European Journal of Endocrinology (2004) **150** 547–556 ISSN 0804-4643

EXPERIMENTAL STUDY

Human thyroid carcinoma cell lines show different retinoic acid receptor repertoires and retinoid responses

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Abstract

Objective: Disturbed expression of retinoic acid (RA) receptors (RAR/RXR) contributes to the pathogenesis and tumor progression of epithelial carcinomas.
Design: To examine whether altered responses to retinoids may correlate with differences in RA receptor equipment, retinoid effects were examined in human thyroid carcinoma cell lines of various differentiation stages in culture and after xenotransplantation onto rodent models.
Methods: Cell growth was assessed by the MTT test, mRNA expression was examined by Northern blot and quantitative competitive RT-PCR, and type I 5′-deiodinase (5′DI) activity was measured by *in vitro* deiodination assay. Nude rats and mice were used for xenotransplantation experiments.
Results: All-*trans*-RA and RAR-selective synthetic retinoids stimulated activity and mRNA expression of the thyroid differentiation marker 5′DI in the follicular thyroid carcinoma cell line FTC-133. In the less differentiated FTC-238 cells, stimulation of 5′DI activity was less pronounced than in FTC-133 cells, and a reduced level of RARβ mRNA was detected. In the anaplastic thyroid carcinoma cell lines HTh 74 and C 643, the activity of 5′DI was not increased by retinoids, and expression of RARα mRNA was reduced. Proliferation of FTC-133 and FTC-238 cells was decreased by all-*trans*-RA. Pretreatment of FTC-133 with RA resulted in a reduced tumor growth in xenotransplantation experiments as compared with untreated control cells. This reduction was less pronounced in the case of FTC-238 cells. Thus, retinoid therapy might be applied to treat follicular thyroid carcinomas. However, tumor-specific RAR repertoires need to be analyzed as a prerequisite for successful intervention with appropriate, probably receptor-selective retinoids.

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Introduction

The vitamin A-derived retinoic acids (RAs) are key regulators of vertebrate morphogenesis, proliferation and differentiation (1). Moreover, they are promising candidates for cancer therapeutics and have already been successfully used for treatment and chemoprevention of hematologic and solid cancers (2) such as thyroid carcinomas (3). Most effects of RAs are mediated by RA receptors (RARs and RXRs). In general, these receptors function as ligand-dependent transcription factors and modulate the expression of RA-responsive genes by interaction with additional protein cofactors (4, 5). A special role in the pathogenesis of epithelial tumors has been ascribed to RARβ, as expression or induction of RARβ after RA treatment is impaired in several epithelial cancers or carcinoma cell lines (6), among them thyroid carcinoma cell lines (7). Furthermore, there is a correlation between dysregulation of RARβ and tumorigenicity of epithelial tumor cell lines in nude mice (8, 9). Overexpression of RARβ induces growth arrest and apoptosis in oral cancer cell lines (10). Finally, RARβ maps to a position on chromosome 3 that may be deleted in epithelial carcinomas (11), although a correlation of the loss of heterozygosity at this locus and RARβ expression was ruled out for esophageal carcinoma (12). This underscores the importance of epigenetic effects for the downregulation of RARβ (13).

Differentiated follicular (FTC) and papillary (PTC) or anaplastic thyroid carcinomas (ATC) arise from epithelial cells of the thyroid gland. The highly malignant ATC may develop from differentiated forms by either of two pathways, PTC or FTC (14). During dedifferentiation, expression of type I iodothyronine 5′-deiodinase (5′DI) is affected. This enzyme consists of two 27 kDa subunits, has a selenocysteine residue in its active site, and catalyzes the deiodination of the prohormone T4 to the biologically active T3 (15). In the healthy thyroid gland, 5′DI contributes considerably to the

total T3 produced in humans. However, in PTC, reduced 5′DI mRNA expression was demonstrated by DNA microarray analysis (16). 5′DI enzyme activity and expression of its 27 kDa substrate-binding subunit are reduced in FTC and very low to undetectable in ATC (17) or in cell lines established from the various tumor types (18). In the cell lines FTC-133 and FTC-238 (19), we described a low basal 5′DI activity, which is markedly increased by RA, but in the cell lines HTh 74 and C 643, which are derived from ATC (20, 21), neither basal nor RA-inducible 5′DI activity is detected. Thus, 5′DI may be regarded as a differentiation marker of the thyroid gland. A similar situation may be encountered in the prostate and the kidney, as both 5′DI enzyme activity and the amount of the 27 kDa subunit are decreased in carcinomas of these tissues, too (22, 23). Recently, it was reported that also type II 5′-DI mRNA and enzyme activity are reduced in PTC (24).

Here, we report that the expression and activity of 5′DI is stimulated by all-*trans*-RA as well as several synthetic receptor-selective retinoid analogs in FTC-133 and FTC-238 cells. This indicates that these carcinoma cell lines regain a differentiated thyroid function which had been downregulated during dedifferentiation. However, the two cell lines differ in the amplitude of the stimulation and in their responses to RA receptor-selective retinoids. We also demonstrate that these differences in 5′DI inducibility are accompanied by a dysregulation of the expression of RARα and RARβ in the cell lines. Finally, we show that RA pretreatment of FTC-133 cells distinctly reduces their ability to produce tumors in athymic nude rats or mice. Again, this reduction is less pronounced in FTC-238 cells. This may have consequences for the use of retinoids for redifferentiation therapy of thyroid carcinomas.

