Influence of recombinant erythropoietin on mesenchymal stem cells proliferation and differentiation during cultivation on allogenic bone matrix

Influencia de la erytropoietina recombinante en la proliferación y diferenciación de células madre mesequímicas durante el cultivo matriz alógena de hueso

Sergey V. Nadezhdin1, Ekaterina V. Zubareva[1], Anatoliy V. Ovsyankin[2], Svetlana A. Guzyukina[2], Elena A. Atamanyuk[3], Ekaterina S. Gnezdyukova[4], Kseniya R. Zhukovskaya[4]
[1]Belgorod National Research University, 308015, Russia, Belgorod, Pobedy St. 85; https://orcid.org/0000-0001-7215-295X
[1]Belgorod National Research University, 308015, Russia, Belgorod, Pobedy St. 85; https://orcid.org/0000-0002-9557-145X
[2]Federal State Budgetary Institution "Federal Center for Traumatology, Orthopedics and Endoprosthetics" of the Ministry of Health of the Russian Federation. 214031, Russia, Smolensk, Stroiteley Ave. 29; https://orcid.org/0000-0002-0779-091X
[2]Federal State Budgetary Institution "Federal Center for Traumatology, Orthopedics and Endoprosthetics" of the Ministry of Health of the Russian Federation. 214031, Russia, Smolensk, Stroiteley Ave. 29; https://orcid.org/0000-0001-5652-9489
[3]Regional State Budgetary Institution of Health "Belgorod Regional Clinical Hospital of St. Ioasaph". 308007, Russia, Belgorod, Nekrasova St. 8/9; https://orcid.org/0000-0002-4095-9473
[4]Belgorod National Research University, 308015, Russia, Belgorod, Pobedy St. 85; https://orcid.org/0000-0002-1672-3495
[4]Belgorod National Research University, 308015, Russia, Belgorod, Pobedy St. 85; https://orcid.org/0000-0002-2835-0630
**corresponding author: Sergey V. Nadezhdin, Belgorod National Research University, 308015, Russia, Belgorod, Pobedy St. 85. Email: nadezhdin@bsu.edu.ru*

Abstract

Effectiveness of mesenchymal stem cells (MSCs) proliferation and differentiation was studied after addition of medicinal product containing human recombinant erythropoietin (rhEPO) into enriched culture medium with components for osteo- and angiogenesis. Cultivation of mesenchymal stem cells under static conditions using "cocktail" of growth factors and rhEPO allowed to populate allogenic discs-scaffolds with connective tissue formations with the participation of osteoblasts and elongated multicellular structures, which formed after endothelium-like cells fusion. In the presence of rhEPO MSCs differentiates more effectively in osteogenic direction than in angiogenic.

Keywords: mesenchymal stem cells (MSCs), recombinant erythropoietin, allogenic bone matrix, proliferation, differentiation.

Introduction

La efectividad de la proliferación y diferenciación de las células madre mesenquimales (MSC) se estudió después de la adición de un medicamento que contiene eritropoyetina recombinante humana (rhEPO) en un medio de cultivo enriquecido con componentes para osteo- y angiogénesis. El cultivo de células madre mesenquimáticas en condiciones estáticas utilizando un "cóctel" de factores de crecimiento y rhEPO permitió poblar los andamios-discos alogénicos con formaciones de tejido conectivo con participación de osteoblastos y estructuras multicelulares alargadas, que se formaron después de la fusión de células de tipo endotelio. En presencia de rhEPO, las MSC se diferencian más eficazmente en la dirección osteogénica que en la angiogénica.

Palabras clave: Células madre mesenquimales (MSC), eritropoyetina recombinante, matriz ósea alogénica, proliferación, diferenciación.

