Abdominal aortic aneurysm (AAA) is a common disease involving segmental expansion and rupture of the aorta, which has a high mortality rate.1,2 Therapeutic options for AAA are currently limited to open or endovascular surgical repair to prevent catastrophic rupture.3 Nonsurgical approaches, particularly pharmacotherapy, are currently lacking for the treatment of AAA,4,5 which is characterized by chronic, chemokine-directed, inflammatory cell infiltration and progressive destruction of the extracellular matrix by proteolytic enzymes such as matrix metalloproteinases (MMPs).5–10 Notably, the molecular mechanisms involved in the regulation of the prolonged, persistent inflammation that characterizes AAA remain largely unknown.

Focal adhesion kinase (FAK, also known as PTK2) is a cytoplasmic tyrosine kinase that plays critical roles in integrin-mediated signal transduction. FAK also participates in signaling through other cell surface receptors.11 In particular, integrin signaling through FAK modulates cell motility, which contributes to the pathogenesis of cancer and other diseases.12–15 We previously demonstrated in vitro that FAK is involved in the inflammatory response to mechanical strain in vascular smooth muscle cells.16 In this role, FAK contributes to the activation of c-jun N-terminal kinase (JNK) and to the upregulation of MCP-1 (monocyte chemoattractant protein-1, also known as CCL2) and MMP-2. Upregulation of MMP-2 by FAK was also suggested by another study.17 These findings led to the hypothesis that FAK promotes AAA progression by maintaining and enhancing inflammatory responses.

Here, we report that FAK has a role in promoting long-lasting inflammation during the progression of AAA. We show that FAK is predominantly activated in macrophages that are recruited to AAA tissue. FAK potentiates tumor necrosis factor-α–induced secretion of matrix-degrading enzymes and chemokines by cultured macrophages. FAK also promotes macrophage chemotaxis. In mice, the administration of a FAK inhibitor that tempers local macrophage accumulation markedly suppresses the development and progression of chemically induced AAA.

**Conclusions**—FAK plays a key role in macrophage behavior, which underlies the chronic progression of AAA. These findings provide insights into AAA progression and identify FAK as a novel therapeutic target. (Arterioscler Thromb Vasc Biol. 2017;37:156-165. DOI: 10.1161/ATVBAHA.116.308542.)

**Key Words:** aorta ◼ aortic aneurysm ◼ chemotaxis ◼ inflammation ◼ macrophage
activated in human AAA tissues compared with nonaneurysmal aortic tissues (Figure 1A). We also determined the localization of FAK and analyzed its association with pathological tissue changes in human AAA walls. An activated form of FAK was found mainly in the outer medial and adventitial layers of AAA walls and was frequently observed near severe cellular infiltration and tissue destruction (Figure 1B). Immunofluorescence staining showed that activated FAK largely colocalized with CD68-positive macrophages in the adventitia but rarely colocalized with α-smooth muscle actin–positive smooth muscle cells in the media (Figure 1C and 1D).

To confirm FAK activation in an experimental model of AAA, we used a mouse model of AAA involving the periaortic application of calcium chloride (CaCl₂) in the infrarenal aorta. This treatment induced inflammatory cell infiltration and the gradual destruction of elastic lamellae in the aortic wall, which continued for ≤6 weeks after treatment. Both the expression and activity of FAK were increased in the aortic walls of CaCl₂-treated mice compared with those of untreated mice. Activated FAK was observed in the media and adventitia of the diseased aorta (Figure 1A and IB in the online-only Data Supplement), consistent with our findings in the walls of human AAA.

