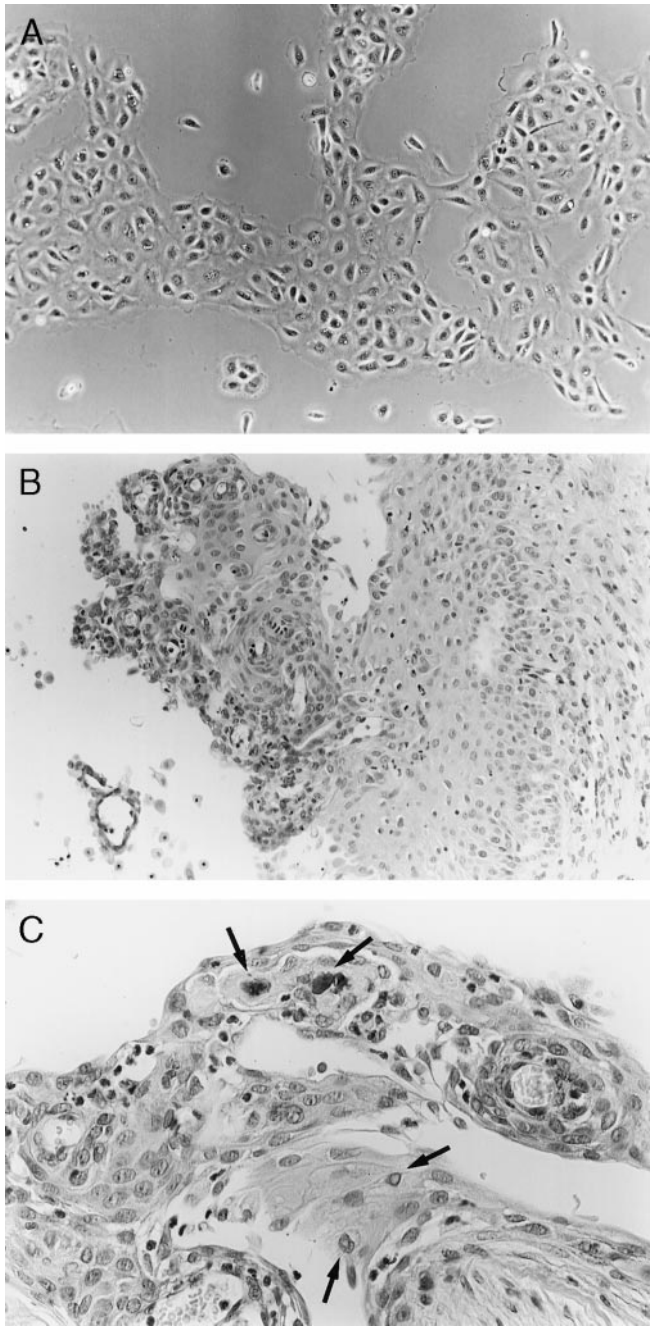

FIG. 1. A, Phase contrast microphotograph of KTC-1 cells *in vitro* (original magnification, ×100). B, Microphotograph of a KTC-1 transplanted tumor (hematoxylin-eosin staining; original magnification, ×80). Note a papillary projection from the cyst wall consisted of tumor cells with squamous metaplasia. C, Microphotograph of a KTC-1 transplanted tumor (hematoxylin-eosin staining; original magnification, ×200). Note the intranuclear pseudoinclusions and psammoma bodies (*arrows*).

