Osteoclastogenic Differentiation of Macrophages in the Development of Abdominal Aortic Aneurysms

Yuichiro Takei,* Teruyoshi Tanaka,* K. Craig Kent, Dai Yamanouchi

Objective—Arterial calcification is common and contributes to the pathogenesis of occlusive vascular disease. Similar to the dynamics of bone, it is a tightly controlled process that maintains a balance between osteogenesis and osteolysis. However, whether calcium homeostasis plays a role in the development of aneurysms has not been explored. We hypothesized that macrophages differentiate into osteoclasts in aneurysmal arteries and that protease byproducts contribute to aneurysm pathophysiology.

Approach and Results—We performed histological and immunohistochemical analyses and showed that macrophages positive for several osteoclast markers, including tartrate acid phosphatase, occur in great numbers in the human aneurysmal aorta, but very few occur in the human stenotic aorta and none in the nondiseased human aorta. Moreover, in situ zymography showed elevated protease activity in these cells compared with undifferentiated macrophages. Tumor necrosis factor-α and calcium phosphate stimulated this osteoclastogenic differentiation process through nuclear factor-κB, MAPKs, and intracellular calcium signaling but not the receptor activator of the nuclear factor-κB ligand. Inhibition of osteoclastogenic differentiation by bisphosphonate inhibits aneurysm development in a mouse model.

Conclusions—These results suggest that differentiation of macrophages into osteoclasts contributes to the pathophysiology of aneurysmal disease. (Arterioscler Thromb Vasc Biol. 2016;36:00-00. DOI: 10.1161/ATVBAHA.116.307715.)

Key Words: aneurysm cardiovascular diseases metalloproteinases osteogenesis osteolysis

Cardiovascular disease is widely recognized as the leading cause of death in the United States. Cardiovascular diseases are usually occlusive, that is, resulting from the narrowing of blood vessels that serve the heart, other critical organs, or the extremities. Alternatively, an abdominal aortic aneurysm (AAA), which is the consequence of arterial dilatation, can be lethal.1 Although several pharmacological therapies have been proposed to prevent AAA growth and rupture, the only clinically available treatment is surgery; thus, there is a need for additional therapeutic strategies guided by a better understanding of AAA pathophysiology.2,3

Calcification is a major feature of atherosclerotic cardiovascular disease. Oclusive atherosclerosis, particularly of the peripheral vessels, is often associated with heavy, rigid, and circumferential calcification. The distribution of calcium associated with aneurysmal disease, however, is different because it is characterized by a thin layer diffusely distributed throughout the arterial wall. Recent reports have revealed that artery calcification is a tightly controlled process similar to that seen in bone, with a balance maintained between osteogenesis and osteolysis.4,5 Osteogenesis is characterized by bone matrix deposition by osteoblasts, whereas osteolysis is the active resorption of the extracellular matrix by osteoclasts, which are derived from monocyte/macrophage lineage cells through osteoclastogenesis. The pathways involved in osteoclastogenesis have been extensively studied.6 The receptor activator of nuclear factor-κB (RANKL) has been shown to induce osteoclastogenesis through its cognate receptor RANK. The RANKL–RANK interaction subsequently activates both tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6) and several downstream signaling pathways, including intracellular calcium ([Ca2+]i), NFκB, and MAPKs.7,8

Because accelerated calcification is a major pathogenic feature of occlusive peripheral arterial disease, many studies have focused on osteogenesis. Alternatively, we hypothesized that the calcification observed in aneurysm walls is significantly less dense and is the result of pathogenic resorption of calcium (osteolysis) by osteoclasts. Because a prominent function of osteoclasts is the production of proteases, in particular, MMPs, which are well-known contributors to AAA, we proposed that increased numbers of osteoclasts in the arterial wall may be an important contributor to the development of AAA.

In this study, we examined whether and how osteoclastogenesis is involved in the pathogenesis of AAA. We report that macrophages undergoing osteoclastogenic differentiation exist in...
human and mouse aneurysms and that pharmacological inhibition of osteoclastogenesis suppresses the development and progression of aneurysmal disease.

Results

Osteoclastogenic Differentiation of Macrophages Is Observed in Aneurysmal Disease

Arterial calcification produced by calcium phosphate (CaPO$_4$) crystals is one of the key pathogenic features of atherosclerosis and is abundantly present in stenotic arteries.$^{9,10}$ Although the pathophysiology of aneurysms differs from that of stenotic disease, we have observed calcification in human AAA specimens as well, albeit to a lesser extent than in stenotic arteries.$^4$ Figure 1A represents representative examples of computed tomographic scans of nondiseased aorta with little calcium, a stenotic artery with dense circumferential calcium, and an aneurysmal artery with a thin, less dense layer of calcium distributed only in a portion of the aortic wall. Histologically, we found dense calcification in the medial layer of human stenotic vessels but moderate diffuse calcification in the media of aneurysm specimens (Figure 1A). A similar distribution of calcification was observed in a mouse model of aneurysmal disease (Figure I in the online-only Data Supplement).$^{11}$

Arterial calcification in stenotic arteries is tightly controlled by a balance maintained between osteogenesis, characterized by the deposition of bone matrix by osteoblasts, and osteolysis, characterized by active resorption of the extracellular matrix by osteoclasts.$^{4,5,12}$ Because macrophages accumulate in AAAs, we hypothesized that differentiation of macrophages into osteoclasts occurs in the aneurysmal aorta, resulting in resorption of calcium and a thinning of the calcified layer. A byproduct of this process might be increased proteolytic activity, with extracellular matrix degradation leading to aneurysmal degeneration.$^2,3$

