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Blocking TNF-α Attenuates Aneurysm Formation in a Murine Model[1]

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Abstract

Abdominal aortic aneurysm (AAA) is one of a number of diseases associated with a prominent inflammatory cell infiltrate and local destruction of structural matrix macromolecules. This chronic infiltrate is predominately composed of macrophages and T lymphocytes. Activated macrophages produce a variety of cytokines, including TNF-*α*. Elevated levels of TNF-*α* were observed in patients with AAA, suggesting that TNF-*α* may play a role in the pathogenic mechanisms of AAA. In the present study, we investigated the role of TNF-*α* in AAA formation. By studying a murine aneurysm model, we found that both mRNA and protein levels of TNF-*α* were increased in aneurysm tissue compared with normal aortic tissues. Therefore, we tested the response of mice lacking expression of TNF-*α*. These mice were resistant to aneurysm formation. Our results show that TNF-*α* deficiency attenuates matrix metalloproteinase (MMP) 2 and MMP-9 expression and macrophage infiltration into the aortic tissue. These data suggest that TNF-*α* plays a central role in regulating matrix remodeling and inflammation in the aortic wall leading to AAA. In addition, we investigated the pharmacological inhibition of AAA. A Food and Drug Administration-approved TNF-*α* antagonist, infliximab, inhibited aneurysm growth. Our data also show that infliximab treatment attenuated elastic fiber disruption, macrophage infiltration, and MMP-2 and MMP-9 expression in aortic tissue. This study confirms that a strategy of TNF-*α* antagonism may be an important therapeutic strategy for treating AAA.

Abdominal aortic aneurysm (AAA)[3] is a common and life-threatening condition. The natural history of large, untreated aortic aneurysms is progressive expansion to a point of

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Disclosures
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[3]Abbreviations used in this paper: AAA, abdominal aortic aneurysm; MMP, matrix metalloproteinase; TNF-BP, TNF-binding protein; SMC, smooth muscle cell; WT, wild type.

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material failure of the aorta. This leads to acute hemorrhage and death in most cases. Analysis of the aortic wall demonstrates that the normal lamellar architecture is altered and that the areas of tissue injury are spatially associated with invading inflammatory cells (1–4). T lymphocytes and macrophages are the predominant inflammatory cells (5, 6). TNF-α is a key inflammatory cytokine, initially produced by T lymphocytes, that mediates a secondary monocyte/macrophage release of TNF-α within 24 h (7). In AAA tissue, TNF-α expression colocalizes mainly with CD68-positive macrophages in the medium and adventitia (8). TNF-α can bind to its receptor and elicit diverse biological responses involved in AAA, such as the activation and recruitment of immune cells to the sites of inflammation, the secretion of proinflammatory cytokines and matrix metalloproteinases (MMPs), and the initiation of programmed cell death in the resident cells (smooth muscle cells and fibroblasts) responsible for maintenance and repair of the structural matrix.

Descriptive studies have identified an associative relationship between human AAA and TNF-α. Circulating levels of TNF-α are increased in AAA patients compared with individuals without AAA (9, 10). TNF-α levels were found to be elevated in AAA tissue than in control aortic tissue without AAA (8). Two separate studies have shown TNF-α to be higher in patients with small aneurysms compared with large aneurysms, suggesting a potential role in an early stage of AAA development (9, 11). This observation is supported by findings of higher TNF-α mRNA levels in the transition zone between the aneurysm neck and the mid-portion of the aneurysm (8). Moreover, in a rat model of AAA, i.v. injection of a TNF-binding protein (TNF-BP) has been shown to prevent the development of elastase-induced AAA (12). These studies clearly implicate TNF-α in AAA.

MMPs are zinc-dependent endopeptidases that play important roles in the control of critical and diverse cellular processes. MMPs have been shown to be responsible for destruction of the orderly elastin and collagen network of the aorta. MMP-9 is one of the most abundant elastolytic proteinases secreted by human AAA tissues (13–16). Activated MMP-2 levels are also elevated in human AAA tissue. It has been shown that macrophage-derived MMP-9 and smooth muscle cells (SMC) MMP-2 are both required and appear to work in concert to produce aneurysms (2).

To gain further insight into the role that TNF-α might play in AAA development, we tested the response of TNF-α null (TNF-$\alpha^{-/-}$) mice to aneurysm induction by $CaCl_2$. We found that TNF-$\alpha^{-/-}$ mice are resistant to aneurysm formation. TNF-α deficiency inhibited both macrophage recruitment and tissue MMP-2 and MMP-9 expression. The ability of TNF-α to induce MMP-2 and MMP-9 expression by SMC and macrophages, respectively, was confirmed by in vitro analysis. Furthermore, a TNF-α antagonist, infliximab, can inhibit aneurysm growth and attenuate elastic fiber disruption, macrophage infiltration, and MMP-2 and MMP-9 expression in aortic tissue. Taken together, these data demonstrate that TNF-α promotes AAA. Considering the elevated TNF-α levels in human AAA, a strategy of TNF-α inhibition may be a viable therapeutic strategy for human AAA.

