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Effects of cell surface α2-3 sialic acid on osteogenesis

Lan Xu · Wei Xu · Guiying Xu · Zhi Jiang · Lei Zheng · Yinghui Zhou · Wenxiang Wei · Shiliang Wu

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Abstract A cell culture model of osteoblast differentiation was applied in our study of the effect of sialic acid on the osteogenesis by using the pre-osteoblast of MC3T3-E1 subclone 14 cells. Following the treatment of different concentrations of α2,3-neuraminidase, which specifically removed the α2-3 sialic acid from cell surface, a significant decrease of α2-3 sialic acid was detected with fluorescein isothiocyanate (FITC)-labeled *Maackia amurensis* lectin (MAL-II) by flow cytometry analysis. von Kossa staining showed that the bone mineralization decreased in MC3T3-E1 subclone 14 cells after the treatment of α2,3-neuraminidase for 2 weeks. However α2,3-neuraminidase did not affect the formation of osteoblasts in MC3T3-E1 subclone 14 cells, which was demonstrated by positive alkaline phosphatase (ALP)-staining. Characteristic biological markers and osteoblast-like cell-related factors of osteoblastic cells were also examined. Both RT-PCR and Western blot analysis demonstrated that the expression of bone sialoprotein (BSP), osteoprotegerin (OPG), and vitamin D receptor (VDR) were significantly decreased when α2-3 sialic acid expression decreased on the cell surface, while the expression of osteocalcin (OC) and osteopontin (OPN) remained unchanged. We propose a hypothesis that α2-3 sialic acid affects bone mineralization but not osteogenic differentiation.

Keywords MC3T3-E1 subclone 14 · Osteoblasts · α2-3 sialic acid · α2,3-neuraminidase

Abbreviations

SA	Sialic acid
MALII	*Maackia amurensis* leukoagglutinin II
ALP	Alkaline phosphatase
OC	Osteocalcin
OPN	Osteopontin
BSP	Bone sialoprotein
OPG	Osteoprotegerin
RT-PCR	Reverse transcription-polymerase chain reaction
CFU-F	Colony forming unit-fibroblast
ECM	Extracellular matrix
BMP	Bone morphogenetic protein
VDR	Vitamin D receptor

Lan Xu and Wei Xu equally contributed to this work.

L. Xu · G. Xu · Z. Jiang · L. Zheng · Y. Zhou · S. Wu (✉)
Department of Biochemistry and Molecular Biology, School of Medicine, Soochow University, Suzhou 215007, China
e-mail: wushiliang@suda.edu.cn

W. Xu
Division of Orthopaedic Surgery, The Second Affiliated Hospital, School of Medicine, Soochow University, Suzhou 215007, China

W. Wei
Department of Cell Biology, School of Medicine, Soochow University, Suzhou 215007, China

W. Wei (✉)
Department of Cell Biology, School of Medicine, Soochow University, Suzhou 215123, China
e-mail: wenxiangw@suda.edu.cn

Introduction

Mammalian cells are covered by a glycocalyx, including glycolipids, glycoproteins, glycophospholipid anchors and proteoglycans [1]. The synthesis of the glycocalyxes starts in the endoplasmic reticulum (ER), which undergoes further modification within the Golgi before being transported to the cell surface. Glycocalyx is essential for development, regeneration and cellular functions such as communication and recognition between cells [2].

Sialic acid is an important member of cell surface sugars and is found at the ends of glycan chains of N- and O-glycans and glycolipids *via* α2-3, α2-6, and α2-8 bonds [3]. Sialic acid functions as a repulsive element due to its

negative charge, and inhibit homophilic interaction between sialylated molecules [4, 5].

Many studies have been published on the functions of α2-3 sialic acid in glycan chains, especially in tumors. Li *et al*. [6] investigated the relationship between glycoprotein chain structure and proliferation and migration by treating the human gastric cancer cell line AGS with different concentrations of α2,3-neuraminidase. They demonstrated that MAL-II could specifically recognize and combine with α2-3 sialic acid residues at the ends of glycoprotein chains. Moreover, the repair capacity and migration of AGS cells were evidently increased following α2,3-neuraminidase treatment, showing that the structure of cell surface α2-3 sialic acid might influence cell–cell signal transduction and affect cell behavior.

Osteoblasts are bone-forming cells that are responsible for bone growth during development and bone formation [7]. During bone formation, multipotential mesenchymal cells proliferate and differentiate into osteoblasts which synthesize and deposit a mineralizing extracellular matrix. Mature osteoblasts eventually become either osteocytes, bone lining cells, or vanish because of apoptosis. Differentiation of multipotential mesenchymal cells into mature osteoblastic cells is regulated by a large number of hormones and locally produced growth factors. As sialic acids are the outermost sugar on the cell surface, we reason sialic acids might influence cell–cell signal transduction and affect osteogenesis, which remains to be addressed.

To study the effects of sialic acid on osteogenesis, we established a cell culture model of osteoblast differentiation using MC3T3-E1 subclone 14 cells as pre-osteoblast. The pre-osteoblast cells were treated with α2,3-neuraminidase, which specifically removes the sialic acids on the cell surface. von Kossa staining showed that the bone mineralization decreased in the treated cells after removal of sialic acids. The expression of osteoblast-like cell-related factors was also affected by the treatment of α2,3-neuraminidase. However, the formation of osteoblasts in MC3T3-E1 subclone 14 cells remained unchanged, as was demonstrated by positive alkaline phosphatase (ALP)-staining. Our study suggests that α2-3 sialic acid on the surface of pre-osteoblast cells affects bone mineralization but not osteogenic differentiation.

