

Effect of BDNF-Layered Hyaluronan Nanoparticles on New Bone Formation

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Abstract

Bone healing is a complicate tissue repair process in the fracture gap. New implants should be able to promote bone formation and reduce side effects as well. Here, BDNF/hyaluronan-NPs were explored for their applicability in bone repair. Microscopy and scanning images showed that the number of human MSCs increased and their differentiation into osteoblast was promoted after application of BDNF. Real-time PCR revealed that the relative mRNA expression of both osteogenic and angiogenic genes significantly increased after stimulated by BDNF. Furthermore, consistent with these results, the administration of BDNF/hyaluronan-NPs induced bone formation *in vivo* and compatible with the surrounding tissue. We conclude that the combination of BDNF and hyaluronan-NPs could efficiently induced osteogenesis and osteoblast activity and facilitate induction of endothelial cells. Thus, BDNF/hyaluronan-NPs might be a promising approach for bone fracture treatment.

Keywords: Bone repair; Osteogenesis; BDNF; Nanoparticle

Introduction

The biology of bone fracture repair is a complex process that recapitulates bone development and can be viewed as a form of tissue regeneration. Indeed, skeletal tissue has the capacity of regeneration, however, this repair process is still facing many challenges and fractures sometimes heal in unfavorable anatomical positions or delay in the healing [1-3]. In past two decades, investigations in both humans and animal models have revealed the pathways that regulate the biological process of fracture repair and provided an insight into formation of new bone mass [4]. Bone fracture repair consists of both endochondral and intramembranous bone healing, which involves a lot of molecular and cellular events [5]. For example, inflammatory response is initiated to recruit inflammatory cells to clear up damaged tissue and promote angiogenesis and even to induce osteogenic differentiation of

Mesenchymal Stem Cells (MSCs) [6-8]. During the healing process, MSCs-derived osteocytes are responsible for bone matrix excretion and ossification as well as bone modeling by sensing the mechanical load of bone [9,10].

The fracture treatments usually consider osteoinductive materials to bridge the fracture gap and allow stable fixation for bone formation by osteocytes [11]. The addition of growth factors to the osteoinductive materials can improve the osteoinductivity and stimulate bone formation. Nanotechnology has been under exploration for its application in the treatment of skeletal-related disorders including bone regeneration for many years. Nanoparticles can offer a range of unique properties including a high ratio of surface to volume and carry of therapeutic agents, for example, mesoporous silica, calcium phosphates, poly(lactic-co-glycolic) acid and chitosan are explored for their use to delivery and sustainable release of drugs [12,13]. For decades, growth factors mainly from the bone morphogenetic protein family have been investigated in the preclinical trials of bone repair. The use of nanoparticles can be an effective approach for the delivery of this growth factor to enhance bone healing [14]. However, significant limitations remain in these approaches, including the lack of synergistic effects in the combination of nanoparticles and active biostimulants, the absence of blood vessel formation and slow rate of bone mineralization [15].

Here, we've investigated the effect of Brain-Derived Neurotrophic Factor (BDNF) delivered by hyaluronan nanoparticles (hyaluronan-NPs) in bone repair. BDNF is known to regulate neuron survival and angiogenesis [16]. Additionally, it also presents in osteoblasts and upregulates during murine and human fracture healing as well as MSC osteogenic differentiation *in vitro* [17-20]. Hyaluronan in previous reports has shown to have many benefits in the tissue repair. It resembles the natural extracellular matrix in terms of high water content and structural stability, which can act as a scaffold to provide a suitable environment for new bone formation [21]. Besides, it can easily incorporate therapeutic agents and adapt to complex-shaped areas with good contact with the native tissue [22]. However, the suitability of combining BDNF and the delivery system on bone repair remains unknown. Thus in the present study, we

evaluated in more detail the effect of BDNF on bone formation in combination with the delivery system of hyaluronan NPs.

Materials and Methods

Cell culture

Primary human MSCs were derived from bone marrow (PromoCell). The cells were cultured in Gibco MEM alpha (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma). The cells were cultivated in a 37°C incubator with 5% humidified CO₂. The culture media was changed every 3 days. The cells were split until approximate 80% confluent. For osteogenic differentiation, the cells were cultured in the growth medium supplemented with 50 g/ml ascorbic acid, 10 mM dexamethasone and 5 mM glycerophosphate. BDNF and hyaluronan were prepared according to the manufacturers' instructions (Sigma) and added to cell culture and injected into the fracture sites.