**FAK Potentiates Tumor Necrosis Factor-α–Mediated Inflammation in Macrophages**

The increase in FAK expression and activity observed in macrophages in AAA lesions prompted us to examine whether FAK modulates the function of macrophages in the context of AAA-related inflammation. First, we extracted thioglycollate-elicited mouse peritoneal macrophages and maintained them in cell culture. We then tested the effects of tumor necrosis factor-α (TNF-α) as a proinflammatory stimulus. Notably, TNF-α is elevated both in serum and in aneurysmal tissue from the aortic walls of patients with AAA. and it has been implicated in AAA pathogenesis. Stimulation of cultured mouse macrophages with TNF-α triggered the activation of JNK and nuclear factor-κB (NF-κB) within 15 minutes; both of these factors are critical for AAA pathogenesis. TNF-α stimulation subsequently caused significant increases in both the expression and activity of FAK, and this was accompanied by sustained activation of JNK and NF-κB and the production of MMP-9 and MCP-1 after 24 hours (Figure IIA in the online-only Data Supplement). Application of a JNK inhibitor, SP600125, blocked TNF-α–induced JNK and NF-κB activation and MMP-9 production; however, it did not affect the enhanced expression and activity of FAK in TNF-α–treated macrophages (Figure IIB through IIG in the online-only Data Supplement). On the contrary, the FAK inhibitor PF573228 completely prevented FAK activation and upregulation, JNK and NF-κB activation, and MMP-9 and MCP-1 secretion, all of which were induced by TNF-α; PF573228 did not, however, affect cell proliferation or death (Figure 2A through 2I). These findings indicated that there was a positive feedback loop that amplified FAK signaling, which in turn activated JNK and NF-κB and subsequently upregulated MMP-9 and MCP-1. These findings were confirmed using 2 other FAK inhibitors, PF562271 and FAK inhibitor 14, and by inhibiting FAK using lentiviral-mediated shRNA (Figures III through V in the online-only Data Supplement).

To extend our findings in cultured macrophages, we applied PF573228 to ex vivo human AAA tissue cultures. Stimulation with TNF-α caused a significant increase in MMP-9 secretion from human AAA tissues, consistent with our previous studies. This result suggested that the viability of human AAA tissues was preserved in ex vivo culture systems after treatment with TNF-α. Interestingly, PF573228 markedly reduced the secretion of MMP-9 and MCP-1 from TNF-α–stimulated human AAA tissues (Figure VI in the online-only Data Supplement). Taken together, these results showed that FAK amplifies its own expression and activity and that it positively regulates the secretion of MMP-9 and MCP-1 by activating JNK signaling, at least in TNF-α–stimulated conditions.

**FAK Activity Underlies AAA Development in Mice**

We next asked whether FAK is critical in the pathogenesis of AAA in vivo using a mouse model in which AAA is induced by periaortic application of CaCl₂. We chose pharmacological inhibition of FAK to investigate this issue because it is clinically relevant to a pharmacological approach in human AAA. After the application of CaCl₂, we treated the mice with PF573228 or with vehicle for 6 weeks. In controls animals, we applied saline (NaCl) as a sham treatment (Figure 3A). There were no differences in body weight changes among the groups, and there were no deaths or wound complications in either group during the study. Morphometric analysis of the aortas after perfusion fixation showed that vehicle treatment caused significant dilation of the aortic diameters compared with the sham treatment (Figure 3B and 3C). Aortic dilation was accompanied by inflammatory cell infiltration in the media and adventitia and disruption of the medial elastic lamellae (Figure 3D), which are pathological hallmarks of human AAA. Treatment with PF573228 completely abrogated the aortic dilation induced by CaCl₂ (Figure 3B and 3C) and largely prevented cellular infiltration and disruption of the elastic lamellae (Figure 3D and 3E). In addition, PF573228 significantly reduced macrophage accumulation, which was quantified as the number of F4/80-positive cells, and protein levels of MMP-9 and MMP-2 within the aortic walls (Figure 3F through 3H). This result suggested that continuous FAK inhibition could abrogate inflammatory responses in macrophages during AAA development. Therefore, FAK is required for the development of CaCl₂–induced AAA.
Phase-Delayed Inhibition of FAK Blocks Aneurysm Progression

