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Potential enhancement of osteoclastogenesis by severe acute respiratory syndrome coronavirus 3a/X1 protein

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Abstract  Severe acute respiratory syndrome coronavirus (SARS-CoV) causes a lung disease with high mortality. In addition, osteonecrosis and bone abnormalities with reduced bone density have been observed in patients following recovery from SARS, which were partly but not entirely explained by the short-term use of steroids. Here, we demonstrate that human monocytes, potential precursors of osteoclasts, partly express angiotensin converting enzyme 2 (ACE2), a cellular receptor of SARS-CoV, and that expression of an accessory protein of SARS-CoV, 3a/X1, in murine macrophage cell line RAW264.7 cells, enhanced NF-κB activity and differentiation into osteoclast-like cells in the presence of receptor activator of NF-κB ligand (RANKL). Furthermore, human epithelial A549 cells expressed ACE2, and expression of 3a/X1 in these cells up-regulated TNF-α, which is known to accelerate osteoclastogenesis. 3a/X1 also enhanced RANKL expression in mouse stromal ST2 cells. These findings indicate that SARS-CoV 3a/X1 might promote osteoclastogenesis by direct and indirect mechanisms.

Introduction

In 2002–2003, over 8,000 people were affected by severe acute respiratory syndrome (SARS) caused by SARS coronavirus (SARS-CoV), and about 10% of these patients died of rapidly progressing dyspnea and systemic failure [1–3]. Pathological characteristics of SARS, such as epithelial cell proliferation and an increase in macrophages with frequent hemophagocytosis in the lung, suggest that dysregulation of proinflammatory cytokine production in macrophages may account for SARS pathogenesis [4]. Many patients were treated with high-dose steroids, and this provided a clinical benefit to some extent.

Sometime after the outbreak, several reports indicated high frequency of arthralgia and reduced bone density in patients who recovered from SARS [5, 6]. Radiological lesions included osteonecrosis of femurs and tibiae and additional nonspecific subchondral and intramedullary bone marrow abnormalities [6]. Although the risk of osteonecrosis was higher in the patients receiving a higher dose of steroids, the other bone abnormalities were independent of steroid dose [6].

The balance between bone resorption by osteoclasts and bone formation by osteoblasts is important for bone homeostasis and remodeling [7]. Osteoclasts are derived from precursor cells of the myeloid lineage, and interaction between receptor activator of NF-κB ligand (RANKL) and RANK on bone marrow monocyte/macrophages is essential for osteoclast differentiation [8, 9]. RANKL is a TNF superfamily cytokine produced by various kinds of cells.
including osteoblasts and activated T cells [7]. A subset of peripheral blood monocytes also differentiate into osteoclasts in the presence of RANKL and M-CSF in vitro [10]. In addition, various inflammatory cytokines such as TNF-\( \alpha \) promote osteoclastogenesis [11, 12]. Since inflammatory responses are associated with SARS, and macrophages are susceptible to SARS-CoV in vitro [13], we hypothesized that SARS-CoV infection might promote osteoclastogenesis.

The SARS-CoV genome encodes several accessory proteins without sequence similarity to known coronavirus proteins [14, 15]. We previously reported that, among the SARS-CoV accessory proteins, 3a/X1 strongly augmented chemokine production by activating NF-\( \kappa \)B, a critical transcription factor for inflammatory responses [16]. Similar effects of 3a/X1 protein on NF-\( \kappa \)B activation, especially in the presence of double-strand RNA, has been reported [17].

In the present study, we demonstrate that SARS-CoV 3a/X1 enhances osteoclast differentiation in a murine osteoclast precursor cell line, RAW264.7. We also demonstrate that 3a/X1 induces TNF-\( \alpha \) expression in human lung epithelial A549 cells and RANKL expression in mouse bone marrow stromal ST2 cells. These findings imply that osteoclastogenesis accelerated by 3a/X1 protein may be involved in the mechanisms of bone abnormalities in patients recovering from SARS, in addition to possible side effects of a steroid therapy.

