Chapter 4

Notoginsenoside R1 can significantly promote in-vitro osteoblastogenesis

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Traditional Chinese Medicine: NGR1 for bone regeneration

Abstract:
Notoginsenoside R1 (NGR1), one of the main effective components of Panax notoginseng, appears to be effective in promoting osteogenesis and treating osteoporosis. However, hitherto, whether NGR1 can directly promote osteoblastogenesis remains to be elucidated. We hereby assessed the effects of NGR1 on the osteoblastogenesis of a pre-osteoblast cell line (MC3T3-E1) in an in-vitro time-course and dose-dependent study. Its efficacy was evaluated by assessing cell viability (indicator for proliferation), alkaline phosphatase (ALP) activity, (a marker for an early osteoblastic differentiation), osteocalcin (a marker for a late osteoblastic differentiation), calcium deposition (a marker for final mineralization) and the expression of a series of osteoblastogenic genes (such as Collagen Iα, Runx2, ALP and osteocalcin) at different time points. NGR1 exhibited a bell-shape dose pattern in promoting the proliferation and ALP activity of pre-osteoblasts with the peak occurring at 50μg/ml. NGR1 could markedly increase the expression of osteocalcin at the concentration of 1000μg/ml in a dose-dependent pattern. Furthermore, 1000μg/ml NGR1 resulted in the highest mineralization 4.3 folds and 5.9 folds on the 21st and 28th day respectively compared with the control group. In conclusion, NGR1 could significantly promote the osteoblastogenesis of pre-osteoblasts, which suggested a promising application potential for bone regeneration.

Key words: Notoginsenoside R1; Osteoblastogenesis; MC3T3-E1; Osteogenesis.
Introduction

Adequate bone volume and appropriate bone quality are of vital importance to achieve a rapid establishment of implants’ functions in dentistry and orthopedics. However, new bone regeneration and implant osteointegration can be affected by various adverse bone conditions, such as large bone defect and low bone density, thus delaying the loading of implants [1]. Autologous bone grafts are still regarded as the “gold standard” to repair bone defects since they contain osteoinductive growth factors and osteogenic cells [2]. However, their usage is still limited due to its intrinsic disadvantages, such as limited availability and donor-site pain. In addition, the efficacy of autologous bone grafts can also be compromised when the patients have low bone density or compromised healing capacity. As alternatives to autologous bone grafts, various bioactive agents that are used either alone or in combination with biomaterials have been resorted to promote bone regeneration [3]. Bone morphogenetic proteins, a group of proteinases growth factors, are the most extensively used drugs for bone regeneration [4]. However, the effective doses of the homodimeric BMPs in clinic to induce bone formation are very high (e.g. up to several milligrams) [5, 6], which leads to not only a substantial economic burden but also a series of potential side effects, such as overstimulation of osteoclastic activity in surrounding milieu and an ectopic bone formation in unintended area [7, 8]. Gene, cell and cytokine therapies have been reported to substitute autografting, but since the high price, they have not been widely used in clinic [9-11]. In comparison, Traditional Chinese Medicine becomes attractive and promising since they possess high bioactivity and less side-effects.

One of such Traditional Chinese Medicine is panax notoginseng saponins (PNS) — a mixture of active compounds that are extracted from panax notoginseng root. It has been widely used as a medicinal herb for over thousands of years in China, which exhibits minimal side effect as its greatest advantage. PNS has been widely used for the treatment of cardiovascular diseases [12]. Recently, PNS was found to protect rabbit bone marrow mesenchymal stem cells from oxidative stress-induced damage and apoptosis through scavenging ROS and regulating the Bcl-2/Bax pathway, and thereby improving the bone formation [13]. Other experiment confirmed that PNS could promote osteogenesis of BMSCs by activating the ERK and p38 signaling pathways [14]. PNS could also promote the proliferation and osteogenic differentiation in NIH3T3 fibroblasts cells by up-regulating phosphorylation of ERK1/2 protein kinase [15]. PNS could also improve the development of osteoblasts [16, 17]. These findings suggested that some effective components in PNS possess an application potential in clinic to promote osteogenesis. Notoginsenoside R1 (NGR1) is one of the main constituents of PNS. Unlike other pharmacologically active saponins in both PNS and other ginsengs, NGR1 exists only in PNS [18, 19]. However, hitherto, whether NGR1 can directly affect osteoblastogenesis remains to be elucidated.