Materials and methods

Chemicals

All chemicals were of analytic or biochemical grade. Cell culture media were obtained from Life Technologies (Eggenstein, Germany) and sera were purchased from Pan Systems (Aidenbach, Germany) or from Life Technologies. Retinoids and rT3 were kindly provided by M. Klaus (Hoffmann LaRoche, Basel, Switzerland) and H. Rokos (Henning, Berlin, Germany) respectively. Retinoids were dissolved in absolute ethanol at a concentration of 1 mmol/l (synthetic analogs Am580, TTNB and Ch55) or 10 mmol/l (RA) and stored at −20 °C. RA solutions were protected from light.

Cell culture and RA treatment

Cell lines FTC-133 and FTC-238 (19) were propagated in Dulbecco's modification of Eagle's medium/Ham's F12 (1:1) (DMEM-F12) with 10% (v/v) and 5% (v/v) fetal calf serum (FCS) respectively. FTC-133 was established from a primary tumor, and FTC-238 from a subsequently developing lung metastasis of the same patient with a FTC. FTC-238 cells show a more dedifferentiated phenotype than FTC-133 cells. They grow faster, have an additional mutation in the Gs_α protein, and are more aggressive in an *in vitro* invasion model (19, 25). Human anaplastic thyroid carcinoma cell lines HTh 74 and C 643 (20, 21) were grown in DMEM-F12 with 5% (v/v) and 10% (v/v) newborn calf serum respectively. For retinoid treatment, cells were split and grown for 1 day in serum-containing medium to facilitate cell adhesion. Then the medium was replaced by serum-free medium, and retinoids were applied as 200–1000-fold concentrates in ethanol. Control cells received ethanol alone.

Cell lines FTC-133 and FTC-238 are deposited at the European Collection of Animal Cell Cultures.

Biochemical assays

Cell homogenates were prepared by sonification of cells in ice-cold buffer (25 mmol/l sucrose, 20 mmol/l Hepes, 1 mmol/l EDTA and 1 mmol/l dithiothreitol, pH 7.4), and 5′DI activity was determined by the release of ^{125}I from ^{125}I-rT3, as previously described (26). Substrate concentration was 50 nmol/l, and incubation was for 2 h. Protein concentrations in cell homogenates were determined by a modified Bradford protein assay (Bio-Rad, Munich, Germany). An immunoluminometric assay kit (Henning, Berlin, Germany) for the determination of human thyroperoxidase was used according to the instructions of the manufacturer.

RNA preparation

Total RNA from thyroid cell lines was isolated as described by Chomczynski and Sacchi (27). PolyA^+-RNA was purified with the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA).

Semiquantitative and quantitative competitive reverse-transcriptase polymerase chain reaction (RT-PCR)

Reverse transcription was performed with 1 μg of polyA^+-RNA or 10 μg of total RNA and 500 ng of oligo-dT (Pharmacia, Freiburg, Germany) using 200 U of Superscript Reverse Transcriptase (Life Technologies), as recommended by the manufacturer. PCR amplification was carried out in a volume of 50 μl with 2.5 U of Taq DNA Polymerase (Amersham or Qiagen). Primer sequences were as follows:

- 5′DI: forward primer: 5′-ccctcctggattatgtagagcctc-3′, reverse primer: 5′-gtgcatgtggtcgtgggtaaa-3′; annealing temperature 67 °C

- TSH-receptor (28, 29): forward: 5′-AATCCCTGTGAATGCTTTTC-3′; reverse: 5′-ACTCAAGGAAAGTGGAAGTT-3′; annealing temperature: 55 °C
- Thyroglobulin (30): forward: 5′-TGTGAGCTGCAGAGGGAAAC-3′; reverse: 3′-CGTAGTCCCCTGAATCCTGA-3′; annealing temperature: 62 °C. β-actin and GAPDH primers were purchased from Stratagene (Heidelberg, Germany) and used as recommended by the manufacturer. PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide.

Truncated variants of 5′DI (Δ5′DI) and GAPDH (ΔGAPDH) cDNAs were constructed by deleting 64 and 82 bp respectively of the PCR target sequence at appropriate restriction sites. ΔGAPDH was used for calibration of cDNAs prepared from FTC-133 cells treated with 1 μmol/l RA (+RA) or ethanol (−RA) as a control, and Δ5′DI was used for competitive quantitative 5′DI RT-PCR. Semiquantitative RT-PCR was performed by comparing band intensities derived from target cDNAs to those obtained from β-actin mRNA during the exponential phase of the reaction. Thirty-five reaction cycles were used for semiquantitative PCR and 40 cycles for competitive quantitative 5′DI RT-PCR.

