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Original Research Article

Effect of β-nerve growth factor on differentiation of endothelial progenitor cells in rats

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Abstract

Purpose: *To investigate the effect of recombinant adenovirus-mediated human β-nerve growth factor (Ad-EGFP-hβ-NGF) on the differentiation of endothelial progenitor cells (EPCs) in rats.*
Methods: *The successfully constructed Ad-EGFP-hβ-NGF and its negative control Ad-EGFP were infected into the isolated and purified rat EPCs to observe their morphological changes. Enzyme-linked immunosorbent assay (ELISA) was conducted to detect the levels of vascular endothelial growth factor (VEGF), von Willebrand factor (vWF) and basic fibroblast growth factor (bFGF) in different rat EPC culture solutions. Western blot was performed to determine the expression of tyrosine kinase receptor A (TrKA) protein in different groups of EPCs.*
Results: *Primary fibrous EPCs were converted into epithelium-like cells. After infection with Ad-EGFP-hβ-NGF for 1 week, some EPCs became round and exhibited neural stem cell-like changes. The expression levels of VEGF, vWF and bFGF in the Ad-EGFP-hβ-NGF infection group were significantly higher than those in the control group ($p < 0.01$). TrKA protein in Ad-EGFP-hβ-NGF infection was also significantly up-regulated compared with that in the negative control and blank control groups ($p < 0.01$).*
Conclusion: *β-NGF up-regulates the expression of TrKA receptor protein and secretion of angiogenic growth factors (i.e., VEGF, vWF and bFGF), thereby promoting the differentiation of rat EPCs, which may contribute to angiopoiesis or vascular repair.*

Keywords: *β-Nerve growth factor, Endothelial progenitor cells, Angiogenic growth factors, Tyrosine kinase receptor A, Cell differentiation*

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INTRODUCTION

Endothelial progenitor cells (EPCs), also known as angioblasts, are bone marrow-derived stem cells characterised by migration; thus, these cells can further proliferate and differentiate into vascular endothelial cells[1]. EPCs play important roles *in vivo* to promote vascular regeneration, repair damaged endothelial functions and improve local blood perfusion [2]. Thus, EPCs are clinically significant. According to our previous clinical studies, autologous peripheral blood stem cell transplantation can effectively improve the outcomes of diabetic peripheral vascular disease [3,4].

Nerve growth factor (NGF) is a nerve cell growth regulator that is firstly found amongst neurotrophic factors. NGF contains three subunits (i.e., α, β and γ), and the active region is the β subunit. β-NGF is a dimer consisting of two single-strands through noncovalent bonds [5].

Recent studies have shown that in addition to its neurotrophic roles, NGF participates in promoting angiogenesis in damaged tissues [6]. In this study, recombinant adenovirus-mediated β-NGF was infected into rat EPCs to investigate its effects on EPC differentiation by determining whether NGF promotes endothelial regeneration through regulating EPC differentiation.

EXPERIMENTAL

Reagents and instruments

Four-week-old male SD rats weighing 60-80 g were provided by the Experimental Animal Center of Tongji Medical College. The following reagents were used in this study: Percoll cell separation solution (Pharmacia, USA), EGM-2MV culture medium (Lonza, Switzerland), rabbit anti-human CD34 antibody and isotype control murine IgG1 antibody (Abcam Inc), rabbit anti-human VEGF ELISA assay kit (R&D, USA), rabbit anti-human vWF ELISA assay kit (China), rabbit anti-human bFGF ELISA assay kit (China), rabbit anti-rat tyrosine kinase receptor A (TrKA; Santa, USA), rabbit anti-rat actin (Santa, USA), secondary antibodies (Beijing Zhongshan) and ECL (Beyotime Biotechnology Co. Ltd). Other reagents were of analytical grade. FACSCalibur flow cytometer (BD Co.), 5810R high-speed low-temperature centrifuge (Eppendorf Co.) and microplate reader (Bio-Rad, Inc) were also employed.

Isolation and purification of rat EPCs

The rats were anaesthetised, decapitated and then soaked in 75 % ethanol for 15 min. Afterward, the femur and tibia were sampled and soaked into 0.9 % precooled NaCl solution (4 °C). The marrow cavity was then repeatedly flushed with saline until the rinsing solution was clear. The cells were pipetted evenly and slowly added into centrifuge tubes containing Percoll cell separation solution. This study was approved by Animal Ethics Committee of Huazhong University of Science and Technology (ref no. 2016005022). All the experimental procedures were performed in accordance with the International Guidelines for Care and Use of Laboratory Animals [7].

