Abstract. The present study aimed to determine the mechanism by which low-intensity intermittent negative pressure affects the differentiation and proliferation of human mesenchymal stem cells (MSCs). Alkaline phosphatase (ALP) activity, type I collagen and vascular endothelial growth factor (VEGF) were detected to analyze differentiation. MTT and flow cytometry were employed to measure proliferation and apoptosis. Western blot analysis was used to examine endoplasmic reticulum (ER) stress-associated factors. This study was divided into two groups, including a normal group (without any treatment) and vacuum group (treated with a vacuum). There was a significant decrease in the proliferation of cells in the vacuum group. The number of cells in S phase was reduced significantly, while the rate of apoptosis and the activity of ALP were markedly increased under vacuum conditions. Expression of collagen type I and VEGF was significantly increased, and the ratio of osteoprotegrin to osteoprotegrin ligand was decreased significantly in the vacuum group. ER stress-associated proteins, p-PRKR-like ER kinase, inositol-requiring enzyme 1 and cleaved activating transcription factor 6, as well as the downstream factors, were activated when treated with negative pressure. In conclusion, treatment with low-intensity and intermittent negative pressure may inhibit the proliferation of MSCs and trigger ER stress-associated cellular apoptosis, further enhancing osteogenesis activity and inducing differentiation to osteoblasts.

Introduction

Fractured bones require a long cycle of healing which results in patient suffering, prolonged hospitalization and increasing economic costs for the individual and society. Bone healing may be delayed or obstructed by various numerous factors, including poor blood, loss of bone and soft tissue, metabolic disease, alcohol drinking, osteoporosis and high intraosseous pressure (1,2). Therefore, in the clinic, the identification of methods to shorten the healing time and improve the healing rate of bone defects and osteonecrosis is important for bone healing.

Vacuum-associated closure (VAC) was first reported by Raffl (3) in 1952, when it was successfully applied to prevent infection and fluidity following flap transplantation. In the clinic, VAC has been used as an effective method to heal the soft tissue. However, its effects on bone tissue have never been investigated. Under specific circumstances, VAC may also promote rapid secondary wound healing by improving the blood supply (4). In soft tissue, VAC stretches cells, increases cell or tissue proliferation, promotes blood flow and reduces bacteria count, which helps to prevent cross infection, and the microenvironment of hypoxia and subacidity caused by VAC promotes angiogenesis and the influx of fibroblasts (5). The aforementioned backgrounds and function of VAC in bone tissue are investigated in the present study.

In a previous study, treatment of a compound fracture and the accompanying serious soft tissue injury by VAC was found to promote fracture union. However, the mechanism of VAC effects must be explored. In addition, the effect of negative pressure on bone healing must be investigated. Mesenchymal stem cells (MSCs) are multipotent stem cells that differentiate into a variety of cell types, including osteoblasts. The availability of MSCs is widespread and the cells are easy to isolate and culture. In modern bone repair clinics, MSCs have been employed as ideal seeding cells for substitution therapy.

The aim of the present study was to investigate the effects of negative pressure on the proliferation and differentiation of MSCs, and the specific mechanisms involved in these processes. Through this investigation, our understanding of the precise mechanisms of MSCs in bone regeneration therapy may be improved.

Materials and methods

MSC isolation and culture. Bone marrow tissue was collected from two patients diagnosed with hip osteoarthritis in the
Detection of apoptosis. Cell apoptosis was detected by flow cytometry analysis that monitored annexin V-FITC binding and propidium iodide uptake simultaneously, according to the manufacturer's instructions (Sigma). The samples were analyzed by fluorescence on a FACScan flow cytometer (Beckman Coulter, Miami, FL, USA). The potential DNA fragmentation was examined by the TUNEL apoptosis detection kit (Beckman Coulter, Miami, FL, USA). The samples were then suspended in D-Hank's liquid, followed by separation on a Percoll gradient (Sigma, St. Louis, MO, USA) via centrifugation at 1,200 x g for 10 min, then the middle layer of white film (mononuclear cells) was drawn and washed with D-Hank's liquid. The cells were then placed in L-DMEM medium in sterile culture bottles and cultured in an incubator at 37°C and 5% carbon dioxide. The third generation cells were digested with trypsin and 2x10^5 cells were incubated with antibodies against CD29, CD34, CD44, CD45 or HLA-DR, at room temperature for 30 min and washed twice with PBS. Next, cells were incubated with a secondary antibody labeled with FITC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 15 min in the dark, followed by suspension in PBS following washing. Cells were examined via flow cytometry.

Western blot analysis of endoplasmic reticulum (ER) stress. In order to investigate the mechanism of MSC apoptosis, several ER stress-associated factors were detected using this assay, including p-PRK-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE-1) and activating transcription factor 6 (ATF6). These proteins are the main factors of the unfolded protein response (UPR) pathway. The downstream factors, which may trigger cell apoptosis, were also detected. A western blot analysis method was employed to analyze the ER stress factors.