Osteoclasts are histologically defined as multinucleated cells that stain positive for tartrate-resistant acid phosphate (TRAP)$^{13}$ and are called osteoclast-like cells if they exist in tissues other than bone.$^4$ Human aneurysmal, stenotic, and nondiseased aortic tissues were stained for TRAP, TRAP-positive cells were identified only in human aneurysmal aorta (100% of samples, N=8) and not in stenotic (N=5) or nondiseased aorta (N=3; Figure 1B; Figures II and III in the online-only Data Supplement). TRAP-positive cells were also abundant in the mouse aneurysm model (100% of samples, N=10; Figure 1C). On the contrary, ALP (an osteoblast marker)-positive cells (stained red) were observed in the vascular intima in the mouse aneurysm model (Figure IV in the online-only Data Supplement). Because our hypothesis is that macrophages transition into osteoclast-like cells in aneurysmal tissues, we stained human aneurysm tissue with CD68 (a marker for macrophages), TRAP, and a calcitonin receptor (CTR; a secondary marker for osteoclast-like cells).$^{15,16}$ Side-by-side staining of TRAP, CD68, and CTR revealed clusters of TRAP-positive cells also positive for CD68 and CTR in human aneurysm (Figure 1D). To further delineate the relative ratio of macrophages (CD68$^+$CTR$^-$) and osteoclast-like cells (CD68$^+$CTR$^+$) in aneurysmal versus stenotic tissues, we performed a flow cytometry analysis of cells disassociated from human specimens, although it is difficult to compare intracellular staining for CD68 with cell surface staining for CTR. We found a significantly greater number of macrophages (CD68$^+$CTR$^-$) and osteoclast-like cells (CD68$^+$CTR$^+$) in aneurysms (18.6% CD68$^+$CTR$^-$ cells and 10.3% CD68$^+$CTR$^+$ cells, respectively) than in stenotic vessels (5.1% and 1.8%, respectively; Figure 1E). Thus, 55% of the macrophages in aneurysmal tissue underwent osteoclastogenic differentiation. Taken together, these results demonstrate the significant activity of osteoclast-like cells in human and mouse aneurysmal tissues, with minimal osteoclast-like activity in stenotic disease.

TNF$\alpha$ Plus CaPO$_4$-Induced Osteoclastogenesis In Vitro

The TNF$\alpha$ pathway as well as the RANKL pathway and its downstream signaling have previously been shown to be involved in osteoclastogenesis.$^{7,18}$ To explore the mechanisms of osteoclastogenesis in aneurysms, immunohistochemical staining of RANKL, osteoprotegerin, a natural decoy receptor of RANKL, and TNF$\alpha$ were performed in human nondiseased, stenotic, and aneurysmal tissues and mouse aneurysms. Immunohistochemical staining showed an absence of RANKL and osteoprotegerin in all of these tissues, suggesting that the RANK ligand does not play a role in osteoclastogenesis in aneurysms. The inflammatory cytokine, TNF$\alpha$ (Figures V and VI in the online-only Data Supplement), was minimally enhanced in stenotic tissues but significantly enhanced in human and mouse aneurysms. There was also a significant increase in TNF$\alpha$ according to Western blotting (Figure VII in the online-only Data Supplement). We evaluated other cytokines known to be involved in AAA, but we did not observe an enhancement of MCP-1 or interleukin-1$\beta$ that was as significant as that of TNF$\alpha$ (Figure VIII in the online-only Data Supplement).

We expanded on this analysis using an in vitro osteoclastogenesis assay using primary cultured and RAW 264.7 mouse macrophages. To explore the role of TNF$\alpha$ in stimulating osteoclastogenesis, we stimulated both types of macrophages with TNF$\alpha$ and observed a minimal enhancement of osteoclastogenesis. We previously reported that CaPO$_4$ crystals,
the critical component of arterial calcification in stenotic and aneurysmal tissues, play an important role in aneurysm formation. Thus, we hypothesized that the presence of calcified crystals might accelerate the differentiation of macrophages into osteoclasts or perhaps that TNFα and CaPO₄ together might synergistically promote osteoclastogenesis.8

Figure 1. Tartrate-resistant acid phosphate (TRAP)-positive macrophages in the aneurysmal aorta. Human nondiseased, stenotic, and aneurysmal aortic tissues were freshly fixed, frozen, and sectioned for histological analysis. A, Representative computed tomography image of nondiseased, stenotic, and aneurysmal aortas (upper). Alizarin red staining shows calcification in the stenotic and aneurysmal aorta but not in the nondiseased aorta. Scale bar, 500 μm. B, Representative images of TRAP-positive staining in human aortic tissues. TRAP-positive cells, identified by purple staining, were observed in every human abdominal aortic aneurysm (AAA) sample (N=8) but not in stenotic (N=5) or nondiseased aorta (N=3). Scale bar, 500 μm. C, Mouse aneurysm 7 days after surgery showing abundant TRAP-positive cells compared with the control (N=10). Scale bar, 500 μm. D, Representative images of double staining for CD68 and calcitonin receptor (CTR) by immunofluorescent staining. After confirming the existence of TRAP-positive cells in side-by-side staining of sequential sections, double stainings for CD68 and CTR were performed. The merged image shows CD68+CTR+ cells. E, Fluorescence-activated cell sorter (FACS) analysis of cells dissociated from a human stenotic and an aneurysmal aorta. The x axis shows the CTR fluorescence level; the y axis shows the CD68 level. AAA samples show a significantly higher proportion of cells positive for both CD68 and CTR (N=3). Values are presented as means±SD. *P<0.05. A indicates adventitia; L, lumen; and M, media.
As shown in Figure 2A and 2B and Figures IX and X in the online-only Data Supplement, CaPO₄ alone (mimicking stenotic aorta) produced minimal osteoclastogenesis in both macrophage cell types, whereas costimulation of macrophages with CaPO₄ plus TNFα (mimicking aneurysmal tissue) yielded TRAP-positive multinucleated cells stained purple-red and an increased cell number. We also observed robust, dose-dependent osteoclastogenesis, as measured by TRAP (Figure XI in the online-only Data Supplement). Furthermore, the combination of TNFα and CaPO₄ induced Trap (also known as Acp5) mRNA 3 days after treatment (Figure 2C).

