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TNF α regulation of Fas ligand expression on the vascular endothelium modulates leukocyte extravasation

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Abstract

It is generally believed that the vascular endothelium serves as an inflammatory barrier by providing a nonadherent surface to leukocytes. Here, we report that Fas ligand (FasL) is expressed on vascular endothelial cells (ECs) and that it may function to actively inhibit leukocyte extravasation. TNF α downregulates FasL expression with an accompanying decrease in EC cytotoxicity toward co-cultured Fas-bearing cells. Local administration of TNF α to arteries downregulates endothelial FasL expression and induces mononuclear cell infiltration. Constitutive FasL expression markedly attenuates TNF α -induced cell infiltration and adherent mononuclear cells undergo apoptosis under these conditions. These findings suggest that endothelial FasL expression can negatively regulate leukocyte extravasation.

The monolayer of endothelial cells that coat the luminal surface of the vessel wall have numerous physiological functions including prevention of coagulation, control of vascular permeability, maintenance of vascular tone and regulation of leukocyte extravasation¹. The inflammatory cytokine TNF α upregulates surface adhesion molecule expression leading to leukocyte adherence to the endothelium². Adherent leukocytes then migrate to the subendothelial space. These processes are key features of the inflammatory response to pathogens³. Furthermore, abnormal leukocyte infiltration of the vessel wall is believed to be a critical early event in the development of atherosclerotic lesions¹.

The Fas-FasL system has been implicated in the regulation of physiological cell turnover, particularly in the immune system.⁴ Fas is expressed on various cell types, but FasL is much more restricted in its expression. Although the expression of FasL was originally considered restricted to activated T cells and NK cells, FasL has been identified in other cell types including Sertoli cells and cells of the eye. It has been proposed that expression of functional FasL by some tissues contributes to their immune-privileged status by preventing the infiltration of inflammatory leukocytes^{5,6}. However, the utility of FasL for transplantation applications may be limited due to its ability to either recruit neutrophils or induce apoptosis in the transplanted tissue^{7–9}. Recently, constitutive FasL expression has been detected on some tumor cells indicating that it may function to induce apoptotic cell death in Fas-expressing immune cells when they attempt to enter the tumor^{10–12}.

Here, we analyzed the role of endothelial FasL expression in controlling leukocyte extravasation. We report that appreciable levels of FasL are expressed on vascular ECs *in vitro* and *in vivo* and that this expression is markedly downregulated by TNF α . The functional significance of this downregulation is indicated by the finding that TNF α -induced leukocyte extravasation can be blocked when FasL is constitutively expressed on the endothelium.

Regulation of endothelial cell FasL expression

To investigate the regulation of FasL expression on ECs, human umbilical vein endothelial cells (HUVECs) were subjected to western immunoblotting assays using an anti-FasL monoclonal antibody. These analyses revealed a single band of 37–40 kDa, which had the same mobility as FasL from extracts of activated Jurkat cells¹³ (Fig. 1a). No FasL was detected in human smooth muscle cell (HUSMC) lysate but a FasL signal was detected in lysates prepared from HUSMCs infected with a replication-defective adenovirus expressing FasL from the cytomegalovirus promoter (Adeno-FasL)^{14,15}. Treatment of HUVECs with TNF α downregulated the FasL expression in a dose-dependent manner (Fig. 1a). Immunoblot analyses also revealed that TNF α downregulated FasL expression on human aortic endothelial cells (HAECs) and human microvascular endothelial cells (HMVECs) (data not shown). In agreement with earlier findings^{2,16,17}, TNF α induced a dose-dependent upregulation of the cell adhesion molecule VCAM-1 (Fig. 1a).

Flow cytometric analyses were performed to examine FasL expression on the cell surface of vascular ECs. Consistent with data from the western blot analyses, incubation with TNF α decreased the expression of FasL on HAECs (Fig. 1b) and HMVECs (not shown). EC expression of the FasL receptor, Fas, was also examined by flow cytometry (Fig. 1c). Fas expression was detectable on HAECs but it was present at lower levels than on Jurkat cells (Fig. 1c). Despite Fas expression on ECs, these cells do not undergo apoptosis when exposed to Adeno-FasL or an anti-Fas antibody that can function as a receptor agonist (Fig. 2).