Materials and methods

Materials and reagents MC3T3-E1 subclone 14 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (China). α-MEM culture medium containing L-Ascorbic acid was purchased from Gibco (USA). Fetal bovine serum (FBS), β-glycerophosphate, dexamethasone, and trypsin were purchased from Sigma-Aldrich (USA). Random hexamers, Taq DNA polymerase, and RevertAid M-MuLV reverse transcriptase were purchased from Fermentas (USA). Reverse-transcriptase polymerase chain reaction (RT-PCR) kits were purchased from TaKaRa (Japan). TRIzol was purchased from Invitrogen (USA). α2,3-neuraminidase was purchased from NEB (USA). MAL-II was purchased from Vector (USA). Streptavidin-PE was purchased from Sigma (USA). Mouse monoclonal antibodies against human osteocalcin (OC), bone sialoprotein (BSP), vitamin D receptor (VDR), osteoprotegerin (OPG), and osteopontin (OPN) were purchased from Santa Cruz Biotechnology (USA). Mouse anti-human IgG-AP secondary antibodies were purchased from Beyotime (China).

Establishment of a cell culture model of osteoblast differentiation MC3T3-E1 subclone 14 cells were cultured in α-MEM medium containing 10 % FBS, 100 mg/L penicillin, and 100 mg/L phytomycin and incubated at 37 °C in a 5 % CO_2 atmosphere. The culture medium was changed every 3 days. After the 50 mL culture flask was completely covered with cells, it was digested in 0.25 % trypsin solution and passaged at a 1:3 ratio. The passaged cells were treated with 0.25 % trypsin, and the cells were replated at 1.5×10^5 cells/well in a 6-well cell culture plate. MC3T3-E1 subclone 14 cells was separated into three groups: control group, cultured with conditioned medium containing L-Ascorbic acid (50 μg/mL), β-glycerophosphate (10 mM/mL), and dexamethasone (10^{-8}M/mL), sample group, cultured with conditioned medium with α2,3-neuraminidase (200 mU/mL), and sample group, cultured with conditioned medium with α2,3-neuraminidase (600 mU/mL). All groups were cultured for 14 days. Growth rates and cell shapes were monitored continuously with an inverted microscope.

Detection of cell surface α2-3 sialic acid by flow cytometry using MAL-II Cells were cultured for 3, 10 and 14 days, the medium was aspirated, and the cells were washed lightly twice with phosphate-buffered saline (PBS) before lectin MAL-II binding was detected according to the kit instructions. Briefly, cells were digested with trypsin first, washed three times with PBS containing 0.05 % Tween 20 (PBS-T), and centrifuged (1,000 rpm, 5 min). The cells were collected, counted, and adjusted to 5×10^5/tube. Each tube was incubated with biotin-labeled MAL-II lectin at 37 °C for 120 min, washed three times with PBS-T, and incubated in streptavidin-R- phycoerythrin from *Streptomyces avidinii*-buffered aqueous solution for 60 min at 37 °C. The fluorescence intensity of each group was measured by flow cytometry.

Alkaline phosphatase (ALP) staining ALP staining was performed according to the manufacturer's instructions at day 10. Briefly, the sample was covered with substrate, sealed with Parafilm wrap, incubated in a humid chamber for 15 min at 37 °C, washed with double distilled water (ddH_2O), incubated with hematoxylin for approximately 10 min, washed again with ddH_2O, and air-dried. Positive samples showed a red color [8].

von Kossa staining At day 14, the medium was aspirated, and the cells were lightly washed twice with PBS, then fixed in 95 % ethanol for 10 min, and washed three times with ddH_2O. They were incubated with 1 % silver nitrate for 45 min in the dark, washed three times with ddH_2O, and then placed under running ddH_2O for 10 min. The sodium thiosulfate reaction was allowed to proceed for 5 min before the cells were fixed in 95 % ethanol for 10 min, dried, and stored. Mineralized nodules were black [9].

Reverse transcription-polymerase chain reaction (RT-PCR) analysis After the cells were cultured for 3 and 10 days, the medium was aspirated, and the cells were lightly washed twice with PBS. Total RNA was extracted from cells following treatment with a TRIzol RNA extraction kit, and RNA amounts were determined quantitatively with a UV spectrometer at 260/280 nm. cDNA was synthesized from 1 μL of total RNA using a TaKaRa reverse transcription diagnostic kit. Special mRNA for each molecule was amplified by PCR using the following primer pairs: β-actin: 5′-GAGACCTTCAACACCCCAGC-3′ and 3′-CCACAGGATTCCATACCCAA-5′; OC: 5′-GAGACCTTCAACACCCCAGC-3′ and 3′-CCACAGGATTCCATACCCAA-5′; OPN: 5′-ATGAGATTGGCAGTGATTTG-3′ and 3′-GTAGGGACGATTGGAGTGA-5′; BSP: 5′-ATGGAGACGGCGATAGTTC-3′ and 3′-GTGGAGTTGGTGCTGGTG-5′; OPG: 5′-TCCTGGCACCTACCTAAA-3′ and 3′-CACCTGAGAAGAACCCATC-5′; VDR: 5′-CCACGGGCTTCCACTTCA-3′ and 3′-GCAGGATGGCGATAATGT-5.′ PCR products were amplified with 30 cycles of denaturation (94 °C, 30 s), annealing (55–60 °C, 30 s), and extension (72 °C, 50 s). RT-PCR products were identified with ethidium bromide staining on 1.5 % agarose gels.