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Materials and Methods

Reagents

Recombinant murine TNF-α and human TNF-α were purchased from BD Pharmingen. Tissue culture medium and FBS were purchased from Life Technologies.

Mouse aneurysm induction model

The homozygous TNF-α gene null (TNF-$\alpha^{-/-}$) mice (on B6129SF2 background) and their wild- type (WT) controls, B6129SF2 mice, were purchased from The Jackson Laboratory. AAA were induced by periaortic application of 0.25 mM $CaCl_2$ as described previously (2). NaCl (0.9%) was substituted for $CaCl_2$ in sham control mice. The aortic diameters were measured before aneurysm induction and at sacrifice 6 wk later. The aortas were collected for zymographic analysis of MMP proteins. For histological studies, the aortas were perfusion fixed with 10% neutral-buffered formalin. Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

Histology and microscopy

After perfusion-fixation with 10% neutral-buffered formalin, aortic tissues were embedded in paraffin and cut into 4-μm sections. For Masson trichrome staining, the slides were stained with hematoxylin, Crocein Scarlet, Acid Fuchsin, and Aniline blue (Sigma-Aldrich. Each staining cycle alternated between fixing and washing procedures. For Verhoeff-Van Gieson staining, the slides were stained with Verhoeff's solution, ferric chloride, sodium thiosulfate, and Van Gieson's solution. Each staining cycle alternated between fixing and washing procedures. The slides were examined and photographed using light microscopy (×40; Nikon).

Immunohistochemistry

Mice underwent AAA induction according to the method described above. Three to five mice in each group were sacrificed at 6 wk for macrophage and T cell staining was performed on paraffin-embedded 4-μm aortic sections. For macrophage staining, the sections were incubated with monoclonal rat anti-mouse Mac3 Ab (BD Pharmingen) diluted 1/500 for 30 min at 37°C. The sections were then briefly washed in citrate solution and subsequently incubated with the secondary Ab, which is a mouse-absorbed, biotin-conjugated rabbit anti-rat IgG. For T cell staining, the sections were incubated with polyclonal rabbit anti-human CD3 (DakoCytomation) diluted 1/50 for 30 min at room temperature. The sections were then washed in citrate solution and subsequently incubated with the secondary HRP-labeled anti-rabbit IgG (DakoCytomation). Macrophage and T cell stainings were examined using light microscopy (×100). Positive controls and nonimmune negative controls were performed. The absolute number of macrophages and T cells in the aortic medium and adventitia was evaluated by a pathologist unaware of the genotype or treatment. Three to four separate sections from each aorta from control and TNF-α null mice were stained and evaluated.

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Isolation of mouse SMC and cell culture

A WT mouse was anesthetized and underwent laparotomy. The aorta then was isolated and minced. Adventitia and endothelium were removed after digestion of the aorta with 175 U/ml type 2 collagenase for 1 h (Worthington). The medium was further digested with a mixture of type 2 collagenase (175 U/ml) and 20 U/ml elastase for 1 h (Sigma-Aldrich). The cells were filtered, centrifuged, resuspended, and plated in Ham's F-12K medium supplemented with 10% HI-FBS, 2 mM L-glutamine, 1.5 g/L $NaHCO_3$, 10 mM HEPES, 10 mM TES, 0.05 mg/ml ascorbic acid, 0.01% mg/ml insulin, 0.01 mg/ml transferrin, 10 ng/ml sodium selenite, and 0.03 mg/ml endothelial cell growth supplement. The cells were grown to confluence and passed after trypsinization with 0.25% trypsin. THP-1 cells were maintained in RPMI 1640 containing 10% FBS. To examine the effect of TNF-α on cellular MMP-2 and MMP-9 expression, cells were incubated with serum-free medium and treated with TNF-α (0–10 ng/ml) for 24 h. Conditioned medium was harvested and analyzed by gelatin zymography.

Western blot analysis and gelatin zymography

Aortic proteins were extracted as previously described (13). The protein concentration for aortic proteins and cultured medium from cells was standardized with a Bio-Rad protein assay. Equal amounts (20 μg) of aortic extracts from NaCl- or $CaCl_2$-treated WT mice were loaded under reducing conditions onto a 12.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). The membranes were then incubated with rabbit anti-TNF-α polyclonal Ab (Cell Signaling). The bound primary Ab was detected with HRP-linked anti-rabbit IgG. Immunoreactive bands were visualized by autoradiography using ECL (Amersham Biosciences). Gelatin zymography was performed as described previously by Longo et al. (2), with 0.8% gelatin in a 10% SDS-polyacrylamide gel. The molecular sizes were determined using protein standards from Invitrogen.

