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PROLIFERATION OF CELLS AND EXPRESSION OF RARS, RXRS AND HPV VIRAL E6 AND E7 PROTEINS IN CERVICAL CANCER CELL LINES AFTER TREATMENT WITH ATRA

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Abstract: All-trans retinoic acid (ATRA) is considered to be a potential chemotherapeutic drug due to its capability to regulate cell growth and differentiation. The effects of ATRA on the proliferation of cells and gene regulation are mediated by retinoid receptors (RAR and RXR), which belong to the nuclear receptor superfamily of ligand-inducible transcription factors. ATRA can act either as a growth inhibitor or growth promoter, according to the functional state of retinoic receptors. Thus, we have established the effect of ATRA on the proliferation of cervical cancer cells line HeLa and CaSki and expression of retinoids receptors as well as the viral HPV oncogenic proteins E6 and E7. ATRA had no effect on proliferation CaSki cells, but it stimulated the growth of HeLa cells, which depended on the incubation time and the concentration of ATRA in cell culture. The overexpression of RAR α in HeLa cells after the administration of 10^{-7} mM ATRA was also observed 72 hours, and the decrease of CaSki by 60–90%. In the study of cervical cancer cell lines, the very low levels of other endogenous RAR and RXR receptors were observed. ATRA does not repress the expression of two viral oncoproteins E6 and E7 HPV16/18, which play a key role in carcinogenesis of the cervix. Our results support the suggestions that the cell response to vitamin A, and other retinoids in the diet, may depend on cell type, and that the cancer cells are differentially resistant to retinoids. Thus, despite the important biological functions of retinoids, the effects of retinoids in a supplementation in supra-physiological doses as well as their physiological action are difficult to define.

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INTRODUCTION

Consumption of vegetable crops provides the human diet with many essential vitamins and minerals important for health maintenance. Vitamin A (retinol) is available in a diet as retinyl esters, mostly from animal products such as liver, eggs, milk, and/or as a carotenoid precursor in plant products. The following vegetables are the main source of carotenoids: carrots, sweet potatoes, spinach and tomatoes. To maximize the availabity of carotenoids in foods, vegetables should be eaten raw or lightly steamed [18]. Vitamin A deficiency in experimental animals has been associated

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with a higher incidence of cancer and with increased susceptibility to chemical carcinogenesis [10, 15]. Vitamin A (retinol) and its natural synthetic analogues (retinoids) have been proved to exert an effect on epithelial cell growth, differentiation, and apoptosis, and retinoids have been shown to have chemopreventive and chemotherapeutic activity for some malignant and premalignant diseases. Therefore, retinoids are currently used as preventive and therapeutic agents in a variety of human cancers such as breast, lung, ovarian, prostate, head and neck, liver and cervical cancers. Retinoids also play a central role in tumour stroma production, tumour progression and invasion due to their ability to regulate the expression of matrix metalloproteinases, transforming growth factor beta, cyclin dependent kinase- p16 or p21, responsible for the cell cycle regulation [10, 11]. One of biologically active form of vitamin A is all-trans retinoic acid (ATRA). Retinoid effects are mediated through two classes of receptors- the RARs and the retinoid Xs (RXRs). These both receptor classes have α, β and γ subclasses with numerous isoforms. These three RAR types have a strong affinity for all-trans and 9-cis isomers of retinoic acid. The three RXR types, on the other hand, have demonstrated especially strong specificity only for the 9-cis isomers. These ligand-activated nuclear receptors induce the transcription of target gene by binding to responsive elements in the promoter region [24, 35]. RARs and RXRs are also capable of interacting with other nuclear receptors, and thus, they expand their spectrum of action on gene expression. Pharmacologically, retinoids have been used as modulators of cell growth, differentiation and apoptosis [7, 19]. However, the efficiency of these compounds will depend on RA receptors and other factors and coafactors interacting with retinoids receptors.