Alkaline phosphatase (AP) activity and mineralization assessment

The osteogenic differentiation was evaluated by the AP activity and mineralization of osteoblasts, which were assessed at days 7 and 21 respectively. For AP activity assessment, cells were washed with PBS 2 times and fixed with 4% PFA and then stained with NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) according to the manufacturer's instructions (Sigma). For mineralization assessment, fixed cell culture was stained by 40 mM alizarin red S (pH 4.1) (Sigma) for 15 min and then washed with distilled water. Stained cell cultures then were scanned and the intensity of colors developed correlated with the AP activity and degree of mineralization.

Gene expression analysis

The gene expression was analyzed by their mRNA level. Quantification of mRNAs was performed by real-time PCR. Total RNA was isolated from cell cultures by TriZol (Invitrogen) and then subject to reverse transcription to obtain cDNA (Promega). The cDNA then was used as the template in the real-time PCR using SYBE Green real-time PCR master mix (TOYOBO). The relative expression level was normalized by GAPDH. The primer pairs for the genes examined were listed as follows. Runx2: 5'-CCCAGTATGAGAGTAGGTGCC-3' and 5'-GGGTAAGACTGGTCAT AGGACC-3'; AP: 5'-ACAAGCACTC CCACTT CATCTGGA-3' and 5'-TCACGTTGTTCTGTTCACTCGT-3'; Osteocalcin (Ocn): 5'GGCAGCGAGGTAGTGAAGAG-3' and 5'-GATGTGGTCA GCCAACTCGT-3'; CD31: 5'AACAGTGTGACATGAAGAGCC-3' and 5 TGTA AACACAGCAGTCATCCTT-3' ; CD34: 5 -AGGTATGCTCCCTGC TCCTT-3' and 5 GAATAGCTCTGGTGGCTTGC-3'; Fli-1: 5 TGGATCAA TCAGCCAGTGAG-3' and 5' -GGCCATTCTTCTCGTCCATA-3'; GAP DH: 5' - AAATCCCATCACCATCTTCCAG-3' and 5' -CATGGTT

CACACCCATGACGAACA-3'.

Histological analysis

To evaluate the effect of BDNF/hyaluronan-NPs on *in vivo* bone repair, the 8-10 week old C57/BL6J mice were used. 20 ng/ml BDNF/hyaluronan-NPs were injected into the fracture site of tibia and fixed. After six weeks, the mice were euthanized, and the tibia treated were retrieved from the mice and evaluated for bone formation by HE and VonKossa staining as previously described. Experiments were conducted after acquiring permission from the local ethical committee for animal research experimentation.

Statistical analysis

The student t-test was used to determine the difference between control and treatment groups compared. Statistical significant was indicated when the p-value was less than 0.05. Data were expressed as mean standard deviation.

Cell imaging of osteogenesis

Differentiation of hMSCs into osteoblasts was evaluated by microscopy. After 3 days of incubation in differentiation medium, no significant differences were observed between cells with or without addition of hyaluronan nanoparticles (**Figure 1a**). Further after addition of BDNF, cell number (**Figure 1b**) and mineralization were visually increased in comparison to that without BDNF as shown by light microscopy after 14 days of incubation as well as alkaline phosphatase (AP) staining (**Figure 1b**) and alizarin red S (ARS) staining (**Figure 1b**) after 7 days and 21 days of incubation respectively.

Results

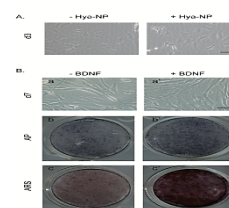


Figure 1: Images of osteogenesis: human MSCs and osteogenic differentiation were depicted after incubation with 40 ng/mL hyaluronan-NPs alone (a): and together with 20 ng/mL BDNF; (b): AP indicates the staining for alkaline phosphatase activity of osteoblast. ARS indicates the staining of Alizarin Red S for mineralization of osteoblasts. Bar: 10 m.