Western blotting After the cells were cultured for 3 and 14 days, the medium was aspirated, and the cells were washed lightly with PBS. Total protein was extracted and quantified by the Bradford method. Then, 10 μg protein samples were boiled for 5 min with Laemmli buffer before 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (10 % SDS-PAGE). Next, proteins were transferred under semidry conditions onto polyvinylidene fluoride (PVDF) membranes and incubated with mouse anti-human OC, OPN, BSP, OPG, and VDR monoclonal antibody at 25 °C for 60 min. After washing, membranes were incubated with HRP-conjugated mouse anti-human IgG-AP at 25 °C for 60 min. Bands were visualized with a BM Chemiluminescence Western Blotting Kit (Boehringer, Mannheim, Germany) according to the manufacturer's protocol.

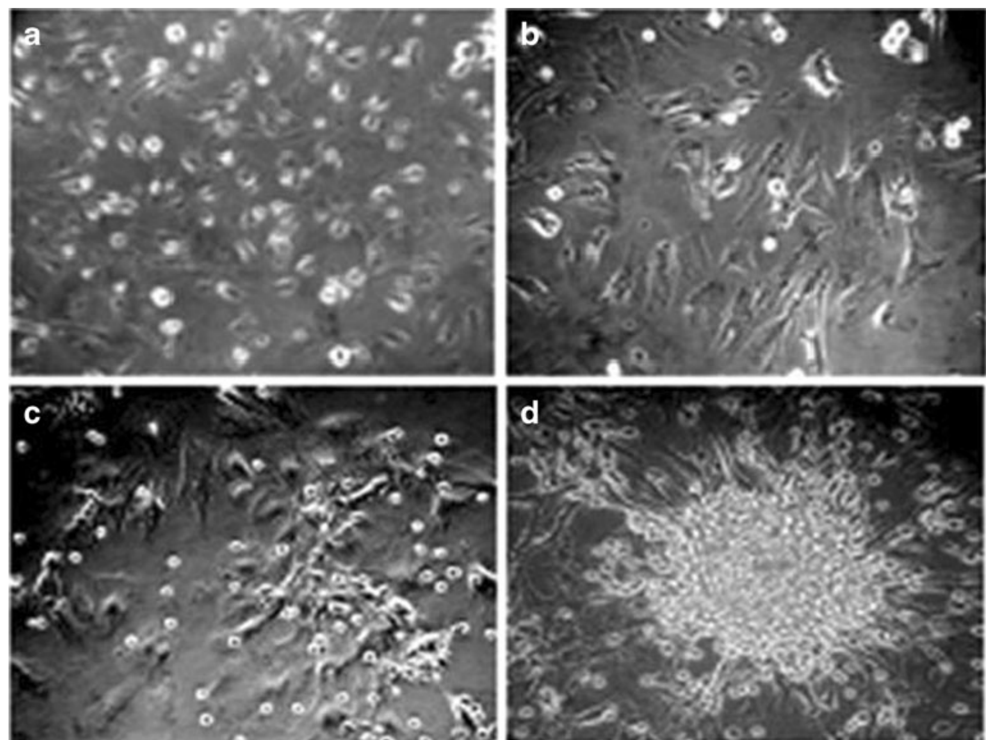

Fig. 1 Osteoblast formation of MC3T3-E1 subclon 14 cells. Cells were cultured in conditioned medium containing L-Ascorbic acid (50 μg/mL), β-glycerophosphate (10 mM), and dexamethasone (10^{-8}M) for 1 day (**a**), 3 days (**b**), 10 days (**c**) or 14 days (**d**), and then were examined and photographed with an inverted microscope

Results

Osteoblast formation of MC3T3-E1 Sub14 cells MC3T3-E1 subclone 14 cells are a pre-osteoblast cell line, and can differentiate into osteoblasts, which was applied in our study to examine the effect of neuroaminadase on the osteoblast formation. We cultured the cells with conditioned medium containing L-ascorbic acid (50 μg/mL), β-glycerophosphate (10 mM) and dexamethasone (10^{-8}M) for 14 days. Growth rates and cell shapes were monitored continuously using an inverted microscope. At 2 h after transfer and inoculation, the cells were treated with conditioned medium. After 24 h, 70–80 % of cells were adherent to the plates with shuttle-like appearance (Fig. 1a). The volume of cells increased 3–4 days later, showing polygonal shapes with bulky protuberances (Fig. 1b). A week later, cells were overgrown, shaping like an acerose shuttle. Cells were monitored in the culture to examine osteogenesis abilities. At day 10, cells began to form clusters and developed spotted nodules (Fig. 1c). Two weeks later, multiple nodules gathered into multi-layer structures that gradually enlarged into brown masses (Fig. 1d).