Cytotoxic activity of endothelial cell FasL

To determine whether FasL expression on ECs is functional, HAECs were co-cultured with P815-huFas target cells that were labeled with BrdU or ³H-thymidine. After 18 hours co-culture, significant target cell DNA fragmentation was observed either by agarose gel electrophoresis and immunoblotting with BrdU-specific antibody (Fig. 3a) or by calculating the specific fragmentation of ³H-thymidine-labeled DNA (Fig. 3b). Prior treatment of the HAECs with TNF α , at a dose that downregulates FasL expression, significantly decreased the cytotoxic activity of HAECs (Fig. 3a and b). HAECs pre-infected with Adeno-FasL, which constitutively express FasL, displayed higher frequencies of target cell DNA fragmentation that was not inhibited by TNF α treatment (not shown). To further demonstrate that the cytotoxic activity of HAECs is mediated by Fas-FasL interaction, HAECs were incubated with a neutralizing anti-FasL antibody for 30 minutes prior to the co-culture assay. Treatment with antibody completely inhibited the cytotoxic activity of HAECs (Fig. 3b), similar to reports that this antibody can inhibit the cytotoxicity of both soluble FasL on W4 cells¹⁸ and simian immunodeficiency virus-infected Jurkat cells¹⁹.

FasL inhibits leukocyte extravasation

FasL expression was detected in the endothelium of the central artery of the rabbit ear as determined by co-localization with CD31, an established marker for vascular endothelial cells²⁰ (Fig. 4a). Analysis of FasL regulation was performed by applying a tourniquet at the base of the ear, to temporarily interrupt blood flow, and infusing TNF α or PBS. Following a 15 minute incubation, blood flow was restored and FasL expression was assessed by immunohistochemistry at 30 hours post-treatment. In the artery treated with TNF α , FasL

expression by the endothelium was markedly downregulated relative to the PBS treated vessels (Fig. 4b). TNF α treatment also upregulated the expression of the adhesion molecule VCAM-1 and robust T lymphocyte and macrophage infiltration was observed under these conditions.

To determine the functional significance of FasL downregulation *in vivo*, rabbit ear central arteries were temporarily isolated and incubated for 15 minutes with Adeno-FasL 12 hours prior to treatment with TNF α . At 30 hours post-cytokine treatment, FasL expression by the endothelium was readily detectable in the Adeno-FasL pre-treated vessels, but not in vessels pre-treated with the control construct Adeno- β gal, a replication defective adenovirus expressing β -galactosidase (Fig. 5). Though VCAM-1 expression was upregulated following treatment with TNF α , T cell and macrophage infiltration was markedly attenuated in vessels that were transduced with Adeno-FasL but not in the Adeno- β gal-transduced vessels (Fig. 5 and Table 1). In control animals, Adeno-FasL-infected vessels treated with PBS were negative for VCAM-1 expression and for T cell and macrophage infiltration (data not shown). TUNEL staining of the Adeno-FasL-treated vessels at four hours post-cytokine treatment revealed mononuclear cells undergoing apoptosis while attached to the luminal surface of endothelium, while mononuclear cell apoptosis was not detected when arteries were pre-treated with Adeno- β gal (Fig. 6).

Discussion

The recruitment of leukocytes at sites of inflammation is a multi-step process that involves tethering, rolling, firm adhesion and the migration of these cells to the subendothelial space². Though much is known about the chemoattractant and adhesion molecules that regulate leukocyte recruitment in response to bacterial infection²¹, relatively little is known about mechanisms that may actively control the transendothelial cell migration. Here we demonstrate functional FasL expression on the vascular endothelium and its downregulation upon exposure to the inflammatory cytokine TNF α . Adenovirus-mediated constitutive FasL expression by the endothelium markedly reduces the leukocyte extravasation that is induced by local treatment with TNF α . Under these conditions, VCAM-1 is upregulated resulting in the adherence of mononuclear cells, but these cells undergo apoptosis rather than diapedesis. These data suggest that the down-regulation of FasL by the endothelium is essential for TNF α -induced leukocyte extravasation.

