ERYTHROPOIETIN INCREASES Epo AND EpoR EXPRESSION IN DLD-1 CELLS

Anna Tankiewicz-Kwedlo[1], Dariusz Pawlak[2,3],
Tomasz Domaniewski[2], Włodzimierz Buczko[1]

[1] *Department of Pharmacodynamics, Medical University of Bialystok, Poland*
[2] *Department of Monitored Pharmacotherapy, Medical University of Bialystok, Poland*
[3] *Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Poland*

ABSTRACT

Introduction. Supplementation of recombinant human erythropoietin (rHuEpo) is one of the methods for the treatment of anemia for patients with colon cancer. However, the results of *in vitro* studies investigating the influence of rHuEpo on cancer cells are contradictory.

Aim. The aim of the present study was an assessment of the effect of rHuEpo on proliferation, as well as Epo and EpoR protein expressions in normoxia and hypoxia conditions on human colon adenocarcinoma cells (DLD-1).

Materials and methods. The cells were cultured in medium with rHuEpo in concentrations of 1 and 3 IU without (normoxia) or with (hypoxia) cadmium chloride for 48 hours. Cell viability was counted using a haematocytometer and trypan blue 0.4% (w/v) dye. Expression of Epo and EpoR protein was assessed by western blot.

Results and Discussion. We observed a decrease in the number of colon cancer cells in hypoxia. Addition of rHuEpo did not modify cell numbers in normoxia and hypoxia. We found a significant increase of EpoR expression in all cells growing in medium with cobalt chloride in comparison with respective normoxic cells. We also noted that rHuEpo in concentration of 3 IU significantly increased expression of Epo and EpoR protein in colon cancer cells in normoxia and hypoxia conditions.

Conclusions. We concluded that Epo and EpoR are constitutively expressed in DLD-1 cells. In hypoxia as well as in the presence of rHuEpo the increase of Epo and EpoR protein was found. However, the expression of Epo and EpoR protein in these cells does not seem essential to their growth.

Key words: erythropoietin (Epo), erythropoietin receptor (EpoR), recombinant human erythropoietin (rHuEpo), colon cancer cells.

Corresponding address: Anna Tankiewicz-Kwedlo, Zakład Farmakodynamiki, Uniwersytet Medyczny w Białymstoku, ul. Mickiewicza 2C, 15-089 Białystok, Poland; phone: +48 85 748 56 01, fax: +48 (85) 748 56 01, e-mail: aniatan@poczta.onet.pl

INTRODUCTION

Erythropoietin (Epo) is a 30.4-kDa glycoprotein hormone produced and secreted in the kidneys of adults and in fetal liver in response to hypoxia as a 166-amino acid peptide [13]. During maturation a carboxy-terminal arginine in position 166 is removed resulting in a circulatory mature 165-amino acid protein. Endogenous as well as exogenous peptide (recombinant human Epo, rHuEpo) has the same composition [15]. The main physiological role of Epo is the stimulation of erythropoiesis. However, the results of numerous *in vivo* studies have shown that Epo protects against ischemia and trauma of the brain, retina, and spinal cord in animals [7, 8, 10]. Moreover, in *in vitro* studies it has been shown to stimulate angiogenesis, cell proliferations and vessel formation [15].

The effect of Epo is mediated by binding to the erythropoietin receptor (EpoR), the expression of which has been shown in nonhematopoietic cells and tissues such as endothelial cells, brain, female genital tract, placenta, myoblasts, kidney, intestine and various cancers [13]. EpoR is a transmembrane protein. The presence of EpoR in these cells may suggest the participation of Epo in autocrine or paracrine mechanisms. The binding of Epo to EpoR activates also others cascades that lead to the enhancement of proliferation, differentiation and survival [13, 16]. Thus, an endogenous Epo/EpoR system plays a prominent role in developing many tissues, including cancers.

