Anti-tumor necrosis factor-α therapies attenuate adaptive arteriogenesis in the rabbit

Sebastian Grundmann, Imo Hoefer, Susann Ulusans, Niels van Royen, Stephan H. Schirmer, C. Keith Ozaki, Christoph Bode, Jan J. Piek, and Ivo Buschmann. Anti-tumor necrosis factor-α therapies attenuate adaptive arteriogenesis in the rabbit. Am J Physiol Heart Circ Physiol 289: H1497–H1505, 2005. First published May 27, 2005; doi:10.1152/ajpheart.00959.2004.—The specific antagonists of tumor necrosis factor-α (TNF-α), infliximab and etanercept, are established therapeutic agents for inflammatory diseases such as rheumatoid arthritis and Crohn’s disease. Although the importance of TNF-α in chronic inflammatory diseases is well established, little is known about its implications in the cardiovascular system. Because proliferation of arteriolar connections toward functional collateral arteries (arteriogenesis) is an inflammatory-like process, we tested in vivo the hypothesis that infliximab and etanercept have antiarteriogenic actions. Sixty-three New Zealand White rabbits underwent femoral artery occlusion and received infliximab, etanercept, or vehicle according to clinical dosage regimes. After 1 wk, collateral conductance, assessed with fluorescent microspheres, revealed significant inhibition of arteriogenesis (collateral conductance): 52.4 (SD 8.1), 35.2 (SD 7.7), and 33.3 (SD 10.1) ml·min⁻¹·100 mmHg⁻¹ with PBS, infliximab, and etanercept, respectively (P < 0.001). High-resolution angiography showed no significant differences in number of collateral arteries, but immunohistochemical analysis demonstrated a decrease in mean collateral diameter, proliferation of vascular smooth muscle cells, and reduction of leukocyte accumulation around collateral arteries in treated groups. Infliximab and etanercept bound to infiltrating leukocytes, which are important mediators of arteriogenesis. Infliximab induced monocyte apoptosis, and neither substance affected monocyte expression of the adhesion molecule Mac-1. We demonstrated that TNF-α serves as a pivotal modulator of arteriogenesis, which is attenuated by treatment with TNF-α inhibitors. Reduction of collateral conductance is most likely due to inhibition of perivascular leukocyte infiltration and subsequent lower vascular smooth muscle cell proliferation. This is the first report showing a negative influence of TNF-α inhibitors on collateral artery growth.

collateral circulation; growth factors

TUMOR NECROSIS FACTOR-α (TNF-α) is a proinflammatory cytokine that plays a crucial role in chronic inflammatory diseases, such as rheumatoid/pсорiatic arthritis and Crohn’s disease. TNF-α, secreted in particular by cells of the monocyte/macrophage lineage (4), evokes pleiotropic immunomodulatory functions, including upregulation of cellular adhesion molecules and secretion of chemokines, such as IL-8 and monocyte chemoattractant protein (MCP)-1, by endothelial cells (28). Because of the prominent role of TNF-α in inflammation, several anti-TNF-α compounds have been developed to therapeutically treat inflammatory diseases. These include the murine-human chimeric antibody against TNF-α infliximab (14) as well as the soluble p75 TNF-α receptor fusion protein etanercept (42).

Whereas the pharmacological effects of TNF-α antagonists have been extensively studied in arthritis and Crohn’s disease (2), little is known about the pharmacodynamic effects within the cardiovascular system. Levine and colleagues (24) provided convincing data demonstrating increased serum levels of TNF-α in patients with advanced heart failure and first postulated a deleterious role of TNF-α in cardiovascular disorders. Subsequent experimental (3, 5) and clinical studies (13, 39) further supported the “cytokine hypothesis” of heart failure (29).

Although several small pilot studies were initiated and demonstrated promising results for therapeutic TNF-α inhibition in patients with heart failure (12), these data could not be confirmed in large-scale randomized clinical trials, which even revealed a worsening of the disease with TNF-α antagonist treatment (10, 27). These unexpected (and, so far, unexplained) results question the paradigm of solely negative functions of TNF-α in the cardiovascular system.

