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Apoptosis of HeLa and CaSki cell lines incubated with All-trans retinoid acid

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Abstract: The aim of the study was to evaluate the concentrations of a soluble form of APO-1/Fas antigen (sFas, CD95) and a soluble Ligand for APO-1/Fas antigen (sCD95L, sFasL) in supernatants from CaSki and HeLa cell line cultures after the incubation with All-trans-retinoic acid. HPV-16 and HPV18 – positive cell lines were cultivated with All-trans-retinoic acid in concentrations of 1×10^{-6} M/L and 1×10^{-8} M/L. The cultures were incubated for 24 hours. Control culture with 3 µl of dimethyl-sulphoxide (DMSO) was incubated under identical conditions. The concentrations of soluble APO-1/Fas antigen and Fas Ligand in cell culture supernatants were estimated using immunoenzymatic methods. The obtained results showed significant decrease of concentrations of soluble APO-1/Fas antigen in supernatants from HeLa cell lines incubated with retinol in comparison with the control culture. Moreover, the concentrations of soluble Ligand for APO-1/Fas antigen in the supernatants of CaSki and HeLa cell lines were significantly lower in the culture incubated with All-trans retinoid acid when compared to the control culture. Higher concentrations of soluble APO-1/Fas antigen in supernatants from HeLa cell line without retinol may constitute a protective mechanism of the cells infected with the virus before undergoing Fas/FasL-dependent apoptosis. Lower concentrations of soluble APO-1/Fas antigen and soluble Ligand for APO-1/Fas in the supernatants from CaSki and HeLa cell cultures incubated with retinol suggest that retinoids can decrease the synthesis of soluble APO-1//Fas and soluble FasL in HPV-16 and HPV – 18 positive cells and that mechanisms protecting infected cells against Fas/FasL-mediated apoptosis become defective under the influence of retinol.

Key words: apoptosis, All-trans retinoic acid, CaSki cell line, HeLa cell line, soluble APO-1/Fas antigen, soluble Ligand for APO-1/Fas antigen

Introduction

The antigen of APO-1/FAS is a member of the TNF/NGF receptor family. It has three extracellular domains, rich in cysteine residues, and intracellular death domain (DD) responsible for transmitting of a pro-apoptotic signal [1-3]. The Ligand for APO-1/Fas antigen is a type II membrane protein that belongs to the tumor necrosis factor (TNF) family and induces apoptosis in Fas-expressing cells. The pro-apoptotic signal is transmitted through the reaction of the receptor with agonistic antibodies or its cognate oligomerizing ligand (CD95L), which is present in cellular membrane or outside the cells in a soluble form [1-3].

The programmed cell death Fas/FasL – mediated takes part mainly in a regulation of an immune response and a body tissue homeostasis. Many studies suggest the additional functions of this system, especially their important role in pathogenesis of many diseases characterized by decreased or excessive apoptosis [1-3].

The mechanisms of signal transmission by APO-1/Fas receptor (CD95) and the role and activity of CD95 Ligand can allow for better understanding of many diseases including neoplastic lesions.

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Recent findings suggest that Fas and FasL antigens are essential for cell death process and that any dysfunction in this system can lead to a breakdown in peripheral tolerance [2-6].

The soluble forms of APO-1/Fas molecule are produced as translation products of alternatively spliced mRNA [4-8]. The secreted receptors represent truncated forms of the membrane-bound receptors that can bind a ligand in a way similar to that of their membrane-bound counterparts [4-8]. As a result, these receptors play an important role in the regulation of normal receptor activity. Cheng *et al*. found that soluble Fas is capable to inhibit Fas-mediated apoptosis *in vitro* [9]. As previously noted, Fas mRNA is expressed by most cells, and to a variable extent all cells expressing Fas may be responsible for the potential sources of any form of soluble Fas [4-8]. Regardless of their sources, higher concentrations of soluble Fas antigen can influence ability of cells to undergo Fas/FasL – mediated programmed cell death process [9-11].

Retinoids belong to immunosuppressive factors negatively regulating activation-induced CD95L expression. They may be natural or synthetic, structurally connected with vitamin A group. They display antiproliferative activity and stimulate differentiation of neoplastic cells. This feature has been used in prophylactics and therapy of various types of human neoplasms, *e.g.* cervical cancer, and precancerous conditions [12-15]. In the studies performed it was speculated that activity of retinoids is related with blocking of mRNA expression for CD95 Ligand. However, lack of responsive elements in CD95L promotor region suggests the existence of indirect mechanisms *e.g.* hyperexpression of IkB and interference with NF-kB [12-15]. Currently it is thought that retinoid activity is the result of their ability of binding to specific nuclear receptors acting as ligand-dependent transcription factors [12-15].

