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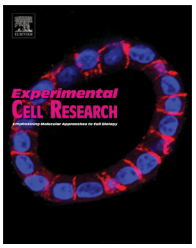

Research Article

Staphylococcal enterotoxin C2 promotes osteogenesis and suppresses osteoclastogenesis of human mesenchymal stem cells

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ABSTRACT

As a super-antigen, staphylococcal enterotoxin C2 (SEC2) stimulates the release of massive inflammatory cytokines such as interferon-gamma (IFN-γ), interleukin-1 (IL-1) and interleukin-2 (IL-2) which are documented to implicate osteoblast differentiation. In the present study, SEC2 was found to significantly improve the osteoblast differentiation by up-regulating BMP2 and Runx2/Cbfa1 expression. Interferon (IFN)-inducible gene IFI16, a co-activator of Runx2/Cbfa1, was also activated by SEC2 in the osteoblast differentiation. In addition, exogenous introduction of SEC2 stimulated OPG expression and suppressed RANKL, suggesting suppression of osteoclastogenesis in hMSCs. Therefore, our results displayed that SEC2 plays an important role in the commitment of MSC to the osteoblast and it might be a potential new therapeutic candidate for bone regeneration.

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Introduction

Staphylococcal enterotoxins (SEs) are a family of heat-stable enterotoxins which elicit a variety of biological activities. As a powerful super-antigen (SAg), SEs can stimulate vigorous proliferation of T-lymphocytes [1] and induce various cytokines such as interferon-gamma (IFN-γ), interleukin-1 (IL-1) and interleukin-2 (IL-2) [2–3], which suppress tumor cell growth in vitro and in vivo [4–5]. Thereby, SEs are potential candidates for antitumor immunotherapy. In China, Staphylococcal enterotoxin C2 (SEC2) has been approved by National Food and Drug Administration for clinical trials as an effective therapeutic agent for patients with malignancy [6–7].

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Osteoporosis is a metabolic disease characterized by loss of bone mass and easily broken. The acceleration of the global population aging makes osteoporosis and osteoporotic fractures to become the major public health problems. Hence, medical intervention is urgently needed to ease the associated economic burden as well as to promote the living standard of these patients. Human mesenchymal stem cells (hMSCs) are multipotential stem cells which can differentiate into various cells such as adipocytes, osteoblasts, and chondrocytes [8]. MSCs also act as osteoblast progenitor to improve the bone formation [9,10]through the transcriptional regulators BMP2 and Runx2/Cbfa [11,12]. Therefore, MSCs may serve as a potential candidate for stem cell based bone modeling and remodeling.

Previous studies have documented that most inflammatory cytokines, such as IFN-γ13-14; interleukin-1β (IL-1β); tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), interleukin-4 (IL-4) improve the osteoblasts differentiation [15,16]. In the present study, SEC2 was found to promote osteogenesis and suppress osteoclastogenesis of human mesenchymal stem cell in vitro. These findings reveal the de novo function of SEC2 on osteogenic differentiation of human MSCs and suggest SEC2 would be a new agent for osteoporosis and bone fracture in clinical practice.

Materials and methods

SEC2 and rSEC2 protein

Wild type SEC2 and recombinant SEC2 protein (rSEC2) were provided by Shenzhen Xielian Gene Engineering Co., Ltd. (Shenzhen, China).

Cell culture and osteogenic induction

Human mesenchymal stem cells (hMSCs) were isolated from bone marrow and identified by cellular surface markers as mentioned before [17]. Briefly, bone marrow was aspirated from healthy donors after formal consents and approval by Joint the Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. Human MSCs were cultured in minimum essential medium alpha medium (α-MEM, GIBCO, Green Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO), 2 mM L-glutamine (GIBCO), 100 U/mL penicillin and 100 μg/mL streptomycin (P/S, GIBCO), and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The hMSCs from the seventh to ninth passages were used in this study. After reaching 60%-80% confluence, cells were stimulated to differentiate into osteoblast as previously described [17]. The osteoblasts were induced by introducing osteogenic inducer (OS) medium containing 10^{-8}M dexamethasone (Dex) (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/mL ascorbic acid 2-phosphate (AsAP) (Sigma) and 10 mM Glycerol 2-phosphate (Gly) (Sigma).

