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Suppressive Effects of Neopterin on Inducible Nitric Oxide Synthase Gene Expression in Ovarian Carcinoma Cells *in vitro*.

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Abstract

In a number of malignant diseases including ovarian carcinomas, increased serum or urine levels of neopterin serve as a valuable marker for the state of activation of the cellular immune system. Reported correlations between elevated neopterin concentrations and shorter survival time of the tumor host might be explained by direct biochemical actions exhibited by the pteridine compound. In the present study, we investigated the influence of neopterin on inducible nitric oxide synthase (iNOS) gene expression in two ovarian carcinoma cell lines. Qualitative and quantitative analyses of iNOS as well as measurements of nitric oxide (NO) metabolites in the cell supernatant revealed an inhibitory effect of neopterin (1000 μM) on NO generation induced by a combination of interferon-γ (100 U/ml), tumor necrosis factor-α (500 U/ml), and interleukin-1β (10 U/ml). Since NO is most likely involved in apoptotic cell death in ovarian carcinoma cells, it is conceivable that suppression of NO synthesis represents a biochemical mechanism mediated by neopterin that is advantageous to tumor progression and thus harmful to the host.

Key words: inducible nitric oxide synthase [L-arginine, NADPH:oxygen oxidoreductases],(EC 1.14.13.39)

Introduction

Neopterin is a pyrazino pyrimidine compound which is biosynthesized from guanosine triphosphate in the pathway leading to tetrahydrobiopterin. Due to a low constitutive activity of the second enzyme within this pathway, 6-pyrovoyl tetrahydropterin synthase, human monocytes/macrophages produce and release large amounts of neopterin following activation with T-lymphocyte-derived interferon-γ, IFN-γ (1, 2). Thus, serum and urinary neopterin levels serve as an indicator in patients with diseases associated with an increased activity of the cellular immune system, e.g. viral and bacterial infections, acute graft rejections, and acquired immune deficiency snydrome (3). In 1981, Hausen and co-workers (4) found urinary neopterin to be a marker for hematologic neoplasms. Subsequently, increased serum or urinary neopterin concentrations have been observed in various malignant diseases such as multiple myeloma, cervical cancer, and hepatocellular carcinoma. Considering ovarian cancer, raised neopterin levels have first been reported in 1987 by Reibnegger et al. (5). They described the prognostic potential of pretherapeutic urinary neopterin levels concerning the risk of death due to the carcinoma. In a study by Haeger et al. (6), significantly higher neopterin concentrations in plasma, ascites, and ovarian cyst fluid were found in patients with malignant ovarian cancer as compared to patients with benign ovarian tumors of the same size. Since neopterin is not produced by the cancer cells themselves, it is not a classical tumor marker but rather reflects the state of activation of the cellular immune system induced by the altered surface of the tumor cells. In this context, the shorter survival time of patients with elevated neopterin levels sounds paradoxical, since one would expect a benefit from the cellular immune activation. One possible explanation is that activated macrophages secrete a number of potential tumor growth factors or substances that promote

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angiogenesis, e.g. tissue growth factor-α (7). Previous investigations have supported the concept that neopterin itself exhibits distinct biochemical properties, most likely via interactions with the cellular redox state (8). These direct actions of neopterin may provide another explanation for the correlation between high neopterin levels and unfavorable cancer prognosis. Recently, we could show variations of inducible nitric oxide synthase (iNOS) gene expression in three different ovarian carcinoma cell lines (9) induced by the pro-inflammatory cytokines IFN-γ, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). In the present study, we investigated whether neopterin affects iNOS gene expression and/or subsequent NO release in two ovarian carcinoma cell lines.

Materials and Methods

Cell cultures

The human ovarian carcinoma cell lines OVCAR-3 and HOC-7 were kindly provided by Dr. C. Dittrich, University of Vienna, and Dr. L. Old, Memorial Sloan Kettering Center, New York, and cultured under standard conditions in Dulbecco's modified minimum essential medium (DMEM) with addition of 10% fetal calf serum, 50 mg/ml streptomycin, and 50 U/ml penicillin.