Studies with a purpose of explaining retinoid activity are usually performed using cellular culture models. The model of latent infection of cervical cells with oncogenic HPV-18 for *in vitro* examinations is HeLa line cells culture. It is used in basic studies of cytotoxic and genotoxic characteristics of medicines, regulation of gene expression and cell reaction to stress.

The aim of this study was to evaluate the concentrations of soluble APO-1/Fas antigen and soluble Fas Ligand in supernatants from CaSki and HeLa cell line cultures after 24 hours of incubation with selected retinoic acid concentrations.

Material and methods

Cell culture and growth condition. HPV-16 – positive cell lines (CaSki cell lines) and HPV-18 – positive cell lines (HeLa cell lines – ECACC 95051229) were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics in a humidified 5% CO_2 incubator at 37°C. The antibiotics were administered in the following concentrations: penicillin 100 IU/ml and streptomycin 100 µg/ml. The number of cells in the culture wells was 10^5/ml. All-trans-retinoic acid (ATRA, Sigma, USA) was dissolved in DMSO and added to the culture in concentrations of 1×10^{-6} M/L and 1×10^{-8} M/L [5-7]. Cell cultures were incubated for 24 hours. Control culture with 3 µl of DMSO was incubated under identical conditions. After incubation the supernatants were centrifuged, in order to be clarified prior to use in the assay. Samples were stored at -20°C.

The concentrations of soluble APO-1/Fas antigen in cell culture supernatants were measured using a quantitative enzyme-linked immunosorbent assay ELISA (Human sAPO-1/Fas ELISA, Bender MedSystems, Diagnostics GmbH, Vienna, Austria). Standard dilutions of sAPO-1/Fas antigen were prepared in concentrations ranging from 16 pg/ml to1000 pg/ml. The measurements were performed in duplicates. Wells designated for samples were filled with 10 µl of each sample of the supernatant, 90 µl of Sample Diluent and 50 µl Biotin Conjugate (10 µl/ml) and then covered with Plate Cover and incubated at 37°C for 1 hour.

After incubation wells had been emptied and washed 3 times with 300 µl Wash Buffer per well. Next, 100 µl of diluted (4 µl/ml) Streptavidin-HRP was added to all wells. Then the plate was incubated again at 37°C for 1 hour.

Wells were emptied again, washed 3 times like before and 100 µl of TMB Substrate Solution was added. After incubation at room temperature for 15 minutes on a rotator, enzyme reaction was stopped by quickly pipetting 100 µl of Stop Solution into each well. Results were read immediately on spectro-photometer using 450 nm wave length.

The concentrations of soluble Fas Ligand in cell culture supernatants were measured using a quantitative enzyme-linked immunosorbent assay ELISA (Human sFas Ligand ELISA, MBL Medical and Biological Laboratories, Co. Ltd., Nagoya, Japan). Standard dilutions of soluble Fas Ligand were prepared in concentrations ranging from 0 ng/ml to 5 ng/ml. The measurements were performed in duplicates. Antibody coated wells were filled with 100 µl of prepared samples of the supernatants and standards and incubated at room temperature for one hour. After incubation wells had been emptied and washed four times with Wash solution. Next, 100 µl of Conjugate solution was added to all wells. Then the plate was incubated again at room temperature for one hour. Wells were emptied again, washed four times like before and 100 µl of Substrate Solution was added. After incubation at room temperature for 30 minutes, enzyme reaction was stopped by quickly pipetting 100 µl of Stop Solution into each well. Results were read immediately on spectro-photometer using 450 nm wave length.

Statistical analysis. Statistical differences between groups were estimated using a standard non-parametric test (Mann-Whitney U test). The results were presented as mean with SD and ranges. Differences at $p<0.05$ were considered as statistically significant. Statistica 7.0 PL software was applied to statistical analysis.

Results

The concentrations of soluble Fas Ligand in the supernatant of HPV-16 positive (CaSki) cell line culture were significantly lower in the culture incubated with 10^{-6} M/L of All-trans retinoid acid when compared to the control culture (CaSki cell line with ATRA 10^{-6} M/L *vs*. control, $p<0.05$).