In this article, we wanted to unveil the effective characteristics of NGR1 on the osteoblastogenesis of a pre-osteoblast (MC3T3E1 cell-line) in a time-course and dose-dependent study. We evaluate its effects by assessing cell viability (an indicator for proliferation), alkaline phosphatase activity (a marker for an early osteogenic differentiation), osteocalcin (a marker for a late osteogenic differentiation), calcium deposition (a marker for final mineralization) and the expression of osteoblastogenic genes (such as Runx2, Collagen Iα, alkaline phosphatase and osteocalcin) at different time points.

Materials and Methods
Cell Culture
MC3T3-E1 (Subclone 14, ATCC CRL-2594) pre-osteoblasts were obtained from ATCC, Manassas, VA, USA. The cells were cultured in a α-Minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) (Gibco®, Invitrogen, Grand Island, NY, USA). The medium was changed every 3 days. Triplicates per group were performed for each parameter per time point. The exponentially growing cells were collected and seeded at a final concentration of 1×10⁴ cells/well in 24-well plates for the cell proliferation assay. For the ALP activity assay, OCN detection and PCR analysis, cells were seeded in 6-well plates at a final concentration of 2×10⁵ cells/well or in 48-well plates at a final concentration of 3×10⁴ cells/well for alizarin red staining. 24h post incubation, the cells were subjected to a low-serum medium (α-MEM containing 2% FBS) for another 24h. Thereafter, the cells were treated with the different concentrations of Notoginsenoside R1 (Nanjing Zelang, China. ZL140310529).

Cell Viability and Proliferation Assay
The cell viability and proliferation of MC3T3-E1 cells in each group were determined using the alamar Blue cell viability reagent (Invitrogen Corporation, Carlsbad, CA, USA) after the treatment for 1, 4 and 7 days. We used a fluorescence spectrometer (SpectraMax M5 Molecular Devices, Sunnyvale, CA, USA) to measure the fluorescent intensity with the excitation wavelength at 540 nm and the emission wavelength at 590 nm.

ALP Activity Assay
The ALP activity and total protein content were measured after the treatment for 4 days and 7 days to assess the early differentiation of pre-osteoblasts. The ALP activity in the whole cell lysate was determined using a LabAssayTM ALP colorimetric assay kit (Wako Pure Chemicals, Osaka, Japan). The ALP activity was normalized by the total protein content that was measured using a commercial BCA Protein Assay kit (Beyotime, China).

OCN Expression Assay
The OCN in the cell culture medium after the 4-day and 7-day treatments was determined to assess the terminal differentiation of pre-osteoblasts. The OCN concentrations were determined using a mouse OCN EIA kit (Biomedical Technologies, Stoughton, MA, USA) [20].

Alizarin Red Staining
To assess the extracellular mineralization of MC3T3-E1 cells, osteogenic medium (10% FBS, 50μg/ml L-ascorbic acid, and 10mM β-glycerophosphate; Sigma-Aldrich, St. Louis, MO, USA) containing different concentrations of NGR1 was used to treat the cells for 21 and 28 days [21]. On the 21st and 28th day, mineralized nodules were stained with alizarin red (Sigma- Aldrich, St. Louis, MO, USA) as previously described [22]. The calcified areas were photographed and then quantified using a software of Image-Pro Plus 6.0.

Isolation of Total RNA and Real-time Fluorescence Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis
On the 4th and the 7th day, the total RNA was extracted using an RNeasy Mini Kit and purified with RNase-Free DNase Set reagent (Qiagen, Germany) according to the manufacturer’s instructions. Total RNA was transcribed to cDNA using a kit of PrimeScript® RT Master Mix (Perfect Real Time, Takara, Japan). A PrimeScript® RT reagent Kit (Perfect Real Time, Takara, Japan) was used to perform RT-qPCR assay. The primers for detecting mRNA transcripts of the Collagen Iα, Runx2, ALP, OCN, and β-actin gene are designed as previously published and shown in Table 1 [1, 4]. Transcripts were normalized to the β-actin transcript levels. The n-fold
upregulation for each gene over the internal control gene (β-actin gene) was calculated according to the delt-delt-Ct method using the formula: $2^{-\left(\text{CT gene of interest-CT internal control}\right)_{\text{sample}}-\left(\text{CT gene of interest-CT internal control}\right)_{\text{control}}}$. 