Reverse transcription and polymerase chain reaction

Cells were washed in sterile PBS and lysed with 4 M guanidine isothiocyanate containing 0.1 M 2-mercaptoethanol. Total RNA was extracted and purified by acid phenol-chloroform extraction. One μg total RNA (concentration was determined photometrically at a wavelength of 260 nm) was reverse-transcribed into cDNA using oligo (dT)15 as primer for reverse transcriptase. RT-generated cDNA encoding for human iNOS was amplified using polymerase chain reaction as desribed recently (9). The amplified products were resolved by 3% agarose gel electrophoresis and visualized by ethidium bromide (0.5 μg/ml) staining. For quantitation of iNOS cDNA, a competetive PCR was performed using a non-homologous DNA fragment derived from the viral oncogene v-Erb B to which the human iNOS primer templates have been added (Competetive DNA MIMIC, Clonetech, Heidelberg, Germany). Densitometric analysis of the scanned photographs was done by using the GelDoc 1000 system (BioRad, Munich, Germany) with regard to the different amount of ethidium bromide intercalation according to the length of the fragments (length of iNOS cDNA is 342 base pairs, length of competitor fragment is 442 base pairs). Results were calculated by detecting the point of equivalence between competitor and iNOS cDNA according to their respective band intensities. Data are given as amol iNOS-cDNA/μg total RNA taking into consideration that 1 μl of the 25 μl RT reaction yield was used per quantitative PCR.

Preparation of cytosolic and nucleic extracts

Cells were rinsed with cold PBS and lysed directly on the culture dishes in 1 ml cold lysis buffer (0.6% Nonidet P-40, 150 mM NaCl, 10 mM Tris pH 7.9, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride). Lysed cells were transferred into a 2 ml Eppendorf tube and incubated for 5 min on ice. The nuclei were pelleted (1250 g, 4°C, 5 min) and the supernatant was collected for determination of cytosolic protein content. Nuclear proteins were extracted in 100 ml extraction buffer (420 mM NaCl, 10 mM HEPES pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 1.5 mM MgCl2, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol) on ice for 20 min. Cellular debris was removed by centrifugation (1250 g, 4°C, 5 min) and the supernatant containing nuclear proteins was stored at -70°C.

Western Blot analysis

Equal protein amounts from each sample (10 μg) were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis using an 8% acrylamide gel. Separated proteins were transferred on a nitrocellulose membrane for 1 h at 250 mA by using a BioRad Transblot apparatus. Washed filters were incubated for 12 h with the polyclonal rabbit anti-iNOS antibody (human, mouse, and rat reactive, corresponding to amino acids 1131-1144 mapping at the carboxy terminus of the enzyme). A secondary antibody was used to visualize the antigen-antibody complexes by an alkaline phosphatase (AP) reaction catalyzing 5-bromo-4-chloro-3-indolyl-phosphate as a substrate. Analyses of the band intensities relative to the incubations with IFN-γ + IL-1β + TNF-α were done by using the Imaging densitometer GS 670 (BioRad Munich, Germany).

Electrophoretic mobility shift assay

Translocation of NF-kB into the nucleus of ovarian carcinoma cells was detected by incubation of nucleic extracts with labeled oligonocleotide probes representing the NF-κB concensus binding sequence located between position -115 and -90 relative to the human iNOS gene transcription initiation site (5′-CCC TAC TGG GGA CTC TCC CTT TGG G-3′). Probes were generated by 3′end labeling of double-strand oligonucleotides (100 ng/μl) with digoxigenin-11-ddUTP in TEN buffer (10 mM TRIS-HCl, 1 mM EDTA, 0.1 M

