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Researh Paper

Effect of the gap junction blocker 1-heptanol on chondrogenic differentiation of mouse bone marrow mesenchymal stem cells *in vitro*☆

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Abstract

Objective: To investigate the effect of the gap junction blocker 1-heptanol on the *in vitro* chondrogenic differentiation of mouse bone marrow mesenchymal stem cells(MSCs) following induction by GDF-5. **Methods:**MSCs were isolated from mouse bone marrow and cultured *in vitro*. After 3 passages cells were induced to undergo chondrogenic differentiation with recombinant human GDF-5(100 ng/ml), with or without 1-heptanol(2.5 μ mol/L). The effect of 1-heptanol on MSCs proliferation was investigated using the MTT assay. Type Ⅱ collagen mRNA and protein were examined by RT-PCR and immunocytochemistry respectively, and the sulfate glycosaminoglycan was assessed by Alcian blue dye staining. Connexin43(Cx43) protein was examined by western blotting. **Results:**GDF-5 induced proliferation and chondrogenic differentiation of MSCs. While 1-heptanol treatment had no effect on this proliferation, it inhibited the expression of both type Ⅱ collagen mRNA and protein. The Alcian blue staining revealed that 1-heptanol also inhibited the deposition of the typical cartilage extracellular matrix promoted by recombinant GDF-5. Western blotting demonstrated that 1-heptanol had no effect on the expression of Cx43. **Conclusion:**These results suggest that mouse bone marrow MSCs can be differentiated into a chondrogenic phenotype by GDF-5 administration *in vitro*. While the gap junction blocker, 1-heptanol, did not reduce gap junction Cx43, these intercellular communication pathways clearly played an important functional role in GDF-5-induced cartilage differentiation.

Keywords: growth differentiation factor-5; gap junction; cartilage; mouse; bone marrow mesenchymal stem cells.

INTRODUCTION

Growth differentiation factor 5(GDF-5) plays a very important role in joint formation and the development of the skeletal structures of the limb[1-4]; and it also can accelerate the differentiation from stem cell to cartilage *in vitro*[2-3,5]. GDF-5 and Cx43 have similar temporal and spatial expressions at the early stage of bone growth when chondrogenic mouse embryonic stem cells gather[3,6]. Cx43 mediated cell-cell communication is involved in the process of acceleration of chondrogenesis by GDF-5[3,7]. The present research uses MSCs induced to differentiate to a chondrogenic phenotype *in vitro* by GDF-5, and the gap junction blocker, 1-heptanol, to study the role of Cx43 mediated cell-cell communication in chondrogenesis.

MATERIALS AND METHODS

Reagents

L-DMEM, trypsinase, and new-born calf serum were obtained from Gibco(USA), GDF-5 factor from Cytolab Co.(USA), mouse collagen Ⅱ monoclonal antibody from Lab Vision(Thermo Scientific, USA), Cx43 polyclonal antibody from Wuhan Bostoer Biological Technology Ltd.(China), Western blotting reagents,SP immunohistochemistry staining and DAB kits from Beijing Zhongshan Biological Technology Ltd. (China), Alcian blue, dexamethasone, insulin and vitamin C from Sigma Chemical Co.(USA), RT-PCR reagents from Beijing Tianwei Time Technology Ltd.(China), and MTT from Huamei Biological Engineering Co. (China).

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Primers were designed and produced by Shanghai Jikang Biological Technology Ltd. (China). Mouse embryos were obtained from mice of the kunming strain provided by the Department of Experimental Animals of Tongji Medical College of Huazhong Science and Technology University.

Separation and culture of MSCs

Cells were removed from the mouse thighbone under an asepsic procedure. The bone marrow was rinsed repeatedly with L-DEM containing 10% newly-born calf serum and fully blown into a single cell suspension. Then, the bone marrow was inoculated into the culture flask at a density of 1×10^6/cm^2, and cultured at 37℃, in 5% CO2 and 100% saturated humidity. The medium was changed for the first time after 48 hours, the unattached cells removed, then changed twice a week. When cells are close to 90% confluent, 0.25% parenzyme was used to detach them for passaging at a 1:3 dilution.