We also confirmed that CaPO₄ and TNFα induced expression of cathepsin K (Ctsk) as well as the CTR (Calcr; Figure 2D and 2E). Moreover, the nuclear factor of activated T cells cytoplasmic 1 (NFATc1), an essential transcription factor for osteoclastogenesis, increased 2-fold by the cotreatment of cells with TNFα plus CaPO₄ (Figure 2F).

To explore the involvement of classical osteoclastogenesis pathways through the RANK ligand and receptor, macrophages were stimulated with CaPO₄ and TNFα or RANKL in the presence of neutralizing antibodies to mouse RANKL. Although RANKL-induced osteoclastogenesis and NFATc1 expression were completely inhibited by the RANKL-neutralizing antibody, TNFα plus CaPO₄-induced osteoclastogenesis and NFATc1 expression were not affected (Figure 2G and 2H). Taken together, these findings demonstrate that the combination of TNFα and CaPO₄ stimulates macrophage osteoclastogenesis; however, the mechanism is not dependent on the RANKL–RANK pathway.

### Signaling Pathway in TNFα Plus CaPO₄–Induced Osteoclastogenesis

Conventionally, osteoclastogenesis is triggered by RANKL through the TRAF6 signaling pathway (Figure XII in the online-only Data Supplement). Thus, we initially investigated TRAF6 levels in TNFα plus CaPO₄–induced osteoclastogenesis. Consistent with our RANKL findings, neither the TNFα nor the TNFα plus CaPO₄ treatments affected TRAF6 mRNA expression (Figure 3A). By contrast, TRAF2 mRNA...
levels were found to be significantly elevated (Figure 3B). To establish the specific role of Traf2 in the transformation of macrophages into osteoclasts, we blocked Traf2 expression with siRNA. Inhibition of Traf2 significantly suppressed TNFα plus CaPO4-induced osteoclastogenesis (Figure 3E). To further evaluate the downstream signaling involved in TNFα plus CaPO4 osteoclastogenesis, we then focused on NFκB and MAPK, both pathways that are involved in the RANK–TRAF6 axis. The TNFα treatment significantly increased IκB phosphorylation levels measured by Western blotting (Figure 3D), as well as nuclear translocation of NFκB p65 (Figure 3E), which was not further enhanced by TNFα plus CaPO4. These results suggest that TNFα induces nuclear translocation of NFκB p65 by activating IκB phosphorylation and degradation. Phosphorylation of p38 MAPK was significantly enhanced by TNFα but not further enhanced by TNFα plus CaPO4 (Figure 3F). Phosphorylated JNK levels also increased after TNFα treatment. Interestingly, phosphorylated JNK levels were higher in the TNFα plus CaPO4 treatment versus the TNFα treatment alone (Figure 3G), suggesting a synergistic effect of CaPO4 on JNK phosphorylation.20 Furthermore, as shown in Figure XIII in the online-only Data Supplement, we performed Western blot analysis to determine the effect of siTraf2 on the phosphorylation levels of IκB, p38, and JNK in RAW 264.7 cells. The phospho-IκB and p38 levels induced by TNFα plus CaPO4 treatment were significantly decreased by siTraf2. The TNFα plus CaPO4-induced phospho-JNK levels tended to decrease, but not significantly, in response to siTraf2. These results suggest that TNFα, using TRAF2 instead of the more traditional pathway of TRAF6, activates downstream NFκB, p38 MAPK, and JNK to induce osteoclastogenesis.

**Figure 3.** Signaling pathway in tumor necrosis factor-α (TNFα) plus CaPO4-induced osteoclastogenesis. A–C, The effect of TNFα and CaPO4 treatment on Traf6 gene expression was evaluated. The effect of TNFα and CaPO4 treatment on Traf6 gene expression levels (A) and Traf2 gene expression levels (B) are shown. C, The effect of Traf2 inhibition by siTraf2 on TNFα plus CaPO4-induced osteoclastogenesis was evaluated, and it was found that Traf2 inhibition suppressed osteoclastogenesis. D–G, The effect of TNFα and CaPO4 treatment on phospho-IκB and IκB protein levels in the cytosol (D), nuclear factor–κB (NFκB) p65 protein levels in the nucleus (E), phospho-p38 and p38 protein levels (F), and phospho-JNK and JNK protein levels (G) are shown. RAW 264.7 cells were treated with TNFα and CaPO4 for 48 h. Values are presented as means±SD for at least 3 replicates. *P<0.05; **P<0.01.
shown in Figure XV in the online-only Data Supplement, we also determined the effects of the CaMK and CaMKK inhibitors, KN-93 and STO-609, on osteoclastogenesis in RAW 264.7 cells. Both inhibitors dose-dependently suppressed TNFα plus CaPO4–induced osteoclastogenesis, supporting the involvement of Ca signaling on TNFα plus CaPO4–induced osteoclast differentiation. Together with the result from anti-RANKL treatment (Figure 2G and 2H), these results suggest that TNFα plus CaPO4–induced osteoclastogenesis is independent of the RANKL–RANK axis but uses a pathway that includes TRAF-2, NF-κB, MAPK, and [Ca2+]i (Figure XVI in the online-only Data Supplement). To examine whether our in vitro results apply to aneurysmal lesions, we performed additional Western blotting using human nondiseased and aneurysmal aorta. Traf2 protein expression and the phosphorylation levels of CaMKIV were significantly increased in AAA patient sections compared with normal human sections (Figure XVII in the online-only Data Supplement), supporting the in vitro results.

Upregulation of Protease Activity by Osteoclastogenesis in an Aneurysm

MMP-9 is mostly secreted from macrophages and has been recognized as an important factor in the development of aneurysms. Because osteoclasts also have an abundant source of MMPs, we evaluated whether Mmp9 expression and secretion of MMP-9 increased in macrophages (RAW 264.7 cells) treated with TNFα and with or without CaPO4.