Retinoids resistance has been associated with loss or down-regulation of retinoids expression in many types of cancers [34, 38]. Pharmacological doses of RA induce cell differentiation and cell cycle arrest in some epithelial tumor cell lines but not others [16, 28]. The success of cancer therapy with retinoids is likely to require a combination therapy with drugs that regulate the epigenome, such as DNA methyltransferase and histon deacylase inhibitors, as well as classic chemotherapeutic agents [35].

The objective of our study was to examine the effects of ATRA – a functional form of vitamin A, on the proliferation of human carcinoma cell lines – HeLa and CaSki and the expression of retinoic acid receptors, as well as the HPV viral oncoproteins E6 and E7.

MATERIALS AND METHODS

Cell Culture. Human cervical cancer cell lines CaSki and HeLa were obtained from the American Type Culture Collection (ATCC). CaSki cells contain an integrated genome of human papillomavirus type 16 (HPV16, about 600 copies per cell) as well as the sequences related to HPV18.

Table 1. Real time PCR primers.

Primer's name	Primer sequences	Product size
RARa1 RARa2	CCCTACGCCTTCTTCTTCC AACTGCTGCTCTGGGTCTC	136 bp
RARb1 RARb2	AAACCCTGCTTCGTCTGCC TCTCGGTGACAAGTGTAAATC	125 bp
RARg1 RARg2	CGGGGCATCAGCACTAAGG TAGAGGCATTGGGGTGGGG	160 bp
RXRa1 RXRa2	GACCTGACCTACACCTGCC TCCACCTCATTCTCGTTCCG	167 bp
RXRb1 RXRb2	TGCCGCTATCAGAAGTGCC CCTCAACGCCCTGGTCAC	184 bp
RXRg1 RXRg2	CAAGAGGACGATAAGGAAGG ACTCTCAGCTCGCTCTCGG	178 bp
GAPDH	CTGCACCACCAACTGCTTAG TTCTGGGTGGCAGTGATG	180 bp

HeLa cells contain human papillomavirus 18 (HPV-18) sequence. The cells were cultivated in RPMI medium supplemented with 6% foetal calf serum (FCS) at 37°C in 90% humidified atmosphere of 5% CO_2. Cells were detached from the flask with trypsin-EDTA and resuspended in a concentration of 10^{-5} cells/ml in MEM. Cell suspension was distributed to 96-wells with a volume of 1 ml each, and after cell monolayer formation, they were used in the cytotoxic characterization analysis assays.

Effect of ATRA on proliferation of HeLa and CaSKI cells. HeLa and CaSki cells were grown on the presence of all-trans-retinoic acid (ATRA, Sigma USA) to examine the effect of ATRA on proliferation of cervical cancer cells. ATRA in the concentrations of 1×10^{-3} mM to 1×10^{-9} mM were analyzed under a microscope after staining with 0.5% toluidine blue.

BrdU cell proliferation assay. The studied cells were placed in 96-well plates (Gibco) at a density of 30,000 cells/well and incubated at 37°C with 5% CO_2 overnight for attachment. In each experimental set, the cells were placed in triplicates and washed and incubated with ATRA with a concentration of 1×10^{-4} mM or 1×10^{-7} mM for 24, 48 and 72 h. Cellular proliferations was measured by colorimetric immunoassay based on BrdU (Cell Proliferation ELISA, BrdU Kit, Roche Molecular Biochemical, Germany) incorporation into the cellular DNA according to the instruction of the manufactuer. Briefly, cells were pulsed with BrdU labelling reagent for 4 h followed by fixation in FixDenat solution for 30 min. at room temperature. Next, the cells were incubated with 1:100 dilution of anti- BrdU-POD for 1.30 h at room temperature. The immunoreaction was detected by adding the substrate solution and the

developed colour was read at 405 nm and 490 nm with a microplate ELISA (BIOTEC Instruments, USA).

