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Substance P Induce Osteogenic Differentiation in Human Adipose-Derived Stem Cells

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Abstract: Substance P (SP) is known to play an important role in bone mineralization. However, little is known about the potential of substance P as an osteogenic inducer in human Adipose-Derived Stem Cells (hADSCs) *in vitro*. Therefore, the aim of this study to investigate the effects of substance P on proliferation and osteogenic differentiation of hADSCs *in vitro*. Human Adipose-Derived Stem Cells (hADSCs) derived from wasted adipose tissue in liposuction operation were cultured. Expression of the SP receptor (NK1) was detected by RT-PCR. Effects of SP on proliferation and differentiation of hADSCs were studied, moreover alkaline phosphatase activity was also assayed. It was found that the NK1 receptor was expressed in hADSCs. SP stimulated the proliferation of hADSCs in a concentration-dependent manner. Higher concentrations (10^{-8} M) of SP increased alkaline phosphatase activity and enhanced differentiated in hADSCs. NK1 receptors are expressed by hADSCs and SP stimulates osteoblast differentiation and function *in vitro*. These findings strengthen the knowledge about the role of SP in hADSCs.

Key words: Substance P, osteogenic differentiation, human adipose-derived stem cells, *in vitro*, RT-PCR

INTRODUCTION

Substance P (SP) is a kind of neurotransmitter widely distributed in the central and peripheral nervous systems. Earlier studies had revealed that SP modulates osteoblastic bone formation through Neurokinin-1 Receptors (NK1-Rs). SP functions are made as a neurotransmitter of nociceptive messages. It is synthesized in unmyelinated sensory neurons and released from their peripheries (Nicoll *et al*., 1980). Several studies have reported that SP was associated with bone metabolism such as osteoblastic bone formation and osteoclastic bone absorption (Fontan *et al*., 2000). The stimulatory effects of SP on osteoclastic absorption of bones was confirmed by earlier studies (Fontan *et al*., 2000). However, the effects of SP on osteoblastic bone formation are still unclear so far. Some studies have showed that SP stimulated osteogenesis in a dose-dependent manner (Shih and Bernard, 1997). But on the other hand, some reports suggested that SP inhibits osteoblastic cell differentiation in rat calvarial osteoblastic cells demonstrated that SP NK1-Rs are expressed in calvarial osteoblastic cells at the late stage of cell differentiation (Goto *et al*., 2007). Yet, the specific molecular targets of SP on modulation of bone formation are still unclear. Thus, it is crucial important to study molecular mechanism of SP for study osteoblastic cells of cell differentiation. Adipose-derived stem cells are a source of multipotent mesenchymal stem cells that can be differentiated into osteogenic, chondrogenic, adipogenic and neurogenic cell types *in vitro* (Zuk *et al*., 2001; Katz *et al*., 1999). These cells can be isolated from wasted adipose tissue and expression of stem cell markers (Madonna *et al*., 2008). Some studies showed that stem cells derived from adipose tissue are able to differentiate into osteoblastoids under high serum conditions and are a potential source of autologous bone produced *in vitro* (De Ugarte *et al*., 2003; Laino *et al*., 2006; Estes *et al*., 2006). This tissue contains progenitor cells that give rise to the bone tissue including osteoblast, osteoclast and other related cells (Zuk *et al*., 2001).

Some studies has been used SP for osteogenic differentiation of MSCs (Wang *et al*., 2009). However, the effects of SP induce osteogenic differentiation in hADSCs is not yet clearly understood *in vitro*. Additionally, SP has been reported to promote the function of osteoblast genesis in the osseous tissue (Gimble and Guilak, 2003). Taken together, researchers hypothesized that cells derived from adipose tissues when treated with SP might optimally induce osteogenic differentiation *in vitro*.

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Therefore, this study was designed to understand the role of different concentrations of SP on proliferation and osteogenic differentiation of hADSCs *in vitro* by a series of investigations.