Induction of the chondrocyte phenotype *in vitro*

Rapidly growing third-passage cells were collected and then inoculated onto the 6-well plates, containing glass coverslips, at a density of 1×10^6/ml. These cells were divided into 3 groups:Control group(group A): serum-free L-DMEM was used as a culture media; GDF-5 group(group B):control group medium+dexamethasone (10^{-7}mol/L), vitamin C(50 μg/ml), insulin(6.25 μg/ml) and GDF-5(100 ng/ml); 1-heptanol group(group C): GDF-5 group medium +1-heptanol group(2.5 μmol/l). The plate was incubated for 72h at 37℃, in 5% CO_2 and100% saturated humidity. The glass coverslips were then removed and used in further assays.

MTT test for the detection of cell proliferation

The proliferation of the cells was observed after culturing for 24h, 48h, 72h. The cell suspension density was adjusted to 1×10^5/ml and wells of a 96 well plate were inoculated with cells in complete medium(100 μl/well) and incubated overnight. The supernatant was then removed and the well filled with representative media of the control, GDF-5, and 1-heptanol groups. After 20h, 44h or 68h incubation, 10 μl MTT(5 mg/ml) was added to each well, and after 4h the medium was removed and replaced with 100 μl dimethyl sulfoxide. Thirty minutes later the absorbance of every well was measured with a plate reader at 570 nm.

RT-PCR detection of Collagen Ⅱ mRNA expression in MSCs

The cells of the 3 groups were cultured for 72hrs and then harvested. The total RNA was extracted by following the instructions of the Trizol kit. After quantification of the RNA, 2 μg of RNA was used in the synthesis of cDNA using reverse transcriptase. The cDNA was then used as a template for PCR amplification of the collagen Ⅱ gene. At the same time, the β-actin gene was used as the internal reference. The sequence of collagen Ⅱ primer was designed(upstream): 5′-AGGGGTACCAGGTTCTCCATC-3′, (downstream):5′-CTGCTCATCGCCGCGGTCCTA-3′, product segment: 225 bp. The reaction conditions were: 94℃ for 3 min, (94℃ for 30 s, 50℃ for 30 s, and 72℃ for 40 s) for 30 recycles, 72℃ 10 min. The amplification product was subjected to 1.5% agarose electrophoresis and scanning by the SensiAnsy gel image analysis system. Semi-quantitative analysis was conducted to measure the ratio between the collagen Ⅱ and β-actin optical density(OD) value.

Detection of Cx43 expression with Western blotting

The cells of the three study groups were incubated for 72 hours and then harvested with lysis buffer. The total protein of each cell preparation was separated by SDS-PAGE electophoresis and transferred to PVDF membranes by electrophoresis. After rinsing, membranes were incubated with anti-Cx43 antibody and anti-βactin antibody and, after rinsing, the antibody was visualized by standard immunology methods, and analyzed using a gel imaging system. The OD value of each Cx43 band was compared with β-actin, and the data calculated as a ratio.

Chemical staining of immunocytes of Collagen Ⅱ

Coverslips of the three groups were taken and cells were fixed with absolute ethyl alcohol and acetone (1:1) for 20 minutes, rinsed with PBS for 3 minutes and incubated for 10 minutes in 3% H_2O_2 deionized water. Cells were then incubated at room temperature for 30 minutes with normal goat serum, the serum removed, and mouse anti-collagen II monoclonal antibody was added(at 1:100), and incubated overnight at 4℃. Biotin conjugated goat anti-mouse lgG was used as the second antibody, and the samples were incubated at 37℃ for 30 minutes, after which a streptavidin-biotin-peroxidase kit was used and the antibody labeling visualized with DAB, following the manufacturer's instructions.