In marked contrast to ECs, Fas-bearing VSMCs are highly sensitive to FasL-induced apoptosis, and the adenovirus construct expressing FasL is a potent inhibitor of neointimal hyperplasia when applied to arteries that have been denuded of endothelium by balloon-injury¹⁴. However, Adeno-FasL does not induce detectable apoptosis in cultured ECs nor in the endothelium of rabbit ear central arteries. Presumably ECs are resistant to Fas-mediated cell death either because they normally express relatively low levels of Fas, or they are unable to transmit an apoptosis-inducing signal following the engagement of Fas by FasL as has been suggested by others²². Thus, it is conceivable that the deregulated expression of FasL, Fas or signaling pathway components of this system may be a feature of EC dysfunction in response to injurious agents leading to inflammatory-fibroproliferative disorders of the vessel wall.

Atherosclerosis is believed to result from a chronic, detrimental immune response at localized regions within the vessel¹. Our data suggest that FasL may serve an atheroprotective function on the endothelium through its ability to induce apoptosis in mononuclear cells attempting to invade the vessel wall in the absence of normal inflammatory stimuli. Human atherosclerotic plaques contain significant amounts of T lymphocytes and macrophages^{1,23–27}. TNF α is expressed by macrophages and smooth muscle cells at the sites of vascular injury and this cytokine is thought to have an important role in the progression of atherosclerotic lesions^{28, 29}. This process is particularly evident in transplant arteriosclerosis, a robust form of

atherosclerosis that is responsible for the majority of deaths in heart transplant recipients surviving for one or more years³⁰. Our data suggest that secretion of TNF α by activated cells within an atheroma^{25,29,31} may downregulate FasL expression in adjacent normal endothelium, promoting more leukocyte extravasation and lesion growth. Consistent with this notion is the observation that blockade of TNF α reduces coronary artery neointimal formation in a rabbit model of cardiac transplant arteriosclerosis, while having no effect on the degree of myocardial rejection³². Therefore, these findings not only suggest an additional function of the vascular endothelium, but may provide insights about the pathogenesis of the atherosclerosis.

Methods

Cells and reagents

HAECs were obtained from Clonetics (San Diego, CA). HMVECs were provided by Joyce Bischoff (Children's Hospital, Boston, MA). Both HAECs and HMVECs were cultured in EGM medium (Clonetics) and were used for this study at less than 9 passages. HUVECs were isolated as described³³ and grown in medium 199 with 20% fetal bovine serum. The human T cell leukemia line, Jurkat clone E6-1, was obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI medium 1640 with 10% fetal bovine serum. Human vascular smooth muscle cells were isolated from a saphenous vein obtained during coronary bypass surgery and cultured as described previously³⁴. Recombinant human TNF α was purchased from R&D systems (Minneapolis, MN). Replication-defective adenovirus constructs encoding murine FasL (Adeno-FasL) or β -galactosidase (Adeno- β gal) under the control of cytomegalovirus promoter were constructed as described previously^{14,15}. Viral titer was measured by standard plaque assay using 293 cells.

Immunoblot analysis of regulation of FasL expression

HUVECs, at approximately 90% confluence, were incubated with basal medium containing different concentrations of TNF α (0, 0.5, 5, 25 ng/ml) for 21 h at 37 °C. Culture medium was removed by washing twice with PBS and cells lysed with a buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin and 2mM PMSF in PBS. The protein content was measured using a BCA protein assay reagent (Pierce, Rockford, IL). The cell lysates (15 μ g for each lane) were analyzed by SDS PAGE using a 10% polyacrylamide gel and were transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk in Tris-buffered saline (TBS), the membrane was incubated with an anti-human FasL mouse monoclonal antibody (1:1000, Transduction Laboratories, Lexington, KY), anti-VCAM-1 goat polyclonal antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-human α -tubulin mouse monoclonal antibody (1:2000, Calbiochem, San Diego, CA). Membranes were washed in TBS supplemented with 0.05% Tween20 (T-TBS) and incubated with horseradish peroxidase-conjugated sheep antibody to mouse Ig or goat Ig (1:3000, Amersham, Arlington Heights, IL). After washing in T-TBS, antibody binding was detected using enhanced chemiluminescence (Amersham).

