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Research Paper

An experiment study of osteogenesis of Ad-VEGF165 transfected human bone marrow mesenchymal stem cells *in vitro*

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Abstract

Objective: To evaluate the effect of osteogenic potential on human marrow mesenchymal stem cells (hMSCs) transferred with human vascular endothelial growth factor (VEGF) gene by adenovirus. **Methods**: hMSCs were isolated from human marrow, cultured *in vitro* and randomly divided into 3 groups: Ad-$VEGF_{165}$ group: adding 1×10^{10}OPU/ml Ad-VEGF in hMSCs culture fluid after incubating 24 hours, changing into ordinary complete culture and continuing culturing; Positive control group: Cultured hMSCs with 1 nmol/L dexamethasone, 10 mmol/L glycerophosphate and 50 mg/L vitamin C, exchanging this conditioned medium twice a week; blank control group: no special treatment but culturing hMSCs in DMEM. To evaluate osteogenesis competence, Von Kossa's staining and a quantitative alkaline phosphates (ALP) activity analysis were performed after 2 weeks treatment. **Results**: The calcified nodes formed after 2 weeks treatment in Ad-VEGF165 group and Positive control group but not in blank control group. ALP activities in Ad-VEGF165 group, Positive control group and blank control group were (7.91 ± 0.90)u/L, (8.18 ± 0.76 u/L) and (3.46 ± 0.49)u/L respectively. The differences were no statistical significance between Ad-VEGF165 group and positive control group ($P > 0.05$), but Ad-VEGF165 group and Positive control group were significantly different with blank control group ($P < 0.05$). **Conclusion**: Adenovirus mediated VEGF165 gene can transfect hMSCs and promote osteogenesis of hMSCs.

Keywords: adenovirus, vascular endothelial growth factor-165, gene therapy, bone marrow mesenchymal stem cells, osteogenesis

INTRODUCTION

Recent research shows vascular endothelial growth factor (VEGF) plays an important role in osteogenesis. Bone tissue rehabilitation relies on angiogenesis as do other tissue, as an important angiogenesis factor, VEGF can induce angiogenesis and adjust osteogenesis. Mesenchymal stem cells (MSCs) are multipotential cells that can differentiate into cells (from mesodermal origin) including fat, cartilage and bone. To evaluate the effect of VEGF165 gene on hMSCs differentiation, we transferred the VEGF165 gene in hMSCs by adenovirus and observed the differentiation of hMSCs *in vitro*.

MATERIALS AND METHODS

Isolation and culture of hMSCs

We drew out 10ml medulla from a 20 year old healthy male volunteer's spina iliaca anterior superior, subsequently adding heparin sodium 5000 u mixed with the same volume Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) without serum. After centrifuged 1 500 rmp of for 20 minutes, cells were collected, cultured in DMEM with 15% newborn calf serum and maintained at 37℃ with 5% CO_2 incubator (Forma, USA). After removing floating cells at 24 hours culture, we changed the culture

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medium every 3 days, and passaged the adherence cells after 70% fusion.

Grouping and treatment

We Selected 50% fusion P_2 generation hMSCs, divided them randomly into 3 groups and passaged in a 48-hole-plate. The Ad-$VEGF_{165}$ group was added Ad-$VEGF_{165}$ (obtained from Doctor Jianing Wang, People's Hospital of Yunyang Medical College) 1×10^{10}OPU/ml in MSCs culture fluid. After incubated for 24 hours, cell were changed into ordinary complete culture and was continually cultured; positive control group cultured with 1 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate and 50 mg/L vitamin C and changed this condition medium twice a week; blank control group was no special treatment except culture in DMEM.

Detecting activity of alkaline phosphatase (ALP) in supernatant of culture medium

On the 12th day, we exchanged culture medium by DMEM without serum, collecting supernatant after 48 hours detecting ALP activity of the culture medium in every group by automatic biochemistry analyzer.

Detection of calcification by von Kossa's Method

This method was adopted from Diane Proudfoot[1]: All groups MSCs were washed with PBS three times, and then fixed in 10% formaldehyde in PBS for 45 minutes at 4℃. The cells were washed with distilled water and exposed to 5% aqueous $AgNO_3$ and strong light for 60 minutes at room temperature. The cells were then exposed to 2.5% sodium thiosulfate for 5 minutes and further treated with Gieson's picrofuchsin to visualize bone collagen nodes (red= positive stain).

