RESEARCH

The inhibition of Wnt signaling attenuates RANKL-induced osteoclastogenic macrophage activation

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Abstract

Abdominal aortic aneurysms (AAAs) have been linked to the activation of osteoclastogenic macrophages. Reports have suggested that Wnt signaling has a dual effect of proliferation and differentiation during osteoclastogenesis. The Wnt/β-Catenin pathway is a critical regulator of cell pluripotency, cell survival, and cell fate decisions. It regulates cell proliferation and differentiation through transcriptional co-activators, CBP, and p300, respectively. The inhibition of β-catenin suppresses proliferation but induces differentiation of osteoclast precursor cells. This study aimed to examine the effect of ICG-001, a β-catenin/CBP-specific Wnt signaling inhibitor, on osteoclastogenesis by inhibiting proliferation without inducing differentiation. To induce osteoclastogenesis, RAW 264.7 macrophages were stimulated with a soluble receptor activator of NF-κB ligand (RANKL). The effect of Wnt signaling inhibition was examined by treating macrophages with or without ICG-001 during RANKL stimulation. The activation and differentiation of macrophages were examined through western blotting, quantitative PCR, and tartrate-resistant acid phosphate (TRAP) staining in vitro. The relative expression level of the nuclear factor of activated T-cells cytoplasmic 1 protein was significantly suppressed by ICG-001 treatment. The relative expression levels of mRNA of TRAP, cathepsin K, and matrix metalloproteinase-9 were significantly lower in the ICG-001-treated group. The number of TRAP-positive cells decreased in the ICG-001-treated group relative to the non-treated group. The inhibition of Wnt signaling pathway via ICG-001 suppressed osteoclastogenic macrophage activation. Our previous studies have shown the importance of osteoclastogenic macrophage activation in AAA. Further research to examine the therapeutic potential of ICG-001 on AAA is warranted.

Introduction

Abdominal aortic aneurysm (AAA) is recognized as the dilatation of abdominal aorta more than 30 mm (1). Older age could increase the prevalence of AAAs. Although AAAs are not common in younger age population (less than 50 years), 12.5% of men and 5.2% of women between 74 and 84 years have AAAs (2). AAAs result in approximately 11,000 deaths per year in the United States. This number might be underestimated, because there are about 5%...
of 200,000 people who are dead suddenly per year, and they have died due to AAAs (3). The pathophysiological features of AAAs have been recognized as the degeneration of arterial media and elastic layers in aortic walls (4, 5). Even though these features have been understood, there is no way to treat AAAs instead of surgical treatments.

Wnt signaling plays a crucial role in embryonic development and is related to many types of diseases including cancer and degenerative disorders (6). Wnt signaling is also important in bone biology such as development and homeostasis (7), and there have been many reports which have shown that Wnt signaling has a direct role in regulating osteoclastogenesis (8, 9). Although these reports have mainly reported the roles of Wnt signaling in osteoblasts, the specific roles of Wnt signaling in osteoclast are not clear. Our group has already reported that osteoclastogenic activation can promote the induction of AAAs (11, 12). Therefore, we hypothesized that Wnt signaling might play a key role to promote the initiation of AAAs initiation.

To examine this hypothesis, this study tests the relationship between osteoclastogenic activation and Wnt signaling using the inhibitor of Wnt signaling, ICG-001. ICG-001 is a small molecule, which modulates Wnt signalling (12). ICG-001 specifically inhibits the interaction between β-catenin and transcriptional co-activator, CREB-binding protein (CBP) (13). By proving this correlation, we would be able to evaluate the potential therapeutic role of ICG-001 for AAAs.

Materials and methods

Cell culture and treatments

The RAW 264.7 mouse monocytic macrophages were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. For induction of osteoclastogenic changes, macrophages were maintained in minimal essential medium-alpha (MEMα) supplemented with 10% charcoal-stripped FBS (cFBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. We stimulated these macrophages with 30 ng/mL recombinant murine soluble RANKL (sRANKL) (Peprotech, Rocky Hill, NJ, USA) with or without 10 µM ICG-001 (Selleck Chemicals, Houston, TX, USA).

