Multifaceted suppression of aggressive behavior of thyroid carcinoma by all-trans retinoic acid induced re-differentiation

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Abstract

Since all-trans retinoic acid (ATRA) has shown promising results in differentiation therapy, the present study was designed to investigate the effects of ATRA on thyroid carcinoma and to evaluate the effectiveness of ATRA in redifferentiation induction of thyroid carcinoma. Therefore, we investigated cell growth rate, morphological and nuclear: cytoplasmic ratio, adherent-dependent growth, response to chemotherapy drug following differentiation, T3 and T4 measurement, and critical genes expression pattern. Papillary cell line showed more growth inhibition by ATRA, in addition, mesenchymal and spindle-shape of 8305C cells changed to polygonal. Additionally, high nuclear: cytoplasmic ratio of anaplastic decreased significantly. Redifferentiation significantly suppressed the anchorage-dependent growth in the both cell lines in a dose-dependent manner, potentiated the arsenic trioxide (ATO) effects in anaplastic and papillary cell lines. Furthermore, reduction in the expression of stemness, and invasion related genes was observed in the both cell lines. Altogether, ATRA treatment could hold the aggressive behavior of thyroid carcinoma in restraint and/or potentiate the effect of chemotherapy drug ATO.

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1. Introduction

Although the majority of differentiated thyroid cancers (DTCs) including papillary (PTC) are cured by radioiodine therapy, surgery and chemotherapy, 10–20% lose the ability to take up iodine or dedifferentiate to a more aggressive type, making treatments ineffective (Vivaldi et al., 2009). These dedifferentiated thyroid cancers, as well as anaplastic thyroid cancer (ATC) and poorly differentiated carcinomas are clinically major problems, lacking responsiveness to conventional radiotherapy or chemotherapy. Some improvements have been reported in individual patients with chemotherapy, external radiation, and thyroidectomy (Ain, 2000; Veness et al., 2004); however, the impact of therapy on survival is poor and death occurs in 2–6 months (Abdoody et al., 2008). Since most tumor cells appear to be relatively undifferentiated or dedifferentiated (Pan et al., 2004); differentiation therapy, which aims to force the cancer cell to resume the process of maturation, seems promising. ATRA, active metabolite of vitamin A, could exert potent effects on cell growth, differentiation, and apoptosis (Siddikuzzaman et al., 2011). Differentiation therapy with ATRA is the first choice drug in the treatment of acute promyelocytic leukemia (APL), and is increasingly used for treatment of various tumors including head and neck carcinoma (Park et al., 2000), bladder cancer (Hameed and el-Metwally, 2008), neuroblastoma (Adamson et al., 2007), and advanced thyroid cancer (Zhang et al., 2007).

Anaplastic cells have abnormally large nuclei, and increased nuclear: cytoplasmic ratio (N:C), owing to less-differentiated state. Additionally, aggressive tumors have high N:C ratio and poor survival (Carvalho et al., 1997). Although ATC arise from follicular cells, the morphology pattern of ATC including giant-cell, spindle shape (Chiacchio et al., 2008), and large nucleus are representative of mesenchymal cells. Tumorigenic and colony formation ability have been attributed to cancer stem cells. The CD133+ cells, maintains the “stemness” of the cells. The CD133+ anaplastic thyroid cancer cells express high levels of OCT-4 gene with high tumorigenic potential in vivo (Trosko, 2006). Most somatic cells...
lack telomerase activity, since they do not express the telomerase reverse transcriptase (hTERT) gene. Telomerase and the maintenance of telomeres are key players in the ability of stem and cancer cells to bypass senescence and be immortal. Stem cells and cancer cells express hTERT, an ability for indefinite cell proliferation (Phatak and Burger, 2009), and its expression is down-regulated during differentiation and silenced in fully differentiated somatic cells.

The aim of the present study was to investigate whether ATRA could effectively induce redifferentiation of two anaplastic (8305C) and papillary (B-CPAP) thyroid carcinoma cell lines by cellular and molecular and morphological changes following treatment. For this purpose, we evaluated the both cell lines growth, morphological changes and N:C ratio measurement in anaplastic cell line, colony-forming capacity of the both cell lines, ATRA-induced sensitization to chemotherapeutically achievable dose of ATO, and mRNA expression of critical genes involved in pathogenesis and malignant behavior of thyroid carcinoma.