In solid cancers, including colon cancer, Epo and EpoR expressions are mainly regulated by hypoxia via hypoxia inducible factors-1 (HIF-1) [1]. HIF-1 is composed of HIF-1A and HIF-1B and under normoxic as well as hypoxic conditions mRNA in both of them are constantly expressed in a number of mammalian cell lines. However, HIF-1A protein is markedly increased by hypoxia, whereas HIF-1B protein is constantly present regardless of oxygen tension [9]. Then, HIF-1A is the main regulator of Epo and EpoR expressions in hypoxic cancer cells.

In vitro studies investigating the role of Epo and Epo–EpoR signaling in tumor growth and angiogenesis have yielded contradictory results. Yasuda et al. observed the inhibition of angiogenesis and tumor cell survival in stomach and melanoma xenografts following the blockade of Epo–EpoR signaling [25]. These results are in opposition to the findings of Hardee et al. who did not observe any effects on angiogenesis and tumor growth in colon and head and neck xenografts after Epo administration [12].

AIM

At present in literature there have been no reports describing the influence of rHuEpo on human colon adenocarcinoma cells (DLD-1). Thus, the aim of our study was to estimate whether rHuEpo might directly affect human colon cells in normoxia and hypoxia. We also assayed the influence of rHuEpo on Epo and EpoR protein expressions in DLD-1 cells in normoxia and hypoxia conditions.

MATERIALS AND METHODS

Reagents

RPMI-1640 medium, fetal bovine serum, penicillin and streptomycin were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA), RhEpo beta (NeoRecormon, Roche) was purchased from Roche (Basel, Switzerland) and cadmium chloride was provided by Sigma (Sigma-Aldrich, St. Louis, MO, USA).

Cell culture

DLD-1 cells were obtained from American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (ATCC), penicillin (50 IU), streptomycin (50 μg/L) in humidified atmosphere (90% relative humidity) with 5% CO_2 at 37°C. The culture media were changed every two days. Cells were generally maintained in 75 cm^2 flasks (Sarstedt Inc., Newton, NC, USA) but for the experiments, they were plated onto 100-mm dishes (Sarstedt) with 6 mL of medium. The cells were incubated for a 24-hour period prior to treatment and allowed to proliferate to 70–80% confluence before the commencement of each experiment, without serum in RPMI-1640 medium. The control was media with PBS only. For all experiments, cells at the 5th to 9th passages were used.

Drug treatment

Prior to each treatment, DLD-1 cells were grown until they reached 50–60% confluence. Then the cells were treated with rHuEpo in the final concentration of 1 IU and 3 IU. Untreated cells were used as control. The incubation time was 48 hours.

Cell number

The DLD-1 cells (5×10^5 cells) were plated on 6-well cluster plates (Nunck, Denmark) in 2 mL medium, and cultured for up to 96 hours at 37°C. Cells were harvested by 0.25% trypsin treatment, and counted at each time point using haematocytometer. Samples were analyzed in triplicate at 48 hours after cell culture was initiated. Cell viability was expressed as a number of viable cells counted using a haematocytometer and trypan blue 0.4% (w/v) dye.

Western blot analysis for Epo, EpoR, and β-actin

Cells were lysed in NP-40 buffer consisting of 50 mmolar Tris-HCL (pH 8.0), 150 mmolar NaCl, 1% Triton X-100 and protease inhibitor cocktail (Roche). The lysate was centrifuged at $10\,000 \times g$ for 20 minutes at 4°C. An aliquot (10 μL) of supernatant was subjected to electrophoresis on a 10% SDS-PAGE, followed by transfer to 0.2 μm pore-sized nitrocellulose membrane (Bio-Rad) according to the method described in the manual accompanying the unit. Blots were blocked for 1 hour at room temperature with 5% non-fat milk (Bio-Rad) in Tris-buffered saline, pH 8.0 (Sigma-Aldrich).