As shown in Figure 5A, Mmp9 expression was enhanced by TNFα plus CaPO4 treatment in a time-dependent manner. The pattern of Mmp9 expression was similar to that of Trap, as shown in Figure 2C. Corresponding to Mmp9 expression, MMP-9 secretion was markedly facilitated by 5 days of treatment with TNFα plus CaPO4 (a 2.6-fold increase versus TNFα alone; Figure 5B). This increase in MMP-9 secretion was suppressed by a pretreatment with the CaPO4 inhibitor, pyrophosphate (Figure 5C). To investigate whether the MMP-9 activity associated with osteoclast-like cells is higher than that of undifferentiated macrophages, we next examined MMP-9 activity in TRAP-positive cells versus non-TRAP–positive macrophages using in situ zymography of a human aneurysmal aorta. After selecting the locations of TRAP-positive cells and non-TRAP–positive macrophages by dual staining of TRAP and CD68, we performed in situ zymography and quantified MMP-9 activity by the relative fluorescence intensity. As shown in Figure 5D and Figure XVIII in the online-only Data Supplement, TRAP-positive macrophages...
produced significantly greater MMP-9 activity compared with non-TRAP–positive macrophages in a human aneurysmal aorta. These results demonstrate an increased protease activity by osteoclast-like cells compared with the undifferentiated macrophages in a human aneurysmal aorta.

Pharmacological Inhibition of Osteoclastogenesis Suppressed Aneurysm Formation

Pharmacological inhibition of osteoclastogenesis uses bisphosphonate for the clinical treatment of osteoporosis.26,27 Bisphosphonate binds to CaPO₄ and locally inhibits osteoclast activity and osteoclastogenesis.28 We hypothesized that controlling the formation and activity of osteoclasts might be an effective strategy for inhibiting aneurysms. Zoledronic acid (ZA) is a nitrogenous bisphosphonate that produces apoptosis of osteoclast-like cells and also interferes with osteoclastogenesis. As shown in Figure 6A–6C, ZA, in a dose-dependent manner, inhibited TNFα plus CaPO₄–induced osteoclastogenesis in vitro and mouse aneurysm formation in vivo, with a nearly complete inhibition of aneurysms at a concentration of 100 μg/kg, which is equivalent to the clinically approved ZA dose of 50 to 100 μg/kg. TRAP staining of vehicle-treated

![Figure 5. MMP-9 expression in abdominal aortic aneurysms.](image)

**Figure 5.** MMP-9 expression in abdominal aortic aneurysms. **A**, Changes in Mmp9 gene transcription in RAW 264.7 cells treated with TNFα and CaPO₄. **B**, Representative western blot and densitometric analysis of secreted MMP-9 expression levels in cultured media from RAW 264.7 cells treated with TNFα and CaPO₄. The cells were treated with TNFα and CaPO₄ for 5 days, and the culture medium was then analyzed. **C**, Representative Western blot and densitometric analysis of secreted MMP-9 expression in culture media from RAW 264.7 cells treated with TNFα and CaPO₄ with 10 μM pyrophosphate (PPi). **D**, Representative images of in situ zymography for gelatinase activity. Human abdominal aortic aneurysm (AAA) tissue samples were stained for tartrate-resistant acid phosphatase (TRAP), and immunohistochemical CD68 staining was performed on an adjacent slide to confirm macrophage infiltration. Green fluorescence shows MMP-9 collagenase activity, and arrows indicate TRAP-positive macrophages. Scale bar, 30 μm. Values are presented as means±SD for at least 3 replicates. *P<0.05.

**Figure 6.** Effect of inhibition of osteoclastogenic differentiation on aneurysms. Mouse model aneurysms were created, and zoledronic acid (ZA) was administered by retro-orbital injection. **A**, The effect of bisphosphonate on osteoclastogenesis was evaluated. RAW 264.7 cells were cultured with tumor necrosis factor-α (TNFα) plus CaPO₄ and ZA, and the number of tartrate-resistant acid phosphatase (TRAP)–positive cells are shown. **B**, Representative images of mouse aneurysms show the fold increase of maximum diameters are shown. ZA (0–500 μg/kg) was injected immediately after surgery. **C**, Representative images of TRAP staining with ZA (100 μg/kg) after 4 weeks. The fold increase was calculated based on the maximum diameter of the artery at the time of the initial surgery and euthanasia. Values are presented as means±SD. *P<0.05. Scale bar, 500 μm.
and bisphosphonate-treated mouse aneurysms showed a near complete inhibition of osteoclastogenesis by bisphosphonate treatment.

**Discussion**

Osteoclast-like cells are defined as multinucleated giant cells that share morphological and histological characteristics with osteoclasts but exist in tissues other than bone. Here we confirm the existence of numerous osteoclast-like cells in both human and mouse aneurysms but very few in stenotic tissues and none in nondiseased aortic tissue. Macrophages were more prevalent in aneurysmal than in stenotic aorta, and almost half of the macrophages had undergone osteoclastogenic differentiation in aneurysms. Although these cells were TRAP-positive, conventional osteoclasts are generally larger in size and with more ruffled borders than the cells we observed, indicating that the macrophages in these aneurysms had undergone osteoclastogenic differentiation. However, the end products of osteoclast-like cells were still distinct from conventional osteoclasts.

To investigate the factors that stimulated this transformation, we screened several cytokines and found elevated TNFα levels in aneurysmal tissue. Further studies revealed that TNFα stimulated osteoclast-like cell formation in vivo. We also identified a novel feedback loop where CaPO4 crystals and TNFα synergistically induced osteoclastogenesis. With regard to signaling, we initially focused on the RANKL–osteoprotegerin pathway, which has been shown to activate TRAP6 and calcium, ultimately leading to the activation of NFATc1, a transcription factor essential for osteoclastogenesis. However, we were not able to demonstrate upregulation of RANKL or osteoprotegerin in aneurysmal tissue. Instead, we identified an alternative pathway independent of RANKL induced by TNFα and CaPO4 and involving TRAF2. Downstream effectors from TRAF2 were similar to those of TRAF6 and included NFκB, MAPK, and intracellular Ca++.