2. Materials and methods

2.1. Cell lines and ATRA treatment

Two human thyroid carcinoma cell lines, papillary (B-CPAP) and anaplastic (8305C) were purchased from the DSMZ (Braunschweig, Germany). These cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Pen-Strep solution. Cells were maintained at 37°C in a humidified incubator, 5% carbon dioxide.

ATRA (Sigma–Aldrich Chemie, Taufkirchen, Germany) was dissolved in DMSO and diluted at desired concentration in the cell culture medium and treated with 5, 10, 20, and 40 μmol/L. Equal volume of DMSO was added in control samples, in which the final concentration of DMSO did not exceed more than 0.1% of total volume.

2.2. Proliferation assay (MTT)

Cell proliferation was assessed by using MTT (3-(4,5-di-methyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma–Aldrich, St. Louis, MO, USA), as described before (Momeny et al., 2010). Briefly, 5000 cells were plated in a 96-well plate in 100 μL/well and treatment was performed after 24 h. The cells were treated for 96 h with ATRA in a dose-dependent manner, and the media was changed every other day. After treatments of the both cell lines, medium was removed, and 100 μL/well MTT was added to each well with final concentration of 5 mg/mL. After 4 h of incubation at 37°C, medium was removed, and 100 μL DMSO was added to each well to solubilize the formazan crystals. Plates were read at wavelength of 570 nm on a Microplate Reader Model 550 (Bio-Rad, Richmond, CA, USA). Reported results are mean of 3 independent experiments. ATRA growth inhibition rate was evaluated using the following equation: inhibition rate (%) = (1 – ODexp/ODcon) × 100, in which ODexp and ODcon represent the optical densitometry of treated and untreated (control) cells, respectively.

2.3. C ratio and morphological analysis

The 8305C cell line was cultured in a 6-well plate and was treated the day after. Cultured cells were treated with 10 and 20 μM/mL of ATRA for 96 h. The medium was changed every 48 h, and after 96 h were fixed in 0.1 M phosphate buffered saline (PBS), 2.5% glutaraldehyde (pH 7.4) for 30 min. The N:C ratio and morphological studies of individual cells were performed under a microscope after Wright–Giemsa (Sigma–Aldrich, Germany) staining, which provided further information about nucleus and cytoplasm. After fixing, sufficient quantity of Wright–Giemsa, which covered all surfaces, was applied according to manufacturer instructions, followed by immersion in a distilled water to wash excess staining. The cytoplasm was stained blue to violet; in contrast, the nucleus was stained red to pink. Image J software (NIH Image, Bethesda, MD) was used to analyze the N:C ratio changes. The N:C ratio was derived from the ratio of the mean areas of the nucleus (Ave_nuc) and mean areas of the cytoplasm (Ave_cyt), according to the relationship below:

\[ \text{N:C ratio} = \frac{\text{Ave}_{\text{nuc}}}{\text{Ave}_{\text{cyt}}} \times 100 \]

For each concentration 1000 cells were analyzed randomly. Stained cells were studied for morphological changes with light microscopy (×40).

2.4. Adherent-dependent colony formation

Subconfluent monolayer cells were trypsinized and collected in growth medium containing serum. The cells were centrifuged (200g, 5 min) and resuspended in fresh growth medium. Dilution of 500 cells/mL were prepared after counting with hemocytometer, and seeded in a 6-well plate with 500 cells/well and incubated in 37°C for 3 h to ensure that cells have been attached to the plate surface. After incubation time, dilution of ATRA with the appropriate concentration were added to the plate, and incubated for 2 weeks. Fresh medium with desired concentration of ATRA were added to the wells after 1 week. To count the number of colonies, medium was removed, and washed with PBS (pH 7.4) solution. Colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v) and the number of colonies was assessed by microscopic counting. The colonies with at least 50 cells were counted. The colony-forming efficiency (CFE) of each cell line was obtained, which was calculated as: CFE (%) = number of colonies/initial seeding density × 100. The data are expressed as colony-forming capacity, which represent the number of colonies in each well after initial seeding.