Measurement of TNF-α mRNA

Aortas from WT mice were collected at 6 wk after treatment with 0.9% NaCl or 0.25 mM $CaCl_2$. Total RNA was extracted using TRIzol reagent (Invitrogen). Levels of TNF-α mRNA were analyzed by RT-PCR. Two micrograms of total RNA was reverse transcribed. The RT-PCR protocol was performed according to the manufacturer's instruction (Invitrogen).

Infliximab treatment

WT mice at the age of 8 wk were treated with $CaCl_2$ or NaCl. The aortic diameters were assessed before aneurysm induction. Experiment mice were injected i.p. with infliximab (10 μg/g body weight) twice weekly beginning at the day of aneurysm induction for 6 wk until sacrifice. For control, 10 μg/g of human IgG was injected.

Statistical analysis

Measurements of aortic diameter are expressed as mean value ± the SE of the mean. A paired Student's *t* test was used to compare original and final diameter. A Fisher's exact test

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or ANOVA (for comparison between multiple groups) was used for all analyses evaluating noncontinuous variables. Statistical significance was accepted at a $p < 0.05$.

Results

TNF-α deficiency in mice prevents AAA formation

Using a murine aneurysm model, which recapitulates key features of the human AAA, including macrophage and lymphocyte infiltration, increased MMP expression, elastin degradation and progressive vessel wall dilatation (2, 17), we found that both mRNA and protein levels of TNF-α were increased in aneurysmal, $CaCl_2$-treated aorta relative to non-aneurysmal NaCl-treated controls (Fig. 1, *a* and *b*). Furthermore, we examined TNF-α mRNA levels at five time points, 3 days, 1wk, 2 wk, 4 wk, and 6 wk after aneurysm induction. We found that induced TNF-α expression in $CaCl_2$-treated aortas compared with NaCl-treated controls starting at 1 wk, reaching a peak at 2 wk and continuing to 6 wk (Fig. 1*c*). The increase of TNF-α in human or murine AAA tissue is not proof that it has a causal role since the increased levels could simply reflect the presence of the local inflammatory response. To determine whether TNF-α contributes directly to aneurysm formation, we used TNF-α null (TNF-$\alpha^{-/-}$) mice to test their response to $CaCl_2$ aneurysm induction. Six weeks after periaortic application of $CaCl_2$, the aortic diameter in the TNF-$\alpha^{-/-}$ mice showed a small increase with only 2 (12%) of 17 of the aortas becoming aneurysmal (>50% increase) (Table I). The histological appearance was similar to the NaCl-treated TNF-$\alpha^{-/-}$ mice (Fig. 1, *f* and *g*). The B6129SF2 WT background control mice showed an 80% increase ($p < 0.01$) in aortic diameter after $CaCl_2$ treatment with 11 (100%) of 11 developing aneurysms (Table I). This difference in aneurysm incidence (2 of 17 vs 11 of 11) is highly significant ($p < 0.0001$). Connective tissue staining of aneurysmal aortic sections from WT mice showed disruption and fragmentation of medial elastic fibers (Fig. 1*e*), while NaCl-treated controls show intact medial elastic lamellae (Fig. 1*d*). These observations demonstrate that TNF-α has a central role in aneurysm development.

Inflammatory cell infiltration is affected by TNF-α

One of the remarkable histological changes in both human AAA and $CaCl_2$-treated aortas is the inflammatory infiltrate in the adventitia and medium (2). In consideration of the possible effects of TNF-α on macrophage recruitment into the aorta, we examined the aortic tissue for the presence of Mac3 (macrophage-specific Ag)-positive cells. As can be seen from Fig. 2, *c* and *d*, and Table II, macrophage infiltration in $CaCl_2$-treated TNF-$\alpha^{-/-}$ mice is markedly decreased compared with $CaCl_2$-induced WT mice. These data demonstrated that TNF-α plays an important role in macrophage recruitment and suggests that the aneurysm inhibitory effects seen in the TNF-$\alpha^{-/-}$ mice are due, in part, to decreased macrophage recruitment to the site of injury. A small number of neutrophils were identified in the aorta of both groups using the NIMP Ab but no differences were noted between WT and TNF-$\alpha^{-/-}$ mice. Using CD3 and c-Kit Abs for lymphocytes and mast cells, respectively, we found that TNF-α deficiency inhibited T lymphocyte recruitment (WT, 6.7 ± 1.3 cell/mm^2 vs TNF-$\alpha^{-/-}$, 1.5 ± 0.8 cell/mm^2; $p = 0.025$; Fig. 2, *e* and *f*) but had no effect on mast cell recruitment between WT and TNF-α knockout mice.