Cell viability

Cells were plated in 96-well plates at density of 2×10^3 per well and treated with different dose of SEC2 and rSEC2 (from 5 μg/ml to 100 μg/ml). After treatment, the cells were continuously maintained for 72 h. The effect of SEC2 and rSEC2 on cell viabilities was determined by MTT assay as previously described [18].

Alkaline phosphatase activity (AKP) assay

Treated with SEC2 and rSEC2 in OS medium for 6 days and 9 days, the cells were harvested and total protein was extracted for AKP activity assay. Intracellular AKP activities were determined with AKP activity kit (Nanjing Jiancheng Biotech, Nanjing, China) [19]. The total protein concentration of cell lysate was determined by Bradford assay (Bio-Rad, USA) which normalized the AKP activity. Each experiment was repeated in triplicate.

Alizarin red staining (ARS) staining

After the cells were fixed in 4% paraformaldehyde (m/v) for 10 min, cells were stained with 2% ARS (pH 4.1) for 15 min and then washed with deionized water. The orange and red spots were recognized as calcium deposits.

Von Kossa staining

For Von kossa staining, cells were stained with 1% silver nitrate solution with UV exposure for 20 min. Then cells were washed and incubated with 5% sodium thiosulfate for 5 min. Black stained spots were regarded as calcium deposits.

Quantitive Real Time PCR (qRT-PCR) analyses

Total RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out with SuperScript™ III Reverse Transcriptase (Invitrogen). The cDNA fragments were amplified with GoTaq® DNA polymerase (Promega, Madison, WI, USA). The qRT-PCR was performed with a 7500 Real Time PCR system (Applied Biosystems, USA). Fold change was calculated as mentioned before [20]. Primers used in qRT-PCR reactions were listed in Table 1. All experiments were repeated in triplicate.

Table 1 – Primers used in the present study.

Gene	Primer sequence FOR(5'-3')	Primer sequence REV(5'-3')
BMP-2	GTATCGCAGGCACTCAGGTC	CACTTCCACCACGAATCCAT
GAPDH	TCCATGACAACTTTGGTATCG	TGTAGCCAAATTCGTTGTCA
OCN	CCTCACACTCCTCGCCCTAT	GTGGTCAGCCAACTCGTCAC
OPN	CTAGGCATCACCTGTGCCATACC	CAGTGACCAGTTCATCAGATTCATC
OPG	TGCTGTTCCTACAAAGTTTACG	CTTTGAGTGCTTTAGTGCGTG
Runx2	ACTTCCTGTGCTCGGTGCT	GACGGTTATGGTCAAGGTGAA

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Identification of the direct interaction between RUNX2/Cbfa1 and IFI16

To identify the interaction between RUNX2/Cbfa1 and IFI16, a flexible docking model, Autodock 4.0, was performed to predict the protein–protein interaction as previously described [21].

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed with SPSS17. The *p*-value below 0.05 was considered to be statistically significant.

Results

SEC2 slightly suppressed viability of human MSCs

We first analyzed wild type SEC2 and recombinant SEC2 (rSEC2) proteins with SDS-PAGE electrophoresis. As shown in Fig. 1, the rSEC2 proteins showed the similar size to wild type SEC2. Next, the effect of SEC2 on cell viability was evaluated. The previous study showed that 10 μg/ml SEC2 suppressed cell growth in Hepa1-6 and H22 tumor cells by more than 60% [22]. The doses from 5 μg/ml to 100 μg/ml of SEC2 were introduced into hMSCs and cell viabilities were measured. Both SEC2 and rSEC2 showed slight suppressive effect on cell proliferation from the concentration of 20 μg/ml to 100 μg/ml (Fig. 2). But they did not induce cell death, suggesting very low toxicity to hMSCs (Data not shown).

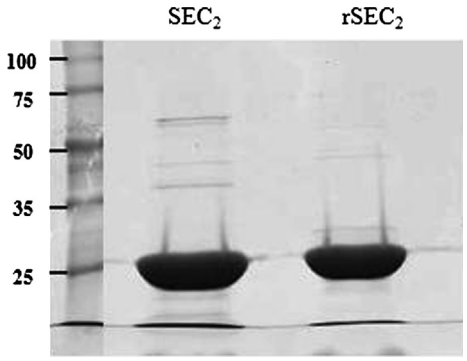

Fig. 1 – The analysis of the purified SEC2 and rSEC2 by SDS-PAGE electrophoresis.