RAR and RXR mRNA expression in HeLa and CaSki cells treatment ATRA (atRA). HeLa and CaSki cells were incubated with ATRA in two different concentrations (10^{-4} and 10^{-7} mM). After 24 or 72 h, total RNA was isolated from the tissue samples using the RNeasy Mini Kit (Qiagen) according to the manufacturer's specifications.

RNA samples were treated with DNase I (Promega), and 1 µg RNA (of each sample) was reverse transcribed with SuperScript TM II RNaseH Reverse Transcriptase (Invitrogen) into cDNA using oligo-dT primers. Real-time PCR was performed in a Light Cycler Real-Time Detection System (Roche Diagnostics) using SYBR-Green I as the detection dye. Target cDNA was quantified using the relative quantification method. The quantity of the RA receptors transcripts in each sample was standardized by either glyceraldehydes-3-phosphate dehydrogenase (GAPDH) or RNA polymerase II (pol II) transcripts level. Real-time PCR reactions were performed in a total volume of 20 µl. cDNA (2 µl) was added to an 18 µl mixture of LC-FastStart DNA Master SYBR-Green I, 1.5 mM $MgCl_2$ and the primers presented in Table 1.

Western blotting. Cells were washed in PBS and then lysed in buffer: Tris-HCl, pH7.5, 1nM EDTA, 15 mM β-mercaptoethanol, 0.1% Triton X-100, 0.5mM phenylmethylsulfonyl fluoride. Protein concentration was determined with Bradfordt reagent (Bio-Rad, Hercules, CA), and 25 µg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylylidene difluoride membrane (Bio-Rad). After blocking in 5% dry milk, the membrane was probed with anti E6 and anti E7 of HPV 16/18 antibodies.

Statistical analysis. The results obtained in the study were submitted to statistical analysis. Arithmetic mean with standard deviation was used for the measurable value. To analyze the differences between study subgroups, non-parametric tests of F-test and Fischer test second χ^2 were used. The values with $p < 0.05$, assuming 5% interference, were considered to be relevant. The results obtained in the study are presented in the Tables and Figures. Statistical analysis was based on computer software STATISTICA v. 9.0 (StatSoft, Poland).

RESULTS

Determination of cell viability in cells treated with ATRA. To characterize the cytotoxic effect of ATRA on cervical cancer cells, HeLa cells and CaSki cells were treated with ATRA in concentration of 10^{-3}–10^{-9} mM for 24–72 h. Cells survival was determined under a microscope after staining with 0.5% of water solution of toulidine blue (Tab. 2).

Table 2. Cytotoxic effect of ATRA in % of dead cells after 72 hours of incubation.

Study cells	Cytotoxic effect of ATRA in % of dead cells						
	10^{-3} mM	10^{-4} mM	10^{-5} mM	10^{-6} mM	10^{-7} mM	10^{-8} mM	10^{-9} mM
HeLa	100	40	15	10	10	5	0
CaSki	100	40	15	10	10	5	0

Table 3. Proliferation of HeLa cells after ATRA treatment.

Concentration ATRA in HeLa cells	Mean ± SD absorbance values (E405)		
	24 h	48 h	72 h
Control	0.162±0.05	0.188±0.06	0.310±0.09
10^{-4}	0.245±0.08*	0.216±0.07	0.446±0.09***
10^{-7}	0.239±0.07**	0.191±0.07	0.348±0.08

SD – standard deviation; p – probability compared to control:
* $\chi^2 = 5{,}023$, $p < 0.05$, Fisher test –$p = 0.029$,
** $\chi^2 = 6{,}421$, $p < 0.05$, Fisher test – $p = 0.032$,
*** $\chi^2 = 5{,}326$, $p < 0.05$, Fisher test – $p = 0.041$.

Table 4. Proliferation of CaSki cells after ATRA treatment.

