N-acetyl-L-cysteine enhances the osteogenic differentiation and inhibits the adipogenic differentiation through up regulation of Wnt 5a and down regulation of PPARγ in bone marrow stromal cells

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ARTICLE INFO

Received 1 March 2011
Accepted 17 April 2011

Keywords:
Bone marrow stromal cells
N-acetyl-L-cysteine
Differentiation

ABSTRACT

Nowadays, the treatment of osteoporosis is still a great challenge in the medical field. The combination of enhancement of osteogenesis and the inhibition of adipogenesis of bone marrow stromal cells (BMSCs) is considered an efficient therapeutic strategy for the treatment of osteoporosis. In the present study, we investigated the effects of N-acetyl-L-cysteine (NAC) on the proliferation, osteogenesis and adipogenesis of BMSCs. NAC treatment enhanced the alkaline phosphatase activity, mineral deposition and mRNA expression levels of osteogenesis markers collagen I, osteopontin, and signal pathway related protein Wingless-type family member 5a in addition to Wingless-type family member 3a during osteogenic induction, and inhibited the accumulation of lipid droplets and the expression levels of lipoprotein lipase, fatty acid binding protein 4 and peroxisome proliferator-activated receptor gamma mRNA during adipogenic induction. Meanwhile, NAC had the same effects as enhancing mineral deposition in regular culture condition. In addition, cell proliferation was also promoted by NAC treatment in regular culture condition. These results suggested that NAC may enhance osteogenic differentiation and inhibit adipogenic differentiation of BMSCs, which is at least partially mediated by up regulating Wnt 5a and down regulating PPARγ. Taking into account the extensive protective effects of NAC and that the maintenance of BMSCs number is an important factor in osteoporosis prevention and treatment, these observations suggested that NAC is a promising potential drug for the prevention and treatment of osteoporosis and its associated diseases.

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1. Introduction

The treatment of osteoporosis is still a great challenge for the medical world. Most people with osteoporosis suffer secondary fractures, which lead to increased morbidity, mortality, and loss of function and therefore greatly impact public health [1–3]. Despite the great effort in developing effective methods to treat this disease, no satisfactory drug has been used in the clinical setting to date [4].

The pathogenesis of osteoporosis is not fully understood. Recently, bone marrow stromal cells (BMSCs) were found to be closely related to the pathogenesis of osteoporosis [5,6]. BMSCs are considered the source of osteoblasts and are thought to take part in bone formation activity [7]. It has been reported that excessive adipogenesis of BMSCs and inadequate osteogenesis contribute to the pathogenesis of osteoporosis [8]. Therefore, the balance between the osteogenesis and adipogenesis of BMSCs provides a new therapeutic target for the treatment of osteoporosis.

2. Materials and methods

2.1. Cell culture

The BMSCs were obtained from New Zealand white rabbits as previously described [12]. Briefly, the rabbits were anesthetized by sodium pentobarbital. Bone marrow aspirates harvested with a transfixion pin from the tibia and femur were poured onto a Ficoll gradient (1.077, Sigma, St. Louis, Missouri) and were centrifuged at 900 g for 30 min. Nucleated cells collected from the middle layer were washed twice with phosphate-buffered saline (PBS) and
incubated at a density of 5 × 10^5 nucleated cells/cm^2 in H-DMEM (Sigma, St. Louis, Missouri) supplemented with 15% fetal bovine serum (FBS, Sijiqing, Hangzhou, China) and penicillin/streptomycin (200 U/mL, Sangon, China) at 37°C in a 5% CO2 humidified incubator. Culture medium was replaced every three days. When grown to 80% confluence, the cells were harvested by incubation with 0.05% trypsin and 0.02% EDTA (Gibco, Karlsruhe, Germany) for 3 min and were then seeded on petri dishes at a density of 1 × 10^4/ cm^2 under the same culture conditions as for expansion.

To determine whether NAC (Sangon, China) affects the proliferation of BMSCs, cells were seeded into 96-well plates at a density of 5 × 10^3/cm^2 and were treated with 20, 40, or 80 μg/mL NAC. After culturing for five days, the cells were subjected to an MTT assay.