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TNF-α deficiency in mice prevents MMP up-regulation during aneurysm induction

Our previous studies have demonstrated that MMP-2 and MMP-9 are essential for the connective tissue degradation in the aortic wall leading to AAA. TNF-α has been shown to modulate MMP expression in a cell-type-specific manner, suggesting another potential mechanism for its role in AAA (18 –21). Aortic protein prepared from control B6129SF2 and TNF-$\alpha^{-/-}$ mice treated with $CaCl_2$ were analyzed by gelatin zymography. As can be seen in Fig. 2*a*, MMP-2 and MMP-9 were dramatically decreased in TNF-α null mice after $CaCl_2$ induction. The effect was equally pronounced for MMP-2 and MMP-9. Some decrease in MMP-9 would be anticipated given the decrease in macrophage number. The large decrease in MMP-2, which is secreted primarily by SMC and fibroblasts, suggests that TNF-α has an important role in stimulating local MMP-2 production through paracrine effects on resident SMC and fibroblasts. We undertook the following in vitro studies to directly assess the impact of TNF-α on SMC MMP-2 and macrophage MMP-9.

TNF-α stimulates synthesis of MMP-2 in mouse SMC and MMP-9 in macrophages (THP-1 cells)

It has been shown that aortic SMC are the primary source of MMP-2 while most MMP-9 is secreted by infiltrating macrophages. We assessed the effects of TNF-α on the expression of MMPs from SMC and macrophages. Mouse aortic SMC and transformed human macrophage THP-1 cells were treated with three different concentrations of TNF-α. Analysis of cell conditioned medium by gelatin zymography demonstrated that SMC MMP-2 was up-regulated by TNF-α. This increase was dose related (Fig. 2*b*). TNF-α did not induce MMP-9 expression in SMC. Macrophage MMP-9 was increased in THP-1 cells in a dose-dependent manner (Fig. 2*b*). By increasing by five times the loading volume of the THP-1 medium, faint MMP-2 bands were identified, confirming the minor role of the macrophage in total tissue MMP-2 production. The low levels of MMP-2 were induced by TNF-α in a dose-dependent fashion (data not shown). This in vitro study further demonstrates that TNF-α can stimulate production of MMP-2 in SMC and MMP-9 in macrophages.

TNF-α antagonist, infliximab, treatment does not result in regression of existing aneurysm, but attenuates AAA formation, MMP expression, and macrophage infiltration

Previous studies using the $CaCl_2$ model of AAA have shown regression of existing AAA by inhibition of JNK signaling (17). Regression of an existing AAA would offer optimal therapy for patients with small AAA. To determine whether aneurysm regression could be achieved, a group of mice were treated with infliximab beginning 6 wk after aneurysm induction at the time that the aneurysm was established. These mice were sacrificed at 12 wk after aneurysm induction. The infliximab has no effect on the aneurysm when treatment was begun after the aneurysm has developed (data not shown).

To determine whether a Food and Drug Administration-approved TNF-α antagonist, infliximab, could inhibit aneurysm development, WT mice underwent $CaCl_2$ aneurysm induction and then were treated with infliximab (10 μg/g body weight) every week. Control mice were treated with human IgG (10 μg/g body weight). Six weeks after, we found that control mice developed aneurysms (five of six) at a rate similar to that of WT mice using the

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standard clinical definition of a 50% increase in diameter (Fig. 3*a*). In marked contrast, however, only one out seven infliximab-treated mice developed an aneurysm. Aneurysm incidence was significantly decreased in the infliximab group by Fisher's exact test (p = 0.029). The mean aortic diameter was also significantly decreased (Fig. 3*a*). Furthermore, control mice exhibit significant disruption of the elastic lamellae of the aortic wall (Fig. 3*b*); the infliximab-treated mice revealed only minor distortion of lamellar architecture (Fig. 3*c*). Infliximab treatment inhibits aortic MMP-2 and MMP-9 production (Fig. 3*d*) and macrophage infiltration (Fig. 3*f*) compared with control (Fig. 3*e*). Taken together, these data demonstrate that inhibition of TNF-α cannot result in aneurysm regression, but may prevent aneurysm growth.

Discussion

Using a murine model of AAA, we have made a detailed investigation of the role of TNF-α. Aneurysm induction is attenuated in the absence of TNF-α. The lamellar architecture is preserved. Among its myriad potential roles in AAA formation, TNF-α could be important in recruitment of inflammatory cells. Immunohistochemical labeling of macrophages showed a reduction in the number of tissue macrophages. TNF-α could also regulate MMPs. MMP-2 and MMP-9 levels in the aortic tissue from the TNF-α null mice were found to be decreased. We assumed that this may be secondary to the decreased macrophage number but considered that TNF-α could also be exerting a direct effect on MMP regulation. In vitro analysis showed that exogenous TNF-α up-regulated MMP-9 and induced SMC MMP-2 expression in a dose-dependent manner. The latter studies demonstrate a direct effect of TNF-α on MMP expression in addition to its effects on cell recruitment.