Staining with Alcian blue

Cells from all three groups were fixed with absolute ethyl alcohol and acetone(1:1) for 20 min and rinsed with distilled water, 1% Alcian was added to the distilled water and cells were stained for 30 min. Then, the cover-slips were rinsed with distilled water, cleared with xylene and sealed with neutral resin.

Statistical analysis

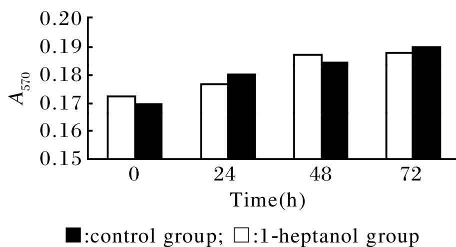
The experimental data was represented as $\bar{x} \pm s$ and analyzed with the SPSS10.0 software. Analysis of variance(two sides, α=0.05) was made among the groups.

Student-Newman-Keuls test was used for pair-wise comparison and a P< 0.05 was considered to be statistically significant.

RESULTS

The effect of 1-heptanol on MSC cell proliferation

Fig. 1 shows the effect of 1-heptanol on cell proliferation. There was no significant difference in absorbance between control and experimental groups after 24 h, 48 h, and 72 h(P> 0.05), indicating that 1-heptanol has no effect on the proliferation of MSCs, or was cytotoxic to these cells.

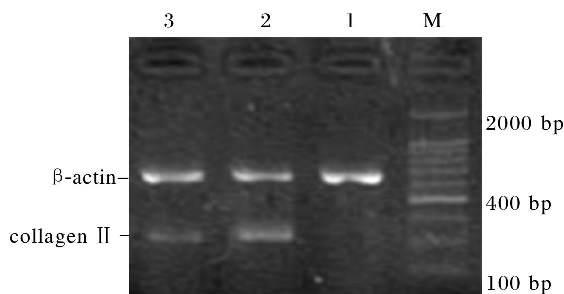


■:control group; □:1-heptanol group

Fig. 1 effect of 1-heptanol on cell proliferation

Collagen Ⅱ mRNA expression

Cells of five replicate experiments were collected and subjected to RT-PCR amplification(***Fig. 2***). In the control group(group A), no collagen Ⅱ mRNA expression was observed. However, in the GDF-5 group cells (group B) and the 1-heptanol group cells(group C) there was expression of collagen Ⅱ mRNA. The mean values of groups B and C were significantly different when the collagen OD was normalized to sample β-actin(0.527 ± 0.013 and 0.386 ± 0.008 in groups B and C respectively). These data show that 1-heptanol inhibited collagen Ⅱ mRNA expression in MSCs differentiated to chondrocytes with GDF-5.

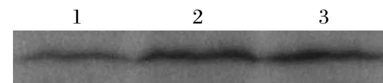


M:100 bp DNA Marker; 1:control group; 2:GDF-5 group; 3:1-heptanol group.

Fig. 2 Detection of Collagen Ⅱ mRNA expression with RT-PCR

Expression of Cx43 protein

Western blotting detected the expression of Cx43 protein after cells were cultured for 72 hours(***Fig. 3***) and the relative densitometry values were:Control group, 0.326 ± 0.004; GDF-5 group, 0.582 ± 0.006;1-heptanol group, 0.576 ± 0.014. The Cx43 protein expression was significantly different when the control group was compared to either the GDF-5 and 1-heptanol groups, but there was no significant difference between GDF-5 and 1-heptanol groups. This indicates that GDF-5 enhanced the expression of Cx43, and that 1-heptanol had no effect on the expression of this connexin.