Flow cytometric analysis of FasL and Fas expression on EC surfaces

HAECs, at approximately 90% confluence, were incubated with either basal medium or 25 ng/ml TNF α at 37 °C, 5% CO₂ for 7 h. HAECs were detached from culture plates using 0.5% EDTA and incubated with anti-FasL monoclonal antibody (A11, Alexis Corp., San Diego, CA) or with rat IgM. Cells were then washed and stained with FITC-conjugated anti-rat IgM antibody (Kirkegaard & Perry Laboratories, Gaithburg, MD). Immunofluorescence staining was analyzed by flow cytometry (Becton Dickinson, Mountain View, CA). To determine Fas expression, HAECs were incubated with an FITC-conjugated anti-Fas monoclonal antibody (clone UB2, Immunotech, Westbrook, ME).

Cytotoxic assay of HAECs on P815-huFas cells

The ability of FasL on ECs to induce apoptosis in Fas-positive target cells was assessed by co-incubating the mastocytoma cells P815 stably transfected with human Fas (P815-huFas, kindly provided by Douglas Green) with HAECs using a technique described previously³⁵. HAECs were plated in triplicate in 24-multiwell tissue culture plates and allowed to reach 90% confluency as a monolayer. HAECs were incubated with either basal medium or medium containing TNF α (10 ng/ml) at 37 °C for 4 h, after which HAECs were washed twice with PBS. P815-huFas cells were labeled with 10 μ Ci/ml ³H-thymidine (NEN, Boston, MA) for 24 h, washed twice in PBS and then incubated at 37 °C for 8 hours to minimize spontaneous release. P815-huFas cells were applied to HAECs at a ratio of 1:4 (HAECs:P815-huFas) in the absence or presence of the anti-FasL antibody (10 μ g/ml, clone 4H9, MBL, Nagoya, Japan), which is capable of neutralizing the cytotoxic activity of human FasL¹⁸. The 24-multiwell culture plates were centrifuged at 200g for 2 min and incubated at 37 °C for 18 h. Cells were harvested by trypsinization. The amounts of the fragmented DNA and the retained DNA were measured by liquid scintillation counting, as described³⁶. The percentage of the specific DNA fragmentation was calculated as $100 \times (\text{spontaneous} - \text{experimental}) / \text{spontaneous}$, when experimental refers to counts per min of the retained DNA in the presence of HAECs and spontaneous refers to counts per min of the retained DNA in the absence of HAECs. All values are presented as mean $\pm$ S.E.M.

Cytotoxicity was also tested by analyzing the ability of ECs to induce a DNA ladder in target cells. The P815-huFas cells were incubated with 100 μ M 5-bromo-2'-deoxy-uridine (BrdU) for 9 h. After washing in PBS, the P815-huFas cells were applied to HAECs and cultured for 18 h. The total genomic DNA from both P815-huFas cells and HAECs was prepared from a well of a 6-well plate as described³⁶ and loaded on a 1.5% agarose gel in 1 $\times$ TBE buffer. The electrophoretically separated DNA was transferred onto a nylon membrane (Hybond-N, Amersham) and UV-crosslinked. The BrdU-labeled DNA from the P815-huFas cells was detected using an anti-BrdU monoclonal antibody (clone BMC 9318, Boehringer Mannheim) as described for the immunoblot analysis.

Local administration of cytokines into rabbit ear central arteries

The effect of TNF α on the endothelial expression of FasL *in vivo* was determined using the rabbit ear central artery which is superficial and therefore easily accessible for isolation and injection³⁷. New Zealand White rabbits (3,500–4,000 g) were sedated by intramuscular injection of xylazine (5 mg/kg) and anesthetized using an intramuscular injection of ketamine (40 mg/kg) and acepromazine (1 mg/kg). The central artery of the rabbit ear was isolated by applying a tourniquet at the base of the ear, temporarily interrupting blood flow. The artery was then cannulated with a 25-gauge needle and one ml of TNF α diluted in PBS (50 ng/ml) or PBS was then infused into the isolated segment and incubated for 15 min. After the restoration of blood flow, rabbits were killed using pentobarbital at either 4 or 30 h after the treatment. The arteries were harvested and snap-frozen in OCT compound (Miles Inc., Elkhart, IN). To study the effect of constitutive FasL expression on mononuclear cell infiltration, the isolated segment of a rabbit ear artery was incubated with 1×10^7 pfu of either Adeno-FasL or Adeno- β gal for 15 min. These replication-defective adenoviral constructs express the murine FasL or *E. coli* β -galactosidase genes from the cytomegalovirus promoter¹⁴. At 12 h post-infection, the artery was treated with PBS or TNF α as described above. The experimental protocol was approved by the Institutional Animal Care and Use Committee and complied with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals".