The concentrations of soluble Fas/APO-1 antigen in the supernatants of HPV-18 positive (HeLa) cell line cultures after the incubation with 10^{-8} M/L and with

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Tab.1. The concentrations of soluble APO-1/Fas antigen in HPV-16 (CaSki) cell line cultures incubated without All-trans Retinoid acid (control) and with different concentrations of All-trans Retinoid Acid (1×10^{-6} M/L, 1×10^{-8} M/L) for 24 hours.

The concentrations of All-trans Retinoid Acid (M/L)	The time of incubation (hours)	The concentrations of soluble APO-1/Fas antigen (pg/ml)	P
control	24	91,99	Control vs RA 10^{-6} NS
10^{-6}	24	59,12	RA 10^{-6} vs RA 10^{-8} NS
10^{-8}	24	54,16	RA 10^{-8} vs control NS

Tab. 2. The concentrations of soluble APO-1/Fas antigen in HPV-18 (HeLa) cell line cultures incubated without All-trans Retinoid Acid (control) and with different concentrations of All-trans Retinoid Acid (1×10^{-6} M/L, 1×10^{-8} M/L) for 24 hours.

The concentrations of All-trans Retinoid Acid (M/L)	The time of incubation (hours)	The concentrations of soluble APO-1/Fas antigen (pg/ml)	P
control	24	298.3	Control vs RA 10^{-6} $P< 0.05$
10^{-6}	24	35.03	RA 10^{-6} vs RA 10^{-8} NS
10^{-8}	24	40.25	RA 10^{-8} vs control $P< 0.05$

Tab. 3. The concentrations of soluble Fas Ligand in HPV-16 (CaSki) cell line cultures incubated without All-trans retinoid acid (control) and with different concentrations of All- trans retinoid acid (1×10^{-6} M/L, 1×10^{-8} M/L) for 24 hours.

The concentrations of All-trans Retinoid Acid (M/L)	The time of incubation (hours)	The concentrations of soluble APO-1/FasL antigen (ng/ml)	P
control	24	0,027	Control vs RA 10^{-6} $P < 0.005$
10^{-6}	24	0,012	RA 10^{-6} vs RA 10^{-8} $P<0.005$
10^{-8}	24	0,026	RA 10^{-8} vs control NS

Tab. 4. The concentrations of soluble Fas Ligand in HPV-18 (HeLa) cell line cultures incubated without All-trans Retinoid Acid (control) and with different concentrations of All-trans retinoid Acid (1×10^{-6} M/L, 1×10^{-8} M/L) for 24 hours.

The concentrations of All-trans Retinoid Acid (M/L)	The time of incubation (hours)	The concentrations of soluble APO-1/FasL antigen (ng/ml)	P
control	24	0,040	Control vs RA 10^{-6} $P < 0.05$
10^{-6}	24	0,023	RA 10^{-6} vs RA 10^{-8} $P < 0.05$
10^{-8}	24	0,034	RA 10^{-8} vs control NS

10^{-6} M/L of retinol were significantly lower when compared to the control (HeLa cell line with 10^{-8} M/L of ATRA *vs.* control, $p<0.005$; HeLa cell line with 10^{-6} M/L of ATRA *vs.* control, $p<0.005$).

The concentrations of soluble Fas Ligand in the supernatant of HPV-18 positive (HeLa) cell line culture were significantly lower in the culture incubated with 10^{-6} M/L of All-trans retinoid acid when compared to the control culture (HeLa cell line with ATRA 10^{-6} M/L *vs.* control, $p<0.005$).

The concentrations of soluble Fas/APO-1 antigen in the supernatants of HPV-16 positive (CaSki) cell line cultures did not differ significantly when compared to the control culture. The results are shown in Table 1-4.

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Discussion

Although the expression of CD95 Ligand (FasL) was initially believed to be confined to activated T cells, several other cell types have subsequently been shown to produce and release this antigen. FasL is constitutively expressed in neutrophils, neurons, thyrocytes, stroma cells of the retina, acinar cells in salivary glands, Sertoli cells and trophoblast cells [10]. Moreover, a variety of cell types can express FasL in response to different stimulatory conditions, including macrophages infected with human immunodeficiency virus, hepatocytes treated with ethanol, leukemia cells exposed to chemotherapy drugs and various cell types upon tumor transformation [10,11]. The functional soluble form of FasL is responsible for killing Fas-sensitive cells through either autocrine or paracrine suicide death [10,11].