Osteoclast differentiation assay

To evaluate osteoclast formation, we conducted tartrate-resistant acid phosphate (TRAP) staining. Briefly, RAW 264.7 cells were seeded in 96-well plates (3000 cells per well) and cultured in MEMα with 10% cFBS. Macrophages were stimulated by 30 ng/mL murine sRANKL with or without 10 µM ICG-001 for 5 days. After that, cells were stained using a commercialized TRAP staining kit (Sigma-Aldrich) according to the manufacturer’s instruction. The total number of osteoclasts (TRAP-positive cells were defined as red, multinucleated (>3 nuclei) cells) in each well was counted.

Western blotting

RAW 264.7 cells were lysed by radioimmunoprecipitation assay buffer with protease inhibitor cocktail (Cell Signaling Technology) at 0–4°C. The protein concentrations for each sample were measured, and 20 µg protein lysates from each sample were subjected to 8 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were transferred onto polyvinylidene difluoride membranes. These blots were blocked with Tris-buffered saline containing 0.05% Tween-20 (TBST), supplemented with 5% non-fat dry milk for 1 h at room temperature, and incubated with primary antibodies (mouse nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) antibody (1:2000, sc-7294, Santa Cruz Biotechnology), rabbit TRAP antibody (1:10000, ab191406, Abcam), mouse cathepsin K antibody (1:1000, sc-48353, Santa Cruz Biotechnology), mouse Cyclin D1 antibody (1:1000, sc-8396, Santa Cruz Biotechnology), and mouse α Tubulin antibody (1:10000, sc-23948, Santa Cruz Biotechnology) overnight at 4°C. After the incubation with primary antibodies, the membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Peroxidase labeling was detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). The relative expression levels of proteins for western blotting were normalized to α Tubulin.

Quantitative reverse transcription polymerase chain reaction

The total RNA was isolated from cultured RAW 264.7 cells using PureLink RNA Mini Kit (Thermo Scientific) according
to the manufacturer’s instructions. For cDNA synthesis, 1 μg of RNA was used to generate cDNA by using iScript cDNA Synthesis kit (Bio-Rad). Quantitative real-time PCR was done using SYBR Green dye with the 7500 Fast Real-Time PCR instrument (ABI, Foster City, CA, USA). PCR primers used for real-time PCR were as follows: Nfatc1, F: 5’-TGG CTA CCC ACA TGT GTT GT-3’ and R: 5’-GAC CAG GGG AGC TAT GAA CA-3’; matrix metalloproteinase-9 (Mmp9), F: 5’-CAT TCG CTT GGA TAA GGA GT-3’ and R: 5’-GTT CTC CTC ATG GTC CAC CT-3’; TRAP, F: 5’-TCC TGG CTC AAA AAG CAG CTG TT-3’ and R: 5’-ACA TAG CCC ACA CCG TTC TC-3’; cathepsin K, F: 5’-CGA AAA GAG CCT AGC GAA CA-3’ and R: 5’TGG GTA GCA GCA GAA ACT TG-3’; cyclin D1, F: 5’-GGC ACC TGG ATT GTT CTG TT-3’ and R: 5’-CAG CTG GCT AGG GAA CTG GG-3’; Gapdh, F: 5’-AAC TTT GGC ATT GTG GAA GG-3’ and R: 5’-ACA CAT TGG GGG TAG GAA CA-3’. The expression of Gapdh served as an internal control, and relative mRNA expression levels for each target gene was calculated using the 2^(-ΔΔCt) method.