2.5. Treatment of the both cell lines after ATRA with chemotherapy drug ATO

The both cell lines were treated with 5, 10, 20, 40 μmol/L of ATRA for 96 h, as described earlier. To have equal cells for ATO treatment, the cells of all doses were trypsinized and collected in the growth medium separately, centrifuged (200g for 5 min), pellet of each dose was resuspended in fresh medium. Appropriate dilution was performed, and 5000 cells/well were seeded on 96-well microtiter plate in triplicate for each dose. After incubation for 24 h in culture medium alone, the medium was replaced with medium containing 2 μmol/L of ATO, a pharmacologically achievable dose in human, for all of the concentrations including the control. The medium was replaced every day, and on the day 3, the MTT proliferation test was performed, as described in the proliferation assay part.

2.6. RNA extraction and quantitative real-time PCR

Total RNA was extracted from ATRA treated cell lines after 96 h by using High Pure RNA Isolation Kit (Roche, Indianapolis, IN, USA), RNA (1 μg) was reverse transcribed with PrimeScript RT reagent kit (Takara), using random hexamer and oligo(dT) primers following the manufacturer’s instructions. Expression of mRNAs was measured by quantitative real-time PCR using StepOnePlus (Applied Biosystem, USA) instrument using SYBER green PrecisionTM 2X
qPCR Mastermix (PrimerDesign Ltd., UK). Reaction mixture included the following: SYBER green master mix (10 μL), cDNA (2 μL), forward and reverse primers (10 μmol), and nuclease free water (7 μL) were added in a final volume of 20 μL. Thermocycling included a single initial heat inactivation and denaturation incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s, and a combined annealing/extension step for 30 s at 60 °C. Melting curve analysis was performed to ensure that all of the primers yielded a single PCR. Hypoxanthine phosphoribosyltransferase 1 (HPRT) was amplified as normalizer, and relative-fold differences of target gene were calculated using the 2^(-DDCT) method normalized to HPRT expression at 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s, and a combined annealing/extension step for 30 s at 60 °C. Melting curve analysis was performed to ensure that all of the primers yielded a single PCR. Hypoxanthine phosphoribosyltransferase 1 (HPRT) was amplified as normalizer, and relative-fold differences of target gene were calculated using the 2^(-DDCT) method normalized to HPRT expression.

2.8. Statistical analysis

Experimental data are expressed by mean ± standard deviation of three independent assays for MTT, qRT-PCR, radioimmunoassay, chemotherapy drug treatment, and duplicate for colony formation assay for both cell lines. An independent t-test was conducted for comparison between groups. Statistical significance was calculated using paired, two-tailed Student’s t-tests. Statistically different values were defined significant at *P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1. ATRA induces growth inhibition

MTT test was used for determining the percentage of cell growth inhibition (GI). As shown in Fig. 1, ATRA could inhibit the growth of B-CPAP cell line in a dose-dependent manner ranging from 24% to 45%. However, the growth inhibition effect of ATRA on the 8305C cell line was lower, ranging from 3% to 10%. The 5 μmol/L concentration did not inhibit proliferation of 8305C cells.

3.2. Significant reduction of N:C ratio and morphological changes

We focused especially on anaplastic cell line after redifferentiation, therefore, the N:C ratio experiment was carried out to determine the volumetric changes of 8305C cell line following ATRA treatment. The high N:C ratio pattern of 8305C cell line altered after ATRA treated cells underwent striking morphological alterations with a decreased N:C ratio. As shown in Fig. 2A panel D, N:C ratio decreased to 66% (*P < 0.0001) compared to the untreated control (DMSO). Data are plotted as mean of three experiments ± SD. Statistical significance were defined at *P < 0.05, **P < 0.01, ***P < 0.001 compared to corresponding control.

Table 1

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larger, partly due to a decrease in the N:C ratio and developed a single small nucleus.

