HYPOXIA INDUCES ERYTHROPOIETIN RECEPTOR EXPRESSION ON K562 CELL LINE

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ABSTRACT
The erythropoietin receptor (EpoR) and erythropoietin (Epo) mediate erythropoietin-induced erythroblast proliferation, differentiation and survival. This study examined the effects of the expression of EpoR on K562 (erythroleukemia) cells upon normoxia and hypoxia. In addition, the impact of the combined effect of recombinant human Epo and hypoxia was investigated. K562 (erythroleukemia) cells and as control group HL60 (promyeloblast) cells were cultured. Hypoxic incubation was performed with 5% O₂, 5% CO₂ and balance Nitrogen for 24 hours. After 24 hours, K562 and HL60 cells were transferred to normoxic and 5% hypoxic conditions. Recombinant Erythropoietin-alpha was added (10 U/ml) to the cells at the beginning of the experiment. Cultured cells were subjected to viability analysis, total RNA isolation, and protein isolation at three timepoints: 3 h, 6 h, and 24 h. Viability was analysed with a trypan blue exclusion using the Vi-Cell automated cell viability system. RT-PCR and western blot results of normoxic and hypoxic K562 and HL60 cell lines with/without rhEPO were compared. We showed that hypoxia upregulates the expression of EpoR on K562 erythroleukemia cells, and longer exposition to hypoxia turns the upregulated EpoR expression to basal levels. Recombinant human Epo (rhEpo) did not produce further impact in hypoxia-induced upregulation of EpoR neither in normoxia nor in hypoxia. Although addition of the rhEpo caused change in expression of cell-cycle regulators, it seems not to effect cell proliferation or cell viability. In the HL60 cell line, however, we detected EpoR mRNA, but not Epo mRNA by RT-PCR. Hypoxia did not alter the level of EpoR mRNA expression. The results of this study suggest that the expression of EpoR might be regulated by hypoxia, but not by Epo, and the expression of cell-cycle regulators might be regulated by both hypoxia and Epo.

Keywords: K562 cell line, hypoxia, EpoR, Epo, HL60 cell line

Introduction
The erythropoietin receptor (EpoR) is a member of the cytokine receptor superfamily and is activated by its native ligand, erythropoietin (Epo) (1, 5). EpoR and Epo mediate erythropoietin-induced erythroblast proliferation, differentiation and survival. In addition to hematopoietic precursor cells, Epo also elicits responses in other tissues depending on the level of expression of its receptors. A growing number of studies demonstrates functional Epo/EpoR signaling events in cancer cells that contribute to disease progression (3). The expression of erythropoietin and EpoR have been recognized in a variety of human cancers, including breast, prostate, colon, ovary, uterine, cervical, glioblastoma, and head and neck squamous cell carcinoma (2, 4). Recent studies have shown that Epo is a pleiotropic cytokine that is proangiogenic and exerts broad tissue-protective effects on nonhematopoietic organs (12). Tumour hypoxia is common among a wide variety of malignancies and is a factor associated with treatment resistance, aggressive clinical phenotype, and poor prognosis (7, 11). Laugsch and his colleagues examined the expression of EpoR under hypoxia in a subset of 10 human cancer cell lines. They reported that hypoxia does not alter the level of EpoR mRNA expression in cancer cell lines (8). K562 cells express the p210 form of the Bcr-Abl gene fusion, which is the predominant form found in CML (9). Epo rescues K562 cells from imatinib-associated cell death and supports their proliferation (6). Here, we show that hypoxia induces EpoR mRNAs as well as its protein in the K562 cell line. We suggest that EpoR upregulation during hypoxia may have a role on the progression of malignancies.