Concentration ATRA in CaSki cells	Mean ± SD absorbance values (E405)		
	24 h	48 h	72 h
Control	0.178±0.08	0.188±0.07	0.373±0.09
10^{-4}	0.175±0.08	0.138±0.05	0.276±0.08
10^{-7}	0.161±0.07	0.134±0.05	0.265±0.07
p	NS	NS	NS

SD – standard deviation, p – probability, NS – not significant.

Based on results of this study, ATRA in concentrations ranged from 10^{-4} (40% cytotoxic effect) to 10^{-7} mM (10% cytotoxic effect) were used for further experiment.

Analysis of proliferation of HeLa cells in response to ATRA treatment. To determine the proliferation of cells after the ATRA treatment (in the concentrations of 10^{-4} and 10^{-7} mM) for 24, 48 and 72 hours, the percentage of S-phase cells was determined by bromodeoxyuridine incorporation assay (Cell Proliferation ELISA, BrdU Kit, Roche Molecular Biochemical, Germany). The proliferation of HeLa cells was increased after 24 h in 10^{-4} and 10^{-7} mM of ATRA, and 72 h after treatment of ATRA in 10^{-4} mM concentration (Tab. 3).

Analysis of proliferation of CaSki cells in response to ATRA treatment. ATRA had no effect on the proliferation of CaSki cells in culture (Tab. 4).

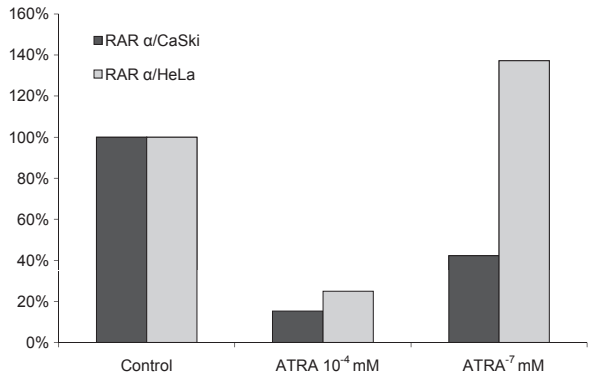

Figure 1. Expression of RAR α receptors in HeLa and CaSki cells.

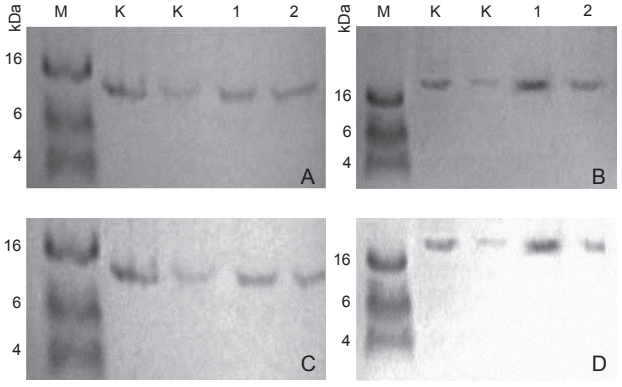

Figure 2. Western blot analysis E6 and E7 HPV16/18 proteins. Total cell lysates from HeLa cells were prepared for Western blot using a E6 (**A**) and E7 (**B**) HPV16/18 antibody. Total cell lysated from CaSki were prepared for Western blot using a E6 (**C**) and E7 (**D**) HPV16/18 antibody. M – marker of molecular weight; K – control cell lysate, line – 1, 2 cells were treated with ATRA 10^{-7} mM.

Identification of RAR and RXR mRNA expression in response to ATRA treatment. To determine the changes in expression of RAR and RXR genes after ATRA treatment, total RNA was extracted from HeLa and CaSki cells and mRNA of RAR and RXR isoforms were analyzed with Real time PCR method. Each reaction was conducted three times. The extreme values obtained in the study were rejected. The arithmetic mean was calculated from approximate values. It was assumed that the number of copies of control transcripts (from cells which were untreated with ATRA) was 100% (Fig. 1).