Statistical analysis

Data were expressed as mean ±SD. Differences were considered statistically significant as analyzed by sample mean *t*-test. *P* value < 0.05 was considered statistically significant.

RESULT

Isolation and culture of hMSCs

The complete bone marrow method culturing hMSCs, was used at first, the culture flask containing a small quantity adherence spindle cells and multiplicity floating hematopoietic cells (***Fig 1***). By 3~4 times exchange of medium, floating cells had been almost completely eliminated. Adherence cells (hMSCs) sped up proliferation, formed colonies, fused each other. After 7~9 days of subcultivation, passaged cells shaped colony and proliferated quickly (***Fig 2***). After different treatment of the second passage cells, hMSCs gradually changed into polygons or irregular shapes, cells enations decreased and proliferation slowed down gradually when the colonies overspreaded on culture plate by 6~7 days. The blank control group had no obvious change as Ad-VEGF165 group and the positive control group.

Activity of alkaline phosphatase(ALP) in supernatant of culture medium

The activity of supernatant in Ad-VEGF165 group and the positive control group were significantly higher than that of the blank control group, but there were no significantly difference between Ad-VEGF165 group and the positive group(***Tab 1***).

Tab 1 **The activity of supernatant in 3 groups**

($\bar{x} \pm s$, u/L, n=16)

Ad-$VEGF_{165}$ group	Positive control group	Blank control group
7.91 ± 0.90*	8.18 ± 0.76*	3.46 ± 0.49

*Compared with blank control group, $P < 0.05$, Ad-VEGF165 group compared with positive control group, $P > 0.05$

Von Kossa's stain

Most cells in the Ad-VEGF165 group and positive control group were stained into red bone tuber(***Fig 3 and 4***), but not in the blank control group(***Fig 5***).

DISCUSSION

Mesenchymal stem cells (MSCs) have been a focus of stem cell-based tissue engineering research, during the last decade. This is due to their capacity to differentiate into multiple skeletal tissues including bone, cartilage and ligaments. Using treatments which often include biochemical and chemical supplements [2], many papers have shown the potential of mesenchymal cells to differentiate to osteoblasts and contribute to bone formation [3-5]. As reported by Gugala *et al*[6], osteogenic cells may derive from the medullary canal because of their proliferation and differentiation ability. MSC potentially useful for bone engineering are mostly derived from the iliac crest, as an appropriate amount of bone marrow stromal is usually collected from this site with a minimally invasive procedure[7-8].

It is well recognized that donor heterogeneity, including age, gender, and disease, may influence the yield and proliferative capacity of stem cells or their osteogenic potential [9,10]. Banfi *et al* [11] has already shown that the bone formation activity of *ex vivo* expanded human MSC decreases with serial passages.

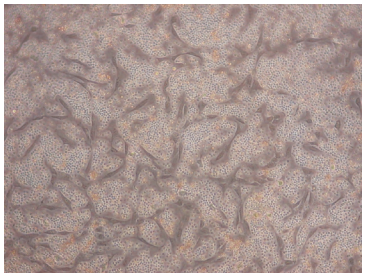

Fig 1 Primary hMSCs,the culture flask contained a small quantity adherence spindle cells and multiplicity floating hematopoietic cells(× 100)

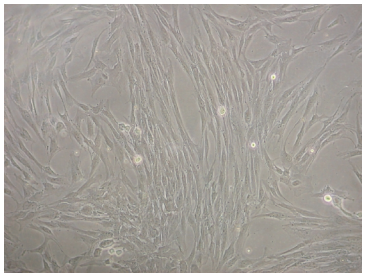

Fig 2 P1 cells, Passaged cells were uniform fusiform shape colony and high speed proliferation (× 100)

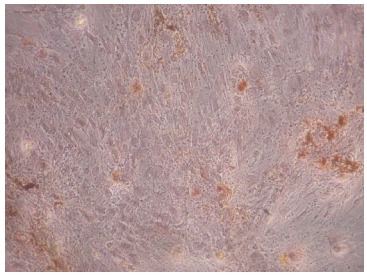

Fig 3 Most cells in Ad-VEGF165 group were stained into red bone tuber (Von Kossa's stain, × 100)