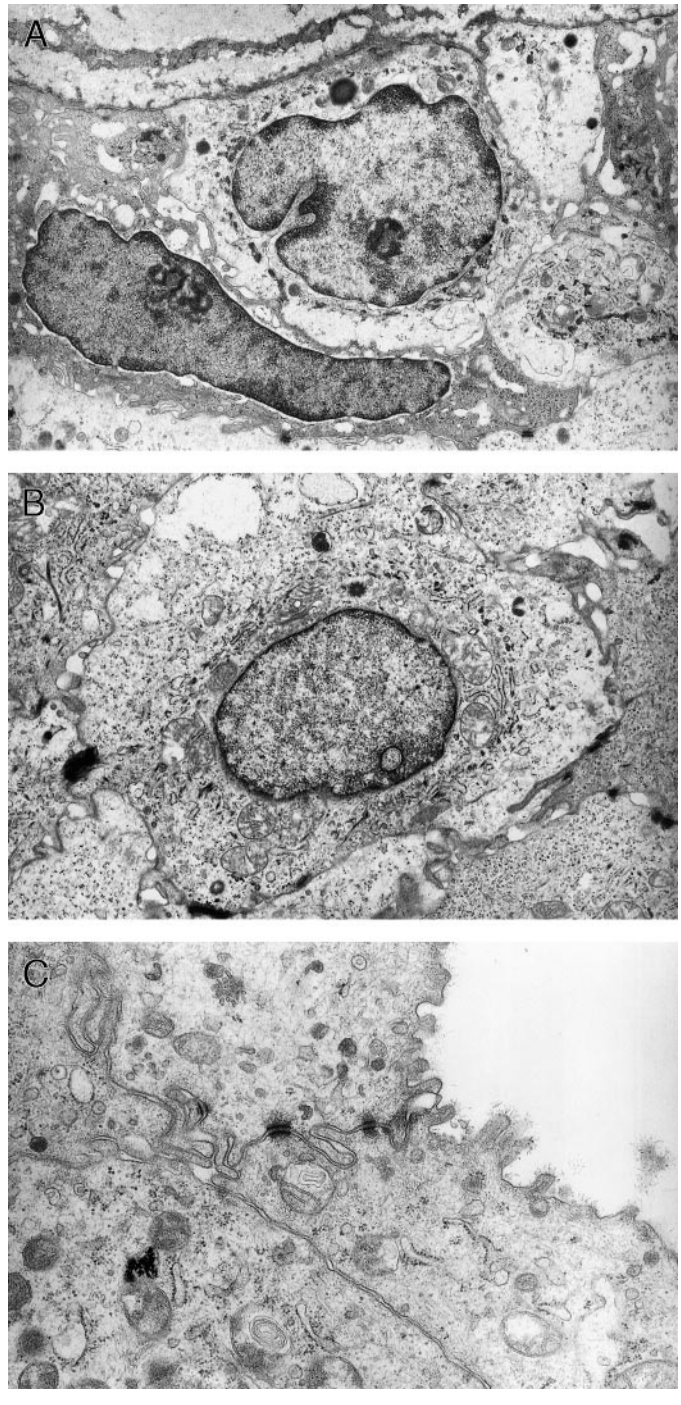

FIG. 2. Electron microphotographs of a KTC-1 transplanted tumor. Note a cytoplasmic invagination (A; original magnification, ×6,000), an intranuclear cytoplasmic inclusion (B; original magnification, ×6,000), and desmosome structures and microvilli (C; original magnification, ×15,000).

characteristics of KTC-1 cells are similar to those of poorly differentiated papillary thyroid carcinoma cells.

Karyotype analysis and p53 mutation analysis

No chromosomal aberration was detected at either the 5th or the 64th passage. This cell line had a normal male karyotype of 46,XY. In addition, no mutation was detected in p53 exons 5–9 by SSCP analysis.

Immunocytochemical analysis

Strong staining for cytokeratin, weak staining for Tg, and no staining for carcinoembryonic antigen in the cytoplasm

were observed in both KTC-1 cell pellets and transplanted tumors.

In vitro and in vivo growth

The population doubling time at the exponential growth phase of KTC-1 cells *in vitro* was approximately 48 h. Subcutaneous injections produced cystic tumors in both male and female nude mice. Tumors, 5 weeks after the cell injections, tended to be smaller in female nude mice (34.1 ± 10.6 mm^3; n = 7) than in male nude mice (482.2 ± 235.6 mm^3; n = 10; $P = 0.14$).

Expression of thyroid differentiation markers, TTF-1, PAX-8, IL-6, and LIF

A low expression level of Tg mRNA was detected by the RT-PCR method, but no mRNA expression of TSH-R, TPO, or NIS was detected (Fig. 3, lane C). In contrast, mRNA expression of all of these differentiation markers was detected in a well differentiated papillary carcinoma that was used as the positive control (Fig. 3, lane B). In addition, mRNA expression of TTF-1 and PAX-8 was detected in the KTC-1 cell line, normal thyroid tissue, and well differentiated papillary thyroid carcinoma, but not in the KPL-4 breast cancer cell line (Fig. 4). Immunoreactive IL-6 and LIF, but not Tg, were detected in the culture medium by the respective enzyme immunoassay. Basal secretion levels of IL-6 and LIF were 162 ± 18 and 152 ± 14 pg/1 × 10^6 cells/48 h (n = 3 each), respectively.