3.3. ATRA-induced suppression of colony-forming capacity

The anchorage-dependent colony forming assay was used to evaluate the long-term growth inhibitory effect of ATRA. As shown in Fig. 3, ATRA inhibited colony forming ability of both cell lines in a dose-dependent manner. Interestingly, ATRA suppressed the 8305C colony forming capacity in a lower dose compared to B-CPAP.CFE in 6-well plate containing medium in the absence of ATRA ranged from 3% for B-CPAP cells to 2.4% for 8305C cells. As evidenced by a duplicate experiment, ATRA at 10 and 20 μM concentration completely inhibited the colony-forming capacity of 8305C (P < 0.006) and B-CPAP (P < 0.002), respectively. ATRA various doses, 5, 10, 20 μmol/L exhibited a 41% and 100% inhibition of anchorage-dependent growth in 8305C and 57%, 90% and 100% in B-CPAP cell line. These results demonstrated that ATRA was able to hinder the anchorage-dependent growth of 8305C and B-CPAP cells, an ability which is mostly attributed to progenitor and stem cells, indicating a persistent down-regulation of growth in the presence of ATRA.

3.4. ATRA pretreatment is capable of sensitizing cells to ATO

Since anaplastic carcinoma shows resistance to conventional chemotherapy, drug sensitivity was evaluated by MTT following ATRA exposure. The MTT assay showed a dramatic dose-dependent sensitization of the 8305C cell line to ATO after ATRA treatment, ranging from 18.4% (P < 0.005) to 84.8% (P < 0.001), compared to the control, Fig. 4. In contrast, the B-CPAP cell line showed a milder sensitization to ATO, which responded from 1.5% (P < 0.3) to 25.9% (P < 0.001). Although 8305C cells were more resistant than B-CPAP cells to ATO, sensitization-induced effect of ATRA made them more vulnerable to chemotherapy-induced reduction of proliferation.

3.5. ATRA-induced down-regulation of stemness, invasion, cell cycle, thyroid transcription factor and thyroid-specific genes accompanied with stimulation of E-cadherin

We evaluated whether ATRA affect the expression of stem cell related markers, OCt-4 and hTERT, cell cycle related, p21, p27, CCND1, B-catenin, thyroid-specific differentiation markers, such as Tg, TSH-R, NIS and TPO, thyroid transcription factors, such as PAX-8, TTF-1, invasion related genes, MMP-2, MMP-9, E-cadherin genes in 8305C and B-CPAP cells. As shown in Fig. 5, exposure to ATRA decreased the expression of OCT-4 in 8305C (panel A) and B-CPAP (panel B) cells. The hTERT expression was reduced in B-CPAP. However, its expression did not reduce in 8305C cell line. To explore whether Cyclin D1 (CCND1) and cell cycle inhibitors p21, p27 are involved in ATRA effects, we examined their expression in the both cell lines. Expression of CCND1 mRNA was reduced in the both cell lines (Fig. 5A and B). The p21 mRNA expression was reduced in the both cell lines in a dose-dependent manner.
Whereas the p27 mRNA was reduced in B-CPAP cells, its expression was induced in 8305C cells in a dose-dependent manner, but showed marginal reduction in 40 μmol/L. Since B-catenin pathway is hyperactive in poorly differentiated and undifferentiated thyroid cancer tissues (Abbosh et al., 2007), we examined its expression when it is accumulated in nucleus, where it activates the transcription of target genes such as c-MYC, CCND1. The mRNA level of B-catenin did not show any difference in 8305C cell line; however, its expression was reduced in B-CPAP cell line in a dose-dependent manner. Moreover, we studied mRNA expression of E-cadherin, to find out whether its expression could prevent probable B-catenin nuclear localization. We could detect its expression marginally in 8305C cell line after ATRA treatment, which was not detectable basally (data not shown). Furthermore, the expression of TTF-1 and PAX-8 were reduced in the both cell lines. Thyroid specific marker, NIS, mRNA expression reduced, too. The expression level of TSH-R and TPO were undetectable in the both cell lines. The c-MYC mRNA expression level reduced in B-CPAP cell line; however, its expression did not change in 8305C cell line. In addition, the mRNA expression of invasion-related genes, MMP-2, MMP-9, reduced in the both cell lines in a dose-dependent manner.

3.6. T3 and T4 determination

Total measurement of T3 and T4 was done to evaluate whether ATRA treatment restores normal function of thyroid cells, i.e. the synthesizing of T3 and T4. In our study, as shown in Fig. 6A and
Fig. 4. The cell viability of 8305C and B-CPAP after ATO treatment. The cells were treated with 5, 10, 20, 40 μmol/L of ATRA for 96 h, and then 5000 cells of the each dose in triplicate after 24 h were exposed to 2 μmol/L ATO for 72 h. Each cell line compared to corresponding control. The results are depicted as a mean ± SD, **P < 0.01, ***P < 0.001.