The expression of endogenous RAR-β, RAR-γ, RXR-α, RXR-β, and RXR-γ mRNA was very low in both types of cells after ATRA treatment, and similarly in the control. The expression of RARα was overexpressed in HeLa cells in a concentration of 10^{-7} nm of ATRA, after 72 h and it was decreased by 60–90% in CaSki cells (Fig. 1).

Western blotting. The viral HPV oncogenic proteins E6 and E7 were identified in protein isolated from study HeLa and CaSki cells treated with ATRA, using a specific antibodies. In both types of cells, the E6 and E7 protein expressions were observed (Fig. 2). This result suggests that ATRA does not repress the expression of two viral oncoproteins, which play a key role in carcinogenesis of the cervix.

DISCUSSION

Chemoprevention, to stop the progression of preneoplastic lesions, is an important strategy in cancer therapy [33]. All-trans retinoic acid (ATRA), 9-cis retinoic acid (9-cRA) and 13-cis retinoic acid (13-cRA) are natural derivatives of vitamin A, currently being used in the treatment of cancer. Studies have reported the antileukemic effects of all-trans-retinoic acid in patients with acute promyelocytic leukemia [37, 22], and also the chemopreventive effects of isoretinoin (13-cis-retinoic acid) in reducing the number of second primary tumours in patients with prior squamous cell carcinoma of the head and neck [20]. In spite of the fact that retinoids have been used in the clinic for many years, their mode of action in the prevention of cervical cancer is still unclear. Retinoids, unlike other signalling molecules, are not strictly endogenous but they are derived from dietary sources of vitamin A or its precursors. Some of them have toxic effects on cells which may be related to the interaction with the retinoid metabolisms or transport of signal transduction [10, 11, 25]. Retinoid signalling is often compromised early in carcinogenesis. The retinoids exert their biological effects through specific receptors RARs and RXRs. These receptors are ligand-dependent transcription factors that either induce or repress the transcription of target genes. The RARs act effectively by heterodimerizing to form an RAR/RXR complex, and act as ligand-inducible responsive elements (RAREs) and retinoid X responsive elements (RXREs) in enhancer regions of RA responsive genes. Both positive and negative regulation of genes by retinoids have been identified [4]. The high level of RARα favours binding of the RXR/RAR heterodimer activated by RAR selective retinoids. However, RAR coactivators and co-repressors are also involved in the efficient transcription of RA-responsive genes [5]. Zeng *et al.* have demonstrated that hADA3 directly binds to the RXR-α and enhances the RXR-α mediated sequence-specific transactivation of retinoid target genes [39].

The level of expression of the various subtypes of retinoid receptors may change during the development of cancer. Alterations in the RARα and RARβ genes and their expression have been found to be associated with acute promyelocytic leukemia and in hepatocellular carcinoma [8, 14, 29].

RAR-β plays a key role in mediating the anticancer effect of retinoids in many different types of cancer cells (lung, head and neck, and cervical cancers). The loss of RAR-β expression has been observed in many cancer cell lines [1, 2, 17]. Although RAR-β silencing is associated with increased tumourigenicity in certain types, other cell contexts depend on different retinoid receptors. For example,

in NT2/D1 human embryonic carcinoma cells, RAR-γ is required for retinoid-mediated differentiation, and RAR-γ repression is not overcome by RA treatment [1, 27].

Other studies have indicated that RAR-γ, RAR-β and RXR are involved in the growth inhibition of immortalized and transformed human bronchial epithelial cells [1]. Thus, specific retinoid receptors confer cell- and tissue-dependent retinoid response.