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primers (F=forward; R=reverse)</th>
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<tbody>
<tr>
<td>Akp2 (ALP)</td>
<td>NM_007431</td>
<td>F: 5'-TGCCCTACCTGGCTGCGTGTA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-TCACCCGAGTTGATTCACAATG-3'</td>
</tr>
<tr>
<td>Osteocalcin (OCN)</td>
<td>NM_007541</td>
<td>F: 5'-AGCAGCTGGCCAGACCTA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-TAGCGCCGGAGTCTGTTCACTAC-3'</td>
</tr>
<tr>
<td>Collagen I</td>
<td>NM_007742</td>
<td>F: 5'-ATGCCGCGACCTCAAGATG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-TGAGGCACAGACGCTGAGTA-3'</td>
</tr>
<tr>
<td>Runx2</td>
<td>NM_009820</td>
<td>F: 5'-CAGTGCCGGTTGGAACAAAGA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-TTTCATAACAGCGAGGCATTTC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_007393</td>
<td>F: 5'-AGGAGCAATGATCTGATCTTT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-TGCCAACACAGTGCTGCT-3'</td>
</tr>
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Table 1 Primers used for RT-qPCR.

Statistical Analysis

Statistical comparisons among the results were made by one-way analysis of variance (ANOVA) with Bonferroni corrections for Post Hoc comparisons. The level of significance was set at p<0.05. SPSS software (version 20) was adopted for the statistical analysis.

Result

Cell proliferation

The effect of NGR1 on cell proliferation exhibited a bell-shape dose-dependent pattern. On the 1st day, a significant increase in the cell numbers was detected under the treatment of 50μg/ml NGR1 (Figure 1). In comparison with the treatment of no NGR1, 5μg/ml, 50μg/ml, 100μg/ml NGR1 resulted in significantly higher cell viability (1.6 folds, 2.2 folds and 1.9 folds respectively), while 200μg/ml and 1000μg/ml resulted in significant lower cell numbers. The cell numbers increased significantly with time for all the groups. On the 4th day and 7th day, 50μg/ml, 100μg/ml NGR1 were associated with a significantly higher cell numbers, while 200μg/ml, 1000μg/ml NGR1 led to significantly lower cell numbers compared to the control group. The highest promoting effect of NGR1 on cell proliferation occurred to 50μg/ml, and its highest inhibitive effect occurred to 1000μg/ml.
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Figure 1. Numbers of murine calvarial pre-osteoblasts (MC3T3-E1 cell line) per well under the different treatments: 1) Control; 2) 5μg/ml NGR1; 3) 50μg/ml NGR1; 4) 100μg/ml NGR1; 5) 200μg/ml NGR1; 6) 1000μg/ml NGR1 for 1, 4 and 7 days. Data are presented as mean values together with the standard deviation (SD). *: p<0.05, **: p<0.01, ***: p<0.001.

ALP activity

Similar to its effect on cell proliferation, NGR1 also exhibited a bell-shape dose-dependent pattern in modulating ALP activity. On the 4th day, NGR1 ranging from 5μg/ml to 200μg/ml significantly enhanced ALP activity with a peak occurring at 50μg/ml, while 1000μg/ml NGR1 did show significant modulating effect in comparison with control. On the 7th day, only 50μg/ml NGR1 resulted in significantly higher ALP activity in comparison with the control group (no NGR1). In contrast, NGR1 of 100, 200 or 1000μg/ml were associated with a significantly lower ALP activity, and the lowest value occurred at 1000μg/ml.
Figure 2. Activity of alkaline phosphatase (ALP) of murine calvarial pre-osteoblasts (MC3T3-E1 cells) under the different treatments: 1) Control; 2) 5μg/ml NGR1; 3) 50μg/ml NGR1; 4) 100μg/ml NGR1; 5) 200μg/ml NGR1; 6) 1000μg/ml NGR1 R1 after for 4 and 7 days. Data are presented as mean values together with the standard deviation (SD).*: p<0.05, **: p<0.01, ***: p<0.001.