For osteogenic differentiation, BMSCs were seeded into 24-well plates at a density of 1 × 10^4/cm^2. H-DMEM supplemented with 15% FBS, 10 mM β-glycerophosphate (Sigma), 50 μg/mL L-ascorbic acid (Sigma), 100 nM dexamethasone (Sigma) and antibiotics served as the osteogenic inducing medium (OIM). For adipogenic differentiation, BMSCs were seeded into 24-well plates at a density of 1 × 10^5/cm^2. H-DMEM supplemented with 15% FBS, 200 μM indomethacin (Sigma), 1 μM dexamethasone, 0.5 mM isobutyl methylxanthine (Sigma), 0.5 μg/mL insulin (Sigma) and antibiotics served as the adipogenic inducing medium (AIM).

To analyse the effects of NAC on the osteogenic and adipogenic differentiations of BMSCs, NAC at a final concentration of 0, 20, 40, or 80 μg/mL were added to the culture medium. The culture medium was changed every three days. Each experiment was conducted with three replicates. After culturing for 10 and 20 days, the osteogenic or adipogenic differentiation of the cells was evaluated.

2.2. MTT assay

At the fifth day, the culture medium was removed, and MTT dye solution (20 μL, 5 g/L, Sangon, China) was added to each well. After four-hour incubation, the supernatant was removed, and 100 μL DMSO was added. Ten minutes later, the optical density of each well was measured using a microplate spectrophotometer (TECAN, Switzerland) at a wavelength of 570 nm.

2.3. Alkaline phosphatase (ALP) activity assay

To determine the ALP activity of BMSCs cultured with OIM and RM, the cells were washed twice with PBS and lysed by 0.3% Triton X-100 (Sigma) at 4°C overnight. The supernatants were subjected to ALP activity and protein content measurements using an ALP assay kit (Nanjing Jiancheng Biotech, China) and a BCA assay kit (Sangon, China), respectively. The experiments followed the procedure provided by the manufacturers.

2.4. Von Kossa staining

To assess the mineral deposition level, the cells were washed with PBS and fixed with 10% formalin for 10 min. After washing three times with deionised water, the cells were incubated with 300 μL of 5% silver nitrate (Sigma, St. Louis, Missouri) for 30 min under sunlight illumination. Then, the silver nitrate was removed, and 300 μL of 5% sodium thiophosphate (Sigma, St. Louis, Missouri) was added for 5 min. The cells were photographed for image analysis using Image Pro Plus software (Media Cybernetics Inc., MD).

2.5. Oil red O staining

To measure the accumulation of lipid droplets in the cells, the cells were fixed as previously and incubated with 300 μL of 0.6% oil red O solution (in 60% isopropanol and 40% water) for 30 min at 37°C. After washing the unbound dye, the cells were photographed for image analysis.

2.6. RNA purification and real-time quantitative RT-PCR

Total RNA was extracted from cells using TRIzol reagent (invitrogen, Carlsbad, CA). The extracted RNA was quantified spectrophotometrically and reversely transcribed by AMV reverse transcriptase (invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed (2 × TaqMan Universal PCR Master Mix, 20 × TaqMan Gene Expression Kits, ABI) and analysed with 7000 SDS v1.0 software. The enthusiasm of the baseline was finely adjusted according to the conditions. β-actin was amplified to serve as an internal control to normalise the PCR efficiency (Table 1).

2.7. Statistical analysis

Statistical analysis was performed using SPSS 12.0 software. P values less than 0.05 were considered significant.

3. Results

3.1. N-acetyl-L-cysteine (NAC) promotes the proliferation of bone marrow stromal cells (BMSCs)

To determine whether NAC can affect the proliferation activity of BMSCs in regular culture condition, the cells were treated with 0, 20, 40, or 80 μg/mL NAC respectively for 5 days. MTT test showed that NAC significantly promoted BMSCs proliferation in a dose-dependent manner (Fig. 1).

3.2. N-acetyl-L-cysteine (NAC) promotes the osteogenic differentiation of bone marrow stromal cells (BMSCs)

To determine whether NAC might affect osteogenesis of BMSCs, the markers characteristic of osteogenic differentiation such as ALP