We next investigated whether inhibiting FAK only during the progression phase of AAA could diminish further aortic dilation. Our intent was to investigate the clinical relevance of treating patients with pre-existing AAA. Toward this end, we waited until 3 weeks after the application of CaCl₂ to administer PF573228 treatment to mice; treatment was then started and continued for 3 weeks (Figure 4A). It has been shown previously that the CaCl₂-induced AAA mouse model is a particularly good model in that it has the same key characteristics seen during the progression of human AAA. Indeed, mouse aortas treated with CaCl₂ showed mild dilatation after 3 weeks, and the increases in diameter continued for ≤6 weeks (Figure 4B and 4C). In addition, both inflammatory cell infiltration and elastic lamellae disruption were observed in the aortas 3 weeks after the CaCl₂ application, and these lesions became more severe during the subsequent 3 weeks (Figure 4D). Notably, treatment with PF573228 3 weeks after CaCl₂ treatment diminished further dilation of the aorta during weeks 4 to 6 after the CaCl₂ application (Figure 4A and 4B). PF573228 treatment also markedly blocked further increases in the severity of cellular infiltration, particularly macrophage accumulation and elastin disruption in the aortic walls, between weeks 4 and 6 (Figure 4D through 4F). Furthermore, PF573228 completely blocked further increases in the levels of MMP-9 and MCP-1, but not in MMP-2, between weeks 4 and 6 after the application of CaCl₂ (Figure 4D and 4G through 4I).

Taken together, these results indicate that pharmacological inhibition of FAK can halt the progression from...
small-sized AAA to larger-sized AAA in a mouse model in which AAA is induced by periaortic CaCl₂ application.

**FAK Plays a Role in Promoting Macrophage Accumulation**

To better understand the mechanism of action underlying FAK-accelerated AAA progression, we delayed PF573228 treatment in mice for 3 weeks after the application of CaCl₂ and then started and continued it for only 4 days (Figure 5A). Interestingly, short-term treatment with PF573228 significantly reduced macrophage accumulation within the aortic walls compared with vehicle treatment (Figure 5B and 5C) without affecting apoptosis (Figure 5D).

These findings led us to hypothesize that FAK modulates macrophage motility in the context of AAA pathogenesis. To test this hypothesis, we used an in vitro transwell migration assay to investigate the role of FAK in regulating the chemotaxis of macrophages collected from mouse peritoneum after thioglycollate elicitation and the chemotaxis of mouse spleen monocytes. Specifically, we quantified the number of cells that migrated through the membrane to the lower well in the presence of MCP-1. Interestingly, PF573228 remarkably inhibited MCP-1–driven chemotaxis of both cell types (Figure 5E and 5F). This result indicates that FAK activity is necessary for macrophage and monocyte chemotaxis in response to MCP-1.

We also addressed the clinical concern that FAK inhibition might comprehensively suppress macrophage function and have unintended immunosuppression effects. Specifically, we examined the level of phagocytosis by quantifying the number of macrophages that took up fluorescent microparticles. We found that PF573228 did not impair macrophage phagocytosis under basal conditions. These results were confirmed using 2 other FAK inhibitors, PF562271 and FAK inhibitor 14 (Figure VII in the online-only Data Supplement).

**Discussion**

The present study shows that FAK is a key regulatory molecule that controls proinflammatory macrophage behavior...
and thereby promotes sustained aortic inflammation and AAA progression. Using human tissue specimens, we demonstrated that both FAK expression and activity were enhanced in AAA lesions, particularly in lesion-associated macrophages. Our in vitro studies using mouse macrophages revealed that FAK stimulated the secretion of MCP-1 and