OCN expression

After a 4-day treatment, NGR1-induced OCN expression showed a dose-dependent increasing trend. With NGR1, OCN expression in pre-osteoblasts significantly increased from the 4th day to the 7th day for each concentration (Figure 3). Different from its effects on cell proliferation and ALP activity, NGR1 showed a dose-dependent increasing pattern in promoting OCN expression. Especially on the 7th day, 1000μg/ml NGR1 significantly increased the OCN expression nearly 5 folds in comparison with the treatment of no NGR1.
**Figure 3.** Expression of osteocalcin (OCN) of murine calvarial pre-osteoblasts (MC3T3-E1 cells) under the different treatments: 1) Control; 2) 5μg/ml NGR1; 3) 50μg/ml NGR1; 4) 100μg/ml NGR1; 5) 200μg/ml NGR1; 6) 1000μg/ml NGR1 for 4 and 7 days. Data are presented as mean values together with the standard deviation (SD). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

**Cell matrix mineralization**

In the control group, rare mineralization was found (Figure 4). On the 21st and 28th day, the mineralization in cell matrix was found in the treatment of NGR1 groups. 100, 200 and 1000μg/ml NGR1 could significantly enhance mineralization in comparison with the control group on the 21st day. 1000μg/ml NGR1 resulted in the highest mineralization, 4.3 folds and 5.9 folds on the 21st and 28th day respectively compared with the control group.
Figure 4. Mineralization of murine calvarial pre-osteoblasts (MC3T3-E1 cells) under the different treatments: 1) Control; 2) 5μg/ml NGR1; 3) 50μg/ml NGR1; 4) 100μg/ml NGR1; 5) 200μg/ml NGR1; 6) 1000μg/ml NGR1. (4-1) Light micrographs depicting the alizarin red staining on the 21st day and 28th day. (4-2) Graph depicting the calcification area on the 21st day and 28th day. Data are presented as mean values together with the standard deviation (SD). *: $p<0.05$. **: $p<0.01$, ***: $p<0.001$.

Expression of osteogenic genes

Runx2 gene was the key to control the pro-osteoblast proliferation and to promote the stage of cell proliferation to osteogenic differentiation [24, 25]. On the 4th day, NGR1 of all the selected concentrations significantly enhanced the expression of Runx2 mRNA, showing a bell-shape dose-dependent pattern. The highest expression of Runx2 mRNA was found in 50μg/ml NGR1 (Figure 5A). On the 7th day, in comparison with the control (no NGR1), 50, 100, and 200μg/ml NGR1 resulted in significantly higher expression of Runx2 mRNA, while 1000μg/ml NGR1 led to significantly lower expression.

On the 4th day, the expression of Collagen Iα gene was significantly enhanced by 50μg/ml NGR1, while significantly suppressed by 1000μg/ml (Figure 5B). On the 7th day, NGR1 of 50 to 1000μg/ml significantly enhanced the expression of Collagen Iα mRNA. 50μg/ml NGR1 was associated with the highest expression on
both time points. Consistent with ALP activity, the highest expression of ALP mRNA was found in 50 μg/ml on both 4th and 7th day (Figure 5C). On the 7th day, 200 and 1000 μg/ml NGR1 significantly down-regulated the expression of ALP mRNA in comparison with control group. Consistent with its effect on the OCN expression, NGR1 showed a dose-dependent increasing pattern in promoting the expression of OCN mRNA (Figure 5D). The expression of OCN mRNA increased significantly with time for all the selected concentrations. 1000 μg/ml NGR1 resulted in the highest expression of OCN mRNA, 1.8 folds and 1.7 folds on the 4th and 7th day respectively compared with the control.

Discussion
Bone regeneration is a delicately orchestrated activity of osteoblasts with coupling bone remodeling by osteoclasts. Many Chinese Medicinal Herbs have been used to promote bone formation in fractures in China for more than one thousand years. In modern Chinese Traditional Medicine, effective compositions are extracted from these Herbs to treat bone diseases. For example, Ligustri Lucidi Fructus, Drynaria fortunei, Du-Zhong cortex, Icariin, can effectively correct the pathological bone metabolism through promoting osteoblastogenesis and osteoblastic activity [26-28]. Other extracts, such as Cinnamomum zeylanicum, can significantly inhibit RANKL-induced osteoclastogenesis and osteoclastic resorption [29]. In an in-vivo osteoporotic model in rats, Cervi Cornu Pantotrichum, the main effective component of antlers, could inhibit the osteoporotic progress and promote bone formation [30]. All these findings indicated their promising potential for clinical application.

