Effect of Recombinant Erythropoietin on Functional Activity of Cultured Human Cells

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Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 2, pp. 83-87, April, 2012
Original article submitted November 8, 2011

We studied the effect of recombinant human erythropoietin on functional activity of skin cells *in vitro*. It was found that erythropoietin stimulated proliferation of mesenchymal and epithelial cells and effectively protected epidermal HaCaT cells from apoptosis. Insignificant effect of erythropoietin on contraction of collagen gel by mesenchymal cells was revealed. These findings suggest that erythropoietin can be a promising component of wound-healing preparations.

Key Words: *erythropoietin; fibroblasts; stromal adipose tissue cells; apoptosis; wound healing*

Erythropoietin (EPO) is a glycoprotein hormone regulating erythrocyte production. The main sources of EPO are the kidneys in adults and the liver in the fetus and newborns [2]; synthesis of this hormone is regulated via mRNA [4]. Up to 90% EPO is synthesized in the renal cortex by interstitial fibroblasts adjacent to the proximal tubular epithelial cells. The rest hormone is produced by hepatocytes and Ito cells (hepatic fibroblast-like cells) [2].

Numerous clinical and experimental studies showed that recombinant EPO is a multifunctional trophic factor, has different expression sites, specific tissue regulation, and different mechanisms of action. EPO receptors are found on membranes of not only red BM cell, but also myeloid lineage cells, lymphocytes, and megakaryocytes as well as on endothelial, mesangial, myocardial, smooth muscle cells and neurons. Moreover, other sites of EPO production were identified (uterus and brain), where EPO expression is regulated in a tissue-specific manner [5].

Recombinant EPO was successfully used for the treatment of renal anemias. EPO is widely used for the treatment of hemodialysis patients [11]. Moreover, recombinant EPO is used in anemia developing during pregnancy and in tumor patients undergoing chemotherapy [8]. Pretreatment of rat heart myoblasts with the hormone inhibited peroxide-induced apoptosis by ~50% [9] and protects myoblasts form damage over 12-h anoxia [2]. The protective effects of EPO (*in vivo* and *in vitro*), in particular, inhibition of apoptosis, were also demonstrated in acute renal ischemia. Renoprotective effects of EPO include also stimulation of regeneration of renal tubular epithelium and functional recovery of the kidney [10].

The brain is also the site of EPO synthesis. EPO production in the brain can be stimulated by hypoxia and other metabolic disturbances (hypoglycemia or changes in neuronal depolarization) leading to mitochondrial dysfunction via activation of hypoxia-inducible factor (HIF-1). Expression of EPO mRNA in astrocytes is dose-dependently stimulated by insulin and insulin-like growth factor [7]. The mechanism of the protective effect of EPO in the brain is poorly studied. A certain role is believed to be played by antiapoptotic activity, suppression of anti-inflammatory cytokines, stimulation of neurogenesis, and activation of the expression of brain-derived neurotrophic factor *in vivo* and *in vitro* [11].

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0007-4888/12/1534-0554

Recombinant EPO stimulates wound healing by promoting angiogenesis and stimulating proliferation and migration of endothelial cells in the presence of fibrin matrix [6]. EPO receptors are detected in many tissues of the body. EPO is a universal tissue protective cytokine. Pleiotropic effects of the hormone (anti-inflammatory, antiapoptotic, angiogenic, *etc*.) substantiate expected positive effects of this preparation (especially recombinant EPO and its analogues) in patients with various pathologies, including skin wounds.

Here we studied the effect of recombinant erythropoietin on functional activity of cultured human cells.

MATERIALS AND METHODS

Immortalized human keratinocytes (HaCaT cells), postnatal human dermal fibroblasts (PF), human embryonic fibroblasts (EF), and adipose tissue stromal cells (ATSC) were used in experiments.