The volume ratio of the rinsing solution to the separation solution was 2:1. After centrifugation at 2500 rpm for 20 min, the milky cloudy mononuclear cells (MNCs) in the middle of the centrifuge tube were gently sucked and moved into a new tube and then rinsed with PBS twice (1300 r/min, 8 min). Subsequently, 20% FBS-containing EBM-2MV culture medium was used to resuspend the cells and then gently pipetted repeatedly to make a single cell suspension.

The cell suspension was seeded into 6 cm dish with a density of 1×10^6 cells/mL and incubated at 37 °C in 5% CO_2 and saturated humidity. After 4 days, the medium was totally changed for the first time. The suspended cells were discarded, and the rest of the cells were stored for culture. The medium was changed every 3 days. Meanwhile, the morphological changes of the cells were observed. On the 14th day (the cells grew to 80 % confluence), 0.25 % trypsin was used to digest the dish wall-adherent cells. The cells were also passaged (1:2), and the culture was continued until the 28th day.

Infection

Adenovirus-mediated human β-nerve growth factor (Ad-EGFP-hβ-NGF) and its negative control Ad-EGFP were provided by Dr Jianing Wang of the Clinical College of Hubei Medical College. The EPCs of rats cultured until the 28th day were divided into three groups and infected with Ad-EGFP-hβ-NGF and Ad-EGFP with 100 multiplicity of infection. Meanwhile, the blank control group was prepared. After 48 h infection, the cell morphological changes were continuously observed.

Expression and detection of TrKA protein through Western blot

After 48 h infection, the cells were washed with precooled PBS, and the supernatant was discarded. Precooled (4 °C) tissue protein extract solution (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % NP-40, 100 μg/ml PMSF, 0.5 % deoxycholate, 0.02 % sodium azide, and 1 μg/ml aprotinin) was added, and the mixture was then incubated on ice for 15 min. The cells were then lysed on ice via ultrasound. The supernatant (obtained by 10 min centrifugation at 4 °C and 12000 rpm) was used to determine the protein concentration with BCA assay, and the protein detected using 12 % SDS-PAGE electrophoresis, membrane transfer, and incubation with primary antibodies (TrKA 1:2000, Actin 1:10000) and secondary antibody (1:10000). ECL chemiluminescence method was used for the coloration, and Syngene gel imaging system was employed to photograph, record and analyse the results.

Detection of VEGF, vWF and bFGF in the culture supernatant by ELISA

After the cells were infected for 6 days, the culture supernatant was collected and

centrifuged at room temperature (2000 rpm, 3 min). VEGF, vWF and bFGF were detected using ELISA kits according to the manufacturer's instructions. The optical density of each well was determined using a microplate reader at 450 nm. Duplicate assays were performed for each sample.

Statistical analysis

SPSS 13.0 software was used for the statistical analysis. Data are expressed as mean ± standard deviation (SD), and multi-group comparisonwas performed using one-way ANOVA and pair-wise comparison (LSD method). $P < 0.05$ was considered statistically significant.

RESULTS

Morphological characteristics of EPCs

MNCs began to adhere to wall and became large when seeded for 24 h. Inverted microscope observation revealed that the transparency of the wall-adherent cells was enhanced. Moreover, the border became clear, and the cells became small rod-shaped or spindle. After cultivation for 7 days, the wall-adherent cells began the 'colony-like' proliferation with numerous spindle peripheral cells. In the second week, the spindle cells increased significantly. When cultured until the 28th day, the spindle cells adhered to one another and exhibited 'paving stone-like' arrangement.

EPCs that formed at different periods are shown in Figure 1. The EPCs cultured for 28 days were infected with adenovirus (the negative control and blank control groups were set at the same time). After 48 h, the cell morphological observation under a fluorescence-inverted phase-contrast microscope revealed the expression of green fluorescent protein in the Ad-EGFP-hβ-NGF and Ad-EGFP groups. However, the control group had no fluorescence. The infection effects in different groups are shown in Figure 2. One week after the infection, some cells exhibited neurosphere-like proliferation. However, the negative control and blank control groups showed no corresponding proliferation, as shown in Figure 3.

Expression and detection of TrKA protein through Western blot

After the cells were infected for 48 h, the total cellular proteins were extracted for immunoblot assay. The results revealed that TrKA was significantly upregulated in the Ad-EGFP-hβ-NGF group than that in the Ad-EGFP and blank control groups ($P< 0.01$), as shown in Figure 4.