PNS, a Traditional Chinese Herbal Medicine, has exhibited a wide range of pharmacological effects, such as angiogenetic, antineoplastic, neuroprotective and anti-inflammatory and immune-modulatory effects.
These well-known effects conferred PNS a strong ability in suppressing pathological bone loss. PNS in combination with granulocyte colony-stimulating factor–mobilized peripheral blood mononuclear cells could cure unreconstructable critical limb ischemia [36]. As an immune-modulator, PNS could significantly decrease the lipopolysaccharide-induced alveolar bone loss and the expression of matrix metalloproteinase-9 in a periodontitis model [37]. Recently, PNS could directly enhance the proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells through modulating intercellular communication activities and upregulating of ERK and p38 MAPK signaling pathways [13, 14, 17]. Canonical Wnt signaling was required for the PNS-induced suppression of RANKL/OPG ratio in bone marrow stromal cells during osteogenic differentiation [38]. NGR1, a unique and abundant component of PNS [39], has already been used to inhibit hypoxia-hypercapnia-induced vasoconstriction and protect cells against intestinal ischemia and reperfusion [40, 41]. Its clinical applications include treatment of vascular disorders and osteoporosis [42]. However, whether NGR1 can directly modulate osteoblastogenesis is unclear now. In this dose-dependent and time course study, we, for the first time, showed that NGR1 could significantly enhance the in-vitro osteoblastogenesis, suggesting a promising clinical application potential for bone regeneration.

The osteoblast phenotype is acquired in two stages. In the first stage, the matrix matures, and specific proteins associated with the bone cell phenotype (e.g., ALP) are detected. In the second stage, the matrix becomes mineralized by calcium deposition. Consequently, layers of spongy bone are formed around the original cartilage [43]. The effect of NGR1 showed a dose-dependent, bell-shaped pattern on modulating cell proliferation (Figure 1) and ALP activities (Figure 2). The optimal concentration occurred to 50μg/ml. 50 and 100μg/ml NGR1 significantly promoted cell proliferation (Figure 1), while 200 and 100 NGR1 significantly inhibited cell proliferation in comparison to the control (no NGR1) (Figure 1). Consistently, 50μg/ml NGR1 also resulted in the peak gene expression of Collagen I (Figure 5B) which is a primary gene product of osteoblasts during bone matrix formation and comprises 85–90% of the total organic bone matrix [44]. On the 4th day, 5 to 200μg/ml NGR1 showed a significant promoting effect on ALP activities with the peak occurring at 50μg/ml. On the 7th day, 100 to 1000μg/ml NGR1 showed a significant inhibiting effect on ALP activities (Figure 2). Analogous with the ALP activity, the peak of ALP gene expression also occurred to 50μg/ml (Figure 5C). On the 7th day, 200 and 1000μg/ml NGR1 inhibited ALP gene expression. Interestingly, the effect of NGR1 on OCN expression showed a dose-dependent increasing pattern (Figure 3). A time-dependent and dose-dependent pattern was also found in mineralization (Figure 4). 1000μg/ml NGR1 significantly enhanced mineralization 5.9 folds (Figure 4), whereas ALP was inhibited (Figure 2). In contrast, the pattern of NGR1 in promoting mineralization (Figure 4) was consistent with that in promoting OCN expression (Figure 3). This result suggested that the promoting effect of NGR1 on mineralization could be partially attributed to its promoting effect on OCN but not ALP activity. These results suggested that high level of NGR1 could significantly enhance the osteoblastogenesis, thereby bearing a promising application potential to facilitate bone regeneration and implant osteointegration for patients.

Hitherto, the molecular mechanisms accounting for the promoting effects of NGR1 on
osteoblastogenesis remain unveiled. Runx2, a key modulator for osteogenic differentiation, controls osteoblast proliferation and promotes a transition from a proliferative to a post-proliferative stage prior to osteoblast differentiation [24, 25]. Runx2 significantly increased under the stimulation of 50μg/ml NGR1, whereas significantly decreased in the presence of 1000μg/ml NGR1 (Figure 5A). This result suggested that the highest mineralization in response to 1000μg/ml NGR1 was not due to the upregulation of Runx2. Previous report showed that the induction of ALP activity was mediated through the activation of a Smad-independent signaling pathway p38 MAPK [45]. OCN is a late differentiation marker for osteoblastogenesis, which is modulated by osterix [46]. It might be plausible that NGR1 of different concentration could modulate p38 MAPK and osterix differentially. Screening the gene expression pattern of pre-osteoblasts under the stimulation of NGR1 in different concentration will help to further understand its signaling pathway.

One limitation of this study was that we only adopted one kind of osteogenic cell line, which might behave differently from primary osteoblasts or bone marrow stromal cells. The test using human primary mesenchymal cells can be more indicative for its clinical application potential.
References