The nitrocellulose was incubated overnight with: mouse anti-Epo (1:400), goat anti-
-EpoR (1:500), mouse anti-Actin (1:3000) antibodies from Sigma-Aldrich in TBS-T
(20 mmol/L Tris–HCl buffer, pH 7.4, containing 150 mmol/L NaCl and 0.05% Tween
20). Secondary antibodies alkaline phosphatase conjugated were goat anti-mouse or
rabbit anti-goat conjugated antibodies (Sigma-Aldrich) were added at concentration
of 1:10 000 in TBS-T and incubated for 1 hour by slowly shaking. Then nitrocellulose
was washed with TBS-T (2×10 min) and submitted to Sigma-Fast BCIP/NBT reagent.
The intensity of the bands was quantified by densitometric analysis using Image J 1.37a
software (National Institutes of Health, USA).

Statistical analysis

Shapiro–Wilk's *W*-test for normality was used for data distribution analysis. In all
experiments, the mean values for six assays with standard deviation were calculated
unless otherwise indicated. The differences between groups were estimated with the
Tukey–Kramer Multiple Comparisons Test. If *P* value was less than 0.05, it was consid-
ered statistically significant.

RESULTS

We observed a significant decrease in the number of cancer cells in hypoxia in compari-
son to normoxia conditions. Medium with rHuEpo in concentrations of 1 and 3 IU did
not indicate a significant impact on a number of these cells growing up under normoxia
as well as hypoxia conditions. Also, no differences in the number of cancer cells were
found in DLD-1 cells growing in medium with solvent of rHuEpo and control (Fig. 1).
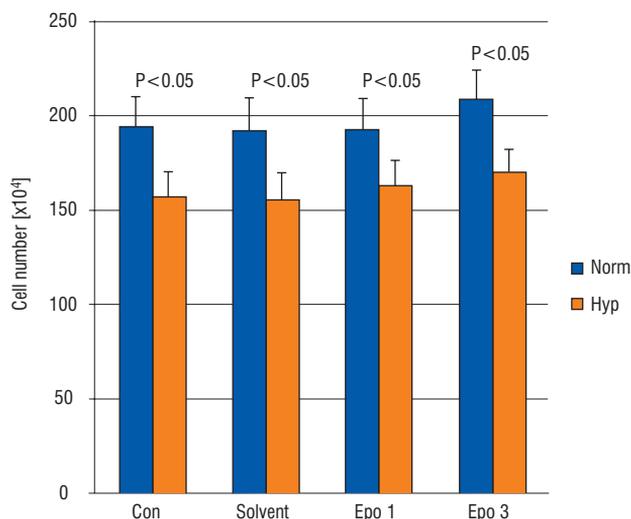

Fig. 1. Effect of rHuEpo on cell number in normoxia (21% of O_2) and hypoxia (250 μmolar $CoCl_2$).

A significant increase of Epo expression was observed in the cells growing in medium with 3 IU rHuEpo in comparison to control. The addition of $CoCl_2$ to medium also caused an increase of Epo expression in comparison to control as well as in comparison to group of cells growing in medium with rHuEpo in concentration of 3 IU in normoxia conditions (Fig. 2).
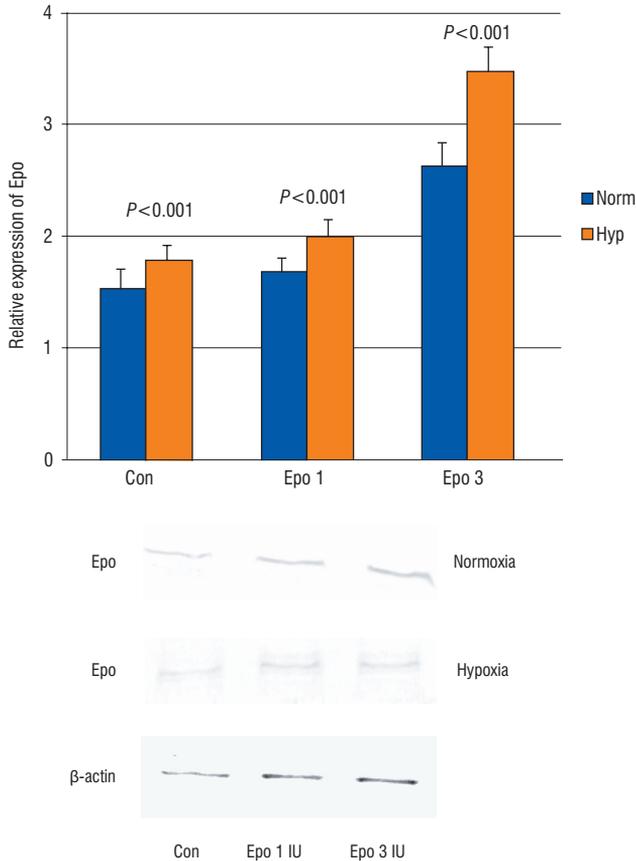