Detection of VEGF, vWF and bFGF levels in the culture supernatants through enzyme-linked immunosorbent assay (ELISA)

After the EPCs were infected for 6 days, the cell culture media were collected for the detection of VEGF, vWF and bFGF through ELISA. All these

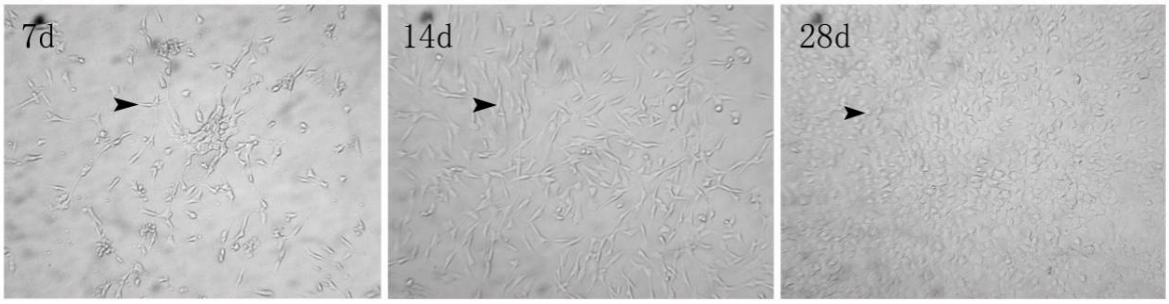

Figure 1: Morphological changes of EPCs at different stages

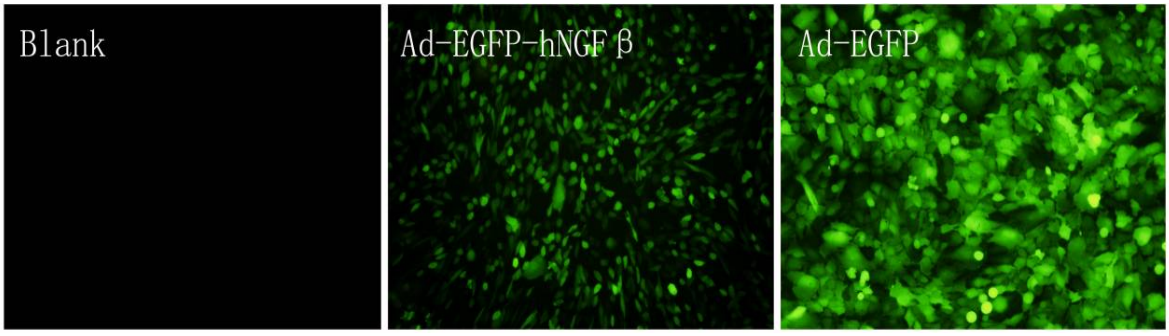

Figure 2: Expressions of green fluorescent protein in different groups

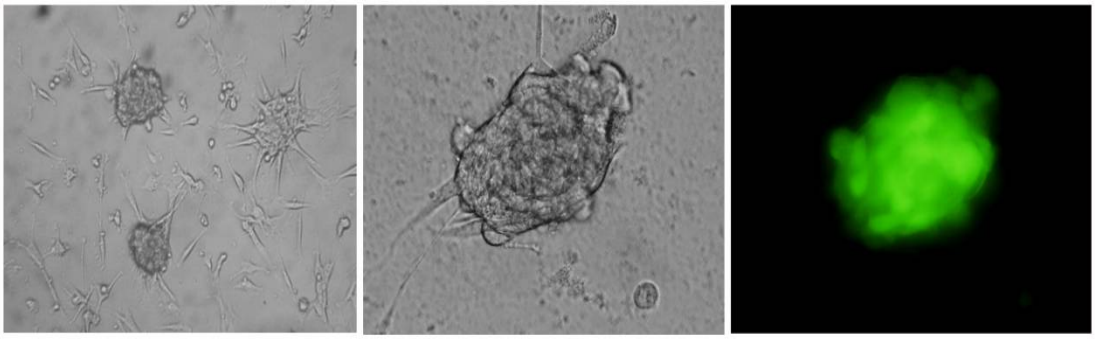

Figure 3: Cells exhibited neurosphere-like proliferation after infection with Ad-EGFP-hβ-NGF for 1 week

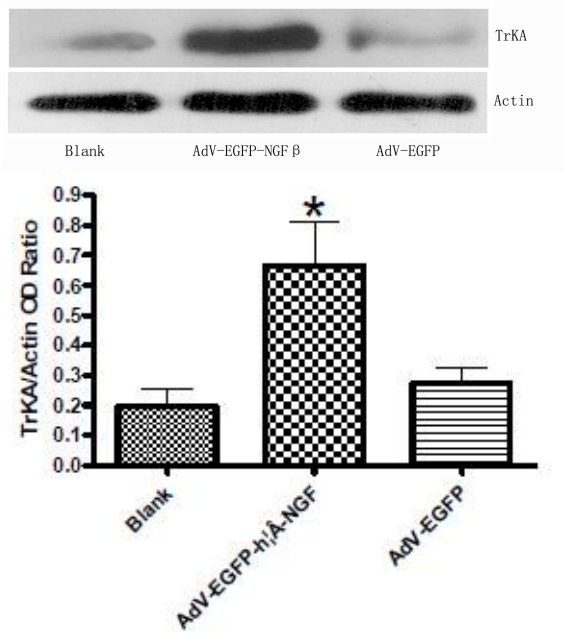

Figure 4: TrKA expression levels in different treatment groups

three factors in the Ad - EGFP - hβ-NGF group were significantly upregulated compared with those in the Ad-EGFP and blank control groups (P< 0.01), as shown in Table 1.