Effects of ATRA on the KTC-1 cell growth and expression levels of Tg, IL-6, LIF, and RARs

The treatment with 0.1–10 μmol/L ATRA for 4 days significantly stimulated the growth of KTC-1 cells (Fig. 5). In contrast, the secretion of both IL-6 and LIF into medium was significantly decreased by the same treatment for 2 days (Fig. 6, A and B). Tg was not detectable in the control and treated culture media.

Relative mRNA expression levels of Tg, but not other thyroid differentiation markers, were up-regulated by the treatment with 1 μmol/L ATRA for 24 h (the relative expression ratio of Tg was 0.25 for control and 0.54 for ATRA, respectively; Fig. 7). In contrast, the expression levels of IL-6 and LIF were down-regulated by the same treatment (0.87 for control and 0.23 for ATRA in IL-6; 0.95 for control and 0.67 for ATRA in LIF, respectively; Fig. 7).

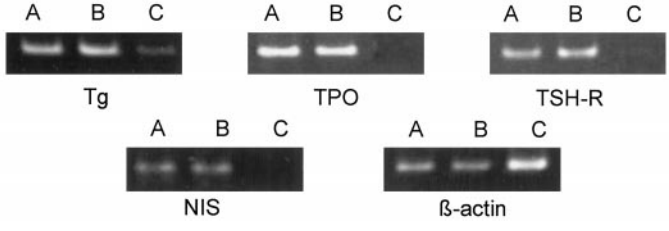

FIG. 3. RT-PCR analysis for Tg, TSH-R, TPO, and NIS in KTC-1 cells (lane C). Note the weak expression of Tg in KTC-1 cells. No expression of TSH-R, TPO, or NIS was detected. Tissue samples of normal thyroid gland (lane A) and well differentiated papillary thyroid carcinoma (lane B) were used as the positive controls.

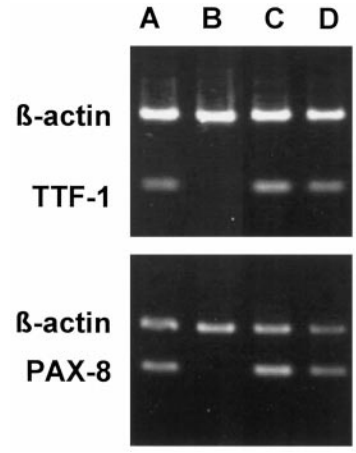

FIG. 4. RT-PCR analysis of TTF-1 and PAX-8 in KTC-1 cells (lane A). A breast cancer cell line, KPL-4 (lane B), was used as a negative control, and tissue samples from normal thyroid gland (lane C) and well differentiated papillary thyroid carcinoma (lane D) were used as the positive controls.

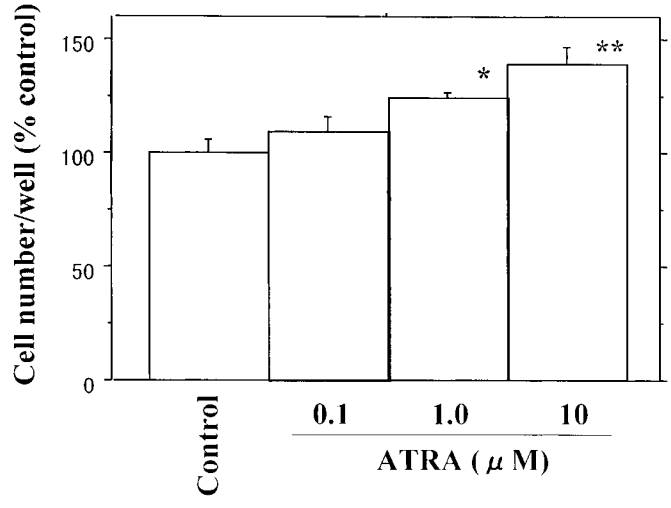

FIG. 5. A dose-dependent growth stimulation of 0.1–10 μmol/L ATRA in KTC-1 cells. KTC-1 cells were incubated with 0.1–10 μmol/L ATRA in phenol red-free RPMI 1640 supplemented with 2% dextran-coated charcoal-stripped FBS for 4 days, and cells were enumerated with a Coulter counter. Values are the mean ± SEM of triplicate wells. *, $P < 0.05$; **, $P < 0.01$.