Introduction

Recent advances in biotechnology allowed to synthesize and produce in an industrial large-scale human recombinant erythropoietin (rhEPO, epoetin)[1] and its analogs (erythropoiesis-stimulating agents (ESAs))[2-4] to use as medicinal products for treatment of various diseases[5,6], rhEPO was characterized as hematopoietic and tissue-protective factor[3,7,8], and besides the properties similar to vascular endothelial growth factor (VEGF) were revealed[6]. It was discovered that erythropoietin could stimulate endothelial cells proliferation in vitro and mobilize endothelial progenitor cells from the bone marrow[9-11], promote circulating endothelial progenitor cells adhesive and proliferative properties[12]. In most research papers it is stated that erythropoietin triggers mesenchymal stem cell differentiation into osteoblasts in vitro and stimulation of bone tissue formation occurs because of increased VEGF expression[13-16]. This raises the question: does erythropoietin induce mesenchymal stem cell differentiation into osteoblasts or endotheliocytes predominantly?

Aim: The research aimed to study the effectiveness of mesenchymal stem cells proliferation and direction of their differentiation after the addition of erythropoietin into culture medium containing supplements for osteo- and angiogenesis.

Materials and methods

The experiment was divided into three parts: during the first part proliferative activity of cells was investigated (6 samples), at the second and the differentiation of the third parts of MSCs was studied in osteogenic (6 samples) and angiogenic directions (n=6) respectively. To perform the study at each part control and experimental groups were formed. Tests performed in triplicates for each group. The cells from the control group were cultured on bone discs-scaffolds in growth medium containing osteogenic and endotheliogenic supplements. The cells from the experimental group were cultured on bone discs-scaffolds in growth medium containing osteogenic and endotheliogenic supplements and recombinant human erythropoietin.

After the end of the cultivation period at the first part of the experiment proliferative activity of hAT-MSCs which were grown on bone, discs-scaffolds was studied using confocal laser scanning microscope DIGITAL ECLIPSE C1 plus (Nikon, Japan) with a fluorescent probe – Calcein AM (Invitrogen, USA). The solution was prepared and the time of cultivation was determined according to the manufacturer's instructions. Fluorescent dye stock solution at a concentration of 5 mM was prepared in dimethyl sulfoxide (DMSO).

During the second and the third parts of the experiment bone discs-scaffolds with cells were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin solution for investigation of the direction of hAT-MSCs differentiation. For these purposes, Osterix (a marker of hAT-MSCs differentiation into osteoblasts) and CD31 (a marker of endothelial cells) were used. Software "Statistica 10" was used for statistical analysis of experimental data.

Main part: Study of hAT-MSCs, osteoblasts and endothelial progenitor cells proliferative potential was performed via measuring of intensive proliferation centers areas because the cells at the 20th day of cultivation on the bone discs-scaffolds united in connective tissue structures. Increase of the area of new formed connective tissue structures in the experimental group (16406.93±235.92 mkm^2) in comparison with the control group (15250.97±182.34 mkm^2) ($p \leq 0.01$) was revealed (Fig. 1).

During the analysis of fluorescent images, it was revealed, that after 20 days of cultivation on bone trabeculae and intertrabecular space of the experimental group samples connective tissue formations appeared which had been formed due to mesenchymal stem cells, osteoblasts, and endothelial progenitor cells proliferation. increase of connective tissue structures area formed by osteoblasts (fluorescent probe Osterix) was noted in the experimental group (17362.94±266.34 mkm^2) in comparison with control (16274.89±247.98 mkm^2) ($p \leq 0.01$) (Fig. 2.A). Besides the bone, connective tissue formations elongated multicellular structures containing endothelial-like cells (fluorescent probe CD31) were discovered in the experimental group samples. increase of the elongated multicellular formations area was revealed in the experimental group (164.19±4.63 mkm^2) in comparison with the control group (147.51±7.34 mkm^2) ($p \leq 0.01$) (Fig. 2.B).