The effects of neuraminidase on the expression of α2-3 sialic acid α2,3-neuraminidase is a highly specific exoglycosidase that can break α2-3 glycosidic bonds in *N*-acetylneuraminic acids containing oligosaccharides [4]. Although α2-6 and α2-8 glycosidic bonds may also be affected by α2,3-neuraminidase, these reactions are much slower and weaker compared with the reaction in breaking α2-3 glycosidic bonds [4]. MAL-II is a hemagglutinin isolectin, which specifically recognizes and binds NeuAcα2,3Galβ1-4GlcNAc/Glc [5]. We added different concentrations of α2,3-neuraminidase to the culture to remove α2-3 sialic acid on the cell surface of MC3T3-E1 subclone 14. The treated cells were examined for the α2-3 sialic acid expression on the cell surface by flow cytometry using Mal-II. The results showed that there was no significant difference between the expressions of α2-3 sialic acid with or without treatment of α2,3-neuraminidase at day 3 (Fig. 2a).

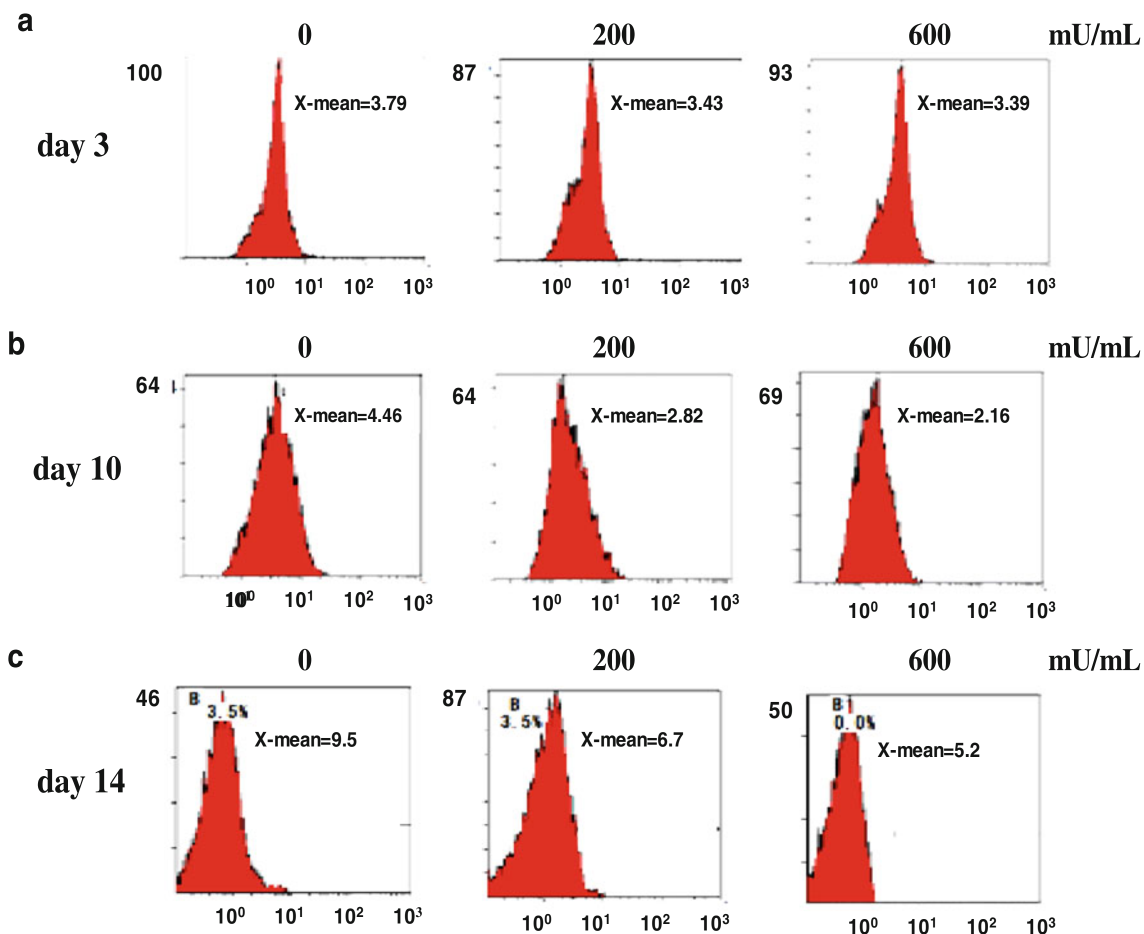

Fig. 2 The effects of neuraminidase on the expression of α2-3 sialic acid. MC3T3-E1 subclone 14 cells were cultured in conditioned medium, conditioned medium with 200 mU/mL neuraminidase, or conditioned medium with 600 mU/mL neuraminidase for 3 days (**a**), 10 days (**b**) or 14 days (**c**). Mal-II was applied for the detection of α2-3 sialic acid on the cell surface by flow cytometry analysis as described in Materials and methods