Fig. 2. Epo expression in DLD-1 cells treated with rhEpo in concentrations of 1 and 3 IU for 48 hours in normoxia (21% of O_2) and hypoxia (250 µmolar $CoCl_2$) conditions by western immunobloting.

In normoxia, an increase in EpoR expression was observed in cells growing in medium with 3 IU of rHuEpo in comparison to control. In all DLD-1 cells growing in hypoxia a significant increase of EpoR expression was found in comparison to the expression observed in cells growing without $CoCl_2$. In hypoxia, an increase in EpoR expression in cells growing in medium with rHuEpo in concentration of 3 IU was observed in comparison to control as well as to expression of EpoR occurrence in cells growing in medium with 1 IU of rHuEpo (Fig. 3).
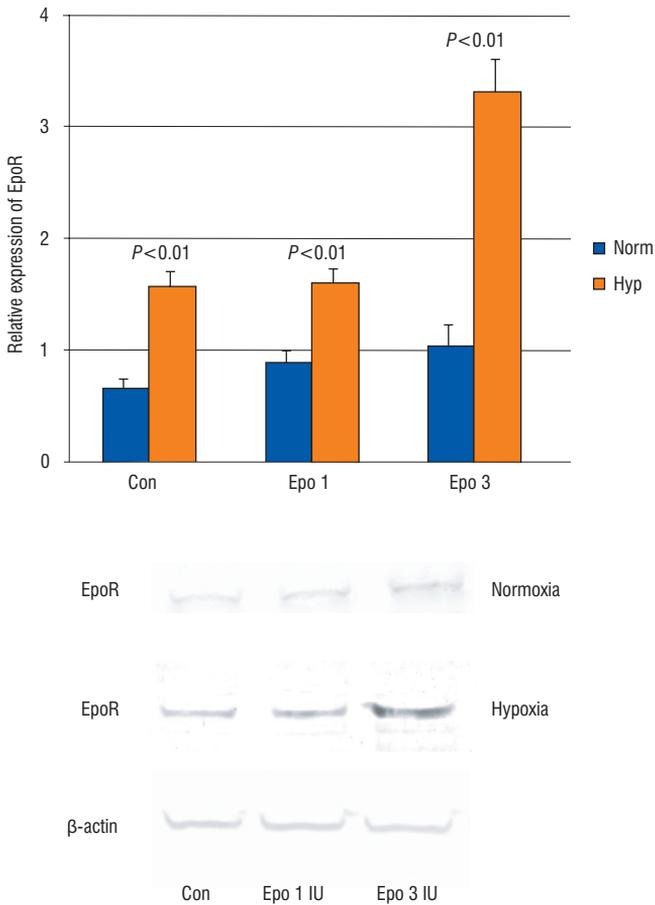
Fig. 3. EpoR expression in DLD-1 cells treated with rHuEpo in concentrations of 1 IU and 3 IU for 48 hours in normoxia (21% of O_2) and hypoxia (250 μmolar $CoCl_2$) conditions by western immunobloting.