Even when IκB was phosphorylated, the levels of IκB were unchanged. The translocation of NFκB to the nucleus was accompanied by the phosphorylation, ubiquitination, and proteasomal degradation of IκBα. Therefore, the opposite trend to that observed in the levels of p-IκB was often observed in the levels of IκBα, which may be increased by TNFα plus CaPO4 stimulation for 48 hours. Traf2 protein expression and the phosphorylation levels of CaMKIV were significantly increased in AAA patient sections compared with normal human sections. These results support the in vitro results and suggest, but do not conclusively prove, the involvement of osteoclastogenesis in AAA development. Further studies are needed to show direct evidence that this is a mechanism underlying the development of AAA.

Because osteoclasts have been identified as an important source of protease during osteolysis, we hypothesized that the TRAP-positive cells contribute to aneurysm formation through their active production of proteases. Among the many proteases, MMP-9 has proven to be one of the most important in aneurysm formation and development. Our in vitro results demonstrate that TRAP-positive macrophages show an almost 8-fold increase in MMP-9 expression relative to TRAP-negative macrophages. In situ zymography consistently revealed higher MMP-9 activity associated with TRAP-positive versus TRAP-negative macrophages in human aneurysmal tissue. Our results from the mouse aneurysm model showed an almost complete inhibition of aneurysm formation as well as inhibition of further expansion of existing aneurysms by the osteoclast inhibitor bisphosphonate. Although a direct effect of bisphosphonate on non-TRAP-positive macrophages cannot be fully excluded, inhibition of osteoclastogenesis seems to be a potentially important therapeutic target for aneurysms. We repeated our studies with bisphosphonate in an alternative AAA model induced by angiotensin II. ZA binds to CaPO4 and exerts a suppressive effect on osteoclastogenesis. It usually requires only a single intravenous injection per year in human, and we, therefore, examined the effect of a single injection of ZA in angiotensin II–induced AAA. However, in contrast to Tsai et al, we found no suppression of AAA (Figure XX in the online-only Data Supplement). These differing results may be because of the amount of ZA binding to CaPO4. We also speculate that the absence of a bisphosphonate effect is related to the lack of overt arterial calcification in aneurysms in this model. Nistala et al also reported negative results for bisphosphonate on aneurysm progression in Marfan syndrome using Fbn1 (mgR/mgR) mice. However, because Fbn1 mice more closely mimic connective tissue disorders, such as Marfan syndrome, we think that the underlying mechanism in this model differs from the pathophysiology of the traditional AAA model.

In females, there is a steep increase in aneurysmal disease and aneurysm rupture and a significant increase in osteoporosis after menopause. It may be that both are related to an enhancement of osteoclast activity in the absence of estrogen. These findings suggest a suppressive effect of estrogen on aneurysm formation, possibly through the inhibition of osteoclastogenesis. We evaluated 3 large clinical data sets to see whether we could determine a relationship between bisphosphonate treatment (which is frequent in postmenopausal women) and the development of aneurysms. Unfortunately, we were not able to obtain a sufficient sample size to establish a relationship. For this reason, a prospective randomized clinical trial to test the effect of bisphosphonate on aneurysms is warranted. If this trial was positive, medical treatment with bisphosphonate would be simple and associated with few complications. Moreover, unlike other pharmacological treatments that require daily dosing, the latest generations of bisphosphonates are effective, with as few as one intravenous injection per year, which could be performed in conjunction with yearly aneurysm screening.

We focused on the differentiation of macrophages into osteoclasts in the pathogenesis of AAA. Recent reports have revealed that artery calcification is a tightly controlled process similar to that seen in bone, with a balance maintained between osteogenesis and osteolysis, and there have been no reports of osteoclastogenesis in AAA. Although we have not determined the extent of calcification and changes in osteoblast-like cells in the pathogenesis of AAA, Nakayama et al reported that a lower extent of calcification correlated with accelerated expansion of AAA, which may be consistent with our results. However, Buijs et al found a trend of increased abdominal aortic calcification in patients with aortic rupture.
Therefore, additional research comparing osteoclasts and osteoblasts is needed to clarify the relation between expansion, calcification, and rupture in AAA.

We used a modified CaCl₂ model in which topical application of CaCl₂ is followed by PBS, which may create the conditions for forming CaPO₄ crystals locally. As described earlier, there was no calcification in alternative AAA models, such as the one generated by angiotensin II infusion. Thus, further experiments are required to demonstrate whether our findings in this study are model-specific by repeating the experiments in additional models, such as the porcine pancreatic infusion model. Similarly, after application of CaCl₂ to the adventitia of the abdominal aorta, aortic dilatation was reportedly accompanied by elastin calcification, loss of vascular smooth muscle cells, and marked infiltration of inflammatory cells, such as neutrophils, lymphocytes, monocytes, and multinucleated giant cells, leading to induction of AAA. The possible physiological differences between these anatomic structures make the carotid artery an imperfect model and are a limitation of this study.

In summary, we have shown conclusively that osteoclastogenic differentiation of macrophages plays an important role in the development of AAAs through novel stimuli (TNFα and CaPO₄) and a host of downstream signaling effectors. Thus, inhibiting this process could prove to be a novel therapeutic approach to the treatment of aneurysmal disease.

Acknowledgments

We thank Jay Yang, MD, PhD (Professor of the Department of Anesthesiology at the University of Wisconsin-Madison), for valuable comments and support. We are also grateful to Chitaru Kurihara, Anesthesiology at the University of Wisconsin-Madison), for technical assistance.

Sources of Funding

This work was supported by a Scientist Development Grant from the American Heart Association project (12SDG9120024) on “The role of osteoclast in abdominal aortic aneurysm formation.”

Disclosures

Drs Yamanouchi and Kent have a US Patent (Methods of treating aneurysm, US 8748410 B2). The other authors report no conflicts.

Drs Yamanouchi and Kent have a US Patent (Methods of treating abdominal aortic aneurysm formation.” American Heart Association project (12SDG9120024) on “The role of osteoclast-like cells in abdominal aortic aneurysm formation.” This work was supported by a Scientist Development Grant from the American Heart Association project (12SDG9120024) on “The role of osteoclast-like cells in abdominal aortic aneurysm formation.”