**Materials and methods**

**Plasmids**

Lentivirus vectors expressing SARS-CoV 3a/X1 (pLenti/V5/X1) were constructed as described before [16]. Empty vector (pLenti6/V5-DEST, Invitrogen) and enhanced green fluorescent protein (EGFP)-expressing vector (pLenti/V5/GFP) served as controls. pGEX-X1 was constructed by subcloning a 3a/X1-coding EcoRI fragment derived from GFP (VSV-G/pLenti/V5/GFP) were generated according to the manufacturer’s protocol (ViraPower™ Lentiviral Expression Kit, Invitrogen). Briefly, HEK293T cells (4 \( \times \) 10\(^6\)) plated in 100-mm dishes were transfected with pLenti/V5/X1 or pLenti/V5/GFP vector (5 \( \mu \)g) together with ViraPower™ Packaging Mix (5 \( \mu \)g), consisting of VSV-G-expression vector, rev-expression vector and gag-pol expression vector. After 48 h, culture supernatants were harvested and filtered through 0.45-\( \mu \)m-pore-size filters. Lentivirus was concentrated ~40-fold by low-speed centrifugation at 6,000\( \times \)g for 16 h, resuspended in 2 ml of RPMI 1640 medium, and stored at -80°C until use.

**Preparation of a lentiviral expression vector**

Lentiviruses expressing 3a/X1 (VSV-G/pLenti/V5/X1) or GFP (VSV-G/pLenti/V5/GFP) were generated according to the manufacturer’s protocol (ViraPower™ Lentiviral Expression Kit, Invitrogen). Briefly, HEK293T cells (4 \( \times \) 10\(^6\)) plated in 100-mm dishes were transfected with the pLenti/V5/X1 or pLenti/V5/GFP vector (5 \( \mu \)g) together with ViraPower™ Packaging Mix (5 \( \mu \)g), consisting of VSV-G-expression vector, rev-expression vector and gag-pol expression vector. After 48 h, culture supernatants were harvested and filtered through 0.45-\( \mu \)m-pore-size filters. Lentivirus was concentrated ~40-fold by low-speed centrifugation at 6,000\( \times \)g for 16 h, resuspended in 2 ml of RPMI 1640 medium, and stored at -80°C until use.

**Flow cytometric analysis**

PBMCs (5 \( \times \) 10\(^5\)) were incubated for 30 min at 4°C with anti-human ACE2 ectodomain antibody monoclonal (mouse IgG2k, R&D Systems, Inc) or with LEAF™ purified mouse IgG2k, \( \kappa \) isotype control (BioLegend) in the presence of Fe-blocking reagent, human (Miltenyi Biotec),
washed twice, and incubated for 30 min at 4°C with goat anti-mouse IgG (H + L)-RPE, human absorbed (Southern Biotechnology Associates). Cells were washed twice and further incubated with FITC-conjugated mouse anti-human CD14 (BD Pharmingen™), washed twice, and resuspended in 1% formalin. Samples were analyzed on a FACS Calibur flow cytometer (Becton Dickinson), and data analyses were performed with CellQuest software (Becton Dickinson).

Reporters assays for NF-κB

Cells (1 × 10⁷) were transfected with NF-κB reporter plasmids κB-Luc (600 ng), renilla luciferase-expressing pRL-CMV (40 ng) and lentivirus vectors expressing SARS-CoV genes (5 μg). Luciferase and renilla luciferase activities were measured from cell lysates 48 h after transfection using the luciferase assay system and the Renilla luciferase assay system (Promega), respectively.

Western blotting and immunoprecipitation

Cell pellets (2 × 10⁶) were suspended in 1 ml lysis buffer (10 mM Tris-HCl [pH 7.4], 2.5 mM MgCl₂, 100 mM NaCl₂) containing 0.1% Nonidet P-40 and protease inhibitor cocktail [CALBIOCHEM, La Jolla, CA], followed by centrifugation at 10,000 × g for 15 min at 4°C. The supernatants were used as cell lysates. For Western blotting, 20 μl of cell lysates or culture supernatants were mixed with 5 μl 5x SDS-sample buffer and treated for 5 min at 95°C and then analyzed by SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and reacted with anti-V5 mouse monoclonal IgG₂a (Invitrogen) and sheep anti-mouse Ig-horseradish-peroxidase-linked F(ab')₂ fragment (Amersham Biosciences), followed by visualization using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and using an LAS-1000 imaging system (Fuji Photo Fm). For immunoprecipitation, cell lysates or culture supernatants were incubated with anti-V5 mouse monoclonal IgG₂a (Invitrogen) for 2 h and then incubated with 30 μl of protein G agarose (GE Healthcare Bio Sciences AB). Immunoprecipitated proteins were separated from protein G agarose by heating for 5 min at 95°C in SDS-sample buffer prior to Western blotting.