Thus, the addition of rhEPO to the culture medium containing inducers of osteo- and angiogenesis leads to enhancement of MSCs proliferation and differentiation in comparison with the control group. This effect could be described because rhEPO is a pleiotropic growth factor which stimulates the proliferation of both mesenchymal and hematopoietic stem cells[17].(reference 26 not valid)

Cultivation of mesenchymal stem cells in static conditions using "cocktail" of growth factors and medicinal product which includes rhEPO allowed to load allogenic discs-scaffolds with cells and accompanied by the formation of connective tissue formations with the participation of osteoblasts and elongated multicellular structures appeared during endothelial-like cells fusion.

Figure 1. Optical slices series. Fluorescence of new formed connective tissue structures in bone disc-scaffold at the 20th day of cultivation (experimental group from the first part of the study, cells were stained with Calcein AM). (DIGITAL ECLIPSE C1 plus, Nikon, Japan, X 200 magnification).

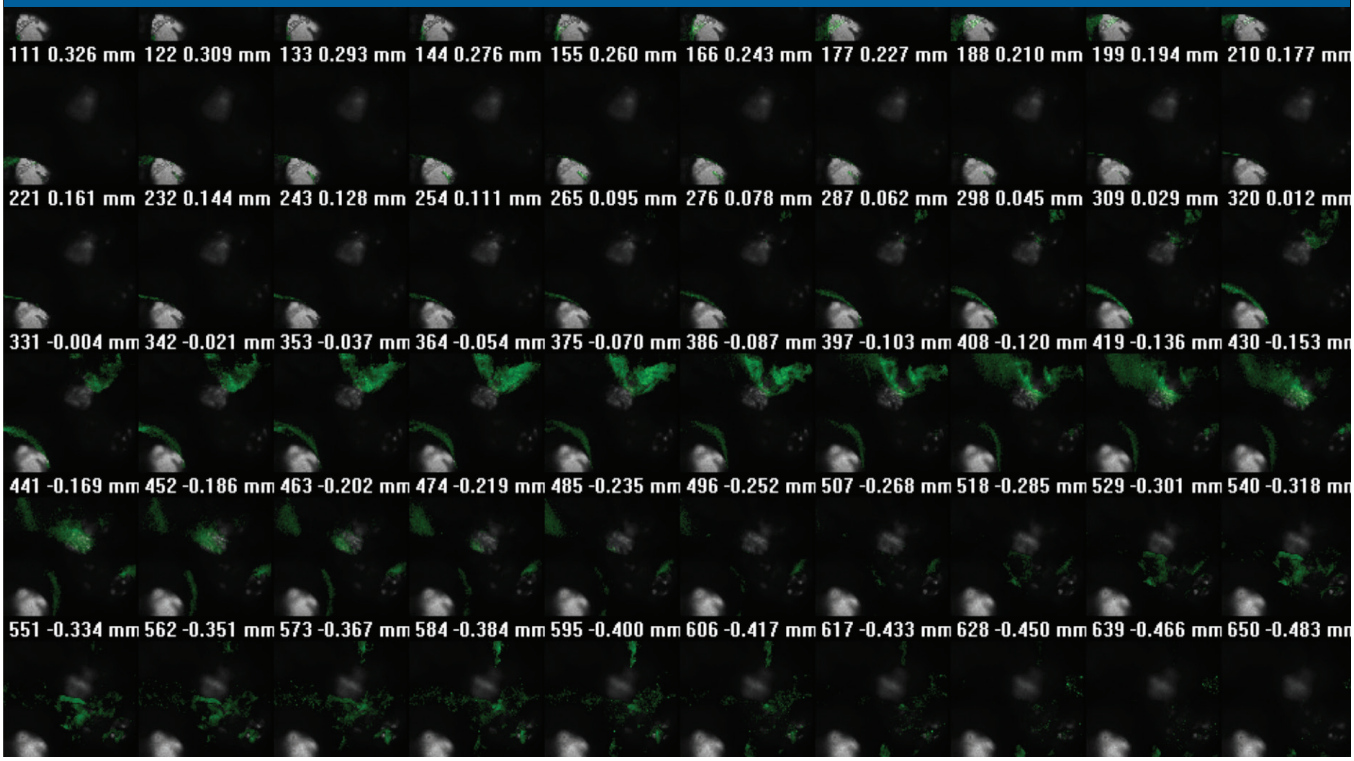
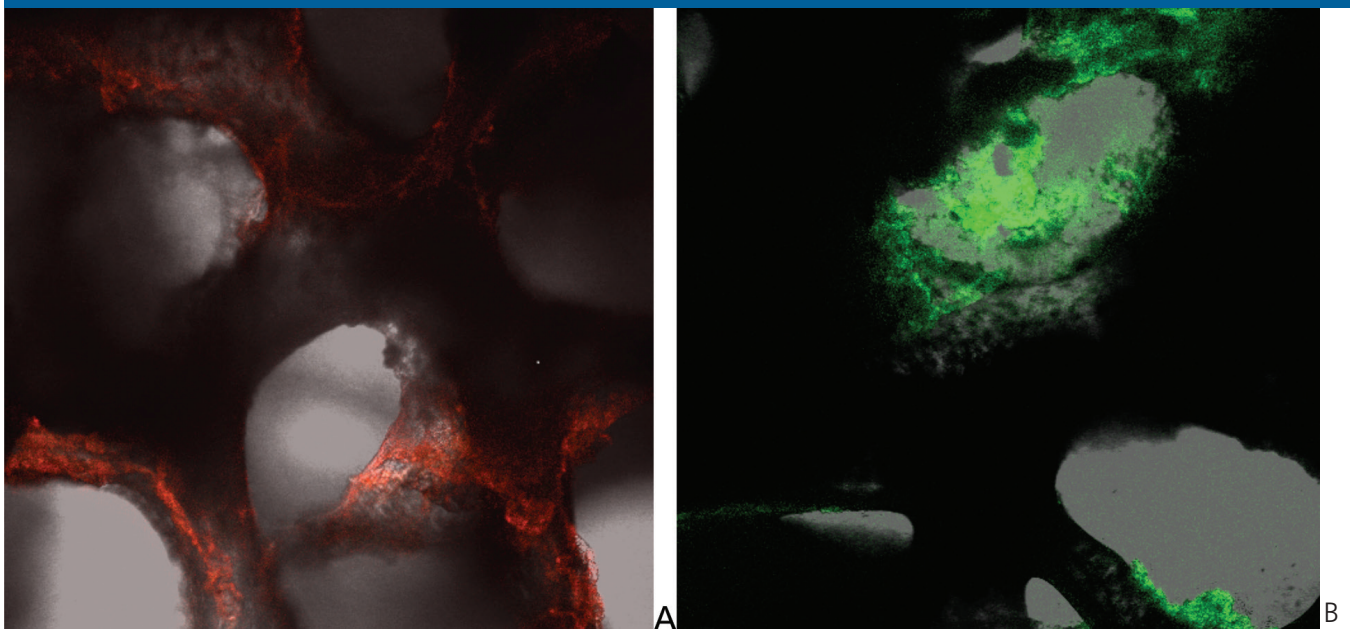


Figure 2. Fluorescence of connective tissue structures – elements of the osteoid matrix (red fluorescence) (experimental group from the second part of the study) and elongated multicellular formations (green fluorescence) on the bone trabecula of disc-scaffold (experimental group from the third part of the study). (DIGITAL ECLIPSE C1 plus, Nikon, Japan, X 200 magnification)



Conclusions

The addition of human recombinant erythropoietin into growth medium increases mesenchymal stem cells proliferation which accompanied by the formation of connective tissue and elongated multicellular structures.

Differentiation of mesenchymal stem cells under the influence of recombinant human erythropoietin is more efficient in osteogenic direction than in angiogenic direction.

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