DISCUSSION

EPCs are responsible for maintaining the dynamic equilibrium of endothelial injury and repair, and their quantity and functions are directly related to the occurrence and development of vascular diseases [8]. Under certain induction conditions, EPCs can promote angiogenesis in ischemic areas and increase collateral circulation. The proangiogenesis mechanisms of EPCs have been confirmed, which mainly include two aspects,(1) Self-proliferation and differentiation to form new blood vessels without reliance on the original organ system, (2) EPCs can secrete cytokines, such as VEGF or bFGF, thereby promoting the proliferation of local vascular endothelial cells via paracrine; in particular, the angiogenetic effects are enhanced, thereby improving blood supply [9]. During EPC differentiation, the regulation of proangiogenic factors and their receptors plays active roles, and they participate in different stages, amongst which the proangiogenetic effects of VEGF, bFGF and vWF have been demonstrated [10].

NGF is a nerve cell growth regulator that is firstly discovered amongst neurotrophic factors, and has important regulatory roles towards the development, differentiation, growth, regeneration and expression of functional properties of the central and peripheral neurons [11-13]. Recent studies have demonstrated that NGF directly promotes angiogenesis mainly through vascular endothelial cell-associated effects [14]. In this study, Ad - EGFP - hβ-NGF and Ad-EGFP were successfully infected into rat EPCs, and we observed that all the cells adhered to wall grew in the control group. The cells also changed from fibroblast-like cells to epithelial cells with time. However, the differentiation rate of the cells in the Ad - EGFP - hβ-NGF group decreased, but the proliferation rate increased. Some cells also exhibited neurosphere-like proliferation. These observations indicate that NGF could maintain and restore the features of EPCs, which are conducive in maintaining the EPC bank and providing adequate cell source for vascular repair and generation. After the EPCs were infected with NGF, cell proliferation was evident, showing similar roles of VEGF, bFGF or vWF; this phenomenon established the foundation for angiogenesis.

Several studies demonstrated that EPCs have two receptors, namely, TrKA and P75 neurotrophin receptor that mediate the signal transduction of NGF. The former possesses high affinity, whereas the latter has low affinity. The TrKA receptor generally mediates positive signals and mainly promotes cell survival and proliferation. When NGF binds the TrKA receptor, it can regulate the affinity of its receptor and the TrKA signal transduction. First, NGF

Table 1: ELISA data for different groups (mean± SEM, ng/ml)

Group	VEGF	vWF	bFGF
Blank	0.185±0.057	0.361±0.058	0.394±0.029
Ad-EGFP-hβ-NGF	0.535±0.097*	0.594±0.025*	0.682±0.068*
Ad-EGFP	0.204±0.076	0.403±0.039	0.349±0.057

*P<0.01

stimulates the expression and secretion of matrix metalloproteinase-2, thereby regulating phosphatidyl inositol 3-kinase (PI3K)/AKT signalling pathway by increasing the phosphorylation of TrKA and the activity of PI3K. Therefore, the main mechanism of NGF in angiogenesis is to exert its biological effects by binding its high-affinity receptor TrKA. At present, the capillaries and aortic endothelial cells in mice, as well as human umbilical vein endothelial cells, can produce NGF and express the TrKA protein. ELISA and immunohistochemical techniques are used to assess ischemic muscles; the endogenous NGF and its high-affinity receptor TrKA are upregulated, and the administration of exogenous NGF can significantly improve the limb ischemic conditions [15].

The results of this study showed that when rat EPCs were infected with Ad - EGFP - hβ-NGF, the cells exhibited neurosphere-like proliferation, and the expression levels of VEGF, vWF, bFGF and TrKA protein in cell culture medium were significantly upregulated. Therefore, NGF may regulate the stem cell properties of EPCs, and the expression levels and secretions of proangiogenic factors (i.e., VEGF, vWF and bFGF) and TrKA protein may be upregulated, thereby promoting angiogenesis. However, the proangiogenetic abilities of NGF should still be confirmed by further experiments.

CONCLUSION

β-NGF upregulates the expression of TrKA receptor protein and the secretion of angiogenic growth factors (i.e., VEGF, vWF and bFGF), thereby promoting the differentiation of rat EPCs. This may contribute to angiopoiesis or vascular repair.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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