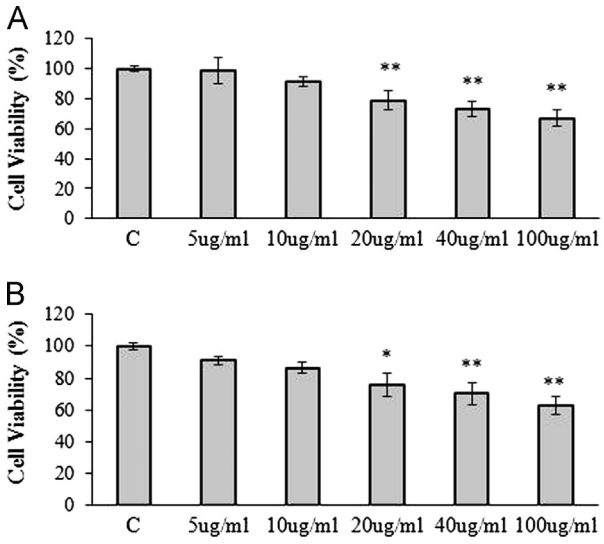

Fig. 2 – Cell viability analyses. A, the SEC2 (B) and rSEC2 (B) have slight suppressive effect on cell viability of hMSCs.

SEC2 promoted osteogenic differentiation of hMSCs

To evaluate the effect of SEC2 on osteoblast differentiation, human MSCs derived from bone marrow were used as cell model. The hMSCs were cultured in an osteogenesis-induced medium and 20 μg/ml SEC2/rSEC2 was introduced. Alkaline phosphatase (AKP) activity, an early marker of osteoblast, was determined at day 6 and day 9. As shown in Fig. 3A, the SEC2/rSEC2 significantly enhanced AKP activity. Moreover, the expression of osteogenic markers OPN and OCN was also significantly increased (Fig. 3B). To further confirm the osteogenic effect of SEC2, the Alizarin Red staining (ARS) and Von Kossa staining were performed and the results showed that SEC2/rSEC2 stimulated osteogenic differentiation of hMSCs (Fig. 3C).

IFI6 implicated in the osteoblast induced SEC2 and rSEC2

Runx2, a well-known transcriptional regulator of osteogenesis, was up-regulated by SEC2 and rSEC2 at day 9 and day 15 (Fig. 4A). IFI16 (P204 in mouse) acts as a co-activator of Runx2/Cbfa1 to enhance osteoblast differentiation. As shown in Fig. 4B, IFI16 was also promoted by SEC2 and rSEC2 at day 9 and day 15. Then we explored their interaction with a docking model which predicted a direct interaction between IFI16 and Runx2/Cbfa1 (Fig. S1). This result was consistent with previous study [23].

SEC2 suppressed osteoclast differentiation of hMSCs

Osteoclast differentiation maintains the balance between the bone formation and resorption. The ratio of OPG to RANKL is a critical indicator of the regulation of osteoclast or osteoblast formation [24]. We found the transcription of OPG was significantly enhanced by SEC2 and rSEC2 at day 9 and day 15 (Fig. 5A). On the contrary, transcription of RANKL mRNA was remarkably reduced by SEC2/rSEC2 (Fig. 5B). Thus, the ratio of OPG to RANKL (OPG/RANKL) increased on both day 9 and day 15, suggesting that SEC2/rSEC2 inhibits osteoclastogenesis (Fig. 5C).

Discussion

In the present study, we identified a novel function of staphylococcal enterotoxin C2 (SEC2) in regulation of osteoblast differentiation of MSCs. We also found that SEC2 suppressed bone resorption through disrupting osteoclastogenesis in hMSCs. These results suggest that SEC2 may be considered as a promising medicine for osteoporosis treatment.