The cells were cultured in DMEM supplemented with 10% fetal calf serum and 4 mM L-glutamine at 37°C, 5% CO_2, and 100% humidity. The medium was replaced after 2 days. For evaluation of the effect of EPO (kindly provided by L. P. Korobitsyn) on proliferative activity, the cell suspension with concentrations of 30,000 cell/ml (PF, EF, and ATSC) and 25,000 cell/ml (HaCaT) were transferred to a 96-well plate (200 μl per well). On the next day, the medium was replaced with a serum-free medium and in 24 h the medium was replaced again and simultaneously EPO in the studied concentrations was added. In series I, EPO was added to a medium with 10% embryonic calf serum (ECS). In series II, EPO was added to a medium with 0.5% ECS (PF, EF, and ATSC) or serum-free (HaCaT) medium. In both series, the medium containing 10% ECS served as the positive control. MTT test was performed in 2-3 days. MTT stock solution was added to each well of the 96-well plate (1:10 MTT-growth medium ratio) and incubated for 3-4 h at 37°C and 5% CO_2. After incubation, the fluid was carefully removed from wells (preserving the formed insoluble formazan crystals), 100 μl DMSO was added to each well, and crystals were dissolved by pipetting until homogenous coloration. Optical density was measured on a microplate reader using the main filter with λ=540-590 nm and a correcting filter with λ=630-690 nm.

Antiapoptotic activity of EPO was evaluated by TUNEL method. The cells were seeded to 48-well plates (55,000 cell/ml, 500 μl per well). After attaining confluence, the cultures were incubated with EPO (0.5 μg/ml) for 24 h. Apoptosis was induced by adding 300 mM H_2O_2 for 30 min. The cells were fixed in 4% paraformaldehyde and permeabilized with proteinase K, Endogenous peroxidase activity was inhibited with 3% H_2O_2 for. Then the cells were incubated with primary antibodies (TdT Ensyme, Br-dUTP; BD) for 1 h at 37°C in a humid chamber and then washed and incubated with second antibodies (Anti-BrdU-Biotin mAb; BD) for 1 h in the dark. Antibody binding was visualized with diaminobenzidine (DAB).

Collagen gel was prepared as follows: sterile 0.34 M NaOH was mixed (1:2) with concentrated (×10) Eagle's medium or medium 199 and then 100 mg glutamine and 9 ml 7.5% sodium bicarbonate per 100 ml mixture were added. The mixture was mixed with cooled collagen solution in acetic acid (1:4), cell suspension was added to a final concentration of 200,000 cell/ml, and the mixture was placed to CO_2-incubator for gelatinization. Contraction of the collagen gel was evaluated by changes in its diameter (measurements were performed daily over 4-9 days).

RESULTS

EPO in a concentration of 500 ng/ml significantly stimulated proliferation of PF and EF (Figs. 1 and 2). The most pronounced effect was observed during culturing in a medium with 10% ECS. Proliferation of HaCaT and ATSC was most effectively stimulated by EPO in the same concentration during culturing in a medium with low ESC content or without it (Figs. 3 and 4).

Contraction of the collagen gel was studied in cell cultures of mesenchymal origin (PF, EF, and ATSC).

TABLE 1. Dynamics of Collagen Gel Diameter (%, *M*±*m*)

Time of observation	PF		EF		ATSC	
	control	+EPO	control	+EPO	control	+EPO
Start of experiment	100	100	100	100	100	100
Day 2	100	100	56±4	52±5	57±4	49±5
Day 4	89±4	80±7	50±6	51±7	52±5	40±3
Day 7	58±3	42±6	33±7	35±6	45±7	30±5

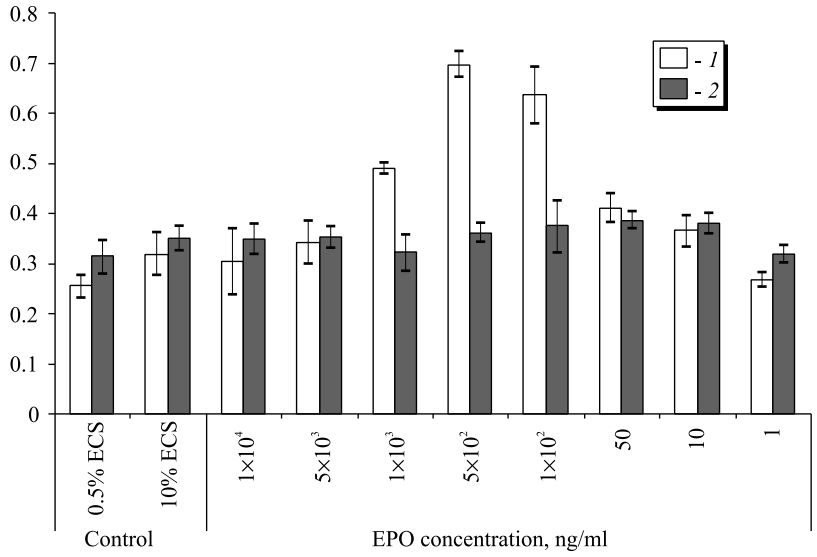

Fig. 1. Effect of EPO on proliferation of PF (MTT test data). Here and in Figs. 2-4: *1*) series I; *2*) series II. Ordinate: optical density units.