MATERIALS AND METHODS

Cell isolation and culture: Human adipose-derived stem cells were obtained from patients in the first hospital of Jilin University (Changchun, China) and an informed consent was obtained from all participants. The collection of stem cells from wasted adipose tissue samples was approved by the Ethics Committee of the Jilin University (Changchun, China). The adipose samples were brought to the laboratory in Phosphate Buffered Saline (PBS) containing 2% antibiotics/antimycotics (a/a; 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin and 0.25 mg mL^{-1} amphotericin B; Invitrogen, USA). The adipose tissues were isolated in the laboratory under laminar hood; cleaned with PBS to remove resident drug and hemacytes.

Then cut the pieces of adipose tissue into plasm. Tissues were then digested in 0.075% collagenase type II 4 mg mL^{-1} (Invitrogen) and dispase 4 mg mL^{-1} (Invitrogen) for 1 h at 37°C. Once digestion was completed the obtained cell suspension was passed through a 7 μm cell strainer (Falcon, USA) and cells were seeded in 6 well culture plates in basic cell culture medium (BM) including of DMEM/F-12 1:1 (Invitrogen), 10% FBS (Invitrogen), l-glutamine and 1% antibiotics/antimycotic (100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin and 0.25 μg mL^{-1} amphotericin B; Invitrogen) and then incubated at 37°C in 5% CO_2.

After 14 days of culture, cells were detached using trypsin in PBS (Lonza) and then cells were expanded in 75 cm^2 culture flasks containing culture medium. Cell culture plates and flasks were monitored daily for cell growth with medium changes taking place three times per week. All assays were performed using cells between passage 3 and 4 and experiments were repeated using cells derived from 3 different donors for hADSCs.

Substance P (Sigma) was dissolved in distilled water to a final concentration of 10^{-3} M and then was aliquoted and stored at -20°C. SP was diluted to the appropriate concentration in the culture medium. Starting on day 1 the mouse cells were continuously stimulated with SP at concentrations of 10^{-8}, 10^{-10} and 10^{-12} M and the medium was changed every 2 days. The concentrations used in this study were based on earlier reports demonstrating *in vitro* efficacy for these concentrations (Adamus and Dabrowski, 2001).

RT-PCR: Total RNA from hADSC cells grown in 6 well plates was extracted by the RNeasy Mini kit (Qiagen, German) and the purity and concentration were determined spectrophotometrically. The cDNA was subsequently synthesized from 1 μg RNA by an Script cDNA Synthesis kit (Takara, Japan). The primer sequences used for amplification were as follows: human β-actin 5' primer, CTA CAA TGA GCT GCG TGT GGC; human β-actin 3' primer, CAG GTC CAG ACG GAT GGC; human NK1 receptor 5' primer, GAAGGCTATCCTCGGTTTCC and human NK1 receptor 5' primer 3' primer, TGTGGCTGAGAATGACTTCG. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and 72°C for 30 sec, 30 cycles in all. The PCR products were electrophoresed in 1.5% agarose gels and visualized with ethidium bromide.

Cell proliferation assay: Cell proliferation assay was done to measure the viability and induction of cell proliferation by SP (Sigma) on hADSCs. The cells were treated with different concentrations of SP (10^{-8}, 10^{-10} and 10^{-12} M) as follows. hADSCs each were seeded at 10,000 cells/well in 24 well plates in BM with different concentrations of SP. The control samples were maintained in BM and blank values were also measured for non-specific binding. The culture media was changed after 3 and 4 days for each media concentration. The plates were incubated at 37°C in a 5% CO_2 containing humidified atmosphere. Cell numbers and viability were quantified at 1, 7 and 14 days time points using the colorimetric reagent WST-1 (Takara, Japan). The absorbance was measured directly with a plate reader Victor 1420 by wavelength of 450 nm.

Alkaline phosphatase activity: Cultured hADSCs were fixed with fresh 10% neutral buffered formalin on days 7, 14 and 21 before each well was assayed for alkaline phosphatase activity. Activity was determined using a commercially available kit (Sigma). The absorbance was measured with a spectrophotometer at 410 nm and the cell number was assayed using crystal violet staining as earlierly described (Cao *et al*., 2005).

Statistical analysis: The statistical analyses of the results were performed with SPSS 13.0 (USA). The data is presented as mean±Standard Error of the Mean (SEM) for all quantitative assays and experiments were conducted in triplicate for cells derived from three donor samples. All statistical analyses were performed at the significance level $p<0.05$. One-way Analysis of Variance (ANOVA) with Dunnett's post hoc test for multiple comparisons was used for the analysis.