NaCl, pH 8.00) by 50 U terminal transferase (DIG gel shift kit, Boehringer Mannheim, Germany). Gel shift reactions were performed by mixing 0.5 ng/µl probe with nucleic extracts (final concentration of proteins: 5 µg/µl), and 1 µg/µl poly(dI-C) to avoid formation of unspecific protein-DIG-labeled DNA complexes. Oligonucleotide-protein complexes were separated by electrophoresis on a non-denaturing polyacrylamide gel for 4 h at 150 V in 0.25 x TBE buffer (89 mM TRIS-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.00). Subsequently, the probes were transferred onto a nylon membrane using a BioRad transblot apparatus (BioRad, München, Germany). Blotting was performed overnight at 15 V and at 4°C. Oligonucleotides were fixed onto the dried membranes by UV-crosslinking (0.121 J/cm2 for 20 sec). The digoxigenin labeled probes were bound to an AP-conjugated anti-digoxigenin antibody (75 mU/ml). Detection of AP was done by autoradiography of the chemiluminescence produced during enzymatic dephosphorylation of CSPD (100 µg/ml). The chemiluminescent signals were recorded by exposure to an X-ray film for 30 min.

Nitrite determinations

Synthesis of the stable NO metabolites nitrite and nitrate was determined in the cell-free culture supernatants following incubation of cells in L-arginine-enriched medium without phenol red. Nitrate was reduced to nitrite by nitrate reductase (0.4 U/ml), in the presence of 10 mM γ-NADPH. Total nitrite accumulation was assayed using the Griess reaction.

Reagents

dNTP-mix, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin-streptomycin, primer sets, trypsin-EDTA, M-MLRV superscript reverse transcriptase, Oligo-(dT) 15, and Taq polymerase were purchased from Gibco Life Tech., Eggenstein, Germany. Guanidine isothiocyanate was from Roth, Karlsruhe, Germany; and nitrate reductase was from Boehringer, Mannheim, Germany. iNOS antibody was from Santa Cruz Biotechnology, Santa Cruz, CA; goat anti-rabbit antibody was obtained from BioRad, München, Germany. Recombinant human IFN-γ, recombinant TNF-α, and recombinant human IL-1β were from TEBU, Frankfurt, Germany. Neopterin was purchased from Schircks Lab. Jona, Switzerland. All other chemicals were from Sigma Chemicals, Deisenhofen, Germany.

Statistics

Results are expressed as mean values ± standard error of the mean (SEM). To test for significance of differences between the mean value of a control vs. the mean value of treated cells, the Student's t-test was used. P-values < 0.05 were considered to be significant.

Results

Fig. 1A and 1B show representative results of the qualitative analyses of iNOS gene expression in