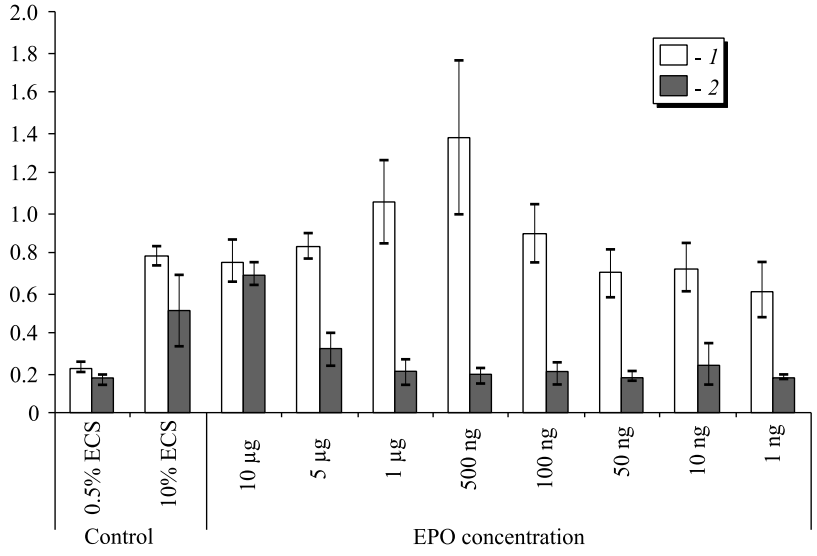

Fig. 2. Effect of EPO on proliferation of EF (MTT test data).

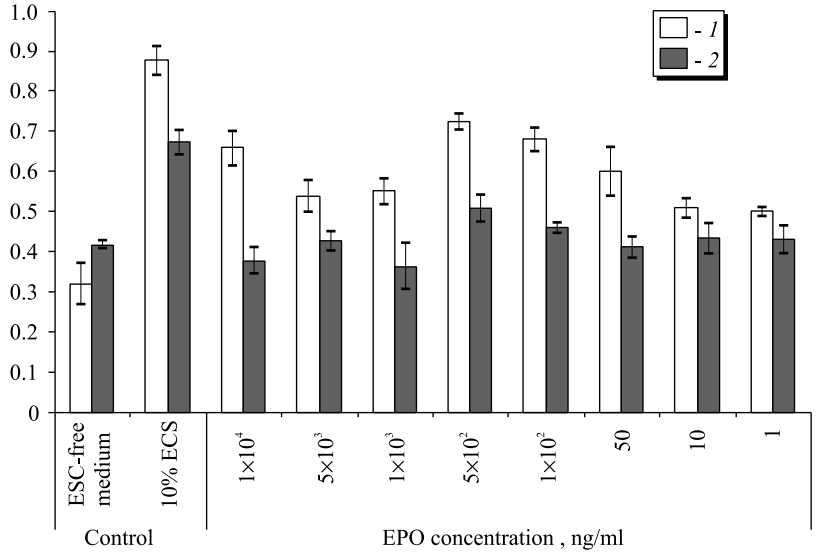

Fig. 3. Effect of EPO on proliferation of HaCaT cells (MTT test data).