Detection of osteogenesis-specific genes expression

After 14 days of induction, cell cultures were harvested for analysis of mRNA specific to the osteogenic markers. The

result of real-time PCR showed that a significant increase in the relative expression of markers like Runx2, AP and Osteocalcin (Ocn) with BDNF/hyaluronan-NPs in comparison to controls without BDNF/hyaluronan-NPs (**Figure 2**).

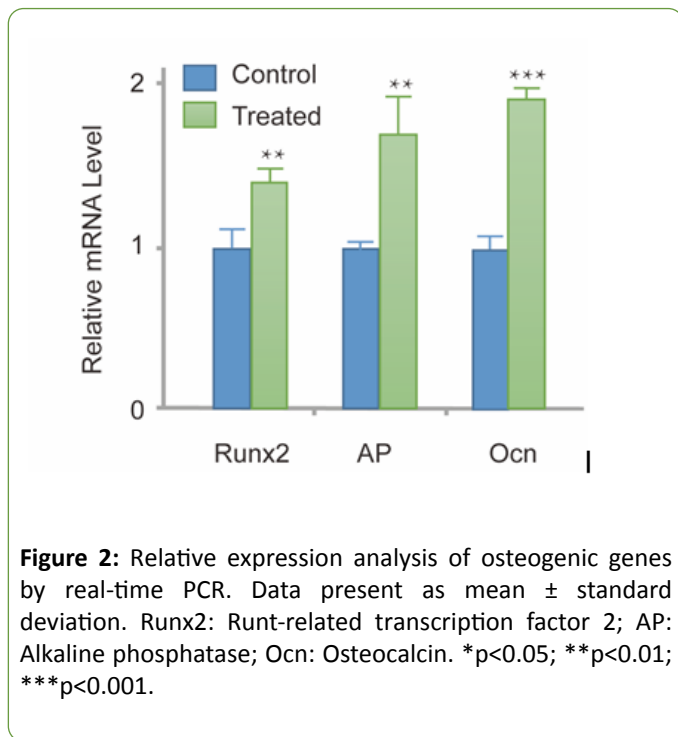


Figure 2: Relative expression analysis of osteogenic genes by real-time PCR. Data present as mean \pm standard deviation. Runx2: Runt-related transcription factor 2; AP: Alkaline phosphatase; Ocn: Osteocalcin. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Detection of angiogenesis-specific genes expression

To evaluate the effect of BDNF/hyaluronan-NPs on angiogenesis *in vitro*, after 14 days of induction, cell cultures were also harvested for analysis of mRNA specific to the endothelial cell markers. The result of real-time PCR showed that a significant increase in the relative expression of the markers, such as CD31, CD34 and Fli-1 with BDNF/hyaluronan-NPs compared to controls without BDNF/hyaluronan-NPs (**Figure 3**).

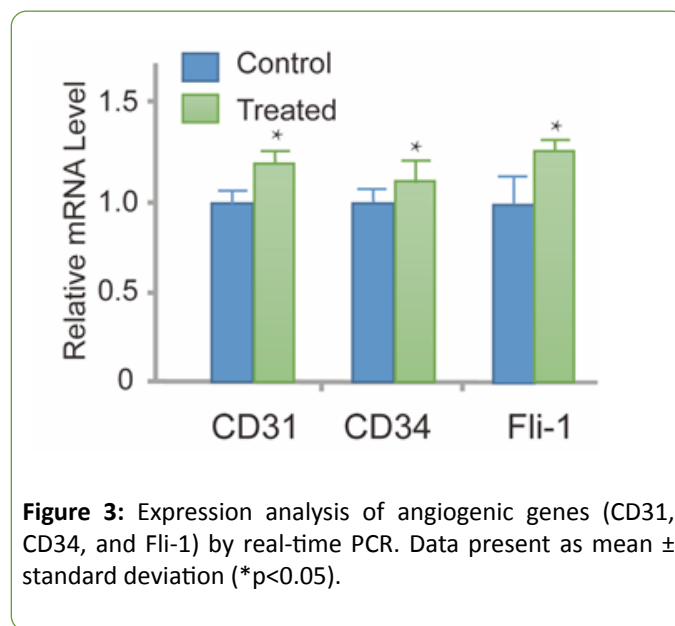


Figure 3: Expression analysis of angiogenic genes (CD31, CD34, and Fli-1) by real-time PCR. Data present as mean \pm standard deviation (* $p < 0.05$).