GST-3a/X1 fusion protein

Competent cells (DH5α, TOYOBO Co., Ltd., Osaka, Japan) transformed by the heat-shock method with pGEX-X1 plasmids, were cultured in 2xYT medium. Protein synthesis was induced by addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Wako, Osaka, Japan) overnight at 20°C. The cells were subsequently centrifuged and suspended in PBS, followed by sonication (3 times for 1 min). The cells were centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatants were filtered and stored at −80°C until use.

Osteoclast differentiation

RAW264.7 cells infected with VSV-G/pLenti/V5/X1 or VSV-G/pLenti/V5/GFP pseudotyped lentiviruses were cultured for 48 h, and then 5 μl of the cell suspension at a concentration of 2 × 10⁵ cells/ml was seeded in the center of the wells of a 24-well plate for spot culture as described elsewhere [23]. After a 30-min incubation, 0.5 ml of growth medium containing 10% FCS and various concentrations of soluble RANKL (sRANKL, Wako Pure Chemical Industries, Ltd.) was added, and the cells were cultured for 5 days. Cells were then stained for tartrate-resistant acid phosphatase (TRAP) using a TRAP staining kit (Primary Cell Co., Ltd.) according to the manufacturer’s instructions. All of the TRAP-positive cells having more than 3 nuclei in a well were counted under a microscope.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using Isogen (Nippon Gene), and cDNA was synthesized from 1.0 μg of total RNA by using Rever Tra Ace for RT-PCR (TOYOBO) with oligo(dT)₂₀ primers. The cDNA was used as a template for PCR in an amplification mixture containing KOD Dash DNA polymerase and dNTP mix (TOYOBO). PCR cycling conditions consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 5 s, and extension at 72°C for 30 s. The primer sets used were 5'-GGGTAATCGAGTCACAACCACAAGTTCCG-3' and 5'-GAAGCAGGAAGGATCATC-3', and 5'-AACACACGGAAGGAATTCCG-3' and 5'-ACGAGGCAAGGATCATC-3'. Total RNA was extracted from cells using Isogen (Nippon Gene), and cDNA was synthesized from 1.0 μg of total RNA by using Rever Tra Ace for RT-PCR (TOYOBO) with oligo(dT)₂₀ primers. The cDNA was used as a template for PCR in an amplification mixture containing KOD Dash DNA polymerase and dNTP mix (TOYOBO). PCR cycling conditions consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 5 s, and extension at 72°C for 30 s. The primer sets used were 5'-GGGTAATCGAGTCACAACCACAAGTTCCG-3' and 5'-GAAGCAGGAAGGATCATC-3', and 5'-AACACACGGAAGGAATTCCG-3' and 5'-ACGAGGCAAGGATCATC-3'.
bands of the PCR products were visualized with ultraviolet light and recorded with Coolsaver (ATTO).

Statistical analysis

Student’s t test was used for evaluating differences between two groups of samples. P values of <0.05 were considered to be statistically significant.

Results and discussion

Cell-surface expression of ACE2 on primary human monocytes

Angiotensin-converting enzyme 2 (ACE2), a cellular receptor for SARS-CoV [26–28], is expressed on the plasma membrane of respiratory epithelial cells and mediates SARS-CoV infection. At first, we addressed the potential susceptibility of osteoclasts to SARS-CoV infection. Since osteoclasts are derived from CD14+ monocytes [29], we examined expression of ACE2 on the surface of CD14+ cells in PBMCs by FACS analysis. As shown in Fig. 1, a certain population of peripheral CD14+ monocytes derived from healthy donors expressed ACE2 on the cell surface. Expression of ACE2 in tissue macrophages in atherosclerotic lesions has also been reported recently [30]. Taken together with previous reports showing that macrophages are susceptible to SARS-CoV in vitro [13], we reasoned that osteoclasts could be infected with SARS-CoV during their differentiation from CD14+ monocytes.