Northern blot

Complementary DNA (cDNA) probes coding for human RARα and RARβ were kindly provided by P. Chambon (Strasbourg, France). A rat β-actin probe was the kind gift of J. Mazoub (Boston, MA, USA). The cDNA probes were labeled with [α-^{32}P]dCTP (Amersham) using the random-primed cDNA labeling kit from Boehringer (Mannheim, Germany). RNAs were separated on denaturing formaldehyde agarose gels (1%), blotted to GeneScreen Plus (Du Pont De Nemours, Bad Homburg, Germany) or Hybond-N membranes (Amersham) and hybridized as recommended by the manufacturers. Exposure was 2 weeks for the detection of RAR signals and 24 h for β-actin signals. RAR signals were normalized for β-actin signals by densitometric analysis on a Vilber Lourmat video densitometer (Fröbel Labortechnik, Lindau, Germany). The results are given as x-fold stimulation in the text.

MTT cell proliferation test

In 96-well plates, 5000 FTC-133 or FTC-238 cells were seeded and cultured with DMEM-F12 medium with or without FCS. After 24 h, all-*trans*-RA or ethanol was added, and cells were incubated for 3 days. Cells were then stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (31) for 2 h at 37 °C and incubated for 30 min with 40 mmol/l HCl in isopropanol at 37 °C. Thereby, a colored formazan salt develops in the mitochondria of living, but not of dead, cells. Optical density at 540 nm was measured with a LIA-mat System 300 (BYK Sangtec, Dietzenbach, Germany). All determinations were done in triplicates.

Xenotransplantation of human thyroid carcinoma cells

FTC-133 cells were xenotransplanted onto adult male, athymic nude rats (HAN rnu/rnu, body weight 252±26 g) (32). The animals received cells grown in serum-free medium and treated for 3 days either with 1 μmol/l RA ($n = 6$) or with ethanol for the same time ($n = 8$). In another, similar experiment, RA- or ethanol-treated FTC-133 as well as FTC-238 cells were transplanted to twelve 6-week-old nude mice ($n = 12$ for each cell line and treatment respectively). Cells were harvested using trypsin/EDTA, centrifuged at 1000 rpm for 3 min, counted and resuspended in DMEM-F12 medium at a concentration of about 3×10^7 cells/ml. The cell suspension was injected subcutaneously at two positions into the axillae of each animal in aliquots containing 1×10^7 cells. Tumor volumes were determined every week by measuring the widest, narrowest and deepest part of each tumor with a micrometer screw-gauge. Human thyroglobulin (hTg) was measured in nude rats by a specific IRMA (mouse antibody against hTg) that does not cross-react with rat Tg (Henning, Berlin, Germany). The lower limit of detection was 0.8 ng/ml plasma.

Protocols involving the use of animals have undergone an ethical review process by the institutional animal care and use of the Medical Faculty of the Martin-Luther-University, Halle-Wittenberg according to the Addendum to Animal Proposal of Dessau Government of 11 November 1998.

Statistical analysis

Experiments were performed at least in duplicate, and the mean values of two experiments or the mean±S.E.M. for three or more experiments is given as indicated. Significance of results was tested using unpaired Student's *t*-test where appropriate.

Results

Synthetic retinoids differentially stimulate 5′DI activity in FTC-133 and FTC-238 cells

We analyzed the effects of RA treatment on the expression of the thyroid differentiation marker 5′DI in thyroid carcinoma cell lines (Fig. 1). By treatment with 1 μmol/l all-*trans*-RA for 3 days under serum-free conditions, 5′DI was stimulated 15-fold in FTC-133 cells, but only sixfold in FTC-238 cells. Both cell lines were incubated under the same conditions with RAR-specific synthetic RA analogs TTNN (selective for RARβ), Am 580 (selective for RARα), and Ch 55 (a nonmetabolizable compound) (33). They all induced

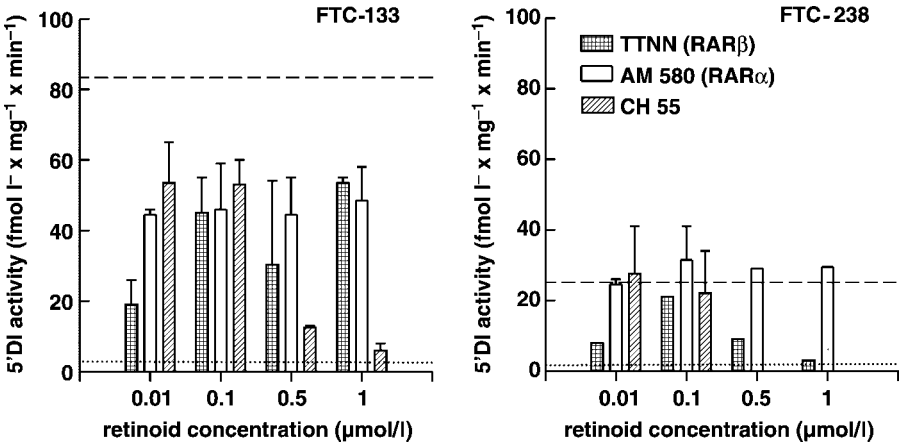

Figure 1 Stimulation of the activity of 5′DI in follicular thyroid carcinoma cell lines by retinoids. FTC-133 (left) and FTC-238 (right) cells were incubated with 1 μmol/l all-*trans*-RA; various concentrations of the retinoids TTNN, AM 580 and Ch 55; and 0.1% ethanol (solvent control). Columns indicate the 5′DI enzyme activity obtained after stimulation with synthetic retinoids; reference lines indicate the 5′DI activity obtained after treatment with ethanol (.....) or all-*trans*-RA (– –).