Fig. 5. Effect of ATRA on mRNA transcriptional level. The relative mRNA expression of OCT-4, hTERT, CCND1, p21, p27, B-catenin, c-MYC, TTF-1, PAX-8, NIS, MMP-2, MMP-9 following ATRA treatment. The relative mRNA expression of each gene was measured by qRT-PCR in the 5, 10, 20, 40 μmol/L ATRA treated 8305C (panel A) and B-CPAP (panel B) cell lines. Levels of expression were determined as a ratio between target gene and the reference gene, hypoxanthine phosphoribosyltransferase 1 (hPRT). Data are presented as mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001. Statistical significance was calculated using paired two-tailed Student’s t-tests.
concentration of ATRA; in addition, to explore morphological astatic papillary thyroid carcinoma cell line (B-CPAP) by various especially anaplastic thyroid carcinoma cell line (8305C) and met-
redifferentiation, inhibit proliferation and malignant behavior, (al., 2004; Willhauck et al., 2008). Our study objective was to induce in vitro, in vivo
tion induction capacity of ATRA have been demonstrated in:
ythroid cancer cells by using compounds accompanied with known metastasis are associated with high mortality ( Toubert et al.,
accounting for nearly 95% of endocrine cancers (Pitt and Moley,
Thyroid cancer is the most common endocrine malignancy
Thyroid transcription factor Tg, TSH-R, NIS, TPO
Invasion MMP-2, MMP-9
Cell cycle p21, p27, CCND1
Epithelial adhesion molecule E-catherin, B-catenin
Multifunctional transcription factor c-MYC

B, we found no significant changes in the level of the T3 (Fig. 6A) and T4 (Fig. 6B) in the both cell lines after ATRA treatment.

4. Discussion

Thyroid cancer is the most common endocrine malignancy accounting for nearly 95% of endocrine cancers (Pitt and Moley, 2010). ATC comprises a small subset of thyroid tumors but accounts for a significant portion of the mortality related to thyroid cancer (Patel and Shaha, 2006). For these patients, surgical management and chemotherapy are the only treatment options. However, ATC patients carry an almost uniformly fatal prognosis (O’Neill et al., 2009) and surgery and standard chemotherapy do not meaningfully improve survival (Liu and Brown, 2010). Although papillary thyroid cancer has good prognosis, multi-metastasis are associated with high mortality (Toubert et al., 2007). Investigators have attempted to induce differentiation of thyroid cancer cells by using compounds accompanied with known differentiation induction potential. The antitumor and differentiation induction capacity of ATRA have been demonstrated in: in vitro, in vivo and clinical studies (Zhang et al., 2007; Clarke et al., 2004; Willhauck et al., 2008). Our study objective was to induce redifferentiation, inhibit proliferation and malignant behavior, especially anaplastic thyroid carcinoma cell line (8305C) and metastatic papillary thyroid carcinoma cell line (B-CPAP) by various concentration of ATRA; in addition, to explore morphological changes. Furthermore, we further aimed to evaluate expression pattern of molecular targets being expressed abundantly or have roles in pathogenesis and malignant behavior of metastatic papillary and anaplastic thyroid carcinoma (categorized in Table 2). ATRA treatment was found to have some biological effects in the both cell lines including: (1) reduction of proliferation; (2) N:C ratio decrement of 8305C in addition to morphological changes; (3) colony-forming capacity suppression in the both cell lines; (4) potentiating the effects of ATO; (5) down-regulation of the sternness markers, in addition to c-MYC; (6) reversing the induction of invasion related markers, in addition to cell cycle related genes. These effects are discussed separately as following:

Proliferation assay of the both cell lines showed that B-CPAP cells were more sensitive to ATRA, showing more growth inhibition rate. On the other hand, the 8305C proved to be capable of resisting ATRA treatment, and showed less growth inhibition rate. Cyclin D1 gene is a positive regulator of the cell cycle, which allows cell cycle progression from G1 to S phase (Wang et al., 1998), and is overexpressed in papillary (Lantsov et al., 2005) and 76% of anaplastic (Wang et al. 2000b) thyroid carcinoma and also good marker for evaluating aggressive behavior of papillary (Lee et al., 2010) and anaplastic thyroid carcinoma. Anti-proliferative effect of ATRA might be partly due to its inhibition of CCND1 in the 8305C and B-CPAP. The p21 is one of known p53responders, and functions as a key regulator of the multiple cellular processes, and its activation can result in cell cycle arrest by induction of p21. Both cell lines that we used in this study have mutation in p53, B-CPAP harbors a Asp259Tyr missense mutation in exon 7 of TP53 (Meireles et al., 2007), and 8305C cells show a point mutation at codon 273 of the p53 gene (Ohnishi et al., 2002). Thep53 gene controls cellular proliferation through a suppressive effect on cell growth. In fact, p53-dependent up regulation of p21accounts in part for p53-mediated G1 checkpoint control, a pivotal role in the pathway that controls cell cycle and proliferation by induction of p21. Therefore, the lack of p53-mediated p21 induction pathway is a common feature in the cell lines that we used in this study, vindicating the lack of p21 induction here. However, abnormality of the ATC cell cycle has been shown by overexpression of p21 and p27 in ATC cells (Wiseman et al., 2007).

It has been shown that stem cells or stem-like cells show a large N:C ratio (Zhou et al., 2004) and the N:C ratio has diagnostic and prognostic values. Our findings showed that the decrease in the N:C ratio between untreated control and ATRA treated could be due to the re-differentiation of the 8305C cells. Additionally, the control and ATRA treated cells exhibited differences in morphology. The progressive decreases of N:C ratio between untreated control and ATRA treated samples were consistent with the morphological changes occurring in the treated cells, thus these data might imply that aggressive behavior of undifferentiated anaplastic thyroid carcinoma is moderated after ATRA treatment.

**Table 2**

<table>
<thead>
<tr>
<th>Biological function</th>
<th>List of gene(s)</th>
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<tr>
<td>Stem cell marker</td>
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<td>Thyroid differentiation marker</td>
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<tr>
<td>Thyroid transcription factor</td>
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<tr>
<td>Invasion</td>
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<tr>
<td>Cell cycle</td>
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<td>Epithelial adhesion molecule</td>
<td>E-catherin, B-catenin</td>
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<td>Multifunctional transcription factor</td>
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</table>
The colony forming capacity has been attributed to small group of the cells, progenitor or stem cells. It has been shown that CD133+ anaplastic thyroid cancer cells, which are enriched with OCT-4, have higher colony-forming capacity (Aboody et al., 2008). As evidenced by Fig. 3C, the number of colony-forming cells in the both cell lines reduced progressively with various concentrations of ATRA, which correlated with OCT-4 down-regulation. Interestingly, the colony-forming capacity of anaplastic cells was suppressed in lower concentration (10 μmol/L) compared to papillary cells (20 μmol/L), which might imply that the number of undifferentiated anaplastic cells with the colony-forming capacity reduced abundantly after ATRA treatment. These data might indicate that cells are losing their malignant features, including colony forming ability (Vivaldi et al., 2009; Freedman and Shin, 1974).

The metastatic and poorly differentiated as well as ATC are clinically major problem and therapeutic options are limited in this group of patients. Therefore, there is a constant effort to find new substances acting against metastatic papillary as well as anaplastic thyroid carcinoma and combine them with drugs already in clinical use. Redifferentiating these tumors in order to make them more responsive to chemotherapy is one of the objectives of thyroid cancer research. Pretreatment or combination therapy of ATRA with chemotherapy has shown good prognosis in patients (Zhang et al., 2007; Koskela et al., 2004). In our study we showed that pretreatment of the both cell lines with various concentrations of ATRA potentiared the ATO-induced growth inhibition, especially on anaplastic cell line. We observed that neither ATRA nor ATO alone was able to significantly inhibit the anaplastic cells growth; on the other hand, pretreatment potentiated the cytotoxic effects of ATO. This may be explained by the reduced number of stem-like/progenitor cells after ATRA treatment, as evidenced by colony formation assay. Although ATRA is well tolerated and initially was promising, tumor regression or its stabilization is seen in 20% of the cases (Antonelli et al., 2008). Cytotoxic chemotherapies for advanced, metastatic thyroid carcinomas have limited effectiveness, with response rates typically 25% or less (Haugen, 1999); however, the combination therapy or pretreatment with ATRA has promising results in neuroblastoma cells (Cernaianu et al., 2008), breast cancer cells (Wang et al., 2000a) and good prognosis in multiple myeloma patients (Koskela et al., 2004). Treatment for patients with metastatic or advanced anaplastic thyroid carcinoma now emphasizes clinical trial opportunities for novel modalities with considerable promise. These data suggest that pretreatment of thyroid cancer with ATRA, not only lowers the threshold for cell killing by chemotherapy agents, but also potentiate the effects of chemotherapy agent.