Activation of NF-κB by SARS-CoV 3a/X1 in the transient expression system

We previously found that the SARS-CoV accessory protein 3a/X1 activated NF-κB in epithelial-like HEK293T cells [16]. Shen et al. [31] reported that 3a/X1 was secreted from SARS-CoV-infected Vero E6 cells. We therefore examined whether 3a/X1 protein secreted from cells could induce NF-κB activation when exogenously added to cells. As shown in Fig. 2, substantial amounts of 3a/X1 were detected not only in cell lysates (Fig. 2a) but also in the culture supernatants (Fig. 2b) of HEK293T cells following transfection with pLenti/V5/X1 plasmids, reproducing the secretion properties of 3a/X1. Then, the biological function of the secreted form of 3a/X1 was addressed using a NF-κB reporter assay. We observed significant activation of NF-κB by direct transfection of the 3a/X1 expression vector, but not by addition of the culture supernatant containing the secreted form of 3a/X1 (Fig. 2c). We also prepared a GST-3a/X1 fusion protein. As shown in Fig. 2d, although E. coli extracts contained substantial amounts of GST-3a/X1 fusion protein, its effect on enhancement of NF-κB activity in HEK293T cells was minimal. Thus, 3a/X1 could activate NF-κB when expressed inside cells, but less effectively in a secreted form, at least in HEK293T cells.

The activation of the NF-κB through 3a/X1 was then examined in a murine osteoclast precursor cell line, RAW 264.7 cells [19, 20]. We transfected RAW264.7 cells with pLenti/V5/X1 or pLenti/V5/GFP (irrelevant control) by electroporation. As shown in Fig. 3, NF-κB activity was significantly augmented by 3a/X1 expression, but not by GFP expression in RAW264.7 cells.

Augmentation of osteoclast differentiation by SARS-CoV 3a/X1

To address possible effects of the 3a/X1 during osteoclast formation, we employed a lentiviral transduction system for forced expression of 3a/X1. Expression of 3a/X1 in RAW264.7 cells infected with VSV-G/pLenti/V5/X1 pseudotyped viruses was confirmed by Western blot analysis (Fig. 4a). At 48 h postinfection, RAW264.7 cells were transferred to further culture with or without sRANKL for 5 days, and then osteoclast formation was evaluated under a microscope after TRAP staining. As shown in Fig. 4b, the number of TRAP+ osteoclasts was higher in the cells subsequently with goat anti-mouse IgG (H + L)-RPE, followed by staining with FITC-anti-human CD14 mAb. The monocyte fraction was gated (left panel) and analyzed by two-color flow cytometry.

Fig. 1 Expression of ACE2 on primary human monocytes. PBMCs from a healthy donor were stained with anti-human ACE2 ectodomain mAb (right) or isotype control antibody (middle) and subsequently with goat anti-mouse IgG (H + L)-RPE, followed by staining with FITC-anti-human CD14 mAb. The monocyte fraction was gated (left panel) and analyzed by two-color flow cytometry.

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transduced with 3a/X1 than in the control cells transduced with GFP. The 3a/X1-mediated enhancement of osteoclast formation was observed in the presence of sRANKL at both optimal (50 ng/ml) and suboptimal (25 ng/ml) concentrations, although the difference was statistically significant only at the concentration of 50 ng/ml. In the absence of sRANKL, expression of 3a/X1 alone was not sufficient to induce osteoclast differentiation. The TRAP$^+$ cells observed in cultures containing sRANKL exhibited large and multinuclear phenotypes similar to those of mature osteoclasts, irrespective of the lentiviruses (Fig. 4c). These results indicate that osteoclast differentiation can be enhanced by SARS-CoV 3a/X1 in cooperation with sRANKL.