In our study we observed decreased concentrations of soluble CD95 Ligand in the CaSki and HeLa cell line culture supernatants incubated with retinol.

It has been observed lately that resistance to apoptosis was associated with an increased release of soluble Fas antigen or with posttranscriptional regulation of CD95 caused by some substances like N-acetyl-L-cysteine, cyclosporine-A and FK506 [3]. Other immunosuppressive agents are also able to negatively regulate activation – induced CD95L expression. Dexomethasone, which induces T cell apoptosis by itself, also inhibits CD95L expression. Retinoic acid shows similar effects. Because no obvious glucocorticoid and/or retinoid response elements were found in the CD95L promoter, it was concluded that dexomethasone and retinoic acid are likely to inhibit CD95L expression by indirect mechanism, involving *e.g.* hyperexpression of IkB and interference with NFkB [12-15].

Most of the studies have described retinol as inhibitor of cell growth. In G1 phase, retinoic acid increases the level of p27 kip1 (cdk inhibitor) due to direct regulation of p27kip1 gene expression by RAR (retinoic acid receptor). Landelfeld reported that retinoic acid induced growth arrest due to enhanced ubiquitin-dependent degradation of cyclin D1 [16]. While, Teixeira presents that retinoic acid reduces the mRNA level of cyclin D1 and cdk-2 [17].

There are some specific effects of retinoids. The major biological activities of retinoids are thought to be mediated by two types of nuclear retinoids receptors, RARs and retinoid X receptors, which are members of the steroid hormone receptor gene superfamily [18]. Retinoids can increase the expression of Fas antigen and augment Fas/FasL-induced apoptosis in cell line with wild-type p53 but not in cell lines having mutant p53 [19]. Furthermore, it has been decribed lately that retinoids inhibit Fas ligand (FasL) expression and repress the activation-induced apoptosis of immature thymocytes and T-cell hybridomas. All-trans-RA represses the trancriptional activity of nuclear factors of activated T-cells (NFAT), which is an important transactivator of the FasL promoter [20].

In this study we are showing that HeLa cell line infected by HPV-18 express high level of soluble APO-1/Fas antigen, which probably protects it against apoptosis and this effect is diminished by retinoid acid. Similar results have been shown in human hepatocellular carcinoma and human lung cancer cell lines [21,22]. The concentrations of soluble Fas/APO-1 antigen in HeLa cell line culture with all-trans-retinoic acid (ATRA) were downregulated when compared with HeLa cell line without ATRA. We did not observed these effects in the CaSki cell line cultures incubated with retinol. These observations suggest different mechanisms of retinoids influence on HPV-16 and HPV-18 infected cell lines.

On the other hand, Zhu *et al.* studied the influence of ATRA on the expressions of bcl-2, Fas and FasL antigen mRNA on murine lymphoma cell line. They observed that ATRA downregulated the expression of bcl-2 without any change of Fas and FasL antigens [23]. Activation of the Fas/FasL system is considered not to be involved in retinoic-induced apoptosis. On the other hand, a number of herpesviruses of gamma type were shown to inhibit all death-receptor-mediated apoptosis by expressing a gene product that directly interacts with caspase-8 and thereby inhibits the formation and activity of the death-inducing signaling complex [24]. Additionally, a number of viruses express a functional Bcl-2 analog, such as E1B by EBV or ORF 16 by HHV-8 [25].

Conclusions

Higher concentrations of soluble APO-1/Fas antigen in supernatants from HeLa cell line without retinol may constitute a protective mechanism of the cells infected with the virus before undergoing Fas/FasL-dependent apoptosis. Decreased concentrations of sAPO-1/Fas antigen in HeLa cell line cultures incubated with retinol may suggest that mechanisms protecting infected cells against Fas/FasL-mediated apoptosis become defective under the influence of retinol.

Decreased concentrations of CD95 Ligand in Caski and HeLa cell line cultures incubated with retinol may suggest that retinoids can inhibit the synthesis of soluble Fas Ligand in HPV-16 and HPV-18 positive cell lines.

Our studies confirm that vitamin A and its analogs inhibit proliferation of cells associated with HPV infection and suggest promising effects of retinoid therapy in inhibiting the progression of early cervical lesions into cancer.

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