To investigate the relative expression levels of RARs and the changes produced by 1 μmol/L ATRA for 24 h, multiplex RT-PCR analysis for RARα, -β, and -γ was also performed. Basal expression levels of RARα and -γ in KTC-1 cells were comparable to those in KPL-4 human breast cancer cells, but the basal expression level of RARβ was much lower in KTC-1 cells than in KPL-4 cells (0.29 for KTC-1 and 0.91 for KPL-4, respectively; Fig. 8). ATRA did not significantly change the expression levels of RARs.

Discussion

Well characterized cancer cell lines are essential research resources for studying cancer cell biology as well as developing new strategies against cancer. Many thyroid carcinoma cell lines have been established for studying thyroid

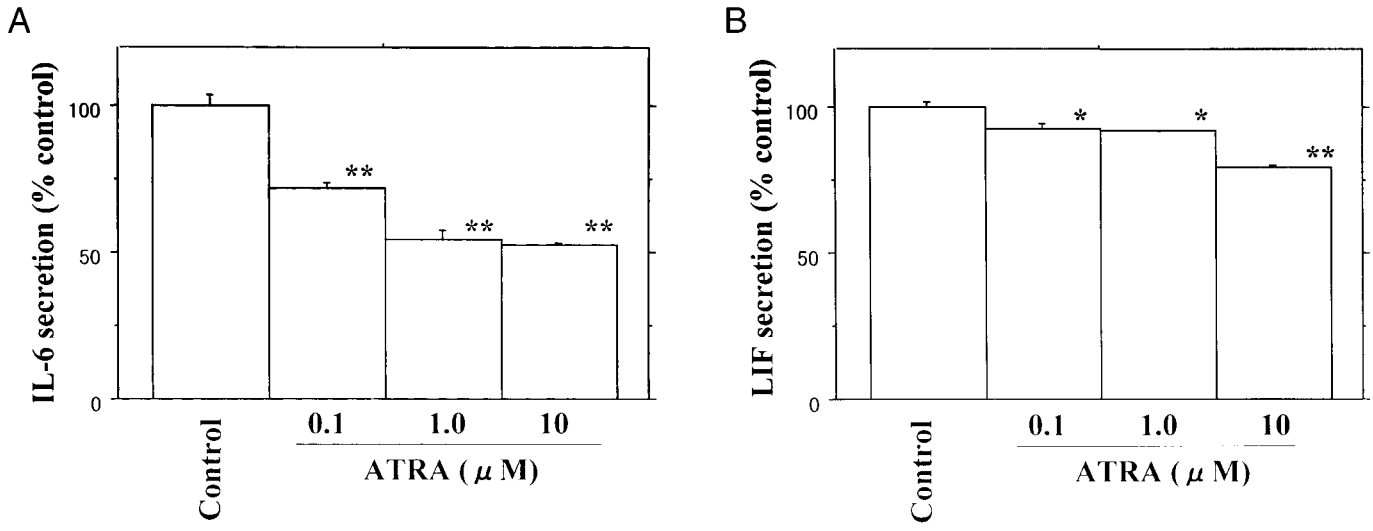

FIG. 6. Inhibition of IL-6 and LIF secretion from KTC-1 cells by 0.1–10 μmol/L ATRA. Semiconfluent KTC-1 cells were incubated with 0.1–10 μmol/L ATRA for 2 days. The culture medium was collected, IL-6 and LIF concentrations were measured by the respective immunoassays, and secretion of each factor was calculated as described in *Materials and Methods*. Values are the mean ± SEM of triplicate wells. *, $P < 0.05$; **, $P < 0.01$.

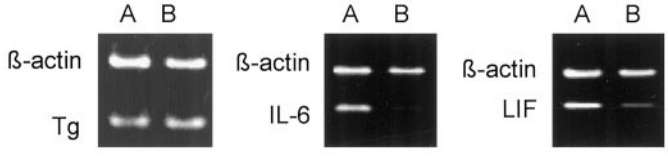

FIG. 7. Effects of ATRA on the mRNA expression levels of Tg, IL-6, and LIF in KTC-1 cells. Lanes A, PCR product of each target gene for control cells; lanes B, that for ATRA-treated cells. To investigate changes in the mRNA expression levels, semiconfluent KTC-1 cells were incubated with 1 μmol/L ATRA for 24 h. After the incubation, KTC-1 cells were collected, and total RNA was extracted. Multiplex RT-PCR for each target gene was performed as described in *Materials and Methods*.