As a superantigen, SEC2 promotes massive activation of immune cells at extremely low concentration, leading to the release of inflammatory cytokines such as IFN-γ, IL-1, IL-6 and TNF-α [25]. Additionally, SEC2 exhibits antitumor effects in vitro and in vivo through immune responsive system [26.27]. Among these secreted cytokines induced by superantigen, IFN-γ is the

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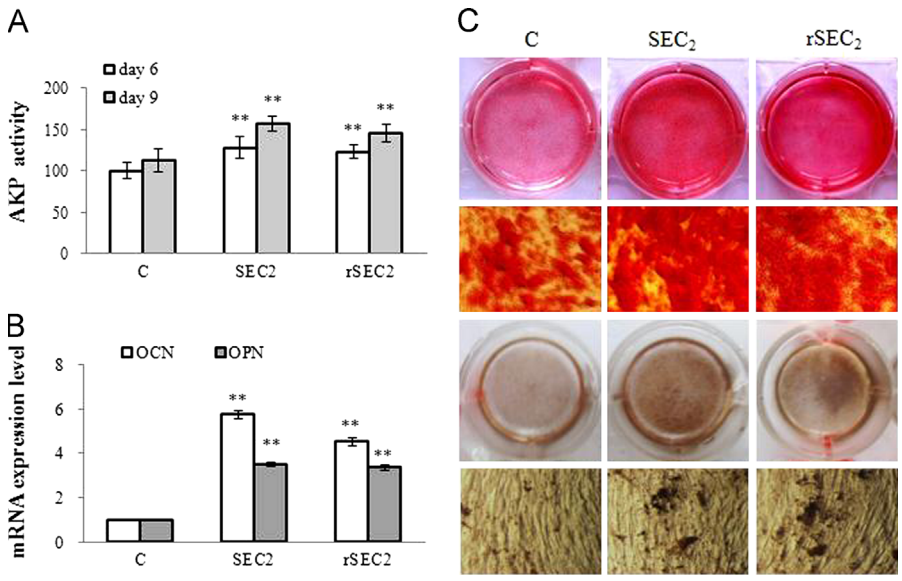

Fig. 3 – SEC2/rSEC2 promoted osteogenesis of human MSCs. A, AKP activities were increased by SEC2 and rSEC2 on day 6 and day 9. $^{**}p<0.01$ ($n=6$). B, mRNA expression levels of osteogenic markers OPN, OCN and regulator BMP2 were up-regulated by SEC2/rSEC2 at day 15. C, Results of ARS (for calcium knots) and Von Kossa (for mineralization) staining at day 15. $^{*}p<0.05$, vs. C; $^{**}p<0.01$, vs. C.

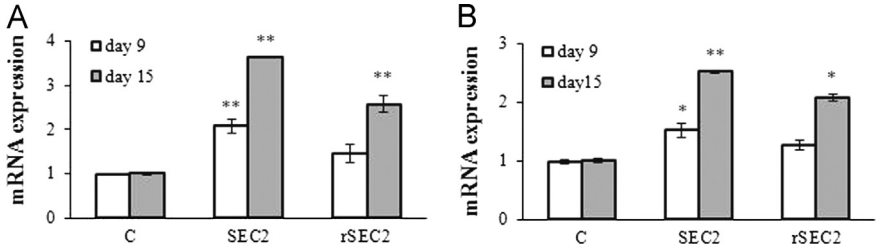

Fig. 4 – IFI16 and Runx2 expression were up-regulated by SEC2 in osteogenesis. A, mRNA expression of Runx2 was improved by SEC2/rSEC2 at day 9 and day 15. B, mRNA expression level IFI16 was enhanced by SEC2/rSEC2 at day 9 and day 15. $^{*}p<0.05$, vs. C; $^{**}p<0.01$, vs. C.

most important one. Recent studies reported the pro-osteogenic effect of IFN-γ in vitro and in vivo [13,14]. Moreover, IFN-γ negatively regulates adipogenesis in vitro and prevents marrow fat infiltration [28]. Therefore, IFN-γ is considered as a therapeutic target for osteoporosis [14]. Thereby, we speculated that SEC2 might stimulate the osteogenesis of hMSCs. Results of AKP activity, calcium knots, mineralization deposits and marker gene expression indicated that SEC2 promoted osteoblastogenesis of hMSCs (Fig. 3).