5′DI activity, however, in FTC-238 cells, and stimulation maximally reached 60% of the values obtained for FTC-133 cells. Interestingly, TTNN, the RARβ-selective compound, elicited weaker responses than Am 580 in FTC-238 cells, whereas in FTC-133 cells, Am 580 and TTNN were about equally potent. Ch 55 stimulated 5′DI at lower doses than the other retinoids, but was cytotoxic at higher concentrations (0.5 or 1 μmol/l). This was accompanied by a markedly decreased protein content and a strongly reduced (FTC-133) or undetectable (FTC-238) 5′DI activity.

RA increases 5′DI mRNA levels in FTC cell lines

To assess the effect of RA on gene expression in thyroid carcinoma cell lines, a semiquantitative RT-PCR assay was performed by comparing band intensities obtained from several thyroid-specific gene products with those of the housekeeping gene β-actin (Fig. 2). Here, 5′DI mRNA levels are increased in FTC-133 and FTC-238 cells after 24 h of incubation with 1 μM all-*trans*-RA from undetectable levels to distinct bands. In FTC-133 cells, the effect was quantified with a competitive RT-PCR assay based on the use of truncated competitor templates. Thereby, a 60-fold increase of 5′DI mRNA expression was demonstrated (Fig. 2B). In the anaplastic thyroid carcinoma cell lines HTh74 and C643, 5′DI-specific amplification products were not detected, either before or after incubation with RA (Fig. 2A).

mRNAs for thyroglobulin (Tg) and thyrotropin receptor (TSHr) were also determined by a semiquantitative RT-PCR approach. Tg and TSHr mRNAs were present in all cell lines, and the most prominent amplification product bands were obtained in FTC-133. No effect of RA on Tg or TSHr mRNA expression was found. Using an immunoluminometric assay, no TPO expression was observed in any of the cell lines, either before or after RA treatment (data not shown).

Retinoids stimulate RAR mRNA expression in FTC-133, but not in FTC-238, cells

Northern blot analysis was performed to investigate which RAR subtypes are expressed in thyroid carcinoma cell lines and might be involved in mediating retinoid effects. All-*trans*-RA elicits a dose-dependent increase of RARα and β mRNA in FTC-133 cells (18). To determine the time course of this stimulation, FTC-133 cells were incubated with 1 μmol/l all-*trans*-RA or ethanol as control for 0, 4, 8 and 24 h (Fig. 3A). Steady-state levels of both RARα and β mRNA are augmented already after 4 h, but there was no marked further elevation after 8 or 24 h (2.1-, 1.2- and 3.1-fold for RARα; 2.2-fold, 1.8-fold and 1.9-fold for RARβ). This increase precedes the increase in 5′DI activity which is measurable after 8 h (1.8-fold) and significant after 24 h (8.2-fold) (18). This is consistent with the notion that α and β RARs are involved in mediating the retinoid-stimulation of 5′DI in FTC-133 cells.

To determine the effects of other retinoids on RAR expression, FTC cells were stimulated for 72 h with synthetic analogs (0.1 μmol/l in the case of Ch 55 and 1 μmol/l in the case of all the other retinoids), and $polyA^+$-RNA of treated cells was used for Northern hybridization. In FTC-238 cells (Fig. 3B), RARα mRNA was detected; however, no stimulation was elicited by treatment with any of the retinoids used. RARβ mRNA was not observed in FTC-238 cells by Northern blot, either before or after retinoid treatment. In contrast, both RARα and RARβ mRNA expression and retinoid stimulation were detected in FTC-133 cells (not shown).

Expression of RARs in anaplastic thyroid carcinoma cell lines

5′DI mRNA was not detected in the anaplastic thyroid carcinoma cell lines HTh 74 and C 643, nor was it induced by RAR treatment (18). Northern blot analysis