DISCUSSION

The influence of rHuEpo on DLD-1 cells in normoxia and hypoxia conditions has not been studied yet. In the present study we observed that induction of hypoxia conditions by cobalt chloride caused a significant decrease in the number of colon cancer cells. The addition of rHuEpo did not modify the cell numbers in normoxia and hypoxia. In all cells growing in medium with cobalt chloride we observed a significant increase of EpoR expression in comparison with respective normoxic cells. We also found that rHuEpo in concentration of 3 IU significantly increased the expression of Epo and EpoR protein in colon cancer cells in normoxia. The same effect was observed in hypoxia conditions.

Our results are in line with the study of Song at al. who demonstrated that hy-poxia inhibits cell proliferation. They postulated that the expression of HIF-1A and caspase-3 resulted in the production of the apoptosis of periodontal ligament cells [19]. Moreover, Yamamoto et al. showed translocation of a pro-apoptotic factor, Bax, from the cytoplasm to the mitochondrial membrane and induction by caspase-3--like activity-dependent apoptosis in response to hypoxia. At the same time, they observed a significant decrease in anti-apoptotic factors such as Bcl-2 and Bcl-xL in epithelial cells under hypoxic conditions [23]. However, the influence of hypoxia on the proliferation of cancer cells is not clear and some of the published results are apparently conflicting. Sahai et al. demonstrated that chronic hypoxia induces prolif-eration of proximal tubule cells mediated by the activation of protein kinase C (PKC) [17]. Yang and Kang found that suppression of HIF-1A gene results in a decrease of pancreatic cancer cell proliferation [24]. In turn, Volm and Koomägi did not observe a relationship between HIF-1A or HIF-1B and proliferation, but found a significant correlation between HIF-1 expression, apoptosis and the pro-apoptotic factors cas-pase-3, Fas, and Fas ligand [20].

In the next set of experiments, our objective was to determine the expression of Epo and EpoR protein. We proved the presence of Epo and EpoR protein in DLD-1 cells in normoxia and hypoxia conditions. The literature data showed no significant measurable Epo-specific binding activity on the surface of the head and neck squa-mous carcinoma cells in normoxia and a significant increase of EpoR expression in hypoxia and rHuEpo treatment [14]. On the other hand, another study reported EpoR expression in DLD-1 cells in normoxia conditions [4].

The presence of EpoR on the cancer cell surface may suggest a potential role of Epo in the activation of specific signal transduction of these cells. Indeed, Hammerling et al. demonstrated a 650% increase in the proliferation of erythroid cell line after administration of rHuEpo in pharmacologically relevant doses (0.01–0.4 IU) [11]. However, 68 tumor cell lines with no observed biological response to rHuEpo have been described [18]. Moreover, rHuEpo (dose range 0.01–100 IU) did not have an impact on human solid tumors (head and neck, lung, breast, stomach, colorectal, hepatocellular, pancreas, ovary, choriocarcinoma, osteogenic sarcoma, glioblastoma, neuroblastoma, prostate, renal) in *in vitro* experiments [6]. Further, another study demonstrated the absence of biological response of cancer cells to this peptide [5]. Therefore, in the next part of the experiments we studied the influence of rHuEpo on cell number as well as Epo and EpoR expressions. In our *in vitro* study we showed the lack of effect of rHuEpo in concentrations of 1 and 3 IU on DLD-1 cells in nor-moxia and hypoxia conditions. Similar results were achieved by Wang et al. who demonstrated no toxicity under normal conditions over a 48-hour period in retinal pigment epithelium cells treated with rHuEpo at concentrations of 0.001–100 IU/mL compared with control [21]. Also results of another study show that in hypoxia con-

ditions rHuEpo in concentration of 30 IU had no effect on head and neck squamous cells survival [14]. Thus, it seems that rHuEpo stimulates proliferation of hematological cancers but in the case of other tumors its effect depends on the types of cells and probably also on conditions of culture.

However, we found that an addition of rHuEpo to the growth medium led to an increase of Epo and EpoR expressions both in normoxia and hypoxia conditions. This observation is in line with findings published by Lo Nigro et al., who demonstrated that the addition of rHuEpo in culture medium increased EpoR expression [14]. Moreover, in an *in vivo* study a positive correlation between the semi-quantitative EPO score and the EPOR score in breast cancer tumor cells [2] and prostate cancer [3] was found.