Materials and Methods
Cell culture and Epo treatments
K562 (erythroleukemia) cells were grown in complete RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (Sigma, St Louis, MO), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM) at 37°C in a humidified atmosphere containing 5% CO₂. HL60 (promyeloblast) cells were grown in complete Iscove’s Modified Dulbecco’s Medium, 20% heat inactivated fetal calf serum, penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (2 mM) at 37°C in a humidified atmosphere containing 5% CO₂. Hypoxic incubation was performed in a humidified incubator (Termo Scientific, USA) with 5% O₂, 5% CO₂ and balance Nitrogen for 24 hours. K562 and HL60 cells were cultured in 6-well dishes the day before. At 8:00 pm the following day, cells were
transferred to normoxic and 5% hypoxic conditions. In parallel, we also tested the effect of recombinant Epo during normoxia/hypoxia in K562 cells. Recombinant Erythropoietin-alpha (NeoRecomon, Roche/Boehringer, Mannheim, Germany) was added (10 U/ml) to the cells at the beginning of the experiment. Cultured cells were subjected to viability analysis, total RNA isolation, and protein isolation at three time-points: 3 h, 6 h, and 24 h. Viability was analysed with a trypan blue exclusion using the Vi-Cell automated cell viability system (Beckman Coulter, USA). RT-PCR and western blot results of normoxic and hypoxic K562 and HL60 cell lines with/without rhEPO were compared.

**Real-time PCR**

Cells in 6-well dishes were washed once with cold PBS and cells were subjected to RNA isolation. RNA was isolated with Roche’s MagNA Pure automated system (Roche, Germany). mRNA was generated by random hexamer priming and Roche’s mRNA RT-PCR kit. EpoR mRNA was quantified in duplicate on Roche LightCycler 2.0 detection system by means of a SYBR green PCR kit (Takara) and program: 95°C for 10 seconds, 95°C for 5 seconds, 56°C for 25 seconds, 72°C for 10 seconds for 35 cycles. Primer sequences for EpoR were forward 5-GAT GCC AGG CCA GAT CTT C-3 (exon 6) and reverse 5-CTC ATC TTC GTG GTG ACG CT-3 (exon 7). For normalization of mRNA data, b2-microglobulin gene product was quantified and used as an internal control. A cloned b2-microglobulin gene was used as an external control. Primer sequences for b2-microglobulin were forward 5-TGC CGT TTC AAC CAT GTG AC-3 and reverse 5-ACC TTC GTG ATG ATG CTT ACA-3. Expression levels were calculated with the Ct method and related to the respective normoxic control cultures. We calculated a relative expression unit (expression of the target gene/expression of b2Mx100) and provided the results as mean (median) relative expression unit±standard deviation. Specificity of the EpoR primer was verified by agarose gel electrophoresis, melting point curve analysis, and also sequence analysis.

**Immunoblotting**

Cells were homogenized in lysis buffer [50 mM Hepes, 150 mM NaCl, 0.5% Triton-X 100, 1mM DTT and protease inhibitor tablet (Roche)]. Samples were centrifuged at 13 000 rpm at 4°C for 10 min, and the supernatant was extracted and stored at -20°C. Cell lysates were normalized according to the Lowry-method. Denatured 20 μg cell lysates were separated by 12.5% SDS-PAGE at 90 volts for 1 h and transferred to PVDF membranes (Millpore) by semidry transfer (Bio-Rad, CA, USA).

Membranes were blocked in tris-buffered saline (TBS; 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5% BSA fraction V) for 30 min at room temperature (RT). Incubation with the anti-EpoR antibody (Santa Cruz, CA, USA) at a concentration of 2.5 μg/ml in TBS was performed at RT for 2 h. After three washes with TBS, membranes were incubated for 45 min with horseradish peroxidase conjugated secondary antibody diluted in TBS [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20]. Chemiluminescence detection was performed by exposure of Biomax MR films (Kodak) to Lumi-Light western blotting substrate-treated membranes (Pierce, USA). The molecular masses of the protein fragments were determined using a protein ladder (Fermentas, Germany).

**Results and Discussion**

Our RT-PCR and western blot results showed a dramatic increase of EpoR mRNA and its protein in the third hour under hypoxic conditions (Fig. 1 and Fig. 2). Our results showed that (as compared to normoxia) hypoxia upregulates the expression of EpoR after 3 hours from the exposition. Longer exposition to hypoxia (6 h & 24 h) leads the EpoR expression to return back to basal levels.