<table>
<thead>
<tr>
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<tr>
<td>Col1</td>
<td>ATGATGACAGAAGATGCGAAC</td>
<td>GCTATCAGAACAACTGTCGAT</td>
</tr>
<tr>
<td>OP</td>
<td>CTCGACGACCTAGTACCC</td>
<td>CTCGCCGCTGCTGACCC</td>
</tr>
<tr>
<td>Wnt 3a</td>
<td>GGAATGCTGTCGGGACTTGC</td>
<td>TGGCGGCTGATTGCAGAAG</td>
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<td>Wnt 5a</td>
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<td>CGATCCTGCAATGACTGTC</td>
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<tr>
<td>LPL</td>
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<td>GCGGAGGCCCTCTGGGAA</td>
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<tr>
<td>FABP4</td>
<td>ATCCGCTGGAGGACTCAAGGA</td>
<td>CAGGAAGGAGGGCCTGAA</td>
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![Fig. 1](image-url) The effect of N-acetyl-L-cysteine (NAC) on the proliferation of bone marrow stromal cells (BMSCs) in regular culture condition. BMSCs were treated with NAC (20, 30 or 80 μg/mL) for 5 days and MTT test was employed to quantify the changes in proliferation. *P < 0.05; **P < 0.01, n = 5.
activity, mineral deposition, collagen I (Col I) and osteopontin (OP), as well as Wingless-type family member (Wnt) 3a and 5a of BMSCs were measured. As shown in Fig. 2, BMSCs exhibited increased ALP activity in response to NAC. Although the ALP activity in OIM group was about ten times higher than RM group, they were all increased two to three folds above basal levels. The mRNA expression levels of osteogenesis markers Col I and OP were observed and found to be promoted in a dose-dependent manner (Fig. 3A, B, D, and E). To determine whether NAC promotes osteogenesis through a Wnt pathway, the mRNA expression levels of Wnt 3a and 5a, respectively canonical and non-canonical Wnts, were measured. Results showed that Wnt 5a mRNA was increased with NAC treatment (Fig. 3C, F), while Wnt 3a mRNA did not get any significant elevation (data not shown). Furthermore, we measured the mineral deposition levels of BMSCs. Calcium deposition was found to be undetectable in RM group and significantly increased by NAC in OIM group (Fig. 4). Taken together, these data indicated that NAC treatment could promote the osteogenic differentiation of BMSCs in both osteogenic inducing and regular culture conditions.

Fig. 2. The effect of N-acetyl-L-cysteine (NAC) on the ALP activity of bone marrow stromal cells (BMSCs). After cells were treated with NAC (20, 30 or 80 μg/mL) for 10 or 20 days, ALP activity was assayed. RM refers to regular medium. OIM refers to osteogenic inducing medium. *P < 0.05, **P < 0.01, n = 4.

3.3. N-acetyl-L-cysteine (NAC) inhibits the adipogenic differentiation of bone marrow stromal cells (BMSCs)

To evaluate whether NAC would affect adipogenesis of BMSCs, cells were stained by oil red O. Cells in regular culture condition were slightly stained too (Fig. 5A). In both RM and AIM groups, BMSCs showed decreased lipid droplet accumulation when treated with NAC at both day 10 and day 20. Then, we calculated the integrated optical density of stained lipid to compare the lipid accumulation. As shown in Fig. 5 C, a significant dose-dependently reduction was observed in cells exposed to NAC. In 20 μg/mL group, NAC reduced adipogenic differentiation rate by about 40%, and in 40-80 μg/mL groups, it blocked the differentiation by about 60-70%. Next, the expression levels of adipogenesis markers LPL and FABP4 mRNA were detected and found to be decreased significantly by NAC (Fig. 6A, B, D, and E). To investigate whether NAC suppresses adipogenesis through a PPAR pathway, the upstream pathway of LPL and FABP4 in adipogenesis of BMSCs, the expression of PPARG mRNA was measured. It was found that PPARG mRNA was strongly inhibited by NAC (Fig. 6E). In RM group, PPARG mRNA showed a similar change (Fig. 6 C).

4. Discussion

Studies have demonstrated that BMSCs can differentiate into multiple cell types, including osteoblasts, myoblasts, chondrocytes, and adipocytes [13]. Adipocytic and osteoblastic cells are reciprocal cell types that are dominant in marrow. It is thought that changes in the ratios of these cells contribute to the pathogenesis of osteoporosis [8] and age-related osteopenia [14]. One mechanism that may account for the reciprocal relationship between decreased bone density and increased fat formation is an imbalance in the production of osteogenesis and adipogenesis in the bone marrow cavity, and an increase in the number of adipocytes occurs at the expense of osteoblasts in osteoporotic disorders [15]. Therefore, it may provide a strategy for the prevention and treatment of osteoporosis to modulate the differentiation of BMSCs to cause a lineage shift away from the

Fig. 3. Quantitative analysis of Col I, OP and Wnt 3a, 5a mRNA expression levels by real-time PCR. Cells were treated with N-acetyl-L-cysteine (NAC) (20, 40 or 80 μg/mL) in OIM or RM for 10 or 20 days. Among them, expressions of Col I (A, D), OP (B, E) and Wnt 5a (C, F) mRNA were significantly increased in both RM and OIM conditions, while expression of Wnt 3a mRNA was not significantly changed (data not shown). *P < 0.05, **P < 0.01, n = 4.

