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Neopterin-induced Suppression of Erythropoietin Production *In Vitro*

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Summary

The production of neopterin increases in several diseases with activation of the cellular immune response. As previously shown serum concentrations of neopterin are inversely correlated with blood hemoglobin concentrations in the anemia of hematological and malignant disorders. Besides the role of chronic immune activation on the disturbed iron metabolism, an inhibitory influence of pteridines on cellular erythropoietin production could not be excluded. To test the possibility that pteridines are able to suppress the hypoxia-induced production of erythropoietin, the effects of neopterin and 7,8-dihydroneopterin on the human cell line HepG2 (hepatocellular carcinoma) were investigated. 24 h incubation with neopterin induced a dose-dependent reduction of erythropoietin production. The erythropoietin concentration significantly decreased by −57.6% with 300 μM and by −34.9% with 100 μM neopterin, respectively. 7,8 dihydroneopterin did not influence erythropoietin production. The inhibitory effect of neopterin on erythropoietin production was a consequence of reduced erythropoietin-mRNA levels. The results of this study show a neopterin-induced suppression of hypoxia-induced erythropoietin formation in HepG2 cultures in a dose dependent manner. We speculate that under in vivo conditions high concentrations of neopterin can aggravate the anemia of chronic disease.

Key words : Erythropoietin, Anemia, Neopterin, 7,8-dihydroneopterin

Introduction

The anemia of chronic disease (ACD) is characterized by relative erythroid bone marrow failure in the presence of moderate shortening of red cell life span in the absence of bleeding, hemolysis, or tumor infiltration in the bone marrow (1, 2). At present the etiology of ACD is not fully understood. Besides the possibility of a functional iron deficiency, as indicated by low serum iron with normal or elevated serum ferritin (2), and an inadequate bone marrow response to secreted erythropoietin (EPO) a suppression of EPO production in relation to the degree of anemia is discussed as further pathogenetic mechanism (4). In fact, in patients suffering from ACD the plasma level of EPO is often lower than expected from the hemoglobin concentration (5-8). Recently, several reports demonstrated that inflammatory cytokines are involved in the pathogenesis of the hypoproliverative anemia of chronic disorders. Cytokines, e.g., interleukin 1 (IL-1), interferons (IFN) α, β and γ as well as tumour necrosis factors (TNF) α and β are potent inhibitors of in vitro growth of erythrocytic progenitors (9-12). Moreover, Jelkmann *et al.* could demonstrate a suppression of EPO production in renal and hepatic tissue by IL-1 and TNF-α (13, 14). Recently, a correlation between the activation state of macrophages, indicated by elevated serum neopterin concentrations, and the degree of anemia in patients with

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hematological disorders and malignant diseases was demonstrated (15, 16).

Besides the role of chronic immune activation in altered iron metabolism a direct influence of the pteridines on EPO production could not be excluded. Thus, we hypothezised that neopterin can reduce EPO formation in vitro. As cell model to test our hypothesis we used the human hepatocellular carcinoma cell line HepG2. HepG2 cells have been previously shown to regulate EPO production in a physiological manner in response to hypoxia and serve as a model system to investigate EPO gene regulation (17). Thus, HepG2 cells appear to reflect the physiological situation of EPO-gene and EPO-protein regulation.

Since a pronounced enhancing effect of neopterin on hydrogen peroxide and chloramine-T-induced chemoluminescence was found recently at slightly alkaline pH (18) we further tested whether alkalosis in addition to exposure to neopterin had an effect on EPO production in HepG2 cells.

Materials and Methods

Cell cultures and incubations

HepG2 cells were obtained from the American Type Culture Collection (ATCC no. HB8065). The cells were maintained in RPMI 1640 medium (Flow Laboratories, Meckenheim, Germany) supplemented with 10% fetal bovine serum (Gibco, Eggenstein, Germany) and $NaHCO_3$ (2.2 g/l) in a humidified atmosphere (5% CO_2 in air) at 37℃ (Heraeus Incubators, Hanau, Germany). Cell monolayers were grown to confluence in 24-well polystyrene dishes (Falcon, Becton Dickinson, Heidelberg, Germany) with a mean density before the experiments of 5×10^5 cells/cm^2. Culture medium (0.5 ml/cm^2) was renewed 24 h prior to the experiments. Substances tested were directly added to the culture medium at the beginning of the 24 h experiments. At the end of the experiments medium was collected and frozen at −20℃ until determination of EPO.

To test whether alkalosis influences the pteridine effects on EPO production, sodium bicarbonate was added to the culture medium to obtain a final bicarbonate concentration of 30 mM and a final medium pH of 7.58.