References


Takei et al Osteoclastogenesis in Aneurysms


---

**Highlights**

- Osteoclastogenic differentiation of macrophages plays an important role in the development of abdominal aortic aneurysms.
- Inhibition of osteoclastogenic differentiation of macrophage by bisphosphonate inhibits aneurysm development in a mouse model.
- Tumor necrosis factor-α and calcium phosphate stimulated osteoclastogenic differentiation of macrophage during aneurysm development through nuclear factor-κB, MAPKs, and intracellular calcium signaling but not the receptor activator of the nuclear factor-κB ligand.
Osteoclastogenic Differentiation of Macrophages in the Development of Abdominal Aortic Aneurysms
Yuichiro Takei, Teruyoshi Tanaka, K. Craig Kent and Dai Yamanouchi

Arterioscler Thromb Vasc Biol. published online July 7, 2016;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2016/07/07/ATVBAHA.116.307715

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2016/07/07/ATVBAHA.116.307715.DC1
http://atvb.ahajournals.org/content/suppl/2016/07/07/ATVBAHA.116.307715.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Materials and Methods

Human Aortic Tissue

Aortic tissues were obtained from eight patients who had undergone open heart surgery at the University of Wisconsin Hospital for degenerative AAA without any known connective tissue disorder, aortic dissection, or infection. Occlusive aortic tissues were obtained from five patients who had undergone open heart bypass surgery for aortic occlusive disease without any known connective tissue disorder, infection, aneurysm, or dissection. Control aortic tissues were obtained from the abdominal aorta of three organ transplant donors. The study protocol was approved by the Institutional Review Committee at the University of Wisconsin Madison (IRB No.2011-0692).

The Mouse Model of Aneurysms and Treatments

Twelve-week-old male C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The procedures for creating our modified CaCl$_2$-induced mouse model of aneurysm were previously described.$^1$ Briefly, 0.5 M CaCl$_2$-soaked gauze was applied perivascularly for 10 min in the abdominal aorta or the carotid artery as indicated. The gauze was replaced with another PBS-soaked gauze for 5 min, and the incised area was sutured. NaCl (0.5 M) was used instead of CaCl$_2$ as a control. The mice were randomly divided into a control group and an experimental group, and different concentrations of zoledronic acid (ZA; Sigma-Aldrich, St. Louis, MO, USA) were retro-orbitally injected into mice immediately after or 7 days after
surgery. At selected time points, the mice were sacrificed, and the arteries were collected after
fixing by perfusion with 4% paraformaldehyde (PFA), as described previously. Anti-TNFα
neutralizing antibody (Cell Signaling Technology, Danvers, MA, USA) or pyrophosphate (PPi,
Sigma-Aldrich) suspended in 25% Pluronic F-127 gel (Sigma-Aldrich) was delivered
periadventitially (10 μg/mL anti-TNFα or 1 mM PPi). For some experiments, an ultrasound was
performed at each time point to measure the size of the artery noninvasively, and observers were
blinded to the study drug given. All animal procedures were conducted in accordance with
experimental protocols that were approved by the Institutional Animal Care and Use Committee
at the University of Wisconsin, Madison (Protocol M02394).

Cell Culture and Treatments

A mouse macrophage cell line (RAW 264.7) was purchased from the American Type Culture
Collection (ATCC, Manassas, VA, USA) and maintained in DMEM containing 10% FBS, 100
IU/mL penicillin, and 100 μg/mL streptomycin. For osteoclast differentiation, 4000 cells were
plated per well in a 96-well plate and maintained in MEMα supplemented with 10% charcoal-
stripped FBS with antibiotics. The cells were then stimulated with 100 ng/mL TNFα (Peprotech,
Rocky Hill, NJ, USA) with or without 2% CaPO₄ crystals. For the crystallization of CaPO₄, a 0.5
M CaCl₂ solution was mixed together with PBS in the experimental tube.

Primary monocytes/macrophages were obtained from 4–6-week-old male mouse BM and
cultured in MEMα with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 20
ng/mL recombinant mouse macrophage colony-stimulating factor (M-CSF). BM-derived macrophages (BMMs) were isolated using Ficoll–Paque medium (GE Healthcare, Piscataway, NJ, USA) and pre-cultured in MEMα overnight. One day later, non-adhesive cells were collected and replated into 96-well plates at 4000 cells per well. The BMMs were then co-administered with 100 ng/mL TNFα, 2% CaPO₄, and 40 ng/mL M-CSF.

**AngII-infused Model Mouse and Treatments**

Retired (over 6 months old) male ApoE-/- mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Ang II (1000 ng/min/kg) was continuously infused by the osmotic pump implanted in the back of mouse. The mice were divided into two group; control group (n=6) and Zoledronic Acid (ZA) administration group (n=6). ZA or the vehicle was by single injection via retro-orbital vein. After 28 days, the abdominal aorta was perfusion fixed by 4% PFA and harvested. The maximum diameter of the abdominal aortae was measured to evaluate the aneurysm formation.