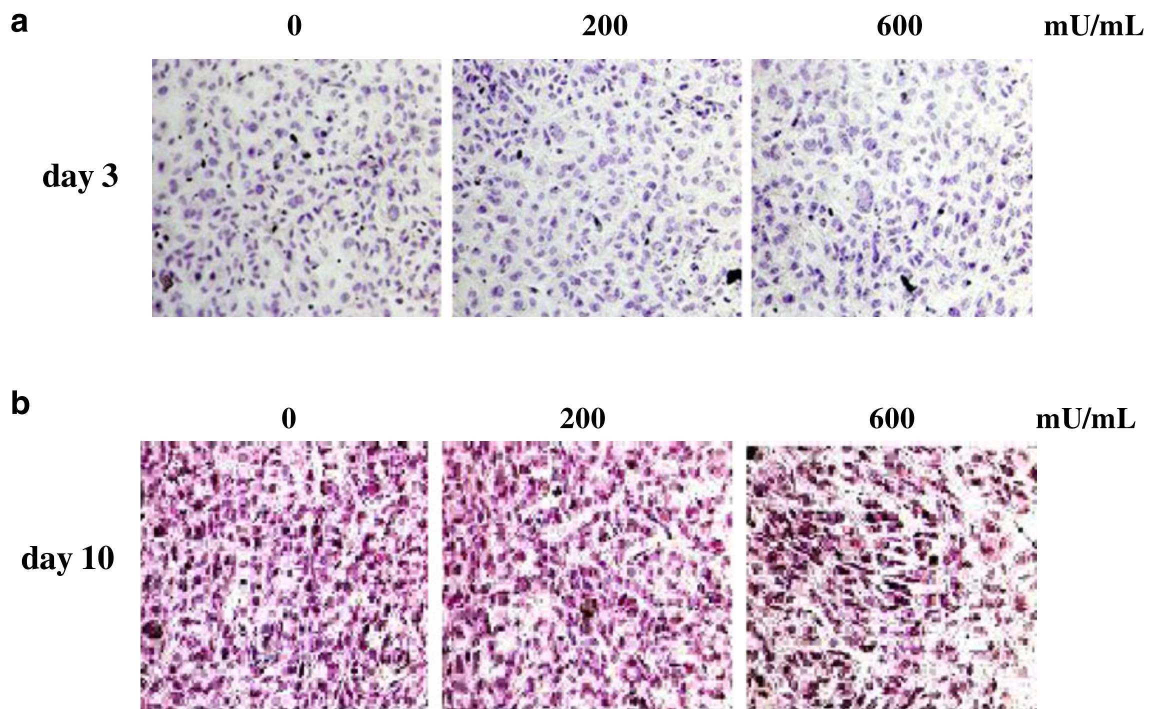

Fig. 3 The effects of cell surface α2-3 sialic acid on osteogenic cells. MC3T3-E1 subclone 14 cells were cultured in conditioned medium, conditioned medium with 200 mU/mL neuraminidase, or conditioned medium with 600 mU/mL neuraminidase for 3 days (**a**) or 10 days (**b**). Cells were subjected to ALP staining as described in Materials and methods. The *red staining* shows the positive expression of ALP

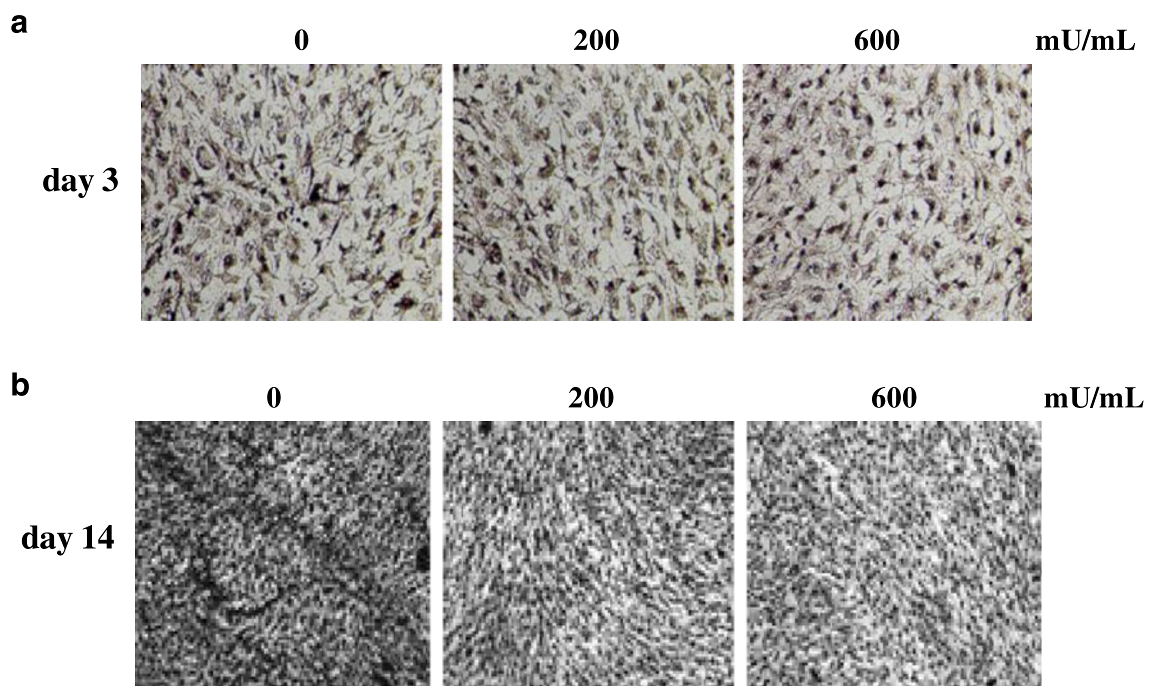

Fig. 4 The effects of cell surface α2-3 sialic acid on bone mineralization. MC3T3-E1 subclone 14 cells were cultured in conditioned medium, conditioned medium with 200 mU/mL neuraminidase, or conditioned medium with 600 mU/mL neuraminidase for 3 days (**a**) or 14 days (**b**). Cells were subjected to von Kossa staining as described in the Materials and methods. Mineralized nodules show *black*

However, at days 10 and 14, α2-3 sialic acid expression decreased with a dose dependent effect after treatment with 200 and 600 mU/mL α2,3-neuraminidase, indicating that neuraminidase treatment is able to reduce the expression of α2-3 sicalic acid (Fig. 2b and c).