Figure 3. The focal adhesion kinase (FAK) inhibitor PF573228 prevents aortic aneurysm development in mice. A, After periaortic application of CaCl₂, male C57BL/6 mice were treated with PF573228 (n=6) or vehicle (n=10) for 6 wk. Control mice were treated with NaCl (n=5) instead of CaCl₂. B, Representative aortas from mice 6 wk after NaCl or CaCl₂ application. C, Quantification of maximal infrarenal aortic diameters 6 wk after NaCl or CaCl₂ application (n=5–10). Values are expressed as means±SD. *P<0.05, **P<0.01, Kruskal–Wallis test with Dunn post-test. D, Histopathologic analysis of aortic sections from mice that were treated as indicated. Sections were stained with hematoxylin–eosin (HE), Elastica van-Gieson (EVG), or antibodies against F4/80, matrix metalloproteinase (MMP)-9, or MMP-2. Bars=30 μm. E, The degree of disruption of the medial layer elastin was graded as follows: none, mild (grade I, white), moderate (grade II, dotted), high (grade III, striped), or severe (grade IV, black) based on EVG-stained sections (8 sections in each mouse, 5–10 mice in each group). **P<0.01, χ² analysis. F, Quantification of macrophages as identified using the F4/80 cell marker; cell numbers were counted in aortic sections that were prepared 6 wk after NaCl or CaCl₂ application (n=5–10). The percentage of stained tissue area was quantified for (G) MMP-9 and (H) MMP-2 staining of aortic wall sections 6 wk after NaCl or CaCl₂ application (n=5–10). Values are expressed as means±SD. **P<0.01, Kruskal–Wallis test with Dunn post-test. A indicates adventitia; M, media; and NC, negative control.
MMP-9 and positively regulated MCP-1–mediated chemotaxis. In a mouse model, pharmacological inhibition of FAK reduced macrophage accumulation and blocked AAA progression. Taken together, these results strongly suggest that FAK is a novel therapeutic target for the pharmacological treatment of patients with AAA.
Figure 5. Focal adhesion kinase (FAK) regulates macrophage accumulation in mice and macrophage migration in vitro. A, Treatment of male C57BL/6 mice with PF573228 (n=8) or vehicle (n=6) was delayed for 3 wk after periaortic application of CaCl₂ and then conducted for only 4 d. B, Histopathologic analysis of aortas stained with hematoxylin–eosin (HE) or with an antibody against F4/80. Bars=30 μm. C, Quantification of F4/80-positive (macrophage) cells in aortic wall sections after treatment (n=6–8). Values are expressed as means±SD. *P<0.05, Student t test. D, Apoptotic cell death in aortic wall sections as assessed by TUNEL (TdT-mediated dUTP nick-end labeling) staining and immunofluorescent staining for TUNEL (red) and F4/80 (green). Bars=30 μm. The percentage of stained tissue area was quantified separately in the media and adventitia after treatment (n=6–8). Values are expressed as means±SD (Mann–Whitney U test). E, Mouse peritoneal macrophages and (F) spleen monocytes were suspended in serum-free medium and added to the upper chamber of a Transwell apparatus with or without PF573228 (20 μmol/L; n=6), in the presence of MCP-1 (monocyte chemoattractant protein-1; 10 ng/mL). After 2 h, the number of cells that migrated to the lower chamber was quantified. Values are expressed as means±SD. *P<0.05, **P<0.01, 1-way ANOVA with Tukey post-test. A indicates adventitia; M, media; and NC, negative control.
Notably, FAK inhibition successfully blocked AAA progression in mice. To understand the mechanisms underlying this phenomenon, we first examined the pathology of aortic walls from mice with experimentally induced AAA. Remarkably, elastic lamellae disruption, which is a direct cause of aortic dilation, was fully abrogated by FAK inhibition. The excessive elastolysis that is mediated by MMPs, particularly MMP-9, is considered a key step in the development and progression of AAA, and macrophages are the major cell type that secretes MMP-9 in AAA walls. This study revealed that FAK is crucial for upregulating MMP-9 secretion in macrophages. Therefore, FAK inhibition may reduce the disruption of elasticity, at least in part, by repressing MMP-9. In addition, we showed that FAK is critical for promoting MCP-1 secretion in macrophages, which can amplify macrophage accumulation. FAK was reported previously to stimulate fibroblast secretion of MCP-1, which subsequently led to macrophage recruitment in fibrotic disorders. MCP-1 plays a crucial role in the development of vascular inflammation and AAA. Taken together, these findings suggest that the FAK–MCP-1 axis plays an important role in promoting macrophage recruitment, which is a key event during the development and progression of AAA.

The unexpected finding in this study was that short-term FAK inhibition rapidly reduced macrophage accumulation within AAA walls. This finding suggests that other mechanisms that do not involve the FAK–MCP-1 axis might also contribute to macrophage accumulation. In general, the level of macrophages in tissue reflects the balance between macrophage recruitment and elimination. FAK has long been known to regulate cell motility. In fact, FAK is thought to control motility in cells that migrate in response to a broad range of stimuli, including chemotactic signals. In this study, we found that inhibiting FAK impaired the MCP-1–driven chemotaxis of macrophages and monocytes. In this context, the results indicate that FAK plays a critical role in facilitating macrophage recruitment by upregulating both MCP-1 secretion and MCP-1–driven chemotaxis. This is supported by findings from a previous study that showed that the recruitment of monocytes and macrophages into thiglycollate-induced peritonitis was impaired in myeloid-specific conditional FAK-knockout mice. The precise mechanisms remain incompletely defined, and it is possible that FAK is involved in macrophage egress from the aorta. Nonetheless, these findings imply that FAK plays a key role in maintaining and accelerating macrophage accumulation in inflammatory lesions.