TNF-α is a nonglycosylated soluble protein of 17 kDa, which is generated by cleaving the 32-kDa transmembrane precursor by TNF-α-converting enzyme. Both soluble and membrane-bound forms of TNF-α are biologically active. The biological function of TNF-α includes the modulation of growth, differentiation, and proliferation of a variety of cell types, but it is also important in regulating programmed cell death (22). Besides these effects, TNF-α is known to augment and regulate both acute and chronic inflammatory responses. It is implicated in the pathogenesis of several chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, and psoriasis (23–25). Increased levels of TNF-α in plasma and aorta is one of the characteristic features of human AAA (8, 10, 26). These observations are corroborated by another study showing TNF-α tissue mRNA levels are higher in the smaller diameter transition zones than at the midpoint of the aneurysm where the diameter is greatest. Taken together, these clinical findings suggest a role for TNF-α in the initial stages of AAA development (8, 9, 11) rather than the later progression of the disease.

It is known that TNF-α can enhance the release of proinflammatory/chemotactic mediators and up-regulate adhesion molecules, such as E-selectin, VCAM-1, and ICAM-1, thus facilitating the migration of macrophages (27–29). Conversely, TNF-α deficiency may affect macrophage infiltration (30, 31). This important role in macrophage recruitment was confirmed by our observations of dramatically decreased tissue macrophages in the TNF-α null mice. Furthermore, TNF-α has been shown to lead to proliferation of lymphocytes

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through direct cell-cell interaction (32). Importantly, we observed a very significant decrease in tissue lymphocytes in TNF-α-deficient mice. We have previously demonstrated an important role for $CD4^+$ lymphocytes in the inflammation associated with AAA (3). The absence of TNF-α and the attendant decrease in tissue macrophages and lymphocytes suggests two potential mechanisms for aneurysm inhibition. The first is that the overall degree of tissue inflammation could be diminished by the inability of the macrophage to invade, recruit other macrophages, and promote lymphocyte proliferation. Another potential mechanism is the reduced levels of tissue MMPs. Macrophages are the most important source of MMP-9 and two separate studies have demonstrated that macrophage-derived MMP-9 is essential for aneurysm formation (2, 33).

We investigated weather TNF-α altered the levels of expression of aortic MMPs. These studies demonstrate that elastic lamellar preservation in TNF-$\alpha^{-/-}$ mice can be accounted for by decreased local expression of MMP-2 and MMP-9, two MMPs essential for AAA development. This is consistent with the observation that TNF-α stimulates MMP production (18, 34). The finding that the TNF-α exerts a major paracrine effect on SMC MMP-2 expression is novel and important since MMP-2 levels are increased in human AAA and SMC-derived MMP-2 is critical for aneurysm formation (2, 13). The observation that TNF-α deficiency inhibits both MMP expression and macrophage recruitment lends further support to the concept that TNF-α has a role in the early stage of aneurysm formation by enhancing extracellular matrix protein degradation and facilitating inflammatory cell infiltration.

Hingorani et al. (12) were the first to show that TNF antagonism could inhibit aneurysm formation. Using TNF-BP, a type I TNF receptor blocker, at 48 and 96 h before elastase aneurysm induction, rats were resistant to aneurysm formation, but TNF-BP had no effect on MMP expression. This study suggested that either inhibition of MMPs was not the major effect of TNF-BP in the rat model or that the major proteolytic changes had passed by the day they harvested tissues (12). In the present study, we evaluated the TNF-α expression levels at five different time points. We found that TNF-α expression was up-regulated 1 wk after aneurysm induction throughout aneurysm development. By studying TNF-α-deficient mice, we have established a causal relationship among TNF-α activity changes, inflammation, MMP expression, and aneurysm growth. Furthermore, we have studied the treatment of a Food and Drug Administration-approved, specific TNF-α antagonist, infliximab, on a murine aneurysmal model. Infliximab is a chimeric mouse-human mAb targeting TNF-α. It has been used effectively in several chronic inflammatory diseases (35, 36). In this study, we find that infliximab not only attenuates aneurysm formation in $CaCl_2$-induced mouse model, but also inhibits aortic matrix degradation and MMP expression. In dealing with any animal model of a human disease, it is important to consider that these results may be specific to the acute model and may not directly translate to AAA in patients. TNF-α antagonism is in clinical use for elderly patients with rheumatoid arthritis and a subset of these patients also have AAA. It would be of particular interest to determine whether aneurysm expansion rates were decreased in this subset.