The MSC lysates were separated by 15% SDS-PAGE and electroblotted onto nitrocellulose membranes. Following blocking with 5% skimmed milk in phosphate buffered saline Tween-20 overnight at 4°C, the membranes were incubated with goat pAb anti-human CHOP (1:2,000), mouse mAb anti-human p-Perk (1:3,000), mouse mAb anti-human IRE1 (1:2,000), mAb anti-human β-actin (1:600) (all Santa Cruz Biotechnology, Inc.), pAb anti-human caspase 3 (1:1,000) (Santa Cruz Biotechnology, Inc.), mAb anti-full length IRE-1 and activating transcription factor 6 (1:2,000) and spliced XBP1 (Stressgen, Farmingdale, NY, USA), mAb anti-human type I collagen and VEGF monoclonal antibodies (Wuhan Bioshide Biotechnology). The cells were washed with PBS, blocked with goat serum and incubated with the primary antibody at a dilution of 1:100 and 4°C overnight. Following washing with PBS, the cells were incubated with the secondary antibody (Santa Cruz Biotechnology, Inc.) at 37°C for 30 min. Next, the ABC reagents were incubated at room temperature for 20 min, washed with PBS and stained using DAB.
the mean ± standard deviation. A statistical analysis was performed using a t-test. *P<0.05 was used to indicate a statistically significant difference.

Results

Proliferation and apoptosis of negative pressure-treated MSCs. Using the flow cytometry analysis, cells were observed to be homogenous and, as is appropriate for MSCs, expressed CD29, CD34, CD44, CD45 and HLA-DR (data not shown). The XTT analysis results indicated that, in the experimental group, the proliferation of MSCs was reduced significantly compared with the control group (Fig. 1A; P<0.05). Flow cytometry detection revealed that the apoptosis rate of the negative pressure-treated group (vacuum) was significantly higher compared with that of the control group (Fig. 1B).

MSCs differentiation. When the MSCs were treated with negative pressure for 2 weeks, the activity of ALP in the vacuum group (16.34±1.47 U/g) was significantly higher compared with that of the control group (7.19±1.18 U/g) (Fig. 2A; P<0.01). The qPCR results illustrated that the OPG mRNA transcription of the control group was significantly increased compared with the vacuum group (Fig. 2B; P<0.01). By contrast, OPGL mRNA transcription was significantly decreased (P<0.01). The ratio of OPGL to OPG mRNA transcription reduced significantly in the vacuum group (Fig. 2B; P<0.01).

Immunohistochemical staining of type I collagen and VEGF. The immunohistochemical staining results indicated that type I collagen was not expressed in the control group.
YANG et al.: FUNCTION AND MECHANISM OF NEGATIVE PRESSURE IN MSCs

1334

(Fig. 3A), but was positively expressed in the vacuum group as a large number of yellow brown particles in the cellular hyaloplasm in those cells (Fig. 3B). In addition, the expression of VEGF in the vacuum group was stronger compared with that in the control group (Fig. 3A and B).

**UPR pathway is involved in the inhibition of MSC proliferation.** To explore the mechanisms of inhibition of proliferation in the negative pressure-treated MSCs, the main UPR factors, p-Perk, IRE-1 and ATF6, were detected by western blot analysis. As shown in Fig. 4A, the treatment of negative pressure triggered the phosphorylation of Perk (p-Perk), the cleavage of ATF6 protein and the activation of IRE-1. The protein expression of p-Perk, cleaved ATF6 and IRE-1 was significantly enhanced by the negative pressure treatment compared with the control group (Fig. 4A; P<0.05).
In addition, the downstream ER stress-associated proteins of the UPR pathway were detected, including the spliced X-box binding protein 1 (XBP1), TNF receptor-associated factor 2 (TRAF2) and eukaryotic initiation translation factor 2α (eIF-2α) proteins. In the negative pressure treatment group, the levels of spliced XBP1, TRAF2 and eIF-2α proteins were significantly increased compared with those of the control group (Fig. 4B; P<0.05).

Discussion

In the clinic, the unique features of the negative pressure therapy method have been hypothesized to contribute to an optimized wound environment, including edema reduction, stimulation of angiogenesis and local blood flow, interstitial fluid flow and exudate management. The method also affects wound perfusion, growth factor, cytokine expression and cellular activity. These roles lead to enhanced granulation tissue formation and improved wound healing parameters (6-8). Therefore, the treatment should be focused on inducing osteogenesis through the effects of mechanical stimulation and growth factors in order to increase the early blood supply and restart bone healing (9). However, its effects on bone tissues and functions in bone repair are poorly understood. In the present study, the effects of negative pressure on osteogenesis in human MSCs and the specific mechanisms were investigated.

