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 availability of carotenoids in foods, vegetables should be eaten raw or lightly steamed [18]. Vitamin A deficiency in experimental animals has been associated...
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 cofactors 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 histone 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>
<thead>
<tr>
<th>Primer’s name</th>
<th>Primer sequences</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>RARα1 RARα2</td>
<td>CTGCACCACCACTGCTTAG</td>
<td>184 bp</td>
</tr>
<tr>
<td>RARβ1 RARβ2</td>
<td>AAAACCCTGGTCTGGCTGCCC</td>
<td>125 bp</td>
</tr>
<tr>
<td>RARG1 RARG2</td>
<td>CGGGGCATCAGCACTAAGG</td>
<td>160 bp</td>
</tr>
<tr>
<td>RXRA1 RXRA2</td>
<td>GACCTGACCTACACTGCCC</td>
<td>167 bp</td>
</tr>
<tr>
<td>RXRB1 RXRB2</td>
<td>TGGCCTATACAGAATGTCAC</td>
<td>184 bp</td>
</tr>
<tr>
<td>RRGB1 RRGB2</td>
<td>CAAGAGGAAGTAAAGG</td>
<td>178 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTGGAGGCCACGCTCTGATG</td>
<td>180 bp</td>
</tr>
</tbody>
</table>

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% CO₂. Cells were detached from the flask with trypsin-EDTA and resuspended in a concentration of 10⁵ 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 × 10⁻⁹ mM to 1 × 10⁻⁷ 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% CO₂ 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 manufacturer. 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
Proliferation and expression of retinoic receptor in cervical cancer cells Hela and CaSki lines after treatment all-trans retinoic acid

Western blotting. Cells were washed in PBS and then lysed in buffer: Tris-HCl, pH 7.5, 1 mM EDTA, 15 mM β-mercaptoethanol, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride. Protein concentration was determined with Bradford reagent (Bio-Rad, Hercules, CA), and 25 µg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene 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 χ² 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 10⁻⁸–10⁻⁴ mM for 24–72 h. Cells survival was determined under a microscope after staining with 0.5% of water solution of toluidine blue (Tab. 2).

<table>
<thead>
<tr>
<th>Study cells</th>
<th>Cytotoxic effect of ATRA in % of dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻⁷</td>
</tr>
<tr>
<td>HeLa</td>
<td></td>
</tr>
<tr>
<td>CaSki</td>
<td></td>
</tr>
</tbody>
</table>

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

Analysis of proliferation of Hela cells after ATRA treatment.

Based on results of this study, ATRA in concentrations ranged from 10⁻⁴ (40% cytotoxic effect) to 10⁻⁵ 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 10⁻⁵ 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 10⁻⁵ 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).
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 corepressors 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 RXRs 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 cervical 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 Hurnenan 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 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 oncprotein 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.

REFERENCES

31. Soprano DR, Soprano KJ: Pharmacological doses of some synthetic retinoids can modulate both the aryl hydrocarbon receptor and retinoid receptor pathways. J Nutr 2003, 24, 277S–281S.