**Proliferation assay of macrophage**

To examine the proliferation of macrophage, the 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted. Macrophage cell lines were seeded in 96-well plates (3000 cells per well) at day 0 and cultured with complete DMEM. The culture medium had been changed from complete DMEM to complete (with 10% cFBS) MEMα every day since day 1. In sRANKL-stimulated group, macrophages were stimulated by 30 ng/mL murine sRANKL, and 10 μM of ICG-001 with murine sRANKL was added to ICG-treatment group. Each groups were cultured by day 4, and MTT assays were done every day using an MTT cell proliferation assay kit (Cayman Chemical). Ten micromolars of MTT reagent per well was added, mixed gently, and incubated for 3 h at 37°C in a CO2 incubator. The medium wastaken out, and 100 μL of dimethylsulfoxide (Sigma-Aldrich) was added to lyse the cells in each well, and the optical density (OD) was read at 570 nm using a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Statistical analysis**

All data represent means ± s.d. Statistical analysis was performed using the GraphPad Prism program, version 7.0 (GraphPad Software, Inc.). Differences between the groups were compared by Student’s t-test or one-way analysis of variance with repeated measures followed by Turkey’s comparison test for multiple comparisons. Statistical significance was set at P < 0.05 between the groups.

**Results**

**ICG-001 inhibits the expression of proteins related to osteoclastogenesis**

NFATc1 is a master transcription factor of macrophage activation (14). NFATc1 stimulates the expression of osteoclastogenesis-associated proteases, such as TRAP, cathepsin K. Then, we stimulated macrophages with sRANKL and examined the effect of ICG-001 in RANKL-induced osteoclastogenesis. The protein expression level of NFATc1 was significantly lower in the ICG-001-treated group than the non-ICG-001-treated group (4.46 ± 0.74 vs 7.13 ± 0.50, P < 0.05) (Fig. 1A). Even though there was not a statistically significant difference in the expression level of TRAP between ICG treatment group and sRANKL-stimulated group (1.78 ± 0.55 vs 2.35 ± 0.38, P>0.24), we found the trend that ICG-001 decreased TRAP expression level (Fig. 1B). Moreover, ICG-001 significantly suppressed the expression of cathepsin K stimulated by sRANKL compared to non-ICG-001 treatment group (4.62 ± 1.60 vs 12.2 ± 1.73, P < 0.05) (Fig. 1C). These results showed that ICG-001 attenuated the protein expression related to osteoclastogenesis.

**ICG-001 inhibits the expression of mRNA related to osteoclastogenesis**

Furthermore, we investigated the expression level of some mRNA associated with osteoclastogenesis. In addition to TRAP and cathepsin K, MMP9 has been known as one of the most important proteases in osteoclastogenesis related to AAA formation (15). Then, we examined the expression level of mRNA to evaluate the effect of ICG-001. The mRNA of Nfatc1 (Fig. 2A), TRAP (Fig. 2B), cathepsin K (Fig. 2C), and Mmp9 (Fig. 2D) was induced by sRANKL stimulation (4.61 ± 0.57, 167.1 ± 5.6, 149.3 ± 14.3, and 323.6 ± 70.0, respectively). With addition of ICG-001, these mRNA levels, Nfatc1 (Fig. 2A), TRAP (Fig. 2B), cathepsin K (Fig. 2C), and Mmp9 (Fig. 2D), were significantly suppressed compared to non-ICG-001-treated group (1.71 ± 0.25 vs 4.61 ± 0.57, P < 0.05, 72.4 ± 5.3 vs 167.1 ± 5.6, P < 0.05, 38.6 ± 7.2 vs 149.3 ± 14.3, P < 0.05, and 175.5 ± 10.5 vs 323.6 ± 70.0, P < 0.05, respectively). These results proved that ICG-001 suppressed the expression level of not only protein but also mRNA that was related to osteoclastogenesis.
ICG-001 attenuates the osteoclastogenic change in macrophages morphologically

For further study, we attempted TRAP staining to make sure of the morphological effect of ICG-001 in sRANKL-stimulated osteoclastogenesis. Even though there were some multinucleated TRAP-positive cells stained purple in ICG-001 treatment group (Fig. 3A), there was a significant difference in the number of TRAP-positive cells between sRANKL-treated group and ICG-001-treated group (131.2 ± 19.4 vs 24.4 ± 7.0, \( P < 0.05 \)) (Fig. 3B). These results showed that ICG-001 decreased the effect of sRANKL in osteoclastogenesis not only functionally (induction of some proteases) but also morphologically.