In conclusion, we have shown that TNF-α has a pivotal role in early aneurysm formation. This finding appears to corroborate evidence from human studies suggesting that TNF-α

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plays a role in the initial stages of aneurysm formation. TNF-α antagonists are currently used therapeutically for a number of chronic inflammatory diseases including rheumatoid arthritis and Crohn's disease (35, 36). The observations presented in this article suggest a strategy through which aneurysm treatment might be achieved using TNF-α antagonism in the early stages of aneurysm formation.

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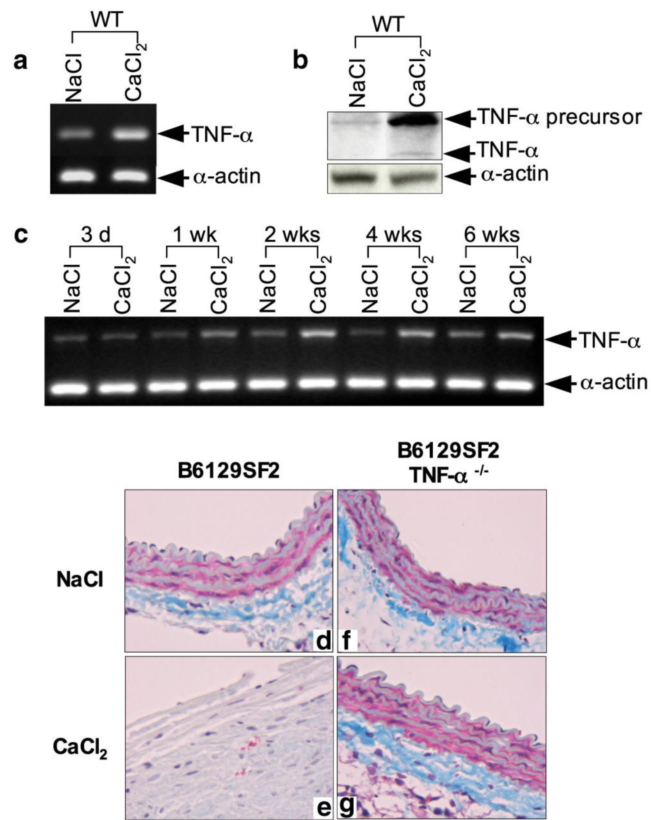

FIGURE 1.

Expression of TNF-α in mouse aorta. Six weeks after 0.9% NaCl or 0.25 mol/L $CaCl_2$ treatment, WT (B6129SF2) mouse aortas were harvested. *a*, RT-PCR: Total RNA from each aorta was extracted and TNF-α mRNA levels were examined by RT-PCR. α-Actin was used as the internal standard. *b*, Western blot analysis: aortic protein extracts were prepared and analyzed by immunoblotting with TNF-α Ab that recognizes both membrane-anchored (precursor) and soluble forms. Aortic extracts were also immunoblotted with an Ab that reacts with actin. Images shown for *a* and *b* are representative of three separate experiments. *c*, RT-PCR: Total RNA from the aortas at five time points, 3 days, 1 wk, 2 wk, 4wk, and 6 wk after aneurysm induction was extracted and TNF-α mRNA levels were examined by RT-PCR. α-Actin was used as the internal standard. *d–g*, Histological changes in mouse aorta 6 wk after treatment (trichrome staining). NaCl-treated (*d*) and $CaCl_2$-treated (*e*) aorta from B6129SF2 (WT) mice; NaCl-treated (*f*), and $CaCl_2$-treated (*g*) aorta from TNF-$\alpha^{-/-}$ mice. Each section shown is representative of three to four samples with similar results.

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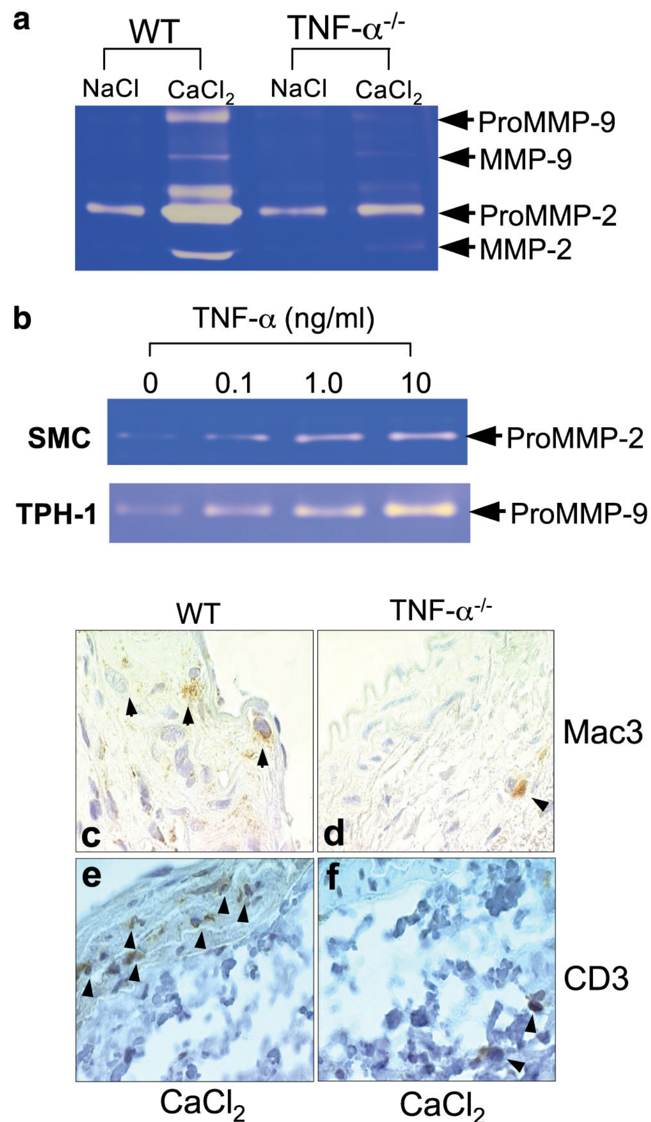