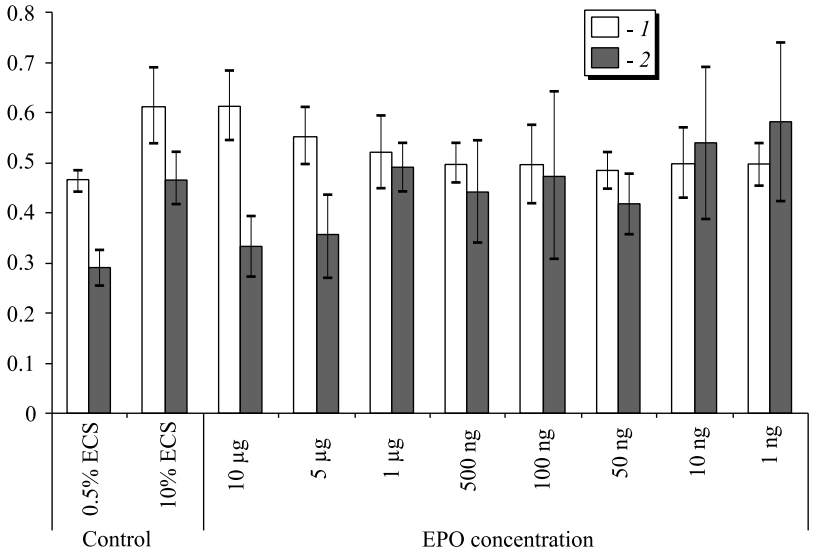

Fig. 4. Effect of EPO on proliferation of ATSC (MTT test data).

This method allows evaluation of functional activity of cells, because their contractile capacity can be thus measured. Mesenchymal cells incorporated into a 3D collagen gel attach to collagen fibers and pull them. This results in shrinkage of the floating gel [1]. EPO had no appreciable effect on the intensity of collagen gel contraction in EF culture (Table 1), but a tendency to contraction stimulation was noted in PF and ATSC cultures. It can be hypothesized that EF are characterized by high activity (including contractile activity) and the influence of external factors is insignificant against this background.

Hence, antiapoptotic activity of EPO was assessed at EPO concentration of 500 ng/ml. The time of incubation with EPO was chosen on the basis of published data [3].

We found that incubation with EPO reduced the relative content of apoptotic cells in HaCaT culture by about 30% (to 0.6013±0.0600% *vs*. 0.981±0.080% in the control). Moreover, zones with high content of mitotic cells were found in cell monolayer cultured with EPO (Fig. 5, *a*, *b*). Mitotic index in the presence of EPO was 1.3608±0.0700 *vs*. 0.6962±0.0800 in the control.

These results suggest that EPO enhanced proliferation of cells of different origin, including skin cells, somewhat stimulated functional activity of mesenchymal cells, and effectively prevented apoptosis of cultured epithelial cells. Thus, EPO can improve not only blood supply to the damaged tissue, but also directly activate its cells.

The study was supported by Program "Development of High-Technological Products of Cell Technologies for Regenerative Medicine", Ministry of Education and Science of the Russian Federation (grant No. 2010-218-02-172).

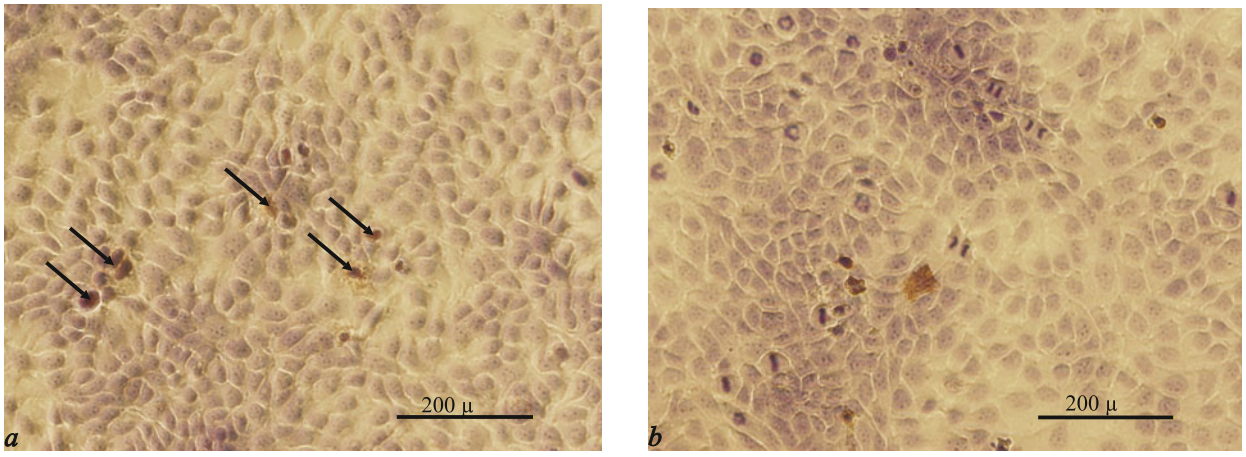

Fig. 5. HaCaT cell culture. *a*) H_2O_2-induced apoptosis. DAB staining and post-staining with Mayer hematoxylin. Arrows: apoptotic cells. *b*) mitotic cells in monolayer after incubation with EPO. Staining with Mayer hematoxylin.

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