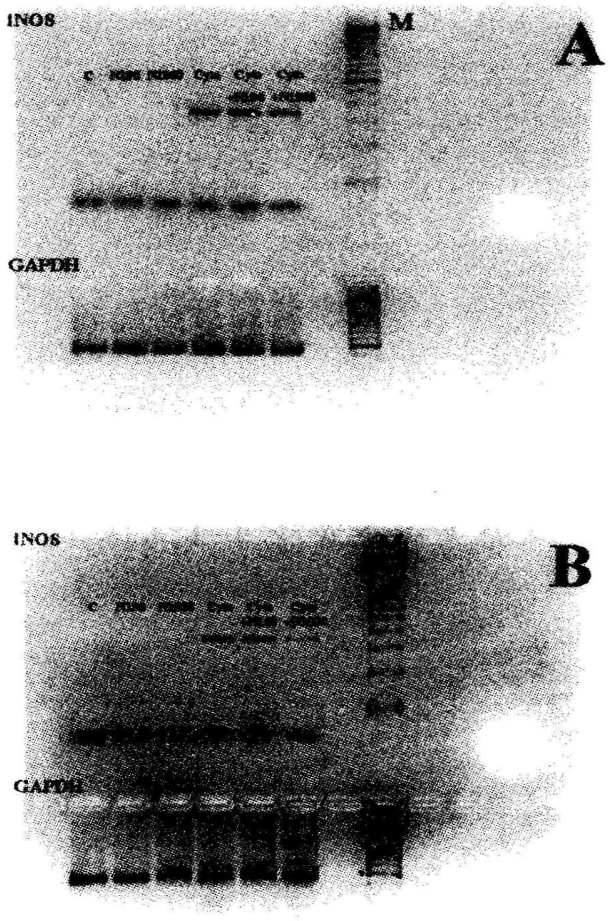

Figure 1: Qualitative analyses of inducible nitric oxide synthase gene expression detected as iNOS cDNA (length of fragment: 342 base pairs) in OVCAR-3 (Panel A) and HOC-7 (Panel B) cells. Cells had been treated for 24 h with neopterin at concentrations of 100 µM (N100) and 1000 µM (N1000), a cytokine mixture (Cyto), or with the combination of the cytokine mixture and 100 µM neopterin (Cyto + N100) as well as 1000 µM neopterin (Cyto + N1000), respectively. C represents the result of a control experiment, M indicates a size standard (100 base pair ladder). Results are representative of five different experiments. Cytokine mixture = interferon-γ (100 U/ml) + interleukin-1ß (10 U/ml) + tumor necrosis factor-α (500 U/ml).

OVCAR-3 (Panel A) and HOC-7 (Panel B) cells. Cells were incubated for 24 h with neopterin (at concentrations of 100 µM and 1000 µM), with the cytokine mixture (IFN-γ (100 U/ml) + IL-1β (10 U/ml) + TNF-α (500 U/ml)), and with the combinations of neopterin plus the cytokines at the given concentrations, respectively. No iNOS cDNA was detectable under control conditions as well as following incubations of OVCAR-3 and HOC-7 with neopterin alone. However, a stimulation of iNOS gene expression could be observed in both cell lines after stimulation with the cytokine mixture as well as the combination of cytokines and neopterin. The results of the quantitative analyses of iNOS cDNA synthesis following administration of the stimuli given above are summarized in Tab. 1. In OVCAR-3, the strongest effect on iNOS gene expression was observed after a 24 h incubation

	OVCAR-3	HOC-7
Control	n.d.	n.d.
Neopterin (100 µM)	n.d.	n.d.
Neopterin (1000 µM)	n.d.	n.d.
Cytokine mixture	1730.0 ± 90.3	102.8 ± 14.1
Cytokine mixture + neopterin (100 µM)	1651.9 ± 53.2	95.2 ± 8.6
Cytokine mixture + neopterin (1000 µM)	280.7 ± 21.1 *	28.2 ± 5.8 *

Table 1. Inducible nitric oxide synthase cDNA concentrations (amol/µg total RNA) in ovarian carcinoma cell lines following 24 h incubations as detected by quantitative polymerase chain reaction.
Data are expressed as mean ± SD (n=5); n.d. = not detectable. * P<0.05 as compared to the respective incubations without neopterin.
Cytokine mixture = interferon-γ (100 U/ml) + interleukin-1ß (10 U/ml) + tumor necrosis factor-α (500 U/ml).

period with IFN-γ + IL-1β + TNF-α. This reponse was not altered by coincubation with cytokines plus 100 µM neopterin. However, when OVCAR-3 were treated with cytokines plus neopterin at a concentration of 1000 µM, iNOS gene expression was significantly decreased as compared to the single application of the cytokine mixture (P<0.05 as compared to the single treatment with cytokines). Comparable effects of neopterin on cytokine-induced iNOS gene expression could be observed in HOC-7, although iNOS cDNA concentrations were lower as compared to those following the respective incubations of OVCAR-3.