**Histological and Immunohistochemical Analysis**

The human aortic specimens and mouse tissues obtained were fixed with 4% PFA overnight. The samples were then embedded with OCT compound (Sakura Tissue Tek, Netherlands), frozen, and sectioned at a thickness of 6 μm per section. Calcification was visualized by 1% Alizarin red S solution. Enzymatic TRAP staining was performed with the Acid Phosphatase kit (Sigma). Immunohistochemical staining enabling a polymer-based detection method was performed using
the ImmPACT kit (ImmPress; Vector Laboratories, Inc., Burlingame, CA, USA) and counterstained by hematoxylin as needed. To retrieve the antigen activity, the sections were microwaved in 0.1 mM citrate buffer (pH 6.0) for 5 min. After blocking with 0.8% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 h, the tissues sections were incubated with primary antibody over night at 4°C. Mouse RANKL (ab45039, Abcam, Cambridge, UK), rabbit OPG (ab9986, Abcam), rabbit TNFα (654250, Merck, Calbiochem, Darmstadt, Germany), rabbit MCP-1(ab9669, Abcam), rabbit IL-1β (ab2105, Abcam) antibodies were used as primary antibodies. Negative control was incubated with an isotype-specific immunoglobulin for each primary antibody. After washing with TBS, the sections were incubated secondary antibody for 30 min at room temperature. Immunofluorescent staining was performed as described previously. Blocking for nonspecific sites was performed using BSA in TBS for 1 hour at room temperature. Primary antibodies, rat CD68 monoclonal antibody (ab53444, Abcam, Cambridge, UK) and mouse calcitonin receptor monoclonal antibody (SM1868P, Acris Antibodies GmbH, Hiddenhausen, Germany), diluted in TBS were applied to arterial sections and incubated for 1 hour at room temperature. Negative control was incubated with an isotype-specific immunoglobulin for each primary antibody. And then, arterial sections were washed with TBS, followed by incubation with secondary antibodies, anti-rat Alexa 488 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and anti-mouse Alexa 568 (Molecular Probes, Invitrogen), diluted in TBS 30 min at room temperature. TO-PRO 3 stain (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was used to identify nuclei. Fluorescent staining was visualized, and digital
images were taken on a Nikon A1R Laser Scanning Confocal imaging system (Tokyo, Japan) with the appropriate argon beam lasers.

**Flow cytometry**

For detaching cells, the human aortic specimens were homogenized in trypsin-EDTA and incubated at 37°C for 30 min. The detached cells were incubated with antibodies conjugated with Alexa 488 or 594. Flow cytometry was performed using the BD LSR II instrument (BD Biosciences, San Jose, CA).

**Western Blotting and Enzyme-Linked Immunosorbent Assay**

Protein from human specimens and cultured cells was extracted with a radioimmunoprecipitation assay buffer with protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA). Nuclear and cytosolic protein was extracted with the CellLytic NuCLEAR Extraction Kit (Sigma-Aldrich, St Louis, MO, USA). Primary antibodies used for western blotting included rabbit TNFα (3707, Cell Signaling Technology, Danvers, Mass), mouse NFATc1 (sc-7294, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit p-IκB (sc-101713, Santa Cruz Biotechnology), rabbit IκB (sc-371, Santa Cruz Biotechnology), rabbit p-p38 (9211, Cell Signaling Technology), rabbit p38 (sc-535, Santa Cruz Biotechnology), mouse p-JNK (sc-6254, Santa Cruz Biotechnology), mouse JNK (sc-1648, Santa Cruz Biotechnology), rabbit p-CaMKIV (sc-28443-R, Santa Cruz Biotechnology), mouse CaMKIV (sc-55501, Santa Cruz Biotechnology), rabbit MMP9 (ab38898, Abcam, Cambridge, UK), and mouse α-tubulin (sc-
23948, Santa Cruz Biotechnology) antibodies. Extracts were resolved on an SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Primary antibodies were detected using a horseradish peroxidase-conjugated secondary antibody and visualized with the Enhanced Chemiluminescence kit (Thermo Scientific, Rockford, IL). The levels of nuclear factor-κB (NFκB) were determined by enzyme-linked immunosorbent assay (ELISA) using the NF-κB p65 (total) ELISA Kit (Invitrogen, Carlsbad, CA, USA).

**Quantitative Real-Time PCR Analysis**

Total RNA was isolated from cultured RAW 264.7 cells with the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Quantitative real-time PCR was performed with SYBR Green in a real-time PCR instrument (ABI, Foster City, CA, USA). Mouse Gapdh primers for quantitative RT-PCR were purchased from Qiagen (#PPM02946E, Valencia, CA, USA). The sequences of other primers used were as follows: Trap F, 5′-AACACCACGAGAGTCCTGCT-3′, and R, 5′-GTACCAGGGCAGAGAGCTGCT-3′; Mmp9 F, 5′-CATTCGCGTGAGATAAAGGAG-3′, and R, 5′-GTACCACCTCATGGTCCACCT-3′; Ctsk F, 5′-CGAAAGAGCCTAGCGAACA-3′, and R, 5′-TGGGTAGCAGCAGAAACTTG-3′; Calcr F, 5′-TCCAAGGAGGTCCACTAGGAGA-3′, and R, 5′-TGGGGCTCTAGGGAGGAACTTG-3′. The expression level for each gene was normalized to the Gapdh expression level in the same sample.

**Intracellular Ca\(^{2+}\) concentration**
Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]_i) was measured by the method reported by Takayanagi et al. using Fluo-4 AM (Invitrogen, Carlsbad, CA, USA) and Fura Red AM (Invitrogen).\(^2\)

**In Situ Zymography**

The procedure for in situ zymography was described previously\(^1\). In brief, 1 mg/ml DQ gelatin (Enzchek Gelatinase/Collagenase Assay Kit, Invitrogen, Carlsbad, CA, USA) was dissolved 1:10 in 50 mM Tris-CaCl\(_2\) and applied to the top surface of the sections. The slides were then incubated at 37 °C for 1 h in a dark, humid chamber. The gelatinolytic activity was observed as green fluorescence (absorption maximum, 495 nm; fluorescence emission maximum, 515 nm) and quantified by the mean fluorescence intensity for five fields.

**Statistical Analysis**

Data are reported as the mean ± S.D. Statistical analysis was performed with the GraphPad Prism program, version 4.00 (GraphPad Software, Inc. San Diego, CA, USA), which first tests the data for normality and equal variance. Parametric tests were analyzed with one-way ANOVA with repeated measures followed by Tukey’s multiple comparison test. P values less than 0.05 were accepted as statistically significant.

Supplemental Figure I

Alizarin red staining (arrow) of the mouse abdominal aorta. Scale bar, 500 μm.
Supplemental Figure II.

Representative images of TRAP-positive staining in human aortic tissues. TRAP-positive cells, identified by purple staining, were observed in every human AAA sample (N = 8) but not in stenotic samples (N = 5). Scale bar, 500 μm; L, lumen; M, media; A, adventitia.
Supplemental Figure III.