D-erythro-neopterin and 7,8-dihydroneopterin were obtained from Dr. Schircks Laboratories, Jona, Switzerland.

EPO was measured in duplicate by a radioimmunoassay as described earlier (19) with the exception that polyethylene glycol (PEG 6000; Merck, Darmstadt, Germany) was used to precipitate antibody-bound EPO. In brief, the assay system included ^{125}I-labeled recombinant human EPO (11-13 TBq/mmol; Amersham Buchler, Braunschweig, Germany), rabbit antiserum against recombinant human EPO and human urinary EPO standard calibrated by bioassay against International Reference Preparation B. The EPO concentration was calculated from log-logit plots of the standard curves. The radioactivity was measured using a gamma-counter (Beckman Instruments Inc., Fullerton, CA USA). The main performance parameters were as follows : lower detection limit 5U/l, intra-assay variance <6%, and interassay variance <12% in the range 20-100 U/l. The rate of EPO production was related to the protein content of the cultered cells. For determination of total cellular protein the cell layer was washed with phosphate-buffered saline (PBS) and lysed with 1 ml SDS-NaOH (5 g/l sodium dodecyl sulfate in 0.1 M NaOH). Protein was measured according to the method of Lowry *et al.* (20), using a microdetermination kit (Sigma Diagnostics, Taufkirchen, Germany).

In order to quantify possible cytotoxicity of the pteridines applied, the cytotoxic effects were assesed by means of the [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl] tetrazolium bromide assay as recently described by Wolff and Jelkmann (21).

RNA isolation and polymerase chain reaction

At the end of the 24 h experiments cells were washed with sterile PBS and lysed with 4 M guanidinium isothiocyanate/0.1 M 2-mercaptoethanol. Total RNA was isolated by acid phenol-chloroform extraction according to Chomczynski *et al.* (22), redissolved in water and determined photometrically at an absorbance of 260 nm.

Qualitative polymerase chain reaction was performed as previously described (17). In brief, 1 µg total RNA was reverse-transcribed into first-strand cDNA using oligo $(dT)_{15}$ as a primer for reverse transcriptase (M-MLRV RT Superscript; Gibco). The efficiency of reverse transcription was estimated as described earlier (17).

PCR was performed in PCR buffer (50 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% mass/vol. gelatine), 200 µM of each dNTP, 300 µM of each 5'and 3'primer and 5 U/ml of Taq polymerase (Perkin-Elmer) in a final volume of 100 µl. PCR was run for 30 cycles after an initial denaturation at 95℃ with an amplification profile of each cycle consisting of denaturation for 1 min at 94℃, primer annealing for 1.5 min at 58℃ and elongation

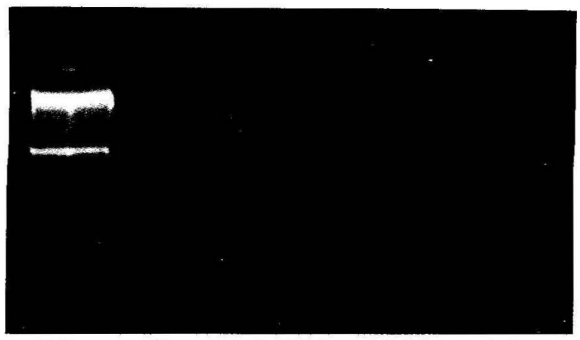

Figure 3. Dose-dependent inhibition of EPO-mRNA levels by neopterin in HepG2 cells. HepG2 cells were incubated for 24 h with neopterin concentrations of 300 µM (N300), 100 µM (N100) or 30 µM (N30). Results from a representative EPO-PCR are shown at a 3% agarose gel stained with ethidium bromide (0.5 µg/ml). Lane M indicates the molecular marker and lane C shows the control experiment.

production at the mRNA level a qualitative EPO-PCR was performed. Figure 3 shows a representative gel of the qualitative EPO-PCR. Compared to the control experiment (=C) lower EPO-mRNA levels were found with higher concentrations of neopterin.

Discussion

The results of this study show a suppressive effect of the macrophages-derived product neopterin on EPO formation in HepG2 cells (Fig 1). The inhibitory influence of neopterin on EPO production was dose-dependent and resulted from decreased EPO-gene expression as indicated by reduced EPO-mRNA (Fig. 3). 7,8-dihydroneopterin did not influence Epo production (Fig. 1).