ICG-001 decreases the proliferation of macrophage

Cyclin D1 is one of the important genes related to cell proliferation (16). Furthermore, it is known that cyclin D1 is one of the main target genes for Wnt/β-catenin signaling pathway (17). Therefore, we hypothesized that blocking of Wnt/β-catenin signaling by ICG-001 attenuated the proliferation of macrophage, following the inhibition of differentiation. Then, we examined the protein expression of cyclin D1 by western blotting. The protein expression level of cyclin D1 was significantly lower in ICG-001 treatment group than sRANKL stimulation group (0.48 ±0.05 vs 1.20 ±0.08, \( P < 0.05 \)) (Fig. 4A). Furthermore, we examined the mRNA expression level of cyclin D1 to compare the differences among the groups. The mRNA expression level of Cyclin D1 was statistically lowered in ICG-treated group compared to sRANKL stimulation group (1.08 ± 0.06 vs 2.08 ± 0.20, \( P < 0.05 \)) (Fig. 4B). Therefore, ICG-001 reduced the macrophage proliferation with the inhibition of cyclin D1 expression. We compared each ODs by MTT assay, and the results revealed that ICG-001 treatment significantly decreased macrophage proliferation compared to sRANKL-treated group (Day 2: 1.44 ± 0.37 vs 2.31 ± 0.52, \( P < 0.05 \), Day 3: 1.46 ± 0.54 vs...
3.04 ± 0.37, \( P < 0.05 \), and Day 4: 2.65 ± 0.69 vs 3.81 ± 0.61, \( P < 0.05 \) (Fig. 4C). These results demonstrated that the blockage of Wnt/\( \beta \)-catenin signaling pathway attenuated the macrophage proliferation.

Discussion

We reported here that the inhibition of Wnt/\( \beta \)-catenin signaling by ICG-001 attenuated osteoclastogenic changes in macrophage stimulated by sRANKL. The attenuative effect for osteoclastogenesis by ICG-001 was induced through the inhibition of macrophage proliferation, following the inhibition of macrophage differentiation. We have already reported that the differentiation of macrophage to osteoclastogenesis is one of the main causes for AAAs formation. Therefore, our report is the first study to confirm the effect of ICG-001 to attenuate osteoclastogenic changes and show ICG-001 as one of the candidates for medical treatment options for AAAs.

The effect of Wnt signaling in osteoclastogenesis has been controversial. Because most of the studies have focused on the effect of Wnt signaling in osteoblasts (9). There have been some literatures that showed the effect of the inhibition of Wnt signaling not in osteoblast but osteoclast; however, the literatures showed the different, opposite results in each other. Albers et al. (18) reported that Wnt signaling negatively inhibits the osteoclastogenic differentiation of macrophage. On the other hand, Santiago et al. (19) showed that Wnt signaling promoted the osteoclast differentiation in RAW 264.7 cell lines. These different results should be derived that Wnt signaling has at least three intracellular pathways, including \( \beta \)-catenin pathway (canonical pathway), planar cell polarity pathway, and Ca\(^{2+} \) pathway (these two pathways were referred to as non-canonical pathway).
(20). Because these pathways interacted each other, and these interactions could make the effect of Wnt signaling in osteoclastogenesis complicated. Therefore, we adopted the specific inhibitor for β-catenin, ICG-001, to examine and make sure of the effect of β-catenin pathway in osteoclastogenesis in this study. ICG-001 treatment reduced the expression levels of protein and mRNA of osteoclast-specific proteases, including TRAP, cathepsin K, and MMP9. Furthermore, cyclin D1 is one of the main markers of increased proliferation, which is one of the main target genes for β-catenin signaling pathway (21). Our results showed that ICG-001 treatment decreased the protein and mRNA expression levels of cyclin D1 and attenuated the macrophage proliferation in MTT assay. These results have proven that the inhibition of Wnt/β-catenin signaling pathway interfered with the osteoclastogenesis in macrophage, and activation of Wnt/β-catenin signaling might contribute to the induction of osteoclastogenic changes in macrophages.