Immunohistochemistry

Cryosections (5 μ m thick) were mounted on microscope slides and fixed in 4% paraformaldehyde for 10 min. Sections were washed twice in PBS and blocked with 5% normal

goat serum and 0.01% Triton-X in PBS for 1 h at room temperature. Biotin-conjugated monoclonal antibody to FasL (1:2000, clone A11, Alexis Corp., San Diego, CA) in PBS with 1% goat serum and 0.01% Triton-X was added to the sections for one h at room temperature. Sections were washed three times in PBS followed by the addition of alkaline phosphatase-conjugated streptavidin for 5 min at 37 °C. Sections were washed three times in PBS and antibody location was determined with the addition of Fast Red substrate (BioGenex Laboratories, San Ramon, CA). Color development was stopped by washing in distilled water. Distributions of ECs, T lymphocytes and macrophages were revealed in adjacent sections using an anti-CD31 monoclonal antibody (1:100, clone JC/70A, DAKO, CA), an anti-CD3 monoclonal antibody (1:2000, clone 6B10.2, Santa Cruz Biotech, Santa Cruz, CA) and an anti-macrophages monoclonal antibody (1:1000, clone RAM11, DAKO, Carpinteria, CA), respectively. Expression of VCAM-1 was revealed using a goat polyclonal antibody against VCAM-1 (1:1000, clone C-19, Santa Cruz Biotechnology, Santa Cruz, CA). Sections were counter-stained with hematoxylin.

TUNEL staining

TdT-mediated dUTP nick end labeling (TUNEL) technique was used to detect apoptotic cells in the rabbit ear artery. Cryosections (5 µm thick) were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4 °C. Terminal deoxynucleotidyl transferase enzyme and dUTP conjugated to a fluorescein cocktail were added to the tissue sections according to the manufacturer's specifications (Boehringer Mannheim, Indianapolis, IN). Nuclei were counterstained with Hoechst 33258 (Sigma) and mounted for fluorescence (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Specimens were examined and photographed on a Nikon Diaphot microscope.

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Fig. 1.

Regulated expression of FasL on human endothelial cells. **a**, Immunoblot analysis of FasL and VCAM-1 regulation in HUVECs. HUVECs were incubated for 21 hours with either basal medium or TNF α at 0.5 ng/ml, 5 ng/ml or 25 ng/ml. Cell lysates (15 μ g) were loaded onto 10% SDS-polyacrylamide gel and analyzed by western blotting using anti-FasL (upper panel), anti-VCAM-1 (middle) or anti- α -tubulin antibody (lower). Lysate of PMA- and ionomycin-activated Jurkat cells and HUSMCs infected with Adeno-FasL (multiplicity of infection of 100) served as positive controls for FasL. Lysate of uninfected HUSMCs were probed to demonstrate antibody specificity. **b**, Flow cytometric analysis of FasL expression on HAECs. HAECs were incubated in either basal medium (left panel) or 25 ng/ml TNF α (right panel) for six hours. Endothelial cells were detached from the culture plate with 0.5% EDTA and incubated with anti-FasL monoclonal antibody (A11, Alexis Corp., San Diego, CA) (filled curve) or with rat IgM (open curve) in PBS with 10% FBS, followed by incubation with FITC-conjugated anti-rat IgM. **c**, Flow cytometric analysis of Fas expression on HAECs and Jurkat cells. Cells were incubated with an FITC-conjugated anti-Fas monoclonal antibody (filled curve) or with an FITC-conjugated mouse IgG (open curve).

Fig. 2.