Effects of cell surface α2-3 sialic acid on osteogenic cells As α2,3-neuraminidase decreases the expression of cell surface α2-3 sialic acid as shown in Fig. 2, we examined the effects of cell surface α2-3 sialic acid on the production of osteoblasts. Alkaline phosphatase (ALP) staining was performed to determine the differentiation of osteoblasts, as ALP is a typical biological marker during the osteogenesis. The results showed that ALP staining was negative in cells that were cultured in conditioned medium for 3 days (Fig. 3a), but strongly positive at day 10, indicating the formation of osteoblastic cells (Fig. 3b). Comparing osteoblast cells with and without treatment of 200 or 600 mU/mL α2,3-neuraminidase, no significant difference was observed between them whether at day 3 or day 10 in culture. These results demonstrate that α2-3 sialic acid did not significantly affect osteogenic differentiation in MC3T3-E1 subclone 14 cells.

Effects of cell surface α2-3 sialic acid on bone mineralization Bone mineralization occurs during the growth and differentiation of osteoblasts. So we examined the effect of neuraminidase on the bone mineralization of MC3T3-E1 subclone 14 cells with von Kossa staining, which demonstrates newly synthesized bone matrix. As shown in Fig. 1, at day 14, cells were forming a light-tight and mineralized node. von Kossa staining showed no dark deposits at the centers of cell clusters at day 3 (Fig. 4a), while showing strong dark deposits after 14 days culture in conditioned medium. Gradually the dark deposits gathered into a mass, showing a positive reaction. When compared with cells cultured in conditional medium only, bone mineralization decreased significantly in the cells treated with 200 or 600 mU/mL α2,3-neuraminidase (Fig. 4b). These results indicate that α2-3 sialic acid is required for bone mineralization.

Effects of cell surface α2-3 sialic acid on osteoblast-like cell-related factors It is known that during the formation of bone, characteristic biological markers and osteoblast-like cell-related factors are expressed. Thus, we examined the effect of neuraminidase on the expression of factors, such as OC, BSP, OPG, OPN, and VDR by RT-PCR. As shown in Fig. 5a, the mRNA expression levels of OC, BSP, OPG, OPN, and VDR were lower after 3 days culture, but significantly increased after 10 days culture in conditional medium (Fig. 5b). Comparing the cells with or without treatment of