Fig. 4. The effect of N-acetyl-L-cysteine (NAC) on the mineral deposition of bone marrow stromal cells (BMSCs). (A) When the cells were cultured in RM, there was no mineral deposition. (B) When the cells were cultured in OIM, the level of mineral deposition was significantly increased by treatment with NAC. (C) Semi-quantitative analysis of mineral deposition. *P < 0.05, **P < 0.01, n = 4.

Fig. 5. The effect of N-acetyl-L-cysteine (NAC) on the lipid droplet accumulation of bone marrow stromal cells (BMSCs). RM refers to regular medium. AIM refers to adipogenic inducing medium. (A, B) Oil red O staining showed significantly decreased lipid droplet accumulation of BMSCs in both AIM and RM groups with NAC treatment. The scale bar represents 50 µm. (C) Semi-quantitative analysis of lipid droplet accumulation. *P < 0.05, **P < 0.01, n = 4.

adipocytes and toward the osteoblasts. Chemicals that are capable of increasing osteogenesis and reducing adipogenesis could be potential candidates for the treatment of osteoporosis. In the present study, we utilized NAC, and demonstrated for the first time that it regulates lineage-specific differentiation of BMSCs in favour of osteogenic and against adipogenic differentiation.

We found that NAC could promote osteogenic differentiation of BMSCs in osteogenic inducing condition as indicated by ALP activity assay, von Kossa staining, real-time RT-PCR of Col I and OP. Meanwhile, BMSCs in regular culture condition also showed increased ALP activity and expression levels of Col I and OP mRNA except mineral deposition. In addition, we found that there exists a dose-dependent relationship between the effect of NAC and osteogenic differentiation. The osteogenic differentiation of BMSCs is represented as three principal phenotypic stages: proliferation, extracellular matrix (ECM) maturation, and mineralization[16]. The most dramatic changes, corresponding to the major transition points in gene expression, occurred during transitions from the proliferation to the ECM maturation stage and from ECM maturation to the mineralization period, with only minor variations in the profiles within each period. ALP is a marker of early ECM maturation. The synthesis and secretion of Col I since the proliferation stage facilitate the eventual formation of mineralized bone nodule [17]. Both of these markers are thought to be signs of early osteogenic differentiation of BMSCs [18]. OP, a marker of late osteogenic differentiation of BMSCs, will reach peak expression when the...
extracellular matrix mature and cells in mineralization phase [16]. ALP and OP are closely related to ECM maturation. Their expression levels were significantly increased, though still about ten times lower in regular condition than that in inducing condition. Mineral deposition was assessed as late marker of mature osteoblast function requiring for complete development of the osteoblast phenotype [17]. These results indicated that NAC can promote the osteogenic differentiation and further maturation of BMSCs in osteogenic inducing condition and the early stage osteogenic differentiation in regular culture condition.

In our study, the expression levels of Wnt3a and 5a mRNA were detected in both osteogenic inducing and regular culture conditions. We found that NAC treatment increased the expression level of Wnt 5a mRNA, whereas the expression level of Wnt3a mRNA was not elevated. Wnt signaling plays a vital role in the regulation of proliferation and differentiation of MSCs. Generally, canonical Wnt signaling functions in maintaining an undifferentiated, proliferating progenitor MSCs population, whereas non-canonical Wnts facilitate osteogenic differentiation [19]. Wnt3a is regarded as canonical wnt classically. Wnt 5a, by activating the non-canonical pathway, has been hypothesized to promote osteogenic differentiation [20]. Thus, it is suggested that this promotion of osteogenic differentiation by NAC is at least partially mediated via non-canonical Wnt pathway.