The interferon-inducible HIN-200 gene family plays important roles in inflammation, autoimmunity, differentiation and apoptosis [29]. As a member of HIN-200 family, the IFI16 (p204) gene has been implicated in multiple transcriptional regulation by protein–protein interactions with other transcriptional factors [30]. Previous studies have demonstrated that p204 is a crucial regulator in the differentiation of myocytes [31], adipocyte [32] and macrophages [32]. Specifically, p204, a co-activator of Cbfa1, stimulates osteoblast and hypertrophic chondrocyte differentiation [33,23]. As noted, Cbfa1 is an essential central regulator of osteoblast differentiation. Hence, we wonder whether IFI16 involves in the osteogenesis induced by SEC2. The prediction suggested that Cbfa1 might directly interact with IFI16, consistent with previous studies [23]. We also found that IFI16 expression was activated by SEC2 (Fig. 4), which indicated that IFI16 was implicated in the SEC2 induced osteogenesis. So we suggested that SEC2/rSEC2 mediated osteoblast, at least partially, through stimulating IFI16.

On the other hand, IFN-γ is considered as a strong inhibitor of osteoclast differentiation and bone resorption [34]. Mice with IFN-γ or IFN-γ receptor deficiency developed bone loss associated with collagen-induced arthritis [35–36]. IFN-γ also suppressed osteoclast differentiation in vitro [27] and induced apoptosis [37]. Furthermore, it has been reported that IFN-γ directly blocks the osteoclast formation by down-regulating the critical osteoclast regulator RANKL signaling pathway [8–39]. Contradictorily, there was also report claiming that IFN-γ stimulated osteoclastogenesis in vivo [40]. Our results showed that the gene OPG was enhanced by SEC2/rSEC2, while the expression of RANKL was suppressed (Fig. 5). The ascending OPG to RANKL ratio indicated that SEC2/rSEC2 suppressed osteoclastogenesis of hMSCs [41].

SEC2 has been used as an effective therapeutic agent in malignant tumor for several years in China with encouraging clinical effects observed [42]. The present study displayed the promising effect on bone formation by improving osteogenesis and suppressing osteoclastogenesis of hMSCs. Although SEC2-based therapeutic to bone regeneration is still in its infancy, our clinical findings on SEC2 are encouraging and suggest that this protein could be a potential therapeutic approach to osteoporosis or bone fracture in near future.

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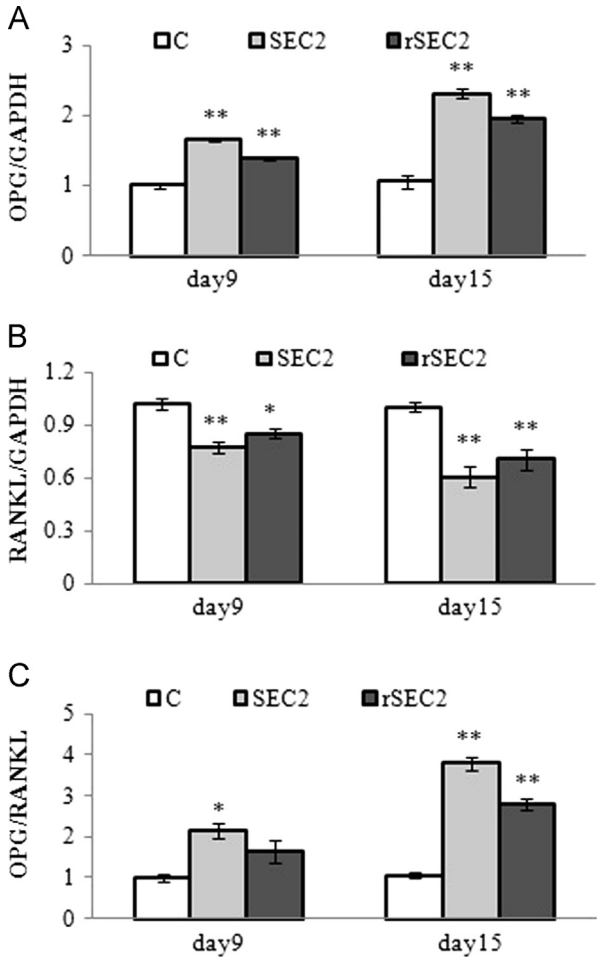

Fig. 5 – SEC2/rSEC2 suppressed osteoclastgenesis of hMSCs. mRNA expression of OPG (A) and RANKL (B) were quantified by qRT-PCR at day 9 and day 15. C, the ratio of OPG and RANKL was assayed at day 9 and day 15. *$p<0.05$, vs. C; **$p<0.01$, vs. C.

Conflict of interest statement

All authors declared no potential conflicts of interest.

Acknowledgment

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2013.12.008.

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