FIGURE 2.

Gelatin zymographic analysis of MMP-2 and MMP-9. *a*, Six weeks after NaCl or $CaCl_2$ treatment, aortas from WT and TNF-$\alpha^{-/-}$ mice were harvested. Aortic proteins were extracted and separated by electrophoresis on a 10% SDS-PAGE containing 0.8% gelatin. The gel shown is representative of three trials with similar results. The decrease in both MMP-2 ($p = 0.003$) and MMP-9 ($p = 0.03$) after CaCl2 treatment was significant. *b*, Up-regulation of the MMP-9 in macrophages and MMP-2 in mouse aortic SMC by TNF-α treatment. SMC and THP-1 cells, a human macrophage cell line, were treated with TNF-α for 24 h at concentrations of 0, 0.1, 1.0, and 10 ng/ml. Conditioned medium was separated on a 10% SDS-PAGE containing 0.8% gelatin for determination of MMP content. The gels are representative of four experiments with similar results. *c* and *d*, Immunohistochemistry of macrophage infiltration in the aortas of WT and TNF-$\alpha^{-/-}$ mice after $CaCl_2$ induction. Aortas were collected from WT and TNF-$\alpha^{-/-}$ mice at 6 wk after $CaCl_2$ treatment. Paraffin sections were immunostained with Mac3 Ab. Arrows indicate macrophage infiltration. Each

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staining represents three to four samples of WT or TNF-$\alpha^{-/-}$ mice with similar results. *e* and *f*, Immunohistochemistry of T cell infiltration in the aortas of WT and TNF-$\alpha^{-/-}$ mice after $CaCl_2$ induction. Aortas were collected from WT and TNF-$\alpha^{-/-}$ mice at 6 wk after $CaCl_2$ treatment. Paraffin sections were immunostained with CD3 Ab. Arrows indicate T cell recruitments. Each staining represents three samples of WT or TNF-$\alpha^{-/-}$ mice with similar results.

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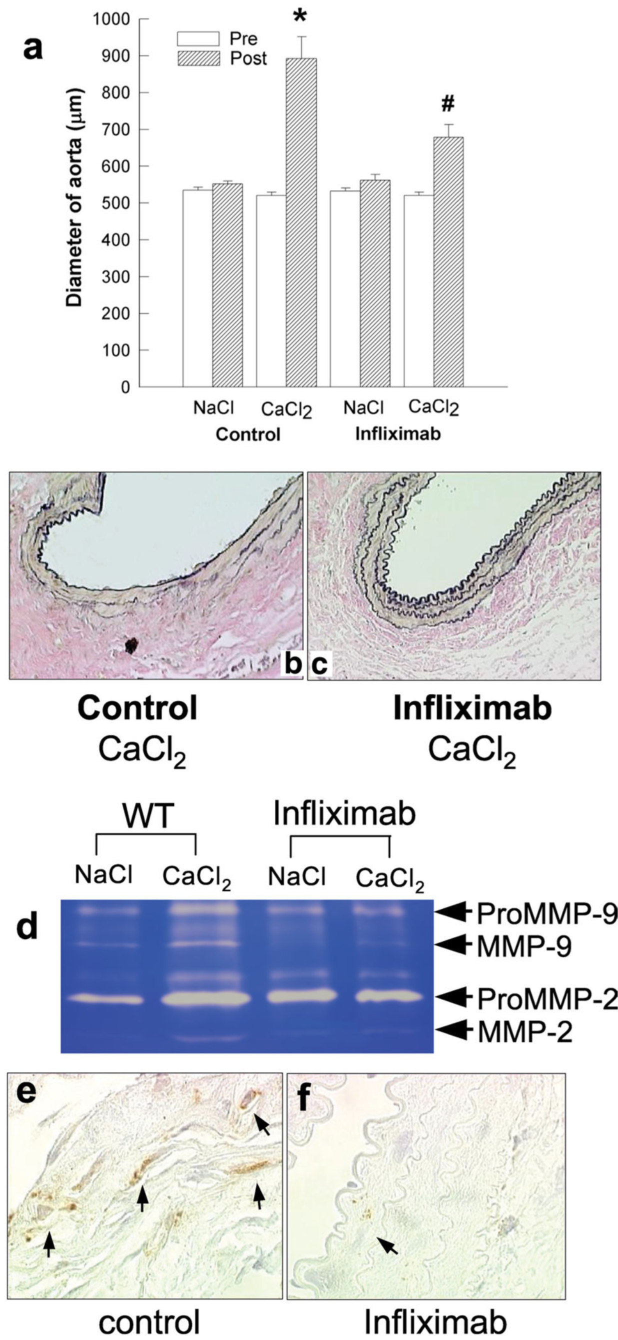