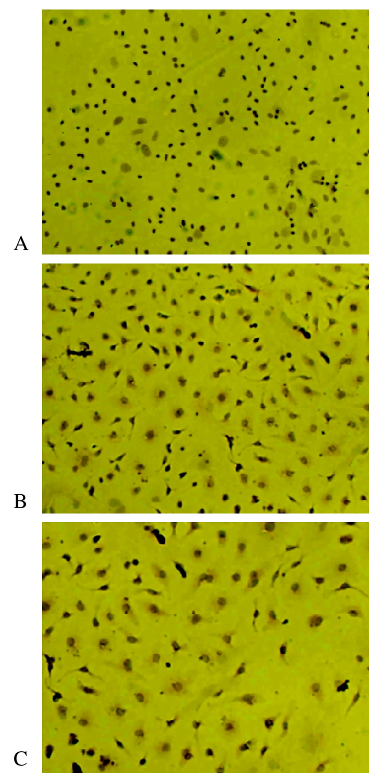


1:control group; 2:GDF-5 group; 3:1−heptanol group.

Fig. 3 Detection of Cx43 protein expression with Western blotting

Immunohistochemical staining of Type Ⅱ collagen

The cells were cultured for 72 hours and stained with the SP method. The control group cells were negative for type Ⅱ collagen, the GDF-5 group cells stained strongly positive and the 1-heptanol group cells stained positive(***Fig 4, Table 1***). This also indicated that 1-heptanol inhibited GDF-5-induced MSCs differentiation into a chondrogenic phenotype, confirming the mRNA data shown above.



A:control group; B:GDF-5 group; C:1−heptanol group.

Fig. 4 Type Ⅱ collagen immunocytochemical staining of cells after 72 h incubation(200 ×)

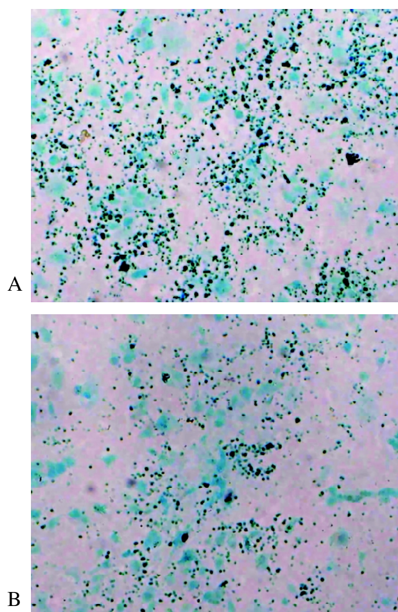
Table 1 **Percentage of cells staining positive for type Ⅱ collagen after 72 h incubation**

GROUPS	positive cells(%)
A	0
B	$63.24 \pm 1.42^{*\#}$
C	$34.68 \pm 3.11^{*\#}$

A:control group; B:GDF-5 group; C:1-heptanol group. As compared with group A, $^{*}P < 0.05$; compared B with C, $^{\#}P < 0.05$.

Staining with Alcian blue

Using Alcian blue stain, cells in group A were negative, while most cells in group B stained blue, and cells in group C stained weakly compared with the cells in group B(***Fig. 5***). These results demonstrated that 1-heptanol inhibits proteoglycan secretion in MSCs induced to differentiate into chondrocytes by GDF-5.



A:GDF-5 group; B:1-heptanol group.

Fig. 5 Alcian blue staining of cells cultured for 72 h after GDF-5(200 ×)

DISCUSSION

Entochondrostosis is the main process of bone formation. It includes mesenchymal cell aggregation and differentiation to cartilage, followed by calcification into developing bone tissue[8]. GDF-5 plays an important role in the aggregation of mesenchymal cells and formation of cartilage in the early stage of limb development. The mouse, which has a natural mutation of GDF-5, shows a bp phenotype, also termed the bp mouse[9]. This mutation causes changes of bone development, leading to severely malformed limbs[10], which includes shortening of the ossa longum and flattening of the phalanges with multi-joint fusion, while axial bones and skull are normal. Humans with this mutation display congenital maldevelopment of cartilage, such as Hunter-Thompson and Grebe's chondrodysplasias. The reasons for such phenotypes include the decrease of bone cell precipitation, and weak communication and signal transduction between cells. *In vitro* experiments it has been shown that GDF-5 can induce the differentiation of mesenchymal cells into cartilage cells, which makes it important to address the cellular and molecular mechanisms by which GDF-5 affects chondrogenesis[11].