In the presented study, we investigated the effects of ATRA on the proliferation of human cervical cancer cells and RARs and RXRs expression. Trans-RA inhibited the growth of CaSki cells and increased the growth of HeLa cells, accompanied by an induction of RAR-α expression. ATRA had no effect on the expression of RAR-β. Many early data indicated that RAR-β plays an important role in mediating the growth of the inhibitory actions of RA. Reduced expression of RAR-β was observed in many types of premalignant lesions and cancers, including cervical cancer [13, 17]. Transcription of the retinoic receptor β gene in HeLa cells was activated in a ligand-dependent manner by the retinoic acid receptor α. We also observed RAR-α expression in HeLa cells, but not in CaSki after ATRA treatment. A sufficient level of RAR-α was also required for the growth inhibition of gastric cancer cells by ATRA [26]. On the other hand, RAR-α protein was reported to be expressed at significantly higher levels in tumours with greater proliferative activity, suggesting that RAR-α expression may be altered with tumour progression [31, 36]. The levels of other retinoid receptors in the studied HeLa and CaSki cells were very low and did not change after ATRA treatment. The results of Soprano *et al.* [32] demonstrated that the growth of inhibitory repressor of squamous cell carcinomas (SSCs) to retinoid treatment was mediated by RARs in general, and RAR-γ in particular. Faluhelyi *et al.* [12]. indicated that SiHa cervival squamous carcinoma cells responded to ATRA treatment in a dose-dependent manner: high dose 10^{-5}–10^{-4} mM but not low-dose 10^{-7}–10^{-6} mM of ATRA induced growth arrest. Similar results was obtained by Hurnanen *et al.* [23]. However, an elevated retinoid concentration increased lipid peroxidation as well as cell death. Our results support suggestions that the cell response to retinoids depends on the cell type, and that the cancer cells are differently resistant to retinoids. This can probably explain the therapeutic effects of retinoids, and it is the major limitation to the application for the cervical cancer treatment.

The etiology of cervical cancer is associated with HPV16/18 infection, and both cervical cell lines examined in the study were transformed by HPV. The viral oncogenes E6 and E7 expressed from high-risk HPV are almost always involved in the transformation of the cervical epithelial cells. The transcription of the viral oncogenes E6 and E7 is controlled by the viral upstream regulatory region (URR), harbouring the tissue-specific enhancer and promoter elements [41]. The viral E6 protein binds to p53 tumour suppressor gene and induces its degradation. The E7 protein binds to the tumour suppressor retinoblastoma gene product-pRb, phosphorylating, and therefore inactivating this protein. Therefore, both viral proteins are responsible for dysregulation of the cell cycle of the HPV positive cells, allowing cells with genomic defects to enter the S-phase – DNA replication phase [9].

Therefore, the next question was whether the ATRA had an effect on the expression of viral HPV E6 and E7 oncogenic protein in studied cells. Our data indicated that ATRA could participate directly or indirectly in the regulation of expression of E6 and E7 viral proteins.

It was indicated that the product of RAR-β gene could inhibit the transcription of the viral oncoprotein E6 and E7, and ATRA mediated growth arrests depending on the coordinate expression of pRB [3].

Thus, the decrease of the expression of RAR-β in the studied cells may be an additional important step on the way towards malignant progression of HPV positive cell. In the studied cells, the expression of RXRs was very low. The biological and therapeutic effect of retinoids is either dependent on RAR homodimers or heterodimer formation with RXRs, which, in turn, bind to retinoic acid response element or retinoid X response elements within their respective target promoters [5]. In the presence of ligands, RARs or RAR/RXR can negatively affect AP-1 protein either by a direct interaction with Jun/Fos family members or by disrupting Jun-Fos dimeryzation [40]. AP-1 is a key factor in a regulatory network, playing not only a fundamental role in transcriptional regulation of various HPVs, but also in cell proliferation and tumor induction [6, 21, 30].

CONCLUSION

The present study indicates that cervical cancerogenesis is very complex and depends on many factors, one of which may be retinoids. Therefore, the importance is the understanding how retinoids work for defining how they may be used as chemopreventive or chemotherapeutic agents in cervical cancer.

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