Previously, we demonstrated that TNF-α acts as a positive modulator of adaptive arteriogenesis (19). In contrast to angiogenesis (the sprouting of small-caliber capillary networks), arteriogenesis results in development of larger arterial blood vessels from small preexisting anastomoses (32), independent of ischemia (11). TNF-α localizes to infiltrating macrophages (1) around proliferating arteries, and mice lacking functional TNF-α or the p55 TNF receptor show a significant reduction in collateral blood flow compared with controls (19). Although the genetic knockout of TNF-α results in a strong impairment of collateral blood vessel growth, the effects of pharmacological cytokine inhibition with the clinically used TNF-α antagonists infliximab and etanercept remain unknown. We therefore tested the effects of infliximab and etanercept on arteriogenesis in a rabbit hindlimb model of femoral artery ligation. Furthermore, we hypothesized that a potential effect might be due to changed patterns of cell adhesion molecule expression.
(37) and/or an increased apoptosis rate of monocytes (7, 26), mechanisms that are critical for adaptive arterial growth.

MATERIALS AND METHODS

Animal model. This study conforms with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). It was performed with the permission of the State of Baden-Wuerttemberg, Regierungspräsidium Freiburg, according to Section 8 of the German Law for the Protection of Animals. Sixty-three New Zealand White rabbits underwent double unilateral femoral artery ligation as previously described (18), with care taken to leave all collateral blood vessel stems intact (18 animals per group). Three animals per treatment group underwent sham operation; i.e., sutures were placed around the femoral artery but not tied. In the rabbit, hindlimb collateral arteries develop from preexisting anastomoses, from the artery profunda femoris and the artery circumflexa femoris to the artery genualis and the proximal arteria saphena, the main supplying artery to the lower limb. Following standard clinical dosage regimes applied in the ATTACK and RENEWAL trials (10, 27) for anti-TNF-α treatment in patients with heart failure, the animals received a single bolus of infliximab (5 mg/kg, n = 21) immediately after surgery or a subcutaneous injection of etanercept (0.33 mg/kg, n = 21) after surgery and again 3 days after the initial operation. A control group of 21 animals received a single intravenous bolus of vehicle (1 ml of PBS).

Hemodynamic perfusion measurements. At 7 days after the initial operation, collateral conductance was assessed in nine animals per treatment group (6 animals after femoral artery ligation and 3 animals after sham operation) as previously described (18). Briefly, the animals were anesthetized with an intramuscular injection of ketamine (4–8 mg/kg) and xylazine (8–9 mg/kg) and treated with heparin (5,000 U). A pump-driven arterial shunt was established between the right carotid artery and the abdominal aorta. The left femoral artery was acutely occluded, and perfusion measurements of the left hindlimb served as an internal control. Differently labeled fluorescent microspheres (15 μm; Molecular Probes, Leiden, The Netherlands) were injected at six different pressure levels into the shunt system, and a blood flow reference sample was withdrawn from the acutely occluded left femoral artery. To avoid interference from neurogenic vascular tonus, a continuous infusion of adenosine (1 mg·kg⁻¹·min⁻¹) guaranteed maximal vasodilation. Total shunt flow and central and peripheral perfusion pressures were recorded on a computer system. After enzymatic digestion of tissue samples, microspheres were counted in a flow cytometer (Beckman-Coulter, Epics XL-MCL, Miami, FL). Conductance indexes were calculated from the slope of the resulting flow-pressure relations (18).

Postmortem angioiography. For postmortem angioiography, hindlimbs of six animals per group were perfused with adenosine and PBS in a warmed (37°C) water bath for 1 min at 80 mmHg and then perfused for 8 min at 80 mmHg with contrast medium based on bismuth and gelatin according to a formula developed by Fulton (15). Subsequently, the contrast agent was allowed to gel on crushed ice. Only collateral arteries with a defined stem, midzone, and reentry following acutely occluded left femoral artery. To avoid interference from neurogenic vascular tonus, a continuous infusion of adenosine (1 mg·kg⁻¹·min⁻¹) guaranteed maximal vasodilation. Total shunt flow and central and peripheral perfusion pressures were recorded on a computer system. After enzymatic digestion of tissue samples, microspheres were counted in a flow cytometer (Beckman-Coulter, Epics XL-MCL, Miami, FL). Conductance indexes were calculated from the slope of the resulting flow-pressure relations (18).

Immunohistology. Frozen tissue samples from the quadriceps muscles were cut into 5-μm-thick sections, fixed in acetone, and incubated overnight with a specific anti-human antibody against TNF-α (1). Equivalent sections from the contralateral unligated hindlimb served as controls. The CD11b subunit of the Mac-1 integrin was used to detect leukocytes (mouse anti rabbit CD11b, clone 198; Serotec, Oxford, UK).