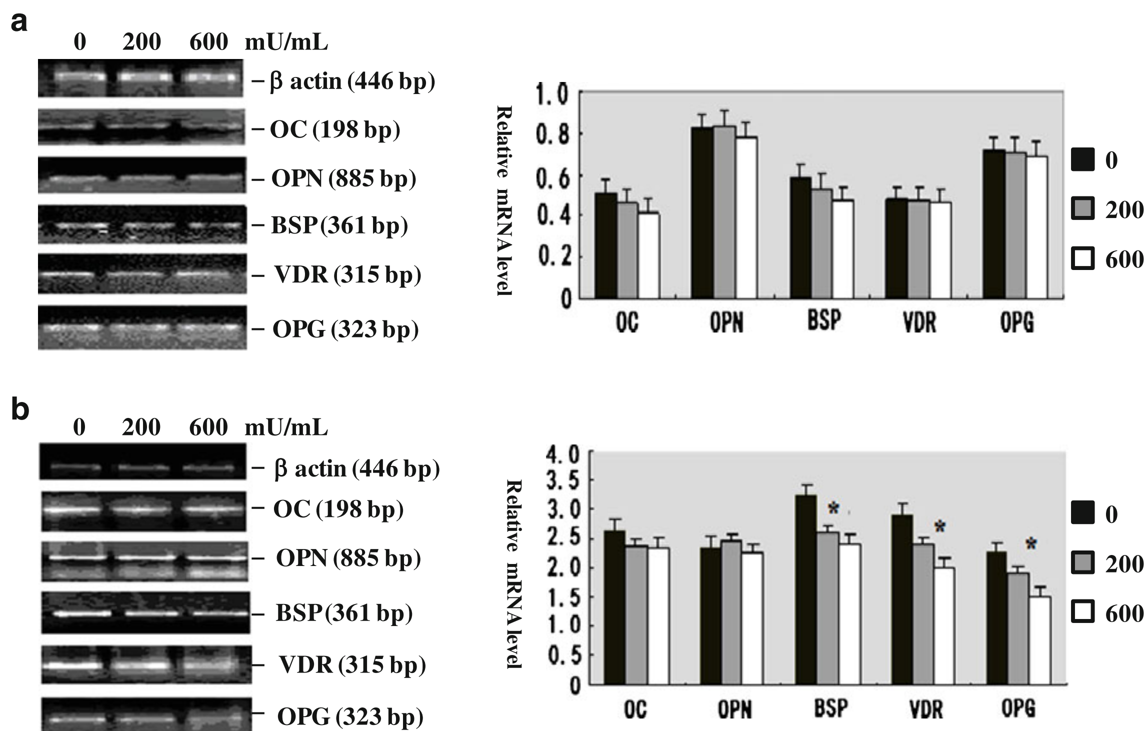

Fig. 5 The effects of cell surface α2-3 sialic acid on the mRNA expression of osteoblast-like cell-related factors. MC3T3-E1 subclone 14 cells were cultured in conditioned medium, conditioned medium with 200 mU/mL neuraminidase, or conditioned medium with 600 mU/mL neuraminidase. Total RNA was isolated at day 3 (**a**) and day 10 (**b**), and RT-PCR analyses were performed and showed to the *left panels*. The Quantity One software was used to conduct the analysis of mRNA expression. The relative expression levels of OC, OPN, BSP, OPG, and VDR mRNA are shown graphically to the *right panels*. *$p<0.05$

neuraminidase, no difference of the expression levels on these factors was observed on day 3. However, the expression of BSP, OPG, and VDR decreased significantly after treatment with 200 and 600 mU/mL α2,3-neuraminidase on day 10 when compared with cells without the treatment (Fig. 5b).

To confirm the expression of characteristic biological markers and osteoblast-like cell-related factors (Fig. 5), Western blot analysis was also applied to examine protein expression (Fig. 6). Total protein was extracted and quantified by the Bradford method on day 3 and day 14. The Western blot results showed that there was no difference on the expression levels of OC, OPN, OPG, and VDR between day 3 and day 14 in the cells. However the expression of BSP was increased at day 14 when compared with BSP expression at day 3 in the cells with or without the treatment (Fig. 6a). Interestingly, the expression levels of BSP, OPG, and VDR were decreased on day 14 after treatment with increasing amount of (200 and 600 mU/mL) α2,3-neuraminidase (Fig. 6b) compared with cells without treatment, which is consistent with the RT-PCR results (Fig. 5).

Discussion and conclusions

Cultured osteoblast cells are an important model for studying bone metabolism and osteogenic mechanisms. Osteoblastic cell lineages can come from several sources: bone marrow stromal cells (also known as colony forming unit-fibroblast cells), pre-osteoblast cells that can directly differentiate into osteoblasts, and strongly proliferative osteoprogenitor cells, which are often found in the bone marrow cavity and can differentiate into pre-osteoblast and cartilage cells [10, 11]. MC3T3-E1 subclone 14 cells were generated by cloning different MC3T3-E1 cell line phenotypes. MC3T3-E1 subclone 14 (ATCC CRL-2594) differentiate into osteoblasts when cultured with ascorbic acid and 3–4 mM inorganic phosphate and formed a highly mineralized extracellular matrix at day 10 [12].

Studies have demonstrated that dexamethasone, β-glycerophosphate, and L-ascorbic acid are required for osteoblast differentiation and bone formation *in vitro*. By