Endothelial cells are resistant to Fas-mediated cell death. HUVECs or Jurkat cells were grown in basal medium, harvested, fixed with 70% ethanol and stained with propidium iodide as described¹⁴. DNA content was analyzed by flow cytometry (*a* and *d*). HUVECs and Jurkat cells were incubated with an agonistic anti-Fas monoclonal antibody (0.5 μ g/ml, clone CH11, MBL, Japan) for 16 hours prior to harvesting and analysis (*b* and *e*). HUVECs and Jurkat cells were infected with Adeno-FasL at a multiplicity of infection of 300 for 48 hours prior to harvesting and analysis (*c* and *f*).

Fig. 3.

Cytotoxic activity of endothelial cells to P815-huFas cells. **a**, P815-huFas cells were labeled with BrdU and applied to HAEC cultures pre-treated with either basal medium or TNF α . Genomic DNA from both P815-huFas cells and HAECs was loaded on an 1.5% agarose gel (left panel). DNA was transferred onto a nylon membrane and the DNA fragmentation of P815-huFas cells was detected by a immunoblotting using anti-BrdU antibody (right panel). Lane 1, 100 bp molecular size standard; lane 2, P815-huFas cells co-cultured with HAECs; lane 3, P815-huFas cells co-cultured with HAECs which have been pre-treated with TNF α (10 ng/ml); and lane 4, P815-huFas cells alone, **b**, P815-huFas cells were labeled with ^3H -thymidine and applied to HAECs that were pre-treated with either basal medium or TNF α (10 g/ml). Cells were harvested after 18 hours co-culture and the percentage of specific DNA fragmentation was calculated as described in Methods. A neutralizing anti-human FasL antibody (4H9) was applied to HAECs 30 min. before co-culturing. All values are presented as mean $\pm$ S.E.M.

Fig. 4.

Regulated expression of FasL on vascular endothelial cell *in vivo*. **a**, A cryosection of the central artery of an untreated rabbit ear was stained for FasL and an adjacent section was stained for CD31, an antigen expressed by endothelial cells. A biotin-conjugated rat IgM was used as an isotype control for the anti-FasL antibody stain, **b**, Central rabbit ear arteries were temporarily isolated by application of a tourniquet and incubated with either PBS or TNF α (50 ng) for 15 min. Thirty hours after blood flow was restored, the arteries were harvested and snap-frozen in embedding compound and cryosections were stained for FasL, VCAM-1, CD3 (T cells) or RAM 11. M Φ , macrophages. Bar, 10 μ m.

Fig. 5. Constitutive expression of FasL prevents $\text{TNF}\alpha$ -induced T cell and macrophage infiltration. Rabbit central ear arteries were isolated and infected with either Adeno-FasL (1×10^7 pfu) or Adeno- β gal (1×10^7 pfu) for 15 min. prior to the restoration of blood flow. After 12 hours, the arteries were again isolated and incubated with $\text{TNF}\alpha$ (50 ng) for 15 min. Thirty hours after blood flow was restored, arteries were harvested and cryosections were analyzed by immunohistochemical methods for FasL, VCAM-1, T cells and macrophages as described in Methods. Bar, 10 μm .

Fig. 6.

Chromatin fragmentation in mononuclear cell adhering to the endothelium following the co-administration of Adeno-FasL and TNF α . Rabbit central ear arteries were infected with Adeno-FasL (1×10^7 pfu) or Adeno-(β gal (1×10^7 pfu) for 15 min. and, after 12 hours, incubated with TNF α (50 ng) for 15 min. Arteries were harvested four hours after TNF α treatment and stained with Hoechst 33258 (blue) to detect total chromatin and TUNEL (green) to detect fragmented chromatin. The internal elastic lamina containing autofluorescent elastin is visible with a filter specific for fluorescein. Bar, 25 μ m.

Table 1

Effect of constitutive FasL expression on mononuclear cell infiltration

	Saline	TNF α	Adeno-FasL +TNF α	Adeno- β gal +TNF α
T cell	0.2 $\pm$ 0.2	31.8 $\pm$ 12	1.5 $\pm$ 0.6	65.0 $\pm$ 23.4
Macrophage	0	4.0 $\pm$ 0.9	0.5 $\pm$ 0.5	7.0 $\pm$ 1.9

The number of mononuclear cells infiltrating into the media (Figs. 4b and 5) was counted in each cross section of the artery (n = 4 for each group).