The different profiles of 24 h iNOS protein generation in OVCAR-3 and HOC-7 are presented in Fig. 2A and 2B. As detected by Western Blot analyses, a 24 h stimulation of OVCAR-3 with IFN-γ + IL-1β + TNF-α as well as with cytokines plus neopterin (at both concentrations of 100 µM and 1000 µM) resulted

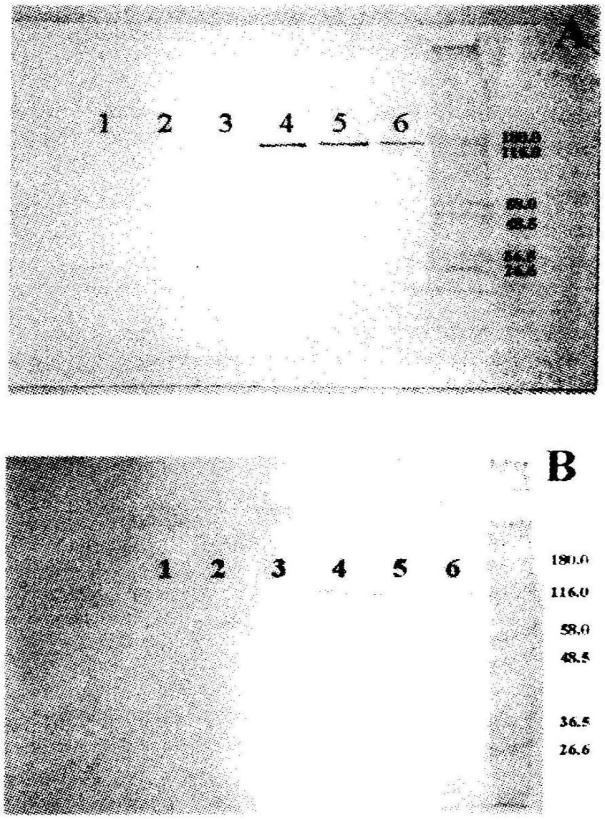

Figure 2: Western blot detection of inducible nitric oxide synthase protein in cytosolic extracts of OVCAR-3 (Panel A) and HOC-7 (Panel B) cells treated for 24 h with: neopterin at concentrations of 100 µM (lane 2) and 1000 µM (lane 3), a cytokine mixture (lane 4), or with the combination of neopterin and the cytokine mixture (lane 5 and lane 6), respectively. Lane 1 shows the result of a control experiment. A prestained kDa-marker was added to identify the molecular weight of the detected protein (~ 130 kDa). Results are representative of five different experiments. Cytokine mixture = interferon-γ (100 U/ml) + interleukin-1ß (10 U/ml) + tumor necrosis factor-α (500 U/ml).

in enhanced cytosolic iNOS protein contents (Fig. 2, Panel A). With regard to band intensities, the highest iNOS protein generation was observed following incubation of cells with the mixture of IFN-γ + IL-1β +

TNF-α. No differences in iNOS protein synthesis were observed following exposure of OVCAR-3 to IFN-γ + IL-1β + TNF-α plus 100 μM neopterin, while addition of neopterin at a concentration of 1000 μM suppressed iNOS protein generation induced by the cytokines (band intensities averaged 25% relative to IFN-γ + IL-1β + TNF-α). In congruence with the results obtained at the transcriptional level, cytosolic iNOS protein in HOC-7 cells (Fig. 2, Panel B) was detected following a 24 h incubation with the cytokine mixture. Considering the band intensities, this response of HOC-7 did not differ following treatment with cytokines plus 100 μM neopterin, but was markedly decreased when cytokines plus 1000 μM neopterin were applied (band intensities averaged 25% relative to IFN-γ + IL-1β + TNF-α).