Another unexpected finding in this study was that both FAK activity and expression were enhanced in TNF-α–stimulated macrophages and human AAA aortic walls. Although several transcription factors, including NF-κB and Nanog, are reported to increase FAK promoter activity, the mechanism underlying the upregulation of FAK expression is not well understood, particularly in macrophages. On the contrary, it is well known that integrin receptor–mediated cell adhesion leads to FAK dimerization and autophosphorylation. Recent studies have indicated that FAK is also activated by receptor tyrosine kinases, cytokine receptors, increased intracellular pH, and mechanical strain. Our findings suggest that TNF-α, a major cytokine that is elevated in inflammatory lesions and in AAA, may contribute to the increase in FAK expression and activity in macrophages in AAA tissues.

Importantly, animal models of AAA contain 2 disease phases: first, the initial development phase, which is model specific and second, the progression phase, which closely recapitulates human disease. An intervention that is applied during the entire experimental period cannot distinguish between effects on the progression phase versus the initial development phase. Notably, our study demonstrated that phase-limited inhibition of FAK could effectively block further progression of pre-existing AAA in a mouse model. Moreover, we demonstrated the effects of FAK inhibition in human AAA tissues in ex vivo cultures. These data strongly support the notion that FAK inhibition might have an important effect on halting AAA progression in humans.

We reported previously that inhibition of JNK caused the regression of established AAA in mice. In the current study, FAK inhibition completely blocked activation of JNK in TNF-α–stimulated macrophages. In contrast, JNK inhibition did not prevent the activation of FAK. These data suggest that FAK may be better than JNK in terms of regulating the proinflammatory macrophage response in AAA tissues, which could imply that FAK inhibition might be more efficacious than JNK inhibition for the treatment of AAA.

FAK inhibition has 2 additional advantages as a therapeutic strategy in AAA. First, this study identified MMP-9 and MCP-1 as potential biomarkers for FAK inhibition therapy. This is an advantage, because biomarkers that enable clinicians to monitor therapeutic efficacy are required for introducing a pharmacotherapy for AAA into practical use. Second, some FAK inhibitors are currently undergoing testing in phase I and II clinical trials of anticancer therapies. Therefore, pharmacological inhibitors of FAK are nearly ready for clinical use; however, further studies are needed to determine the efficacy and safety of FAK inhibition in patients with AAA.

In conclusion, this study provides new insights into the mechanisms that accelerate macrophage accumulation and lead to prolonged inflammation in AAA. Although potential off-target effects remain to be investigated, our findings demonstrate that pharmacological inhibition of FAK halts the progression of AAA in mice by regulating proinflammatory macrophage behavior. FAK inhibition therapy may provide a nonsurgical therapeutic option for treating AAA, a disease that is frequently fatal.

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Disclosures

None.

References


**Highlights**

- Both focal adhesion kinase (FAK) expression and FAK activity are enhanced in macrophages in abdominal aortic aneurysm tissue.
- FAK promotes the secretion of MCP-1 (monocyte chemoattractant protein-1) and matrix metalloproteinase-9 by cultured macrophages and positively regulates MCP-1–mediated macrophage chemotaxis.
- Pharmacological inhibition of FAK reduces macrophage accumulation and halts the progression of abdominal aortic aneurysm in a mouse model.
- FAK may play a key role in facilitating chronic inflammation and abdominal aortic aneurysm progression by controlling the proinflammatory behavior of macrophages.
- FAK inhibition therapy may provide a nonsurgical therapeutic option for treating abdominal aortic aneurysm.
Focal Adhesion Kinase Promotes the Progression of Aortic Aneurysm by Modulating Macrophage Behavior
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