To verify specific binding of infliximab and etanercept in the rabbit, a fluorochrome antibody-labeling kit (FluoSprio 498, EMP Biotech, Berlin, Germany) was used to conjugate the IgG1 domain of both substances with an FITC-like fluorochrome. The labeled TNF-α antagonists were used as diagnostic antibodies on cryostat tissue sections from control animals in double-staining studies with TNF-α or CD11b. A mouse anti-rat Ki-67 antibody with cross-reactivity to rabbit tissue (clone MIB-1; Dako, Glastrup, Denmark) and an FITC-conjugated antibody against smooth muscle actin (Sigma, St. Louis, MO) were used to quantify vascular cell proliferation rates as percentage of Ki-67-positive nuclei per total number of vascular smooth muscle cells per section. A Cy3-labeled anti-mouse IgG1 antibody was used as secondary agent (Amersham Biosciences, Uppsala, Sweden) for TNF-α, Ki-67, and CD11b staining (1 h of incubation at room temperature). Hoechst 33342 (Molecular Probes, Eugene, OR) was used for nuclear staining. Negative controls for all immunologic detections were performed by omission of the primary antibody. For quantification of cell proliferation and leukocyte accumulation around collateral vessels, a total of 36 sections per animal were analyzed at ×400 or ×200 magnification, respectively, with ≥100 μm between the sections. For additional measurements of collateral diameter, the minimal and maximal diameters of two defined collateral arteries in the vastus intermedius quadriceps were measured in 36 sections per animal with ≥100 μm between the sections, and a mean diameter was calculated for each animal.

Flow cytometric analysis of monocyte apoptosis and monocyte CD11b expression. To evaluate the apoptosis rate of circulating monocytes, EDTA-treated blood samples were obtained 3 and 5 days after the initial operation. Monocytes were identified by CD14 antigen expression (mouse anti-human CD14, phycoerythrin conjugate, cross-reactive to rabbit; Dako), and FITC-conjugated annexin V (Alexis Biochem, Lausen, Switzerland) was used for detection of apoptotic cells. The cell population positive for annexin V and CD14 was identified as apoptotic monocytes in flow cytometric analysis and expressed as percentage of all CD14-positive monocytes.

For determination of Mac-1 adhesion molecule expression on circulating monocytes during TNF-α inhibitor treatment, expression of the Mac-1 subunit CD11b was measured with an FITC-conjugated specific antibody (monoclonal mouse anti-rabbit CD11b, clone 198; Research Diagnostics) by a double-staining method with CD14. To assess potential differences in response to leukocyte-activating agents under infliximab or etanercept treatment, heparin-treated blood samples were incubated for 2 h with 100 ng of LPS as a potent inducer of Mac-1 expression and again analyzed by fluorescence-activated cell sorting.

Statistical analysis. Values are means (SD). Differences between treatment groups were assessed using ANOVA with Bonferroni’s post hoc test for multiple comparisons. P < 0.05 was considered to be statistically significant.

RESULTS

No animal suffered gangrene or gross impairment of hindlimb function due to ligation of the femoral artery. Body weight of all animals remained stable during the treatment period.

Hemodynamic perfusion measurements. At 1 wk after unilateral femoral artery occlusion, PBS-treated animals (control group) showed an approximately fivefold increase in collateral conductance compared with the acutely occluded contralateral hindlimb: 10.5 (SD 3.2) vs. 52.4 (SD 8.1) ml·min⁻¹·100 mmHg⁻¹. Infliximab- and etanercept-treated animals demonstrated a significant (>30%) reduction of collateral conductance compared with control animals 7 days after the initial operation [11.0 (SD 2.0) vs. 35.2 (SD 7.7) ml·min⁻¹·100 mmHg⁻¹ after acute occlusion vs. 1 wk of treatment with infliximab (P < 0.001 vs. PBS) and 10.2 (SD 1.5) vs. 33.3 (SD 10.1) ml·min⁻¹·100 mmHg⁻¹ after acute occlusion vs. 1 wk of treatment with etanercept (P < 0.001 vs. PBS)], which was comparable between the two TNF-α antagonists (Fig. 1A).
Perfusion measurements of the acutely occluded left hindlimb served as an internal control and showed no difference between groups. Vascular conductance 7 days after sham operation was comparable in all treatment groups: 531.7 (SD 51.1), 533.1 (SD 37.9), and 537.7 (SD 63.6) ml·min⁻¹·100 mmHg⁻¹ for conductance without femoral artery occlusion and with PBS, infliximab, and etanercept, respectively (P = not significant).