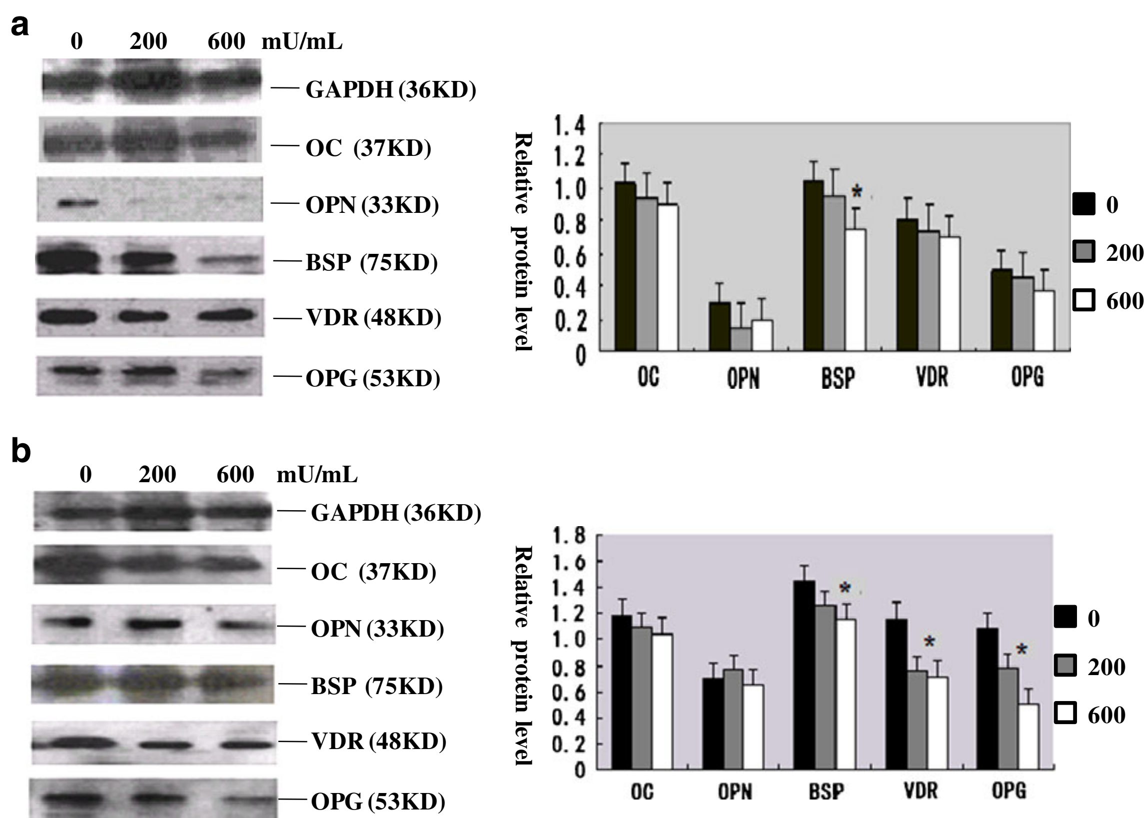

Fig. 6 The effects of cell surface α2-3 sialic acid on the protein expression of osteoblast-like cell-related factors. MC3T3-E1 subclone 14 cells were cultured in conditioned medium, conditioned medium with 200 mU/mL neuraminidase, or conditioned medium with 600 mU/mL neuraminidase. Cell lysates were prepared for Western blot analysis (*right panels*) with antibodies against OC, OPN, BSP, OPG, and VDR at day 3 (**a**) and day 14 (**b**). The Quantity One software was used to conduct the analysis of protein expression which was shown graphically to the *right panels*. *$p<0.05$

inhibiting stromal cell proliferation, dexamethasone increases alkaline phosphatase activity and promotes transformation into osteoblasts [13]. β-Glycerophosphate provides phosphate as a substrate for ALP, which promotes the deposition of physiological calcium salts and subsequent mineralization [14]. L-Ascorbic acid may accelerate collagen synthesis and mineralization *in vitro*, and it may also regulate the activities of adenosine triphosphate (ATP) and ALP or influence their synthesis.

We successfully established a cell culture model of osteoblast differentiation using the pre-osteoblast of MC3T3-E1 subclone 14 cell line, which showed positive ALP staining at day 10, indicating osteoblastic cell formation, and showed positive von Kossa staining on day 14, indicating mineralization of newly synthesized bone matrix. The expression of characteristic biological markers of osteoblastic cells was also regulated during osteogenesis of MC3T3-E1 subclone 14 cell line, as we observed.

BMP, BSP, and OPN play important roles in bone formation. BSP, in particular, has recently been studied as a new bone growth-inducing factor. However, these proteins are glycoproteins, and previous studies have focused on genomics and proteomics. Researchers have come to realize that saccharides are also important biologic information molecules and are a 'continuation' of genetic information [15]. A recent study found that glycosylation was necessary for the biological activity of protein molecules and altering protein glycosylation produced new protein biological activities [16].

We treated MC3T3-E1 subclone 14 cells with α2,3-neuraminidase, which decreased α2-3 sialic acid expression on the cell surface. The expression of mRNA and protein levels of BSP, OPG, and VDR were examined for the effect of neuraminidase in the osteogenesis. Wuttke *et al*. [17] transfected human embryonic kidney cells with a BSP expression plasmid and compared the recombinant BSP with bone-derived BSP. They found that both BSP had similar secondary structures, whereas bone-derived BSP had a higher affinity for hydroxyapatite than recombinant BSP. The authors believed that this was because of greater numbers of *N*-acetylneuraminic acids on BSP oligosaccharides. Hydroxyapatite is a natural mineral that constitutes 60–70 % of bone tissue and plays a role in bone induction. OC plays an important role in bone composition and metabolism and is a sensitive indicator for bone formation. Because OC appears primarily during mineralization, it has been considered as one of the differentiation indicators of osteogenic cells undergoing mineralization. The synthesis of OC is regulated by vitamin D [18, 19], which competes with TBP for the TATA/VDRE binding site to inhibit transcription. BSP, CRE, and FRE are significant elements in initiating BSP transcription. One of the functions of vitamin D is to directly stimulate osteoblasts to promote bone mineral deposition, but the mechanism is as yet unclear. Osteoblasts deficient in vitamin D can still synthesize bone matrix and collagen fibers, but they are incapable of calcification. Recent studies suggest that bone mineral density is affected by polymorphisms in the VDR gene. Morrison *et al*. reported that VDR polymorphisms could be a useful genetic marker for predicting bone mineral density, which has a 26.3 % genetic influence, and vitamin D deficiency could lead to bone matrix damage and osteomalacia [20, 21]. Our results demonstrate that α2-3 sialic acid affects bone mineralization but not osteogenic differentiation in MC3T3-E1 subclone 14 cells. A possible mechanism may exist that sufficient α2-3 sialic acid on the cell surface of osteoblast supports the expression of BSP, VDR and OPG, which in turn promote the bone mineralization rather than differentiation.

In conclusion, we successfully established a cell culture model of osteoblast differentiation using pre-osteoblast of MC3T3-E1 subclone 14 cells. Cell surface α2-3 sialic acid structures were effectively recognized with FITC-labeled specific lectin MAL-II. Using this model, we found that α2-3 sialic acid affected bone mineralization, but not osteogenic differentiation.

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