Wnt signaling plays a key role in embryogenesis and organogenesis which include cell differentiation, proliferation, apoptosis, survival, and migration (22). Therefore, the aberrant activated Wnt signaling could induce many kinds of diseases, including malignant diseases (23), organ fibrosis (24), and skeletal bone and muscle diseases (25). In the field of vascular diseases, it has been reported that atherosclerosis could be induced by extraordinary activation of Wnt signalling (26). Wnt5a is proven to be of importance in atherosclerotic lesions. Malgor et al. (27) reported that the expression levels of mRNA and protein were higher in advanced atherosclerotic lesions than less-advanced atherosclerotic lesion. Furthermore, Wnt5a stimulates the endothelial cells and vascular smooth muscle cells to induce endothelial dysfunction (28). This stimulation could lead to arterial calcification. However, there has been no report which shows the relationship between Wnt signaling pathway and arterial aneurysmal diseases, such as AAAs. We have already proven that the differentiation of macrophages to osteoclastogenesis plays a key role to induce AAAs formation (10, 11). Therefore, we have tried to examine the effect of Wnt signaling pathway for aneurysmal formation in point of the osteoclastogenesis and showed that the decrease of macrophage proliferation by inhibiting Wnt/β-catenin signaling might attenuate the induction of AAAs. Our study is the first report showing the relationship between Wnt signaling and arterial aneurysmal disease. Interestingly, some studies have reported that NFATc1, transcriptional factor for the activation of macrophage, coactivates CBP, and this coactivation contributes a critical role to induce NFATc1-dependent gene expression (29, 30). Therefore, we should examine the coactivation between NFATc1 and CBP, which might be related to the osteoclastogenesis, arterial aneurysmal formation.

It is important to consider that the dysregulation of Wnt/β-catenin signaling can change over time, as observed in tumorigenesis where early Wnt-dependent events can be altered by desensitizing Wnt targets as the tumor progresses (31). A similar phenomenon might occur in aortic disease, and future studies should investigate this possibility and develop strategies to overcome potential issues related to changes in Wnt signaling over time.

There are some limitations of this study. First, we have not conducted in vivo experiments to make sure of the attenuative effect of ICG-001 in the induction of aneurysmal disease yet. To confirm the effect of ICG-001, further experiments are necessary. Second, there might be other mechanism which attenuate osteoclastogenic change in macrophage by ICG-001, as mentioned above. Wnt signaling pathways which contain three main pathways, affect each other, and there should be other mechanisms which correlate to osteoclastogenesis. Therefore, other mechanism attenuating osteoclastogenic change with ICG-001 should be studied. Lastly, while Wnt signaling is more commonly associated with osteoblasts, it is important to consider the potential effects of ICG-001 on these cells as well (32). Further studies should explore the impact of ICG-001 on osteoblasts and the balance between osteoblasts and osteoclasts in the context of aortic disease. Bone homeostasis is regulated by the coupling action of osteoclasts and osteoblasts in the bone microenvironment. To better understand the therapeutic potential of ICG-001 for AAA, it would be informative to investigate the effect of ICG-001 on the coupling action of osteoclasts and osteoblasts, and how this may contribute to aortic disease progression.

Conclusions

Our study showed that (1) ICG-001, a Wnt/β-catenin signaling-specific inhibitor, suppressed the osteoclastogenesis in macrophages stimulated by sRANKL treatment and; (2) ICG-001-attenuated macrophage proliferation, resulting in the reduction of macrophage activation. These results suggest that ICG-001 might be a potential candidate for the treatment of AAAs. Further studies, including animal experiments, should be conducted to confirm the effect of the inhibition of
Wnt/β-catenin signaling in osteoclastogenesis, to investigate the mechanisms of ICG-001 in both osteoclasts and osteoblasts, and to determine its in vivo effects to evaluate ICG-001 as a potential alternative treatment option for AAAs. Additionally, future research should investigate the effect of ICG-001 on osteoclasts and the coupling action.

Declaration of interest
The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

Author contribution statement
YD contributed to the conception and the design of the study, critical revision of the manuscript, and final approval of the submitted manuscript.
KI contributed in the conception and the design of the study, acquisition of data, and drafting the article.

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