Bone ossification assay

To evaluate the effect on the bone formation *in vivo*, the BDNF/hyaluronan-NPs were injected on the tibia fracture and fixed. 6 weeks later, the tissue was subject to histologic evaluation and the compact bone was found to form as evidence by HE staining (**Figure 4a**) and vonKossa staining (**Figure 4b**). Moreover, no remnants of the hyaluronan particles were found present there. The osteocytes were frequently found toward the bone side representing active bone formation (**Figure 4a**).

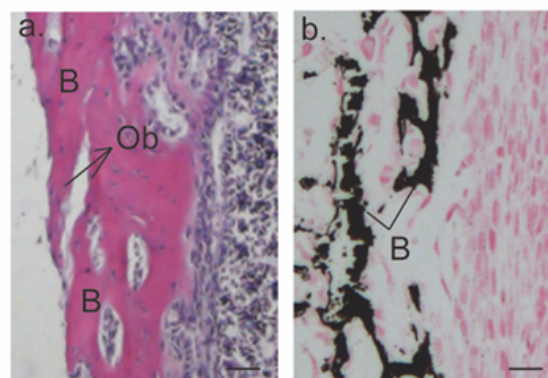


Figure 4: *In vivo* bone formation induced BDNF/hyaluronan-NPs. Hematoxylin and eosin (HE) staining (a) and vonKossa staining; (b): B indicates bone formed and Ob indicates osteoblasts. Bar: 100 μ m.

Discussion

The purpose of this study was to investigate the potential of BDNF/hyaluronan-NPs on bone repair and the compatibility of the tissue. The study was based on cell imaging, real-time PCR as well as *in vivo* bone formation assay.

Using light microscopy, human MSCs were observed to expand more frequently with the stimulation of BDNF but not with hyaluronan material. Additionally, The BDNF/hyaluronan-NPs could significantly enhance osteogenic differentiation of these cells *in vitro* as shown by increased AP activity and mineralization (**Figure 1b**). To verify this observation, real-time PCR was performed to quantify the mRNA amount of Runx2, AP and Ocn, which are specific osteogenic markers and correspond to the initial differentiation through terminal osteoblast stage [23,24]. To evaluation of the potential of BDNF/hyaluronan-NPs to induce bone formation and repair

the fracture, an *in vivo* pilot test was performed at the mouse tibia fracture. After 6 weeks of injection, BDNF/hyaluronan-NPs were found to induce bone formation and have osteoblasts spread along. Moreover, hyaluronan-NPs was already degraded and no remnants left (**Figure 4**). These observations indicate hyaluronan-NPs were compatible with the tissue and could be used a promising drug delivery system. In addition to the biocompatibility [25], hyaluronan-NPs used as the drug delivery system have many benefits as it offers the possibility to adjust the concentration and duration of growth factor release at the fracture site. Several growth factors are known to enhance bone formation, especially from the Bone Morphogenetic Protein (BMP) family [26,27]. However, unlike the BMPs that mainly regulate developmental bone formation, and some protein like BMP2 that can induce inflammation [28,29], BDNF has the ability to induce endothelial cells in addition to promote osteoblast differentiation [16,20].

Conclusion

These combinational features specially benefit to bone repair as the implanted BDNF/hyaluronan-NPs have the potential to reduce side effects and induce vessel formation and deliver nutrients and other essential elements for the newly regenerated tissue. However, in present study, the concentrations and release of the BDNF/hyaluronan-NPs remain to be optimized.

Overall in this study, the application of BDNF/hyaluronan-NPs induced osteoblast differentiation and mineralization *in vitro* and bone formation *in vivo* as well as increased induction of endothelial cells.

Authors' Contribution

Conceived and designed the experiments: Guokang Xu. Performed the experiments: Bin Li and Fei Chen. Analyzed the data: Yi Liu. Wrote the paper: Bin Li. The authors declare no conflict of interest.

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