Then, we determined whether NAC treatment affect the proliferation of BMSCs in regular condition. The result showed that the proliferation level was increased to the greatest degree of about 1.5 folds above basal levels with 40-80 μg/mL NAC treatment. It is usually known that the proliferation and differentiation are mutually exclusive. Actually, researchers have been unceasingly reporting substances promoting both proliferation and differentiation. For instance, it is reported that tigogenin inhibit adipogenic differentiation, induce osteogenic differentiation and enhance the proliferation in mouse BMSCs [21]. IGF-I stimulated proliferation of rat skeletal muscle satellite cells to a small degree but demonstrated a more pronounced stimulation of differentiation [22]. Insulin-like growth factors have been reported to be stimulators of differentiation, as well as proliferation of myoblasts [23,24]. In BMSCs, proliferation is a necessary early stage in osteogenic differentiation accompanied by sequential changes in protein composition of the nuclear matrix [16]. Exerting dual effects of promoting proliferation and osteogenic differentiation of BMSCs, NAC would help achieving larger cell population and higher differentiation efficiency.

Furthermore, in this study, we assessed the effect of NAC on adipogenic differentiation in BMSCs. Our results showed that NAC could inhibit adipogenic differentiation of BMSCs in both adipogenic inducing and regular culture conditions manifested by oil red O staining and real-time RT-PCR of LPL, FABP4 and PPARG. PPARG is commonly referred to as the master regulator of adipogenesis, because no factor has yet been identified that can induce normal adipogenesis in its absence. All critical cell signaling pathways involved in adipogenesis converge on PPARG and most factors that stimulate adipogenesis ultimately exert their effect through regulation of this transcription factor [25,26]. BMSCs adipogenesis is also characterized by a dramatic increase in PPARG expression [26]. This increased expression of PPARG in adipogenesis directly activates or induces the expression of the majority of genes that characterizes the adipocyte phenotype including LPL, FABP4, Glut4, the insulin receptor and so on [27]. Many reports support an inverse relationship between adipogenesis and osteogenesis. For example, PPARG-deficient mice have decreased bone marrow adipogenesis as well as enhanced osteogenesis and increased trabecular bone volume [28]. Consistently to our result, a report demonstrated that Wnt5a-mediated non-canonical signaling inhibits adipogenesis in ST2 cells by attenuating the transcriptional activity of PPARG [29]. These evidences suggested that this decrease of adipogenic differentiation of BMSCs by NAC is mediated partially by inhibiting PPARG expression via a cross-talk with non-canonical Wnt pathway.

The differentiation of BMSCs into adipocytes rather than osteoblasts is at least in part responsible for reduction in the number of osteoblastic cells in osteoporosis and aging [8]. Our results described NAC’s effect of simultaneously promoting osteogenesis and inhibiting adipogenesis in vitro. This suggested that NAC may have a potential effect of diverting the adipogenesis of BMSCs to osteogenesis, and implies that NAC can be used in the prevention and treatment of osteoporosis. Many kinds of drugs, such as statins [15,30], Tigogenin [21] and alendronate [31], have been proven to act on BMSCs to stimulate osteogenic differentiation and inhibit adipogenic differentiation. Simvastatin is reported inhibiting the proliferation of BMSCs [32]. Although many studies...
demonstrated that statins have the effect of reducing risk of osteonecrosis, it is known that decreasing the number of BMSCs may lead to the development of osteonecrosis [33]. NAC is an antioxidant. A recent study reported the beneficial effects of an antioxidant beverage on modulation of experimental osteoporosis by increasing bone density of the middle and the epiphysis of femur in the rats [34]. Previous studies have shown that NAC exerts cellular protective effects through various pathways, including extracellular inhibition of mutagenic agents, inhibition of the genotoxicity of reactive oxygen species, protection of DNA and nuclear enzymes, and prevention of the formation of carcinogen–DNA adducts [34]. Clinical trials of NAC treatment showed good compliance in treated patients and a low frequency of side effects [35]. These properties make NAC helpful for cell therapy and be a promising potential drug for the prevention and treatment of diseases associated with osteoporosis and aging. Taken together, the above results indicated that, in both inducing and regular culture conditions, NAC exerts dual influences on BMSCs by promoting osteogenic differentiation and inhibiting adipogenic differentiation. In addition, NAC was able to promote cell proliferation in regular culture condition. We may deduce that NAC might have protective effect and be helpful in the prevention and treatment of osteoporosis by stimulating osteoblast formation and inhibiting adipocyte formation from BMSCs.

316 Disclosure of interest

The authors have not supplied their declaration of conflict of interest.

319 Acknowledgments

We thank Bo Tao and GuangChuan Wang for technical support. We also thank Wenjing She for language editing.

322 References