In both cell lines, treatment with substances that stimulated iNOS gene expression and protein synthesis resulted in an increase of nitrite/nitrate concentrations in the cell-free culture supernatants following 24 h incubation periods (Tab. 2). This indicates that the cells were activated to produce and release NO. With regard to the different agonists, the combination IFNγ+ IL-1β + TNF-α had the strongest impact on nitrite/ nitrate accumulation, with HOC-7 being less susceptible than OVCAR-3. No significant change in nitrite production could be seen in response to cytokines plus

	OVCAR-3	HOC-7
Control	2.84 ± 0.27	1.38 ± 0.26
Neopterin (100 μM)	2.49 ± 0.27	1.49 ± 0.17
Neopterin (1000 μM)	3.46 ± 0.58	1.74 ± 0.17
Cytokine mixture	139.21 ± 2.19*	7.57 ± 0.43*
Cytokine mixture + neopterin (100 μM)	142.34 ± 2.84*	7.48 ± 0.66*
Cytokine mixture + neopterin (1000 μM)	37.72 ± 1.57*,#	2.46 ± 0.11 *,#

Table 2. Nitrite/nitrate concentrations determined as accumulated nitrite (nmol/106 cells) in the cell-free culture supernatants of ovarian carcinoma cells following 24 h incubations.
Data are expressed as mean ± SEM, n=8. * P<0.05 as compared to unstimulated controls, # P<0.05 as compared to incubations with the cytokine mixture.
Cytokine mixture = interferon-g (100 U/ml) + interleukin-1ß (10 U/ml) + tumor necrosis factor-a (500 U/ml).

100 μM neopterin, but nitrite concentrations were significantly lower in those cells treated with cytokines plus 1000 μM neopterin.

The results of the electrophoretic mobility shift assays using the NF-kB concensus binding sequence located between position -115 and -90 relative to the human iNOS gene transcription initiation site as a probe are shown in Fig. 3. In control experiments (lane 1) as well as after stimulation of cells with the cytokine

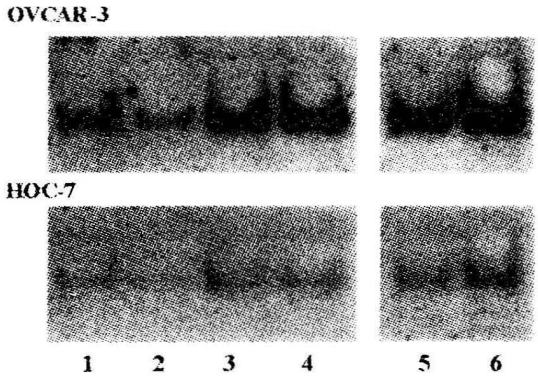

Figure 3. Activation of NF-kB detected by electrophoretic mobility shift assay in nuclear extracts of ovarian carcinoma cell lines following 1 h incubations. Lane 1 = unstimulated controls; lane 2 = cytokine mixture; lane 3 = neopterin (100 μM); lane 4 = neopterin (1000 μM); lane 5 = cytokine mixture + neopterin (100 μM); lane 6 = cytokine mixture + neopterin (1000 μM). Results are representative of 3 different experiments. Cytokine mixture = interferon-g (100 U/ml) + interleukin-1ß (10 U/ml) + tumor necrosis factor-a (500 U/ml).

mixture (lane 2), with neopterin (lanes 3 and 4) or with the combination of cytokines plus neopterin (lanes 5 and 6), translocation of NF-kB into the nucleus of OVCAR-3 and HOC-7 could be detected. As compared to controls, NF-κB binding activity was lesser following incubation of cells with the cytokine mixture. In contrast, a prominent activation of NF-κB transfer is present in those cells treated with 100 μM and 1000 μM neopterin, either following application as a single stimulus or together with the cytokine mixture.

Discussion

The present study demonstrates that neopterin inhibits cytokine-induced iNOS gene expression and NO-synthesis in ovarian carcinoma cell lines *in vitro*. These observations may serve as an explanation for the significant correlation between increased neopterin in various body fluids and poor prognosis in ovarian cancer disease, i.e. patients with elevated neopterin concentrations were charaterized by a shorter survival time (5, 6, 10-12). Apart from being a marker for an inappropriate immune response or the production of