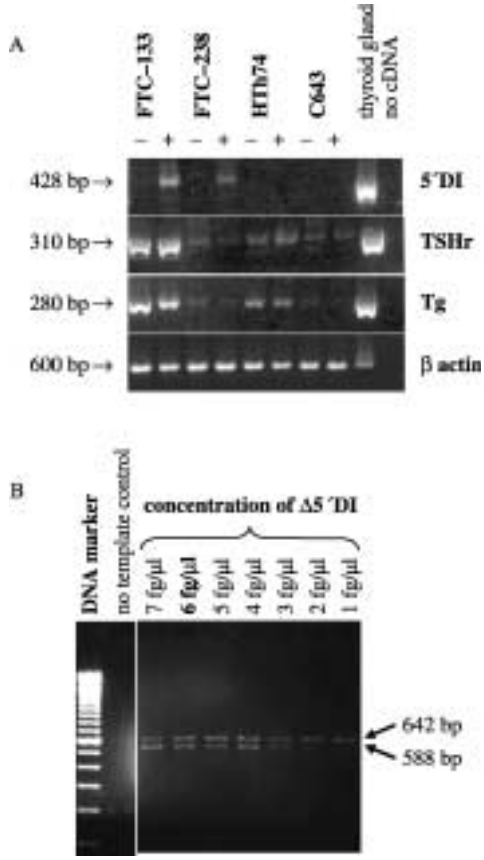

Figure 2 (A) Semiquantitative RT-PCR analysis of the expression of thyroid-specific genes in human thyroid carcinoma cell lines. Cell lines were incubated with ethanol (control) and with 1 μmol/l all-*trans*-RA for 24 h in serum-free medium. A volume of 1 μg polyA$^+$ RNA (cell lines) or 10 μg total RNA (thyroid gland) was reversely transcribed. Resulting cDNAs were used for PCR amplification with primers specific for 5′DI, TSHr and Tg, as well as β-actin primers for calibration. Products were separated on agarose gels and stained with ethidiumbromide. As PCR reactions result in markedly higher product amounts in the case of FTC-133 cells and goiter samples, corresponding lanes are heavily overloaded and show running artifacts, which simulate a smaller fragment size in these cases. (B) Quantitative competitive RT-PCR analysis of 5′DI mRNA expression in FTC-133 cells. Shown is an assay using cDNA prepared from FTC-133 cells after an incubation with 1 Δmol/l all-*trans*-RA for 24 h in serum-free medium. PolyA$^+$ RNA prepared from these cells was reverse transcribed and used for competitive PCR amplification in the presence of truncated Δ5′DI DNA in various concentrations. Equal amounts of products amplified from the wild-type target sequence and the truncated template were obtained when Δ5′DI DNA was added at a final concentration of 6 fg/μl (boldface) as a competitor to cDNA prepared from RA-treated FTC-133 cells, or if 0.1 fg/μl Δ5′DI DNA was added to untreated FTC-133 control cells (not shown). This indicates a 60-fold increase of 5′DI mRNA expression after incubation with RA. Treated and untreated cDNAs were previously calibrated for equivalent DNA content by competitive PCR using a truncated ΔGAPDH competitor.

revealed increased expression of RARβ mRNA after 3 days of incubation with 1 μmol/l all-*trans*-RA (Fig. 3C). The factor of stimulation was 2.9-fold for HTh74, but no factor could be calculated for C643 cells, as there was no signal detectable before RA stimulation. The size of the RARβ transcript differed in the two cell lines. A higher molecular weight species is present in C 643 cells, whereas HTh 74 cells exhibit a lower molecular weight mRNA. RARα mRNA expression is barely detectable, and its expression is not further stimulated by RA.

Growth of follicular thyroid carcinoma cell lines is inhibited by all-trans-RA

The effects of all-*trans*-RA on growth of follicular thyroid carcinoma cells were determined by an MTT test (Fig. 4). Cell proliferation was lower in the absence than in the presence of serum. In both cases, increasing amounts of RA led to a lower cell number than with controls. At the highest concentration (1 μmol/l RA), cell number was reduced by about 24% in FTC-133 and 23% in FTC-238 cells after 3 days. Signs of cytotoxicity of RA in thyroid carcinoma cell lines were observed only at 10 μmol/l or higher concentrations, but not at the concentrations used in this study.

RA pretreatment reduces tumorigenicity of xenotransplanted FTC-133 and FTC-238 cells

To investigate whether RA treatment alters the tumorigenicity of human follicular thyroid carcinoma cells in an *in vivo* model, HAN rnu/rnu athymic nude rats were used for xenotransplantation experiments. FTC-133 cells were grown in the presence of either 1 μmol/l all-*trans*-RA or 0.1% ethanol (solvent control) for 3 days. Collected cells were injected into adult male animals, and tumor volume was measured weekly (Fig. 5, left panel). After 5 weeks, the first small tumors were detectable in control animals ($n = 8$). Their mean volume reached about 7 cm^3 after 8 and about 14 cm^3 after 9 weeks. No metastases were observed. Serum hTg levels were significantly increased from 6 weeks on, as detected by an IRMA specific for the human protein (Fig. 5, right panel). This was due to secretion of hTg by the growing tumors. The maximal serum concentration of hTg was about 90 ng/ml after 9 weeks. No hTg above the detection limit was observed 1 week after injection. Tumor tissue stained weakly positive for hTg (data not shown). In rats xenotransplanted with RA-treated FTC-133 cells ($n = 6$), tumor volumes did not exceed starting values (that is, the volume of the injected cell suspension) after 9 weeks, and no increase in serum hTg was detected.