TRAP-positive cells 7 days after surgery in the mouse abdominal aorta. Scale bar, 100 μm; L, lumen; M, media; A, adventitia.
Supplemental Figure IV

Alkaline phosphatase (ALP) staining in mouse abdominal aorta. Scale bar, 100 μm; L, lumen; M, media; A, adventitia.
Supplemental Figure V.

Representative images of immunohistochemical staining for RANKL, OPG, and TNFα in human non-diseased, stenotic, and aneurysmal aorta. Scale bar, 500 μm; L, lumen; M, media; A, adventitia. There was no difference in RANKL or OPG expression between groups, whereas TNFα stained more intensely in AAA samples than in the stenotic or non-diseased aorta.
Supplemental Figure VI.

Immunohistochemical staining of RANKL, OPG, and TNFα in mouse carotid artery. Scale bar, 1 mm.
Supplemental Figure VII.

Western blot analysis of TNFα expression levels in human non-diseased (N = 3), stenotic (N = 4), and aneurysmal aorta (N = 4). Relative expression levels for western blotting, quantified by densitometry. Values are presented as means ± SD for at least three replicates. *P < 0.05.
Supplemental Figure VIII.

Inflammatory cytokines in human non-diseased, stenotic, and aneurysmal aorta. Representative images of immunohistochemical staining of MCP-1 and IL-1β in human non-diseased, stenotic, and aneurysmal aorta. We did not observe positive staining of MCP-1 or IL-1β (brown) that was as significant as that of TNFα. Scale bar, 500 μm; L, lumen; M, media; A, adventitia.
Supplemental Figure IX.

Representative images of TRAP staining after osteoclastogenic differentiation of primary cultured mouse macrophages. Macrophages were cultured with TNFα and/or CaPO$_4$ for 6 days. Scale bar, 100 μm.
Supplemental Figure X.

Representative images of TRAP staining after osteoclastogenic differentiation of RAW 264.7 cells. The cells were cultured with TNFα and/or CaPO₄ for 6 days. Scale bar, 100 μm.
Supplemental Figure XI.

**TNFα-plus-CaPO₄-induced osteoclast differentiation in a dose-dependent manner.** Representative images of TRAP staining after osteoclast differentiation in RAW 264.7 cells cultured with TNFα and/or CaPO₄. TRAP-positive cells were counted after 5 days. Scale bar, 100 μm. Values are presented as means ± SD for at least three replicates. *P < 0.05.
Supplemental Figure XII.

**Schematic representation of RANKL–RANK-induced osteoclastogenesis.** Osteoclastogenesis is evoked by RANKL, which is expressed on the cell membrane in osteoblasts. When RANKL binds to RANK, TRAF6 is recruited and activates NF-κB and MAPKs (p38 and JNK), which is followed by the activation of AP-1. The induction of NFATc1, which is a master regulator of osteoclastogenesis, depends on AP-1 and NF-κB. RANK also activates the non-receptor tyrosine kinase Syk through ITAM and then phosphorylates BLNK (also known as SLP-76). Btk and Tec, which are activated by RANK, bind to BLNK and phosphorylate PLCγ, which produces IP3 and induces Ca^{2+} release from the estrogen receptor, followed by Ca^{2+} oscillation. CN activated by Ca^{2+} signaling contributes to the dephosphorylation of NFATc1. The dephosphorylated NFATc1 translocates into the nucleus and binds to its own promoter, resulting in the autoamplification of NFATc1 expression. NFATc1 acts with other transcription factors, such as AP-1 and CREB, to induce various osteoclastic proteins, including TRAP, cathepsin K, calcitonin receptor, and MMP9.
Supplemental Figure XIII.

The effect of Traf2 inhibition by siTraf2 on TNFα-plus-CaPO₄–induced phospho-Iκb (A), p38 (B), and JNK (C) levels in RAW 264.7 cells. Values are presented as means ± SD for at least three replicates. *P < 0.05.
Supplemental Figure XIV.

Measurement of intracellular Ca\(^{2+}\) levels by Fluor4 in RAW 264.7 cells with TNF\(\alpha\) and/or CaPO\(_4\) treatment for 48 h are shown. CaPO\(_4\) alone and TNF\(\alpha\)-plus-CaPO\(_4\) treatment significantly increased the intracellular calcium ([Ca\(^{2+}\)]) level.
Supplemental Figure XV.

The effect of the CaMK (A) and the CaMKK inhibitors, KN-93 and STO-609 (B), on TNFα-plus-CaPO4–induced osteoclastogenesis in RAW 264.7 cells. Values are presented as means ± SD for at least three replicates. *P < 0.05; **P < 0.01.
Supplemental Figure XVI.
Possible mechanism by which TNFα plus CaPO₄ induces osteoclastogenesis in abdominal aortic aneurysms. TNFα binding to the TNF receptor recruits TRAF2, which activates NF-κB and MAPKs (p38 and JNK). The incremental increase in [Ca²⁺]i resulting from the addition of CaPO₄ crystals activates downstream CaMKIV and CN. These two pathways contribute to NFATc1 induction, resulting in the differentiation into osteoclasts.
Supplemental Figure XVII.

Traf2 protein expression and the phosphorylation levels of CaMKIV in human non-diseased and aneurysmal aorta. Values are presented as means ± SD. *P < 0.05.
Supplemental Figure XVIII.

Gelatinase activity quantified by fluorescence intensity with TRAP-positive macrophages and non-TRAP-positive macrophages. Values are presented as means ± SD for at least three replicates. *P < 0.05
Bisphosphonate had no effect on the incidence of AngII-induced aneurysms. Images of aortic dissection in ApoE KO mice after 4 weeks of AngII administration. AngII (1000 ng/min/kg) was continuously infused by an osmotic pump implanted in the back of the mouse. ZA (100 μg/kg) was injected immediately after implantation. Values are presented as means ± SD.
Osteoclastogenenic Activation of Macrophage

Aneurysm Formation