Effects of 3a/X1 on expression of TNF-$\alpha$ and RANKL mRNA

The differentiation of osteoclasts from their precursor cells requires interaction with osteoblasts in the bone marrow,
which express RANKL [7]. Inflammatory cytokines such as TNF-α also promote osteoclastogenesis in the presence of RANKL [11, 12]. Finally, we addressed the effect of the 3a/X1 on RANKL and TNF-α expression. For this purpose, we employed A549 cells derived from human lung cancer, as we found that A549 cells expressed ACE2 (Fig. 5a). We then transfected A549 cells with pLenti/V5/X1 and evaluated TNF-α and RANKL mRNA expression by RT-PCR. As shown in Fig. 5b, the level of TNF-α transcripts was significantly increased by expression of 3a/X1, whereas RANKL expression remained at a low level. We also examined the effects of 3a/X1 on RANKL expression in human T-cell line Jurkat cells and mouse bone marrow stromal ST2 cells, as both cell lines have been reported to express RANKL upon stimulation [9, 32]. In Jurkat cells, stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, but not 3a/X1, induced RANKL mRNA expression (Fig. 5c). In ST2 cells, however, 3a/X1 enhanced RANKL mRNA expression (Fig. 5d). These findings suggest that 3a/X1 has a strong ability to induce TNF-α expression in epithelial cells and might potentially enhance RANKL expression through indirect mechanisms in some stromal cells.

RANK-stimulation by RANKL activates NF-κB and JNK pathways [33], both of which are also activated by

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**Fig. 3** Activation of NF-κB by SARS-CoV 3a/X1 in RAW264.7 cells. The NF-κB reporter plasmid (κB-Luc) (closed bar) and pRL-CMV (open bar) were introduced together with pLenti/V5/X1 (3a/X1) or control pLenti/V5/GFP (GFP) vectors into RAW264.7 cells by transfection, and luciferase activities were measured approximately 48 h after transfection. Data represent means ± S.D. of fold increases against GFP controls in three independent experiments.

**Fig. 4** Augmentation of osteoclast differentiation by SARS-CoV 3a/X1. a RAW264.7 cells were infected with pseudotyped viruses expressing 3a/X1 (VSV-G/pLenti/V5/X1) or GFP (VSV-G/pLenti/V5/GFP), and the cell lysates 48 h after infection were subjected to Western blot analysis with anti-V5 antibody after immunoprecipitation with the same antibody. Arrows indicate the size of 3a/X1. b RAW264.7 cells were infected with VSV-G/pLenti/V5/GFP (open bar) or VSV-G/pLenti/V5/X1 (closed bar) pseudotyped viruses for 48 h, and 1,000 cells/spot were further cultured for 5 days with sRANKL at the indicated concentrations. The cells were stained with TRAP, and the osteoclast-like TRAP⁺ multinuclear cells in each well were counted under a microscope. Data are expressed as means ± SD of triplicate wells. *p < 0.05. c Representative images after TRAP staining of untreated RAW264.7 cells (left) and VSV-G/pLenti/V5/GFP-infected (middle) or VSV-G/pLenti/V5/X1-infected (right) RAW264.7 cells in the presence of 50 ng/ml sRANKL.
SARS-CoV 3a/X1 expression [16]. The common pathways downstream from these two molecules explain the enhancement of osteoclastogenesis by 3a/X1 expression in concert with RANKL. Since circulating monocytes express ACE2, SARS-CoV infection of these cells may accelerate differentiation to osteoclasts accumulating in the bones. In addition, we found that 3a/X1 induced TNF-α transcription in lung-cancer-derived epithelial A549 cells expressing ACE2. This is consistent with the notion that 3a/X1 might be involved in the robust inflammatory cytokine production in SARS patients. 3a/X1 enhanced RANKL expression in ST2 cells but not in A549 or Jurkat cells, suggesting that RANKL up-regulation in ST2 cells by 3a/X1 might represent indirect effects. It has been reported that inflammatory cytokines such as IL-1 and TNF-α induce RANKL expression in synovial fibroblasts [34].

In conclusion, SARS-CoV 3a/X1 potentially promotes osteoclastogenesis by accelerating osteoclast differentiation from precursor monocytes/macrophages, and also indirectly through TNF-α and the RANK/RANKL system. These findings partly explain the unusual bone abnormalities observed in patients following recovery from SARS.

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