In a further xenotransplantation experiment, nude mice were used instead of nude rats. Here, tumors deriving from ethanol-treated FTC-133 cells ($n = 12$) were first seen after 5 weeks, and they reached a mean volumme of 1.3 cm^3 after 9 weeks. Tumors deriving from RA-treated FTC-133 cells ($n = 12$) became visible after 6 weeks and did not get bigger than 0.1 cm^3 after 9 weeks. In contrast, tumors developing

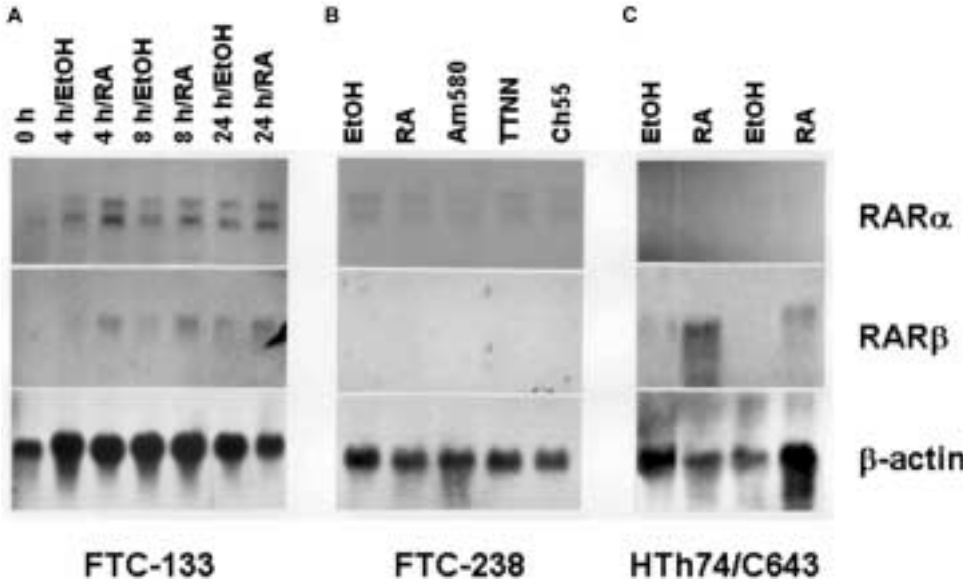

Figure 3 Expression of RARα and RARβ mRNA in human thyroid carcinoma cell lines. An amount of 20 μg of total RNA was loaded per lane and hybridized to cDNA probes specific for RARα, RARβ and β-actin. (A) Time course of RAR mRNA stimulation by all-*trans*-RA in FTC-133 cells. FTC-133 cells were incubated for various time intervals with 1 μmol/l all-*trans*-RA or ethanol (control). (B) RAR mRNA expression in FTC-238 cells. FTC-238 cells were incubated for 3 days in serum-free medium with ethanol (control) or various retinoids, as indicated at a concentration of 1 μmol/l (0.1 μmol/l in the case of Ch 55). (C) RAR mRNA expression in anaplastic thyroid carcinoma cell lines. HTh74 and C 643 cells were incubated for 3 days with ethanol (control) or with 1 μmol/l all-*trans*-RA.

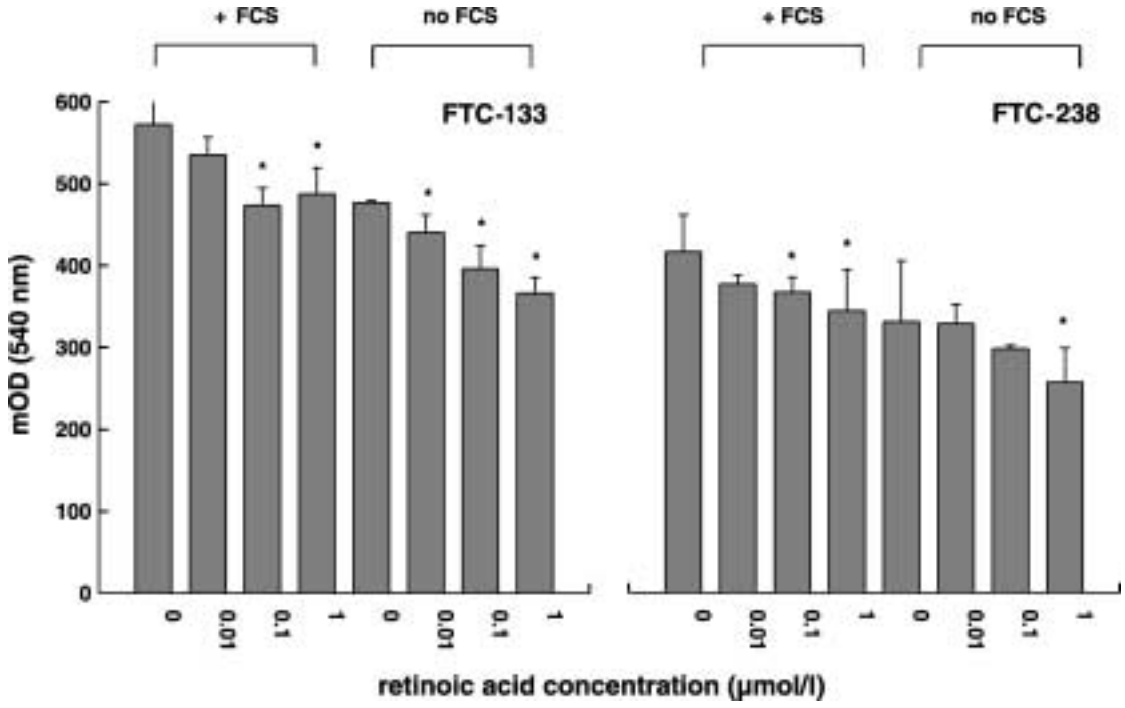

Figure 4 Growth and viability of human thyroid carcinoma cell lines under treatment with all-*trans*-RA. Per well of a 96-well plate, 5000 FTC-133 (left graph) or FTC-238 cells (right graph) were seeded and treated for 72 h with the indicated concentrations of all-*trans*-RA either in the absence or in the presence of serum (FCS). The number of living cells is proportional to the concentration of colored formazan salt produced in an MTT assay and measured at a wavelength of 540 nm. $^*P < 0.05$ vs control (ethanol).