macrophage-derived mediators that promote tumor growth, augmented levels of neopterin may indicate direct interactions between this pteridine compound and tumor metabolism. Previous studies have shown a number of biochemical properties exhibited by neopterin in various cell lines. It was found to induce TNF-α-synthesis in human macrophages (13) and rat vascular smooth muscle cells (14). In addition, neopterin provided pro-apoptotic activities with superinducing effects on cell death when applied with IFN-γ and TNF-α in coincubation experiments in rat alveolar type II epithelial cells and in rat vascular smooth muscle cells, respectively (15, 16), and in a hepatocellular carcinoma cell line, neopterin was found to suppress hypoxia-induced erythropoietin synthesis (17). From these data, one may conclude that neopterin exhibits distinct biochemical functions via interactions with cellular redox mechanisms and the promotion of oxidative stress (18). Therefore, neopterin plays a role as a modulator during host defence reactions. In this context, the question remains concerning the usability of *in vitro*-neopterin concentrations exceeding those found in serum under clinical conditions. The amounts of neopterin applied to the cells in the present study (100 μM, 1000 μM) are much higher than those found in blood samples of patients with ovarian cancer (averaging 10-40 nM, see Ref. 11). However, plasma values do most likely not reflect the concentration of pteridine compounds within the tissues, where continous production and release as well as cell to cell interactions may lead to an accumulation of neopterin in the cellular microenvironment.

The effects of neopterin-induced suppression of NO release on tumor metabolism remain to be elucidated. Some evidence can be drawn from the fact that endogenous as well as exogenous NO and NO-derived reactive oxygen intermediates e.g. peroxynitrite are reported to act as a trigger for apoptotic cell death in various cell types, including ovarian carcinoma (19-22). In OVCAR-3 and HOC-7, iNOS gene expression induced by the same pro-inflammatory cytokines used in the present study is correlated with the degree of apoptosis in these cells. Moreover, inhibition of iNOS protein activity by aminoguanidine significantly suppressed apoptotic cell death in both cell lines (data not shown). Thus, the decreasing effects of neopterin on iNOS gene expression and NO generation may inhibit apoptosis in ovarian carcinoma cells susceptible to NO.

However, inhibition of iNOS gene expression does not seem to be a general property of neopterin occuring in all cell lines. In contrast to the present study, we could describe stimulation of iNOS gene expression and NO-synthesis in rat vascular smooth muscle cells in response to incubation with neopterin (23). One possible explanation for these discrepancies might be the different interactions between neopterin and intracellular signaling mechanisms known to be influenced by oxidative stress. In previous studies, neopterin was reported to activate NF-κB in rat VSMC (24) which is most likely the biochemical mechanism whereby neopterin stimulates iNOS gene expression in these cells. Similar obeservations were made in the present study, where addition of neopterin to ovarian carcinoma cells led to a translocation of NF-κB into the nucleus. In contrast to VSMC, this was accompanied by a suppression of iNOS gene expression. Transcription of the iNOS gene can not exclusively be attributed to the activation of NF-κB, the interaction between neopterin and other transcription factors might be involved in the decrease of NO production. Apart from interfering with NO synthesis, NF-κB is probably another indicator for the tumor-promoting effects of neopterin. It has been shown that NF-κB plays an important role in cell survival, i.e. it protected immortalized cells from killing by TNF-α, ionizing radiation, and cancer chemotherapeutic compounds (25-27). Recently, constitutive activation of NF-κB was reported to cause resistance to apoptosis in a human T-cell lymphoma cell line (28). In the present experiments with OVCAR-3 and HOC-7, the degree of NF-κB activation was correlated to the amount of neopterin applied to these cells. Thus, neopterin-induced activation of NF-κB might be involved in the survival of ovarian carcinoma cell lines, too.

In conclusion, neopterin-induced inhibition of NO synthesis in ovarian carcinoma cells might serve as an indicator for anti-apoptotic effects exhibited by this pteridine within the tumor tissue. Therefore, increased serum neopterin levels in patients with ovarian carcinomas not only represent a marker of an activated cellular immune system but might be advantageous to tumor progression and thus directly harmful to the host.

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