In the process of embryonic bone development, the earliest morphological changes taking place as cartilage begins to be formed include a decrease in the distance between mesenchymal cells, and cell-cell contact. This is an important stage before the formation of cartilage[12]. The gap junction is a specific membrane to membrane contact between adjacent connected cells. It constitutes a straight passage communication between cells and is composed of connexin molecules. The signal transduction between cells is mainly accomplished by these gap junctions. In the early stage of limb bone development, gap junction protein, mainly Cx43, is highly expressed in aggregated mesenchymal stem cells and cartilage cell membranes. With the process of differentiation, its expression proportionally decreases. Thus, evidence shows that the gap junction, mediated by Cx43, promotes cell-cell attachment, and signal transduction and coordination between cells[13]. Moreover, both of GDF-5 and Cx43 are expressed early on the development of cartilage and are their expressions are highly correlated, suggesting that the effect of GDF-5 depends on the Cx43-mediated intercellular communication through gap junctions[6].

Intercellular communication mediated by gap junctions is readily interrupted by disease and chemical agents. 1-heptanol is a hydrophobic uncoupling agent of gap junctions that can reversibly suppress the functioning of gap junctions between the cells. However, at high concentrations it will also lead to cell the toxicity[14]. This study used a low concentration of 1-heptanol(2.5 μmol/L) to block gap junction to observe GDF-5 induced differentiation of cartilage *in vitro*. The MTT results showed that 2.5 μmol/L 1-heptanol had no effect on the proliferation of mouse marrow stem cells (MSCs), suggesting this concentration was not cytotoxic.

In the present studies, mouse MSCs were induced to form cartilage. Cells in serum-free medium acted as a control, while those in the GDF-5 group received dexamethasone, vitamin C, insulin and 100 ng/ml GDF-5, and those in the1-heptanol group received the same treatment as those in the GDF-5 group, but with the addition of 2.5 μmol/L 1-heptanol. Alcian Blue staining, RT-PCR and immunocytochemistry methods were used to confirm that GDF-5 induced the differ-

entiation of MSCs into chondrocytes at the early stage of morphological development. The gap junction blocker, 1-heptanol decreased the secretion of cartilage cell specific type II collagen and proteoglycan matrix. These results showed that 1-heptanol could attenuate the effect of GDF-5 by blocking the gap junction. Moreover, GDF-5 induced the expression of Cx43, while 1-heptanol had no effect on the upregulation of this connexin. This confirms the possibility that GDF-5 can upregulate the Cx43 to increase the communication between cells through gap junctions to induce the cartilage phenotype. While 1-heptanol had no effect on the expression of Cx43, it is known to function by blocking gap junction communication. Some studies have shown that the communication through the gap junction, mediated by Cx43, participates in the process of the bone morphogenetic protein(BMP) family induction of cartilage formation *in vitro*[15-16]. The present study shows that the Cx43-mediated gap junction plays an important role in the induction of cartilage formation by GDF-5, a member of the BMP family.

In conclusion, this study investigated the roles of GDF-5 and Cx43 gap junctions in the *in vitro* differentiation of MSCs to cartilage producing cells. We have shown that GDF-5 regulates the aggregation of these stem cells and their differentiation to a cartilage-producing phenotype by a process that involves Cx43 mediated gap junctions. However, the mechanisms of the role of GDF-5 in bone development and cartilage phenotype inducement *in vitro* are still uncertain. Besides the increase of the gap junctions mediated by Cx43, the Smad and MAP kinase pathways also participate in the chondrogenesis, and require further study.

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