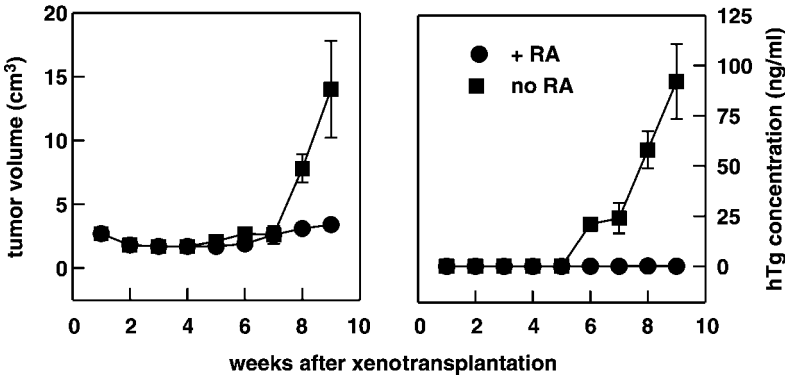

Figure 5 Influence of RA on tumorigenicity of FTC-133 cells in nude rats. FTC-133 cells, pretreated for 3 days either with all-*trans*-RA (SC) or with ethanol ((SB); control), were xenotransplanted onto athymic nude rats. Tumor growth (left graph) and production of hTg (right graph) were analyzed for up to 9 weeks.

from FTC-238 cells grew faster and got bigger, even those from RA-treated cells. Control cells gave detectable tumors after 3 weeks that attained a mean volume of $2.20 \pm 0.09\,cm^3$ after 7 weeks ($n = 12$). RA-treated cells caused tumors of a mean volume of $0.1\,cm^3$ after 4 weeks and of $1.18 \pm 0.14\,cm^3$ after 7 weeks ($n = 12$; data not shown). The difference was statistically significant according to the Mann–Whitney U-test ($P < 0.001$).

Discussion

We report on a stimulating effect of RA on the steady-state levels of the thyroid differentiation marker 5′DI in FTC-133 and FTC-238 follicular thyroid carcinoma cell lines, but not in the anaplastic lines HTh 74 and C643. Recently, we described a similar effect, that is, an upregulation of the mRNA coding for the human sodium iodide symporter (NIS) by RA in human FTC cell lines (34), while van Herle *et al.* described an increase in iodide uptake after treatment with 13-*cis*-RA in the FTC cell line UCLA-RO 81 (35). Expression of Tg, TSHr and TPO seemed not to be enhanced in our cell culture models, but an increase in Tg mRNA expression after incubation with 1 μmol/l all-*trans*-RA for 24 h was reported by Kurebayashi *et al.* (36) in the papillary thyroid carcinoma cell line KTC-1. Furthermore, there is an influence of RA on other genes or cellular properties, among them expression of alkaline phosphatase, ICAM-1, E-cadherin and CD97 as well as cell structure (reviewed in Ref. 3). Thus, RA stimulates (in a cell type-specific manner) a subset of thyroid differentiation markers and regulates other differentiation-relevant compounds, and therefore seems able to direct thyroid carcinoma cell lines into a more differentiated and functional stage.

We also demonstrated an inhibitory effect of all-*trans*-RA on the growth of the human follicular thyroid carcinoma cell lines FTC-133 and FTC-238, and similar results were also described for other cell lines (37, 38). Furthermore, when FTC-133 cells pretreated with RA were xenotransplanted onto athymic nude rats and nude mice, a clearly reduced tumor growth was detected, as compared with animals that had obtained untreated control cells. This effect was observed without further treatment of the animals with RA. This suggests that in addition to growth retardation a differentiation program was initiated by RA *in vitro* that was irreversible even after removal of the drug and xenotransplantation of the cells.

However, RA effects differed between the two cell lines FTC-133 and FTC-238. In xenotransplantation experiments, FTC-238 cells were less susceptible to RA effects. Tumor growth inhibition by RA pretreatment was less efficient in FTC-238 cells, as tumors grew faster and got larger than tumors derived from FTC-133 cells in the nude mouse model. Furthermore, the amplitude of 5′DI stimulation was smaller in FTC-238 than in FTC-133 cells. As the 5′DI promoter contains RA-responsive elements (39, 40), a direct action of RA receptors in transcriptional activation of the 5′DI gene is conceivable. The same holds true for the NIS gene (41). By Northern blot analysis, expression and RA-triggered upregulation of RARα and β by RA was observed in FTC-133. In contrast, although RARα mRNA was detected in FTC-238 by Northern blot, and minimal expression of RARβ mRNA was observed by RT-PCR (42), neither of the two receptor mRNAs was increased by RA treatment as in FTC-133. This variation in receptor equipment and regulation in the two cell lines might be a reason for their differential retinoid responsiveness and may also contribute to the more dedifferentiated phenotype of FTC-238.