FIGURE 3.

Infliximab treatment on the aneurysm formation in $CaCl_2$-induced aneurysm model. *a*, Aortic diameter before (□) and 6 wk (▫) after NaCl and $CaCl_2$ treatment in WT mice treated with infliximab (10 μg/g body weight) or human IgG (control; 10 μg/g body weight) twice weekly beginning at the day of aneurysm induction for 6 wk until sacrifice. *b* and *c*, Histological changes in $CaCl_2$-induced mouse aorta 6 wk after Ab treatment using Verhoeff-Van Gieson staining. Human IgG-treated (*b*) and infliximab-treated (*c*) aorta from B6129SF2 mice. *d*, Gelatin zymographic analysis of mouse aortic MMP-2 and MMP-9. Six

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weeks after NaCl or $CaCl_2$ induction and Ab treatment, aortas were harvested. Aortic proteins were extracted and separated by electrophoresis on a 10% SDS-PAGE containing 0.8% gelatin. The gel shown is representative of three trials with similar results. *e* and *f*, Immunohistochemistry of macrophage infiltration in the aortas of $CaCl_2$-induced and Ab-treated B6129SF2 (WT) mice, human IgG-treated (*e*), and infliximab-treated mouse aorta. Each staining represents three to four samples with similar results. Aortic diameters were measured before NaCl or $CaCl_2$ incubation (pretreatment) and at sacrifice (posttreatment). Measurements of aortic diameter were expressed as mean ± SE. The development of an AAA was defined as at least a 50% increase relative to the original aortic diameter. The percent increase was represented as a percent compared with pretreatment.*, $p < 0.01$, Student's *t* test, compared with pretreatment value. #, $p < 0.0001$, Fisher's exact test, aneurysm.

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Table I

Changes in aortic diameter in B6129SF2 and TNF-$^{-/-}$ on B6129SF2 background mice after treatment with NaCl and CaCl2[a]

	B6129SF2		TNF-$\alpha^{-/-}$	
Treatment	**NaCl**	**$CaCl_2$**	**NaCl**	**$CaCl_2$**
No.	19	11	11	17
Pretreatment (μm)	558 ± 9.6	543 ± 8.1	544 ± 9.1	536 ± 8.0
Posttreatment (μm)	582 ± 9.1	979 ± 31.5[*]	570 ± 9.4	665 ± 22.7
AAA development	0	11/11 (100%)	0	2/17 (12%)[#]
% increase	4.6 ± 2	80.2 ± 4.6[‡]	5.1 ± 2.3	24.3 ± 4.2

[a]Aortic diameters were measured before NaCl or $CaCl_2$ incubation (pretreatment) and at sacrifice (posttreatment). Measurements of aortic diameter were expressed as mean ± SE. The development of an AAA was defined as at least a 50% increase relative to the original aortic diameter. The percent increase was represented as a percent compared with pretreatment.

[*]$p < 0.01$, Student's t test, compared with pretreatment value.

[‡]$p < 0.001$, Student's t test, compared with $CaCl_2$-treated TNF-$\alpha^{-/-}$ mice.

[#]$p < 0.0001$, Fisher's exact test, aneurysm.

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Table II

Macrophage infiltration in the $CaCl_2$-treated aorta of B6129SF2 and TNF-$\alpha^{-/-}$ mice[a]

B6129SF2	TNF-$\alpha^{-/-}$
12.0 ± 2.6*	3.5 ± 1.9

[a] Macrophages were evaluated and scored in cross-section of aorta. The values reflect the mean cells/mm^2 ± SE.

* $p = 0.0161$, Student's t test, compared with TNF-$\alpha^{-/-}$ mice.