RESULTS AND DISCUSSION

Human ADSCs were isolated by Enzyme-Digestion Method. Both cell types exhibited initial triangular, stellate or spindle shape cell morphology after initial plating. The time required to form confluent cell cultures varied from 1-2 weeks for hADSCs. The hADSCs appeared spindle or stellate in shape as observed under phase contrast microscope as shown Fig. 1. To characterize NK1 receptor mRNA expression during osteogenic differentiation, RT-PCR was performed to quantify mRNA levels of NK1 receptors in adherent hADSCs. As measured by RT-PCR, NK1 receptor mRNA was expressed in hADSCs, human osteoblast and rat osteoblast which was used as a positive control (Fig. 2). The effects of different concentrations of SP at 14 days on proliferation of hADSCs was analyzed. The addition of SP to hADSCs induced increase in cell proliferation when compared to the cells treated without the SP as shown in Fig. 3. ALP staining was performed to identify osteoblast differentiation in hADSCs during the period of cultivation, lower concentration and high concentration of SP treatment hADSC cells showing a positive ALP activity.

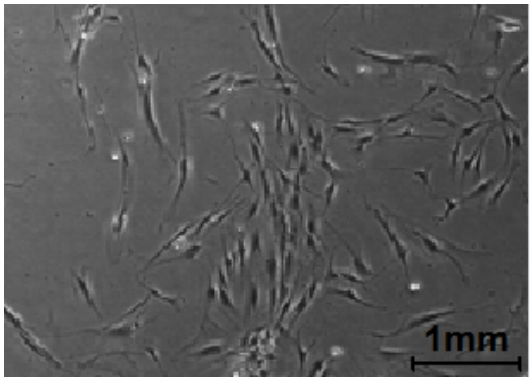

Fig. 1: Light microscopic appearance of hADSC (scale bar = 1 mm)

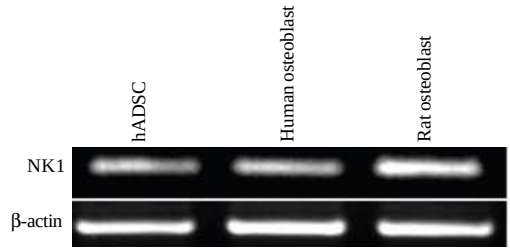

Fig. 2: NK1 receptor mRNA is present in hADSC, human osteoblast and rat osteoblast

However, treated with higher concentration SP ALP activity is higher than those of lower concentration SP throughout the period of cultivation (Fig. 4). The present study shows that osteoblast differentiation in hADSCs was stimulated by SP. Differentiation of MSCs such as those derived from bone marrow tissue into osteoblast like cells is induced *in vitro* by treating the cells with Dex, ascorbic acid and β-glycerophosphate as reported in many studies (Zhou *et al*., 2006; Gupta *et al*., 2007). In addition, earlier study showed that SP may be superior to dexamethasone as an agent that induces osteogenic differentiation in mesenchymal stem cells (Huang *et al*.,

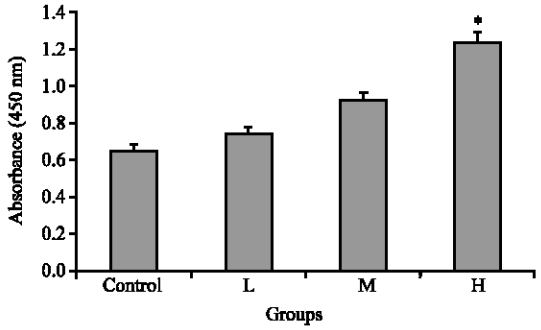

Fig. 3: Effect of different concentration SP on cell proliferation. Cell numbers were analyzed in hADSC at 14 days. Columns represent mean±SEM (n = 5); statistically significant difference when treated samples were compared to the control of each time point, *p<0.05; L: Lower concentration of SP (10^{-12}); M: Middle concentration of SP (10^{-10}); H: Higher concentration of SP (10^{-8})