Accordingly, in the stimulation of 5′DI activity, the RARβ-selective retinoid TTNN is, at any given concentration, less efficient than the RARα-selective retinoid Am 580 in FTC-238, whereas the two compounds, except for the lowest concentration, were roughly equally potent in FTC-133. The stimulatory action exerted by TTNN in FTC-238 can be explained by both the small residual amount of RARβ expression and a minor affinity of this RARβ-selective (but not totally RARβ-specific) compound for RARα, resulting in a reduced but still detectable effect on 5′DI activity.

Another situation was realized in the anaplastic thyroid carcinoma cell lines C 643 and HTh 74.

They did not respond to retinoid treatment with increased expression of 5′DI mRNA and protein or of NIS mRNA (18, 34). In these cell lines, RARβ was readily stimulated by RA, as shown by Northern blot and by RT-PCR, but RARα expression was absent, as judged by Northern blot analysis and inhibited by RA treatment in RT-PCR experiments (42).

Thus, in general, retinoid receptors are present in thyroid carcinomas and functioning with respect to DNA and ligand binding in thyroid carcinoma cell lines (42). However, the pattern of expression is variable. For example, by immunohistochemistry and Western blot, Rochaix *et al.* (7) demonstrated reduced expression of RARβ in PTC samples, a RA receptor whose loss of expression is involved in the tumorigenesis of several carcinomas (6), but other RA receptors are also affected (36, 37, 42). It is conceivable that these alterations are associated with the degree of dedifferentiation of a tumor (43); Vice versa, if RARβ is reintroduced into epidermoid lung cancer or oral cancer cell lines that have lost its expression, this may have a tumor-suppressive effect via the regulation of cell growth and/or apoptosis (9, 10).

A loss or dysregulation of RA receptors may even have therapeutic consequences. Several clinical trials have already assessed RA redifferentiation therapy for thyroid cancer (44–50). In the most extensive study (45) so far, patients with poorly differentiated, inoperable thyroid carcinomas lacking radioiodide accumulation were treated with 1–1.5 mg 13-*cis*-RA per kg body weight and day over 5 weeks. Twenty-one of 50 patients showed an increase in radioiodide uptake. Furthermore, tumor size regressed or remained stable in 22 of 50 RA-treated patients. This means that RA treatment causes growth reduction of tumors and an increase in the expression and functionality of a thyroid differentiation marker, NIS, in some patients, effects comparable to those that have also been observed in *in vitro* models.

On the other hand, only some of the RA-treated patients responded to RA redifferentiation with a stabilization or an improvement of clinical parameters (45, 49). Therefore, we may ask whether the RAR repertoire of tumors might also be important for retinoid therapy of thyroid cancer. If progressive dedifferentiation leads to loss of RAR expression, a tumor might no longer be responsive to retinoids. A further important question is whether the expression of accessory activators of RARs (5) is disturbed in thyroid carcinoma cells, as has been shown, for example, for *AIB*3 in breast cancer (51).

In addition to retinoids, many more potentially redifferentiating compounds have emerged. Among them, inhibitors of DNA methylation have shown a potential for the redifferentiation of thyroid carcinomas (52). Especially interesting in this context, opening the perspective of a redifferentiation therapy with a combination of compounds, is the observation that there is a certain cross talk between methylation-related and RA-regulation-dependent events. It has been shown that RARβ is silenced by aberrant methylation in epithelial cancers, such as those of the breast (13). In addition, cellular RA-binding protein I, which regulates responses to RA by delivering RA to nuclear receptors, may be downregulated by hypermethylation in PTC (53). Re-expression of these genes after demethylation might enhance the effect of a concomitantly applied RA dose by reactivating these two central compounds involved in RA signal transduction.

In summary, retinoid effects in thyroid carcinoma cell lines apparently affect essential components and properties that are relevant to thyroid-specific function, growth, differentiation and metastasis. These multiple and synergistic actions of retinoids on several aspects of tumor cell biology may lead to an integrated antiproliferative response and partial regaining of differentiated thyroid epithelial cell function which may be exploited for therapy. However, to access as much thyroid carcinomas as possible by retinoid redifferentiation therapy, the RA receptor equipment of individual tumor entities may have to be considered and probably compensated for by the use of ligands specific for certain receptor subtypes. Alternatively, the combination with other redifferentiating drugs may be advisable to potentiate treatment by retinoids. Our data provide a possible explanation for the effects of RA treatment in different clinical studies previously reported.

Acknowledgements

We would like to thank P Chambon and J Mazoub for RAR and β-actin plasmids, K Bienert for excellent technical assistance and M Mager for critically reading the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Wi 231/9-3, TP1) and the European Community Biomed program (BMH1-CT920081).

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Received 26 August 2003
Accepted 13 January 2004