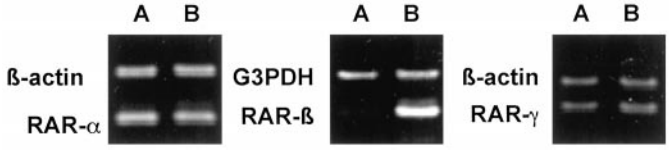

FIG. 8. Basal mRNA expression levels of RARs in KTC-1 cells (lane A) and the KPL-4 human breast cancer cells (lane B). Multiplex RT-PCR for each target gene was performed as described in *Materials and Methods*.

cancer cell biology (10–30). However, most of these cell lines were categorized as undifferentiated or well differentiated. Only one cell line, named SMP, was reported to be poorly differentiated, and no data on the expression of thyroid differentiation markers and the morphology of this cell line were presented (15).

KTC-1 cells in nude mice showed morphological characteristics similar to those commonly observed in papillary thyroid carcinoma. Histological findings indicated transplanted tumors to be a poorly differentiated papillary thyroid carcinoma. In addition, KTC-1 cells express TTF-1, PAX-8, and Tg, but no other thyroid differentiation markers, and secrete cytokines, IL-6, and LIF into culture medium. It has been reported that undifferentiated thyroid carcinoma cells secrete cytokines, such as IL-6 (11, 20). These findings support the idea that the KTC-1 cell line is originated from a poorly differentiated papillary thyroid carcinoma. There is no report that a thyroid carcinoma cell line secretes LIF, one of the cachexia-associated cytokines (31), into culture medium.

This KTC-1 cell line was found to have characteristics unique from those of previously reported thyroid carcinoma cell lines, including 1) a normal karyotype and no p53 mutation; 2) tumorigenicity in both male and female nude mice, producing cystic tumors; 3) morphological characteristics similar to those of papillary thyroid carcinoma; and 4) ability to express Tg but no other thyroid differentiation markers on the other hand secreting IL-6 and LIF into culture medium.

Karyotype analysis was informative in 9 published reports of the 21 reports cited above (10, 11, 18, 19, 23, 25, 26, 29, 30). None of the cell lines showed a normal karyotype. However, minute chromosomal aberrations are difficult to detect using conventional Giemsa staining. Comparative genomic hybridization of the KTC-1 cell line is under investigation.

Mutation analysis of p53 proved informative in only 2 of the 21 reports. A papillary thyroid carcinoma cell line, B-CPAP, showed immunoreactivity against mutant p53 protein (16). SSCP analysis of p53 in exons 5–8 was performed in 3 undifferentiated and 1 poorly differentiated thyroid carcinoma cell lines. Only 1 undifferentiated thyroid carcinoma cell line, KOA2, showed p53 mutation (15). Otherwise, some reports have suggested that p53 mutations are associated with poorly differentiated or undifferentiated thyroid carcinoma (3, 4).

Tumorigenicity was informative in 10 of the 21 reports (10, 11, 14, 16–19, 25, 29, 30). All of the cell lines were tumorigenic in various immunodeficient mice, such as athymic nude mice and SCID (severe combined immunodeficiency) mice, and produced solid tumors. The KTC-1 cell line is the only line to produce cystic tumors in nude mice. No study has compared the growth of transplanted tumors between male and female nude mice. It should be noted that the KTC-1 cell line, which was derived from a male patient, seemed to grow

faster in male nude mice than in female nude mice. Further studies are needed to clarify this interesting phenomenon.

Morphological analysis of transplanted tumors was informative in the same 10 reports (10, 11, 14, 16–19, 25, 29, 30). Only 1 report indicated the existence of a cytoplasmic invagination in transplanted tumor cells of a papillary thyroid carcinoma cell line, B-CPAP (16). In contrast, not only cytoplasmic invaginations, but also intranuclear cytoplasmic inclusions in KTC-1 cells, were demonstrated by the electron microscopic study. Psammoma bodies were also observed by light microscope.