The OCT-4 expression is associated with an undifferentiated cell phenotype and is down-regulated when cells differentiate (Sylvester and Scholer, 1994), and CD133+ cells in ATC cell lines express OCT-4 profoundly (Aboody et al., 2008; Zhang et al., 2006). For most tumors it is not clear whether hTERT expression is due to their origin from telomerase positive stem cells or reactivation of the gene during tumorigenesis (Ducrest et al., 2002) and has been shown that its expression is overexpressed in thyroid carcinoma (Asaad et al., 2006). We know that critical levels of OCT-4 and hTERT expression are required to sustain stemness. We observed that re-differentiation of the 8305C and B-CPAP cells resulted in the appropriate down-regulation of OCT-4 and hTERT. Down-regulation of OCT-4 by ATRA has been shown in other studies (Ahn and Lee, 2010; Gupta et al., 2008). Our observation that re-differentiation of the both cell lines resulted in the proper down-regulation of OCT-4 was entirely consistent with the suppression of colony forming capacity in the both cell lines. This data is in accordance with the other study that cells with colony forming capacity retain high expression of OCT-4 (Todaro et al., 2010). Similarly, hTERT expression was down-regulated in the B-CPAP cell lines. However, its expression did not show significant changes in the 8305C cell line. The major contributor to the regulation of the telomerase activity is transcriptional control (Horikawa and Barrett, 2003), and Sp1 interaction with p53 negatively regulates the hTERT expression. Thus, inactivation of the p53, which happens in 8305C, may allow Sp1 to function as an activator in hTERT expression. The c-MYC transcription factor is positive regulator of hTERT gene expression (Horikawa and Barrett, 2003) and its down-regulation in B-CPAP cell line resulted in down-regulation of hTERT expression. On the other hand, c-MYC basal expression correlated to the no changes in the hTERT mRNA expression. This finding might suggest that proliferation of these precursor cells or maturation into more differentiated cells is affected by ATRA, as evidenced by down-regulation of OCT-4 and hTERT.

Beneficial effects of ATRA have been documented in thyroid cancer differentiation and its anti-metastatic potential has been suggested (Lan et al., 2009). Metastatic papillary, B-CPAP (Baldini et al., 2004; Liang et al., 2010) and 8305C anaplastic thyroid cancer (Baldini et al., 2004) are ascertained to be characterized by a significant expression of MMP-2 and MMP-9, associated with the possible progression of a tumor process and concurrent with poor clinical and morphological signs, such as low tumor cell differentiation (Delektorskaiia et al., 2010). In our study, we observed that ATRA treatment significantly down-regulated the expression of both MMP-2 and MMP-9 in the both cell lines in a dose-dependent manner. The present study demonstrates the influence of ATRA on important determinants of metastatic behavior in thyroid carcinoma cell lines. These findings may add to the explanations for beneficial effects of ATRA in the treatment of metastatic thyroid carcinomas.