For our clinical practice, VAC cured soft tissue defects when applied at a pressure of -50 kPa for 30 min at a frequency of 2 times per day. In the compound injuries, bone healing was also improved when treated with negative pressure. We hypothesized that the negative pressure treatment may promote bone healing through mechanical stimulation, by increasing blood supply, recruitment of osteogenitor cells and inhibiting the proliferation of the osteogenitor cells (MSCs). MSCs are usually used as the seeding cells for gene and cell therapy of skeletal diseases, owing to their potential for differentiation into osteogenitor cells (10,11). The oxygen concentration in vitro culture is ~20%, whereas in vivo oxygen concentration may decrease to only 0.4%-4.7% (12). Therefore, in the present study, the intermittent vacuum incubator (with 2% oxygen tension) was selected to incubate the MSCs with only a few cell cultures, including DMEM. Previous studies have indicated that the disorders correlated to bone repair often appear in older patients or those in which the ability of cells to proliferate has decreased (13,14). Therefore, the target cells (MSCs) from the older patients were selected, and the effects of apoptosis on the proliferation of MSCs were investigated.

The present study also illustrated that the proliferation of MSCs was inhibited under the low-intensity and intermittent negative pressure. This inhibition may be attributed to the temporal hypoxia caused by negative pressure and the apoptosis caused by specific stress stimuli for the MSCs. A number of factors may affect the differentiation and proliferation of the MSCs, including mechanical stimulation and apoptotic responses. In the present study, the MSCs in the vacuum group exhibited significantly higher levels of apoptosis compared with the control group (P<0.05). The apoptosis inhibited the proliferation of the MSCs and triggered differentiation into osteoblasts in a subsequent step (15,16). Our previous study also indicated that negative pressure may lead to upregulation of HIF-1, which contributes to the maintenance of oxygen homeostasis in physiological or pathological conditions. HIF-1 plays a significant role in the regulation of acute and intensive hypoxia (17,18). Towler (19) demonstrated that HIF-1 signaling in the development and differentiation of osteoblasts was central to the coupling of angiogenesis and long bone development in mice, and thus strategies promoting HIF-1 signaling in osteoblasts are likely to augment bone formation and accelerate fracture repair. Therefore, the treatment of negative pressure was hypothesized to be capable of triggering differentiation into osteoblasts.

In order to investigate the effects of the negative pressure on the differentiation of MSCs, ALP activity, type I collagen and VEGF expression were also detected. The results demonstrate that the three factors were enhanced significantly compared with the untreated control group, and promoted and induced differentiation into osteoblasts. This effect of negative pressure may be attributed to mechanical stimulation and cellular hypoxia. Mechanical stimulation plays a basic role in the cell development. Various mechanical stimuli have been revealed to act through numerous signaling pathways that result in changes in gene expression and proliferation of osteoblasts. Previous studies (20,21) have indicated that mechanical stimulation promotes the osteogenic differentiation of human bone marrow stromal cells. Wiesmann et al (22) also reported that the collagen type I and osteonectin expression levels were significantly enhanced by the mechanical stimulation. Compared with other studies, the shorter 30-min intermittent negative pressure treatment time at a frequency of two times per day was hypothesized to increase the expression of type I collagen and VEGF, which are mediated by HIF-1 and promote bone formation.

The present study demonstrated that intermittent negative pressure decreased OPG and OPGL mRNA expression in MSCs. OPG plays a key role in the physiological regulation of osteoclastic bone resorption and acts by binding to OPGL. Negative pressure decreased the ratio of OPG to OPGL in vitro and may therefore correlate with osteogenesis and osteoclastogenesis, which may associate with temporal hypoxia. A pronounced decrease in OPG/OPGL caused by mechanical stimuli results in an increase in bone formation and an inhibition of bone absorption (23,24). The results of the present study revealed a concomitant change of OPG and OPGL mRNA expression in response to a variety of mechanical stimuli. However, these observations are inconsistent with a study by Rubin et al which demonstrated that the change was attributed to decreased mRNA expression of OPGL only.

To investigate the specific mechanism of cell proliferation of negative pressure-treated MSCs, ER stress-associated proteins (UPR pathway) (25,26), including p-Perk, IRE1 and cleaved ATF6 levels, were detected. Three proteins were demonstrated to be activated when treated with negative pressure. Therefore, the UPR pathway may be involved in ER stress-associated apoptosis. Furthermore, the downstream factors, including spliced XBP1, TRAF2 and eIF-2α proteins were also detected. The activation of Perk may phosphorylate eIF-2α proteins and the results markedly indicated the emergence of an ER stress (or UPR pathway) following treatment with negative pressure in the MSCs. The observation that treatment of negative pressure triggered ER stress of MSCs
is of great importance. Therefore we hypothesize that, when treated with negative pressure, the proliferation of the MSCs may be blocked by the ER stress-triggered apoptosis and induce differentiation of MSCs.

In conclusion, treatment with low-intensity and intermittent negative pressure may inhibit proliferation of MSCs and trigger cellular apoptosis, further enhancing osteogenesis activity and inducing differentiation of osteoblasts. The negative pressure may improve bone formation and differentiation by decreasing the ratio of OPGL/OPG mRNA, inducing type I collagen and VEGF expression and decreasing the ratio of OPGL and OPG mRNA. Therefore, this method may be promising for the treatment of bone regeneration disorders.

References