Only recent studies have conducted a systemic analysis of thyroid differentiation markers. In the 21 reports, Tg expression was detected in 10 human papillary, follicular, or Hurthle cell thyroid cancer cell lines by enzyme-linked immunosorbent assay, immunohistochemistry, RT-PCR, or Northern blotting (10, 13, 16, 22, 25). A weak TSH-R expression was detected by Northern blotting in 1 undifferentiated cell line, Hth 74 (21), of 12 cell lines examined. No expression of TPO was detected by Northern blotting in 11 cell lines tested (12, 13). No expression of NIS was detected by Northern blotting in 4 cell lines (12). In general, expression levels of thyroid differentiation markers, except Tg, are very low in human thyroid carcinoma cell lines. It is possible that long-term culture *in vitro* decreases the expression levels.

IL-6 is a multifunctional cytokine and was reported to be involved in cancer-associated cachexia (39, 40). It was reported that only two undifferentiated thyroid carcinoma cell lines secrete IL-6 into medium (11, 20). The regulation of IL-6 secretion from these cell lines has yet to be investigated. LIF was also reported to be involved in cancer-associated cachexia (31). There is no report of LIF expression in thyroid carcinoma cell lines. Secretion of cytokines, such as IL-6, LIF, colony-stimulating factors, and PTH-related protein, from advanced thyroid carcinoma may induce various paraneoplastic syndromes, such as cachexia, hypercalcemia, and asthenia, and may worsen the patient's quality of life. Therefore, the regulation of expression of these factors in thyroid carcinoma cells should be investigated to develop new therapeutic strategies against these paraneoplastic syndromes. In the present study ATRA significantly inhibited the secretion of IL-6 and LIF from KTC-1 cells. Anticachexic effects of retinoids might be expected in patients with advanced thyroid carcinoma.

ATRA has been under investigation as a redifferentiation agent for the treatment of patients with advanced dedifferentiated thyroid carcinomas that no longer respond to TSH suppression therapy and radioiodine therapy (32–34). Some experimental studies have supported the efficacy of this strategy (41–43). It has been reported that retinoids increased radioiodine uptake and TSH-binding activity in a follicular thyroid carcinoma cell line (41); induced 5′-deiodinase activity, a functional differentiation parameter, in a follicular thyroid carcinoma cell line, but not in an undifferentiated thyroid carcinoma cell line (42); and increased NIS expression in two follicular thyroid carcinoma cell lines, but not in an undifferentiated thyroid carcinoma cell line (43). These findings prompted us to investigate the redifferentiation activity of ATRA in the KTC-1 cell line.

Unexpectedly, treatment with 0.1–10 μmol/L ATRA for 4 days significantly stimulated the growth of KTC-1 cells. Because a recent study suggests that treatment conditions strongly influence the antiproliferative effects of retinoids (44), we decided to use phenol red-free medium supplemented with 2% dextran-coated charcoal-stripped FBS to reduce the effects of impurity of phenol red and unknown factors in serum. However, no remarkable difference in the growth-modulating effects of ATRA was observed between phenol red-containing medium supplemented with 5% FBS (data not shown) and the above-mentioned medium tested. Several studies have suggested that cell growth inhibition accompanied redifferentiation by retinoids in various cell lines (44–54). Recently, it has been suggested that the expression of RARβ in some cancer cell lines is required and sufficient to confer retinoid-mediated growth inhibition (45, 46, 55, 56). The relative expression level of RARβ of KTC-1 cells was much lower than that of a human breast cancer cell line, KPL-4. The low expression level of RARβ in KTC-1 cells might be responsible for the unexpected growth stimulatory effects of ATRA. However, some other reports suggest the complex interaction of retinoids with peroxisome proliferation-activated receptor-γ1, Stat1, and RARγ1 (57–59). Further studies are needed to elucidate the mechanisms responsible for the growth stimulatory effects of ATRA on the KTC-1 cell line.

In contrast, ATRA increased the mRNA expression levels of Tg in KTC-1 cells, but not those of other thyroid differentiation markers. Because discrepancy in the effects of certain agents on cell differentiation and proliferation has been reported (60–62), these results are not surprising. Signaling pathways responsible for cell growth and differentiation might dissociate in the KTC-1 cell line. It should be noted that in the present study only short-term (24-h) effects of ATRA were investigated. Long-term exposure of retinoids to the KTC-1 cells might produce other findings, as indicated by a recent report (63). However, it has been suggested that a restoration of Tg synthesis can be addressed as a redifferentiation parameter in advanced thyroid carcinoma patients treated with retinoids (32). Further studies of the induction of Tg expression by other differentiation inducers are warranted.

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