**Fig. 1. RT-PCR results of K562 EpoR mRNA**

The black bars represent normoxic K562 cells; The grey bars represent hypoxic K562 cells

**Fig. 2. Western blot analysis of EpoR protein in hypoxic and normoxic K562 cells.** Anti-EpoR antibody has interacted with the 60 kDa EpoR protein

**Fig. 3. K562 cells with exogeneous rhEPO during hypoxia and normoxia**
We also tested the exogeneous effect of Epo on K562 cells, which exhibited hypoxia-induced upregulation of EpoR expression. We added 10 U/ml of recombinant human Epo (rhEpo) to the cultured K562 cells during hypoxia, but there was no additive effect of exogeneous Epo on expression level of its receptor, EpoR. RT-PCR results showed downregulation of EpoR expression after addition of rhEpo (Fig. 3).

We could detect Epo mRNA in K562 cells by RT-PCR (Fig. 4), but we could not detect any Epo protein by western blot (data not shown).

We compared HL60 myeloid cell line as a control in this experiment. We detected EpoR mRNA in the HL60 cell line, but not Epo mRNA by RT-PCR. Hypoxia did not alter the level of EpoR mRNA expression (Fig. 5). There was no significant change in viability of both K562 and HL60 cell in normoxic and hypoxic condition within 48 hours (Fig. 6).

In order to see any change in proliferation of both K562 and HL60 cells, the expression of main cell-cycle regulators (Cyclin D1, p21 and p27) was analysed. In the expression of Cyclin D1 and p21, same pattern of change was demonstrated (Fig. 7). Similar pattern was also shown in the expression of p27 (data not shown).

The expression of Epo receptor (EpoR) in cancer cells has raised the possibility that exogenous recombinant Epo (rhEPO) may have direct effects on tumour cells, such as the potential for stimulation of proliferation, resistance to apoptosis, and modulation of sensitivity to chemoradiation therapy (2, 7, 12, 14).
Recombinant human Epo (rhEPO) therapy is widely used for the prevention and treatment of chemotherapy-related anaemia in cancer. Although, EPO is known to regulate physiological erythropoiesis, it was concerned, due to its expression on cancer cells, that therapeutic rhEPO might stimulate tumour growth or protect cancer cells from drug-induced apoptosis. In some researches, Epo was shown to stimulate proliferation of tumour cells and their protection from apoptosis (14). In this study, we investigated the possible effects of recombinant human erythropoietin (rhEPO) on K562 cell line. K562 cells expressed elevated EpoR in hypoxic conditions, but HL60 did not. To elicit the effects of Epo on its receptor during hypoxia, we also incubated the K562 hypoxic cells with recombinant Epo. We found that hypoxia induces increased level of EpoR mRNA and protein in K562 cells, but rhEpo did not produced further impact in the expression of EpoR neither in normoxia, nor in hypoxia. Our results showed that rhEPO caused a transient increase in EpoR mRNA expression. To investigate the physiological consequences of this increase, we analyzed cell viability and expression of cell-cycle regulators (CyclinD1, p21 and p27). The presence of rhEPO did not significantly affect cell viability. Expression of cell-cycle regulators, however, was effected dramatically by the presence of rhEPO. This effect of rhEPO seems like it is repressive at normoxic condition. Expression of cell-cycle regulators was negatively affected by hypoxic condition as well. Interestingly, hypoxia-dependent decrease in expression of cell-cycle regulators seems to be prevented by the presence of rhEPO. This prevention might be through protection of mRNA against hypoxia-dependent degradation (10, 13), rather than increased transcription.

Since rhEPO exhibited its effect on both cell-cycle activator (CyclinD1) and inhibitors (p21 and p27) in a similar manner, we concluded that rhEPO does not inhibit or stimulate proliferation of K562 cells. Those results support the safety of rhEPO therapy in leukemia, especially erythromyeloid type.

This is the first report which shows that hypoxia induces EpoR in the human K562 erythroleukemia cell line.

**Conclusions**

Hypoxia upregulates the expression of EpoR on K562 erythroleukemia cells, longer exposition to hypoxia returns the upregulated EpoR expression back to basal levels, and rhEPO

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Fig. 7. The effect of Epo, without (left) and with (right) hypoxia, on the expression of Cyc D1 and p21 throughout 48 hours

A: K562, normoxia; B: K562, hypoxia; C: HL60, normoxia; D: HL60, hypoxia Hx: Hypoxia
did not produce further impact in the expression of EpoR neither in normoxia nor in hypoxia.

Acknowledgements
This work was supported by the Research Fund of the University of Istanbul, Project no: 3094.

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