Neopterin and 7,8-dihydroneopterin are produced in excess by human monocytes/macrophages upon activation by interferon-γ (23). Several studies indicate a diagnostic value of neopterin in many diseases, e.g., malignant and infectious diseases such as AIDS and hepatitis (reviewed in 24, 25). Chronic infectious, inflammatory and malignant diseases are regularily associated with anemia. The pathogenesis of ACD is multifactorial including several aggravating factors that are at present unknown. In most anemias hemoglobin concentration in plasma determines EPO production which in turn stimulates bone marrow erythropoietic activity. Each mechanism leading to a disruption of the expected feedback mechanism of EPO production causes an inadequately low EPO concentration. In fact, in many cases of ACD circulating EPO was observed to be relatively low in relation to the degree of anemia, sometimes leading to a loss of the inverse correlation between hemoglobin and EPO (4).

Recently an association between elevated levels of neopterin (serum or urine) and the degree of anemia was described in patients with HIV infection (26), with gynecological cancer (15) and with malignant hematological disorders (16). Moreover a correlation was found between neopterin and serum ferritin and an inverse correlation between neopterin and serum iron, respectively. From these studies it appeared that persistent immune activation is involved in the transfer of serum iron into storage sites thus aggravating ACD (27). We report for the first time a direct influence of neopterin on cellular regulation of EPO production. HepG2 cells grown to confluence continuously produce EPO since under conventional culture conditions cultured cells are permanently hypoxic due to diffusion-limited O_2 supply (28). Neopterin at 300 µM and 100 µM concentrations significantly suppressed the continous EPO production in HepG2 cells, whereas 7,8-dihydroneopterin had no effect (Fig. 1). Serum concentrations of neopterin in healthy individuals usually range from 3-8.7 nmol/l and may reach approximately 300 nmol/l in severely ill patients with, e.g., end stage HIV infection or with uremia (29, 30). Neopterin concentrations at the local site of production may well reach and even exceed the level found to be effective in our test system. Moreover, neopterin levels are approximately 200-fold concentrated in the kidney before excretion(29).

At present no data are available concerning the signalling pathway of pteridines in HepG2 cells. In human monocytic cells both, neopterin as well as 7,8-dihydroneopterin, increase intracellular calcium concentrations possibly by activating calcium channels (31). Only a few studies investigated the influence of calcium on EPO production. McGonigle *et al.* (32) described an enhanced EPO secretion in hypoxic rats after application of calcium channel blockers. This result was confirmed in human renal carcinoma cells which produced more EPO in the presence of low extracellular calcium (33). If, on the other hand, elevated cell calcium can inhibit EPO production is at present unknown. Thus, we only can speculate that the neopterin-induced EPO suppression in HepG2 cells is induced by an altered cell calcium level.

Recently, Überall *et al.* (34) reported that both pteridines, neopterin and 7,8-dihydroneopterin, particularly together with cyclic guanosine monophosphate (cGMP), induce c-fos gene expression. In a study by Ohigashi *et al.* (35), hypoxia induced EPO

production in the hepatocellular carcinoma line Hep3B was in part mediated by elevated cGMP levels and reduced by LY 83583, an inhibitor of the soluble guanylate cyclase. If in our study the reduction of EPO by neopterin was associated with the generation of cGMP or an induced c-fos gene expression, needs further investigations.

A recent study by Weiss *et al.* (18) showed that neopterin but not 7,8-dihydroneopterin pronounced the hydrogen peroxide (H_2O_2) and chloramine-T-induced chemoluminescence at slightly alkaline pH. The data suggested that neopterin may enhance the formation of reactive molecules originating from H_2O_2 or chloramine-T or, that neopterin itself could be converted into a reactive intermediate upon reaction with H_2O_2 or chloramine-T. Fandrey *et al.* (36) reported an inhibitory effect of exogenous as well as endogenous H_2O_2 on EPO production in HepG2 cells. They suggested that an H_2O_2-generating heme protein might be part of the O_2-sensor controlling EPO production. In our study neopterin plus alkalinisation lead to a shift in the dose-response indicated by a significantly reduced EPO production even at a neopterin concentration of 30 µM. 7,8-dihydroneopterin in an alkalotic medium did not influence 24h EPO production. Thus, one possible mechanism for neopterin-induced EPO suppression could be mediated by hydrogen peroxide formation. In conclusion, neopterin but not 7,8-dihydroneopterin suppresses EPO-gene expression. Thus, neopterin may be involved in the cellular regulation of EPO production. We speculate that in several clinical syndromes associated with an inadequate EPO response to anemia and activated cellular immune response, neopterin can aggravate the anemia.

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