In our *in vitro* study we proved the presence of EpoR in DLD-1 cells, but we did not observe evidence of the biological action of rHuEpo on these cells except for an enhancement of Epo and EpoR expressions. Westphal et al. suggested that only 60% of the newly synthesized EpoR is processed to the glycosylated receptor protein in the Golgi apparatus. It is possible that the performed staining showed both mature and also premature protein [22]. Thus, not all receptors detected by western blot possess biological activity and, despite an increase of EpoR expression, we did not observe any effects of rHuEpo.

CONCLUSIONS

We conclude that Epo and EpoR are constitutively expressed in DLD-1 cells. In hypoxia, as well as in the presence of rHuEpo, the increase of Epo and EpoR protein was found. However, the expression of Epo and EpoR protein in these cells does not seem essential to their growth.

ACKNOWLEDGMENTS

REFERENCES

1. Acs G., Acs P., Beckwith S. M., Pitts R. L., Clements E., Wong K., Verma A.: *Erythropoietin and erythropoietin receptor expression in human cancer*. Cancer Res., 2001; 61 (9): 3561–3565.
2. Arcasoy M. O., Amin K., Karayal A. F., Chou S. C., Raleigh J. A., Varia M. A., Haroon Z. A.: *Functional significance of erythropoietin receptor expression in breast cancer*. Lab. Invest., 2002; 82 (7): 911–918.
3. Arcasoy M. O., Amin K., Vollmer R. T., Jiang X., Demark-Wahnefried W., Haroon Z. A.: *Erythropoietin and erythropoietin receptor expression in human prostate cancer*. Mod. Pathol., 2005; 18 (3): 421–430.
4. Arcasoy M. O., Jiang X., Haroon Z. A.: *Expression of erythropoietin receptor splice variants in human cancer.* Biochem. Biophys. Res. Commun., 2003; 307 (4): 999–1007.
5. Berdel W. E., Danhauser-Riedl S., Oberberg D., Zafferani M.: *Effects of hematopoietic growth factors on malignant nonhematopoietic cells.* Semin. Oncol., 1992; 19 (2 Suppl 4): 41–45.
6. Berdel W. E., Oberberg D., Reufi B., Thiel E.: *Studies on the role of recombinant human erythropoietin in the growth regulation of human nonhematopoietic tumor cells in vitro.* Ann. Hematol., 1991; 63 (1): 5–8.

7. Brines M. L., Ghezzi P., Keenan S., Agnello D., de Lanerolle N. C., Cerami C., Itri L. M., Cerami A.: *Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury.* Proc. Natl. Acad. Sci. U. S. A., 2000; 97 (19): 10526–10531.

8. Celik M., Gökmen N., Erbayraktar S., Akhisaroglu M., Konakc S., Ulukus C., Genc S., Genc K., Sagiroglu E., Cerami A., Brines M.: *Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury.* Proc. Natl. Acad. Sci. U. S. A., 2002; 99 (4): 2258–2263.

9. Chun Y. S., Choi E., Kim G. T., Choi H., Kim C. H., Lee M. J., Kim M. S., Park J. W.: *Cadmium blocks hypoxia-inducible factor (HIF)-1-mediated response to hypoxia by stimulating the proteasome-dependent degradation of HIF-1alpha.* Eur. J. Biochem., 2000; 267 (13): 4198–4204.

10. Grimm C., Wenzel A., Stanescu D., Samardzija M., Hotop S., Groszer M., Naash M., Gassmann M., Remé C.: *Constitutive overexpression of human erythropoietin protects the mouse retina against induced but not inherited retinal degeneration.* J. Neurosci., 2004; 24 (25): 5651–5658.

11. Hammerling U., Kroon R., Wilhelmsen T., Sjödin L.: *In vitro bioassay for human erythropoietin based on proliferative stimulation of an erythroid cell line and analysis of carbohydrate-dependent microheterogeneity.* J. Pharm. Biomed. Anal., 1996; 14 (11): 1455–1469.