E-cadherin is a marker of thyroid differentiation (Brabant et al., 1993), and is only weakly expressed in papillary thyroid carcinoma and the absence of E-cadherin expression in ATCs has been reported in the literature (Liu and Brown, 2010). In our study, although E-cadherin expression was not detectable in control group of 8305C cell line, we could detect its expression marginally after ATRA treatment (data not shown). Over-expression or abnormal accumulation of B-catenin in the nucleus activates the transcription of target genes, such as CCND1 and c-MYC, and derangement of the E-cadherin/B-catenin complex is associated with the aggressive behavior of thyroid carcinoma (Wiseman et al., 2006). We observed that the expression of B-catenin was reduced in a dose-dependent manner in B-CPAP cell line, and the down-regulation of its responsive genes, CCND1 and c-MYC correlated to the mRNA expression of B-catenin. In 8305C cell line, however, B-catenin expression did not show any significant changes and did not correlate with its responsive gene, CCND1, which might imply that B-catenin did not accumulate in the nucleus and might have been coupled with E-cadherin, as evidenced by detection of E-cadherin expression. This result is in accordance with other study that showed an increased expression of E-cadherin after ATRA treatment (Lan et al., 2010). The results of the performed study suggest that ATRA could reverse the aggressive behavior of metastatic papillary and anaplastic thyroid carcinoma in vitro, partly by suppression of MMP-2, MMP-9, B-catenin and c-MYC.

Furthermore, we evaluated the gene expression of NIS, PAX-8, TTF-1, TSH-R, Tg and TPO to investigate whether their expression is affected after ATRA treatment. We observed that there was no expression of TSH-R and TPO either basally or after ATRA treatment, in addition, the expression of Tg was very low in the both cell lines even after ATRA treatment. The expression of two known thyroid transcription factors, PAX-8 and TTF-1, decreased after ATRA treatment in the both cell lines, in addition, the expression of their putative target genes, Tg and NIS, was correlated with
PAX-8 and TTF-1 in the both cell lines. The expression of thyroid specific genes, Tg, TPO, and NIS depends on cooperation between TTF-1, TTF-2, and PAX-8 (Fabbro et al., 1994; Pasca di Magliano et al., 2000); thus, reduced expression of these thyroid transcription factors correlated with down-regulation of thyroid specific genes, Tg and NIS. It has been reported that ATRA treatment is able to increase the expression of NIS, Tg, PAX-8, and TTF-1 (Vivaldi et al., 2009), however, the degree of differentiation of thyroid carcinoma cell lines is very heterogeneous (Fabbro et al., 1994). Interestingly, it has been shown that forced expression of TTF-1 was unable to induce NIS expression, and even co-transfection of TTF-1 did not induce the expression of TPO, Tg or TSH-R in thyroid carcinoma cell line (Pasca di Magliano et al., 2000; Akagi et al., 2008), indicating that the presence of other factors and their cooperation is necessary to induce expression of thyroid specific genes. The transcriptional absence of these genes suggests that factors present within such cell lines provide transcriptional inhibition for the expression of these genes, and their identification and regulation are necessary to proceed to fully functional thyroid cells. Furthermore, 8305C and B-CPAP cells showed no significant changes in the level of total T3 and T4, even in the presence of 10 μM/mL human Thyrotropin, which might suggest that production of T3 and T4 need cooperation between all thyroid specific factors. During malignant transformation of thyroid carcinoma, cells lost: (1) differentiated phenotype including TSH dependence for proliferation (Pasca di Magliano et al., 2000); therefore, TSH independence growth is a common feature of thyroid carcinoma including the cell lines that we used in this study; (2) T3 and T4 secretion by induction of TPO gene expression (Fabbro et al., 1994), as we observed that ushering the thyroid carcinoma cells with ATRA to thyroid hormone-secreting cells was not possible. It has been shown that human thyroid tumor cell lines do not respond to TSH, in addition, the expression of thyroid specific genes approximately have been lost in the all cell lines and could not be restored after forskolin treatment (van Staveren et al., 2007). There have been some tumor cells that fail to maintain physiological functions after redifferentiation, therefore, an important aim is to: (1) reduce the level of malignancy as we observed in growth inhibition, N:C ratio, colony formation and morphological phenotype; (2) sensitize the cells to chemotherapy, and morphological and ultrastructural characteristics of a tumor. Arkh Patol. 72, 3–6.

Taken together, these effects confirm the antitumor potential of ATRA in metastatic papillary and anaplastic thyroid cancer cells. These observations suggest a possible role of ATRA in the pretreatment for thyroid carcinoma, and might provide experimental evidence for clinical application. Thus, pretreatment with ATRA may improve the clinical prognosis of anaplastic and papillary thyroid carcinoma and may be useful in multimodal therapy programs.

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