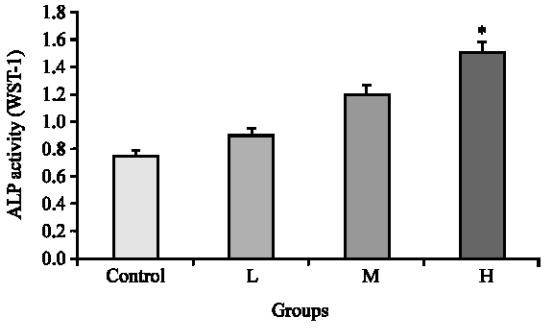

Fig. 4: ALP activity of hADSC was assessed after 14 days of differentiation by different concentration of SP treated. Columns represent mean±SEM (n = 5). Statistically significant difference when treated samples were compared to BM of days 7 and 14, *p<0.05; L: Lower concentration of SP (10^{-12}); M: Middle concentration of SP (10^{-10}); H: Higher concentration of SP (10^{-8})

2006). Considering the earlier reports it was important to confirm the effects of SP with or without the addition of Osteogenic Supplements (OS) on proliferation and osteogenic differentiation of hADSC. In this study, the data showed that SP can induced osteogenic differentiation of hADSC which was comply with previous results that SP could inhibit osteoblastic cell differentiation in rat calvarial osteoblastic cells (Goto *et al*., 2007).

Bone is abundantly innervated by small diameter sensory nerves in the periosteum, bone marrow and vascular canals (Imai and Matsusue, 2005; Mach *et al*., 2002). In addition to conducting pain and information about thermal, mechanical and chemical stimuli that have the potential to cause tissue damage, these skeletal sensory neurons produce a variety of peripherally released neurotransmitters including Substance P (Sp), Calcitonin Gene-Related Peptide (CGRP) and somatostatin. It has been reported that capsaicin induced depletion of neuropeptides such as SP and CGRP in the unmyelinated sensory neurons of adult rats is accompanied by bone loss and increased bone fragility (Offley *et al*., 2005). Earlier investigators have reported conflicting results regarding the presence of the SP NK1 receptors in bone and bone cells (Goto *et al*., 1998; Togari *et al*., 1997). Some investigators have reported that SP treatment can stimulate Bone Marrow Stromal Cell (BMSC) proliferation, protein production and mineralization (Shih and Bernard, 1997; Adamus and Dabrowski, 2001). Paradoxically, SP inhibitory effects have been observed on alkaline phosphatase activity, mineralization and osteoblastic gene expression and in addition, SP had no effect on cAMP production in osteoblastic cell lines and in mouse calvarial osteoblastic cells (Azuma *et al*., 2004; Adamus and Dabrowski, 2001; Bjurholm *et al*., 1992). Goto *et al*. (2007) demonstrated that SP treatment of calvarial osteoblasts increased mineralization. In the current study, researchers examined osteogenic differentiation of hADSC and human osteoblasts, the results showed SP increased cell proliferation and osteoblast differentiation of hADSC and human osteoblasts.

The low concentrations of SP required for its osteogenic effects *in vitro* are close to the SP concentrations observed in human serum (10^{-11} M) and synovial fluid (10^{-10} M) (Arai *et al*., 2003; Grimsholm *et al*., 2005) and in rat trabecular bone (10^{-9} M) (Huang *et al*., 2006), data suggesting that SP could be a physiologic activator of bone formation *in vivo*. Higher concentrations of SP are observed in the synovial fluid of rheumatoid and osteoarthritis arthritis patients (Pritchett, 1997; Grimsholm *et al*., 2005) and enhanced SP signaling has the potential to induce osteoclastogenesis and bone resorption in the arthritic joint. The results showed that 10^{-8} SP had increased effects on the proliferation and activity of hADSC compared to low concentration of SP which is agree with earlier study (Pritchett, 1997; Grimsholm *et al*., 2005).

CONCLUSION

Researchers demonstrate that neuropeptide SP stimulates osteoblastic activity and increased ALP activity in the hADSC cultures. These results clarify the role of SP in the osteoblastic differentiation process at the molecular level and will hopefully provide a basis for future studies on links between the nervous system and bone formation.

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