12. Hardee M. E., Arcasoy M. O., Blackwell K. L., Kirkpatrick J. P., Dewhirst M. W.: *Erythropoietin biology in cancer.* Clin. Cancer Res., 2006; 12 (2): 332–339.

13. Larsson A. M., Jirström K., Fredlund E., Nilsson S., Rydén L., Landberg G., Påhlman S.: *Erythropoietin receptor expression and correlation to tamoxifen response and prognosis in breast cancer.* Clin. Cancer Res., 2009; 15 (17): 5552–5559.

14. Lo Nigro C., Maffi M., Fischel J. L., Monteverde M., Catarsi P., Tonissi F., Lattanzio L., Riba M., Etienne-Grimaldi M. C., Formento P., Milano G., Merlano M.: *Impact of erythropoietin on the effects of irradiation under hypoxia.* J. Cancer. Res. Clin. Oncol., 2009; 135 (11): 1615–1623.

15. Maiese K., Chong Z. Z., Li F., Shang Y. C.: *Erythropoietin: elucidating new cellular targets that broaden therapeutic strategies.* Prog. Neurobiol., 2008; 85 (2): 194–213.

16. Remy I., Wilson I. A., Michnick S. W.: *Erythropoietin receptor activation by a ligand-induced conformation change.* Science, 1999; 283 (5404): 990–993.

17. Sahai A., Mei C., Zavosh A., Tannen R. L.: *Chronic hypoxia induces LLC-PK1 cell proliferation and dedifferentiation by the activation of protein kinase C.* Am. J. Physiol., 1997; 272 (6 Pt 2): F809–815.

18. Sinclair A. M., Todd M. D., Forsythe K., Knox S. J., Elliott S., Begley C. G.: *Expression and function of erythropoietin receptors in tumors: implications for the use of erythropoiesis-stimulating agents in cancer patients.* Cancer, 2007; 110 (3): 477–488.

19. Song Z. C., Shu R., Li X. T., Hu J. C., Zhang X. L.: *Influence of cobalt chloride-induced hypoxia on the proliferation and apoptosis of periodontal ligament cells.* Shanghai Kou. Qiang. Yi Xue., 2009; 18 (5): 489–492.

20. Volm M., Koomägi R.: *Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer.* Anticancer Res., 2000; 20 (3A): 1527–1533.

21. Wang Z. Y., Shen L. J., Tu L., Hu D. N., Liu G. Y., Zhou Z. L., Lin Y., Chen L. H., Qu J.: *Erythropoietin protects retinal pigment epithelial cells from oxidative damage.* Free Radic. Biol. Med., 2009; 46 (8): 1032–1041.

22. Westphal G., Niederberger E., Blum C., Wollman Y., Knoch T. A., Rebel W., Debus J., Friedrich E.: *Erythropoietin and G-CSF receptors in human tumor cells: expression and aspects regarding functionality.* Tumori, 2002; 88 (2): 150–159.

23. Yamamoto K., Morishita R., Hayashi S., Matsushita H., Nakagami H., Moriguchi A., Matsumoto K., Nakamura T., Kaneda Y., Ogihara T.: *Contribution of Bcl-2, but not Bcl-xL and Bax, to antiapoptotic actions of hepatocyte growth factor in hypoxia-conditioned human endothelial cells.* Hypertension, 2001; 37 (5): 1341–1348.

24. Yang L., Kang W. K.: *The effect of HIF-1alpha siRNA on growth and chemosensitivity of MIA-paca cell line.* Yonsei. Med. J., 2008; 49 (2): 295–300.

25. Yasuda Y., Fujita Y., Matsuo T., Koinuma S., Hara S., Tazaki A., Onozaki M., Hashimoto M., Musha T., Ogawa K., Fujita H., Nakamura Y., Shiozaki H., Utsumi H.: *Erythropoietin regulates tumour growth of human malignancies.* Carcinogenesis, 2003; 24 (6): 1021–1029.