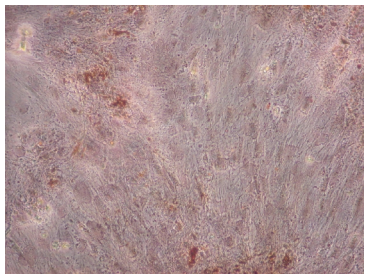

Fig 4 Most cells in positive control group were stained into red bone tuber(Von Kossa's stain, × 100)

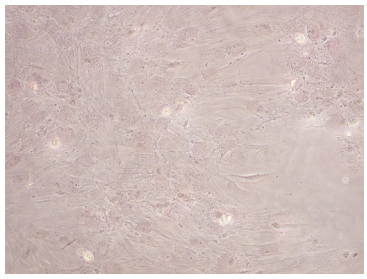

Fig 5 Cells in black control group were not stained into red bone tuber(Von Kossa's stain, × 100)

MSC from old patients cannot be expanded for more than 5 passages without the risk of losing their bone-formation potential. In this study, it's appropriate we selected the 2nd passages cells as treated cells.

Bone formation can not take part with angiopoiesis. In addition to osteogenesis, blood supply is a crucial component of bone formation and fracture healing. Vascular endothelial growth factor (VEGF), one of the best-characterized angiogenic factors, plays an important role in bone growth, via the endochondral ossification pathway. This is because VEGF not only mediates bone vascularization, but also affects the differentiation of progenitors into hypertrophic chondrocytes and osteoblasts [12-14]. Haeusler [15] reported that the expression of VEGF was limited to the chondrocytes of the hypertrophic zone, and osteoblasts and osteoclasts in the human growth plate. Zelzar *et al* [16] suggested that VEGF stimulates bone formation by increasing the activity of osteoblasts both in intramembranous and endochondral bones. Natsuko Kakudo *et al*[17] demonstrated that the blood supply is an important factor for promoting heterotopic osteoinduction by rhBMP-2, especially in producing large masses of bony tissue. Blood vessel regeneration is a very important process in bone formation, and the administration of VEGF may enhance blood vessel regeneration these conditions. Peng H *et al* [18] proved that with the addition of VEGF, there was a significant increase in the density of the bone formed rather than the addition of BMP-4 alone. The multiple roles of VEGF in bone remodelling is evidenced also by studies implicating VEGF in fracture healing, blood vessel formation (in endochondral bone formation) chondrocyte apoptosis, cartilage remodeling and endo chondral growth plate ossification[19]. Zelzer E *et al* [20] have revealed that VEGF may play a role in osteoblast biology in several different ways:Firstly, VEGF, expressed by osteoblasts, could couple angiogenesis to bone formation by adjusting the angiogenic response to osteoblastic activity. Secondly, VEGF could serve as a messenger in bilateral regulation. By expressing VEGF, osteoblasts could induce cells in the vicinity to express factors that, in turn, regulate osteoblastic differentiation. Thirdly, VEGF could be an autocrine regulator of osteoblastic differentiation and activity.

Complete bone marrow culture hMSCs gives quick proliferation and shorten cultivation cycle time, which is ideal for use in clinic. In the process of culture, the floating hemopoietic stem cells can be removed by exchanging culture medium, which can raise purity of hMSCs in culture medium.

Adenovirus is a common carrier for gene transfection as it has high transfection efficiency. Destination genes mediated by adenovirus are not confirmed in

chromosome, that will be only expressed in cytoplasm 4~8 weeks, which can satisfy the need of the gene therapy and avoids the side-effect produced by excess expression[21].

ALP can measure the activity of bone formation. To avoid the ALP in culture medium interference with the experiment result, the culture medium was substituted by DMEM without serum in our experiment. Mineralization of bone tuber gave the strongest supporting evidence indicating bone formation. In our experiment, Ad-VEGF165 group and positive control group had significant difference on ALP and mineralization bone tuber as compared with the blank control group, which testified Ad-VEGF165 gene transfection can promote hMSCs to transfer into osteoblast.

Ad-VEGF165 transfected hMSCs can promote bone formation following angiogenesis. It demonstrated feasibility in treating bone defects and disunion of fracture. In this experiment, the bone formation competence of Ad-VEGF165 group has no significant difference as to the positive group, but the induce time of the positive group lasted 2 weeks while only 24 hours in the Ad-VEGF165 group, which illustrated Ad-VEGF165 can shorten the induce time of bone formation. In clinic, we found it able to shorten treatment cycle, degrade risk of cells pollution and expend the range of indication.

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