Postmortem angiography. Postmortem angiography showed several large collateral arteries spanning from the deep femoral artery and the circumflex femoral artery to the popliteal artery, bypassing the site of femoral artery occlusion (Fig. 1B). There was no statistically significant difference in the number of detectable collateral arteries after treatment with etanercept or infliximab compared with PBS-treated controls: 17.2 (SD 1.5), 17.3 (SD 2.0), and 16.8 (SD 1.7) with PBS, infliximab, and etanercept, respectively.

Immunohistology. To verify cross-reactivity of infliximab and etanercept to the rabbit species, both substances were conjugated with an FITC-like fluorochrome and incubated on histological sections of rabbit collateral arteries. Double staining verified a localized binding of labeled infliximab and etanercept to cells in the adventitia around proliferating collateral arteries (Fig. 2). Staining with the modified infliximab and etanercept localized primarily to CD11b-positive leukocytes in the perivascular tissue (Fig. 2), which was also strongly positive for TNF-α. The proliferation index of vascular smooth muscle cells after application of infliximab or etanercept was significantly reduced compared with the PBS-treated group 7 days after femoral artery ligation (Fig. 3): 30.1 (SD 9.1), 12.9 (SD 7.5), and 12.3 (SD 3.6) %Ki-67-positive cells with PBS, infliximab, and etanercept, respectively (P < 0.05). Treatment with either TNF-α antagonist resulted in significantly fewer transmigrated CD11b-positive leukocytes around collateral arteries than in the PBS-treated group at the same time (Fig. 4). After solvent treatment with PBS, 44.6 (SD 6.2) CD11b-positive leukocytes/mm² around the collateral artery could be stained; this number decreased to 28.1 (SD 11.0) and 27.0 (SD 11.5) after infliximab and etanercept treatment, respectively.
Hematoxylin-and-eosin staining revealed no differences in collateral blood vessel morphology between the groups, but mean diameter of collateral arteries in the vastus intermedius quadriceps was significantly reduced after treatment with TNF-α inhibitors: 127.2 (SD 35.3), 97.7 (SD 15.3), and 100.5 (SD 18.5) μm with PBS, infliximab, and etanercept, respectively (P < 0.05).

Flow cytometric analysis of monocyte apoptosis and monocyte CD11b expression. Fluorochrome-labeled infliximab and etanercept bound to monocytes and neutrophils from rabbit perivascular cells that are also strongly positive for TNF-α and represent infiltrating leukocytes, as verified by CD11b staining. Single-filter images of double stainings do not reveal TNF-α expression by vascular tissue 7 days after femoral artery ligation (Ab). Overlay images show limited binding of infliximab and etanercept to leukocytes positive for TNF-α (Bd and Bf). HE, hematoxylin-eosin.
blood samples after activation with LPS in vitro and membrane permeabilization (data not shown).

Because infliximab is known to induce apoptosis of circulating monocytes (which are known to be key mediators of arteriogenesis), apoptotic cells were detected by annexin V staining of CD14-positive cells 3 and 5 days after femoral artery ligation. Only infliximab treatment resulted in a significant increase in apoptotic cells (expressed as percentage of all CD14-positive monocytes) compared with the control animals at both times: 2.2% (SD 0.8) and 4.4% (SD 2.4) with PBS and

Fig. 3. Proliferation of vascular smooth muscle cells (SMC) 7 days after femoral artery occlusion as percentage of Ki-67-positive nuclei of Hoechst 33342-positive nuclei (blue) at ×400 magnification. Immunofluorescent staining of the proliferation marker Ki-67 (red) in a double-staining study with α-smooth muscle actin as marker for vascular smooth muscle cells (green) revealed significantly fewer proliferating cells with infliximab or etanercept treatment (P < 0.05).

Fig. 4. Leukocytes accumulating around collateral arteries after treatment with TNF-α inhibitors 7 days after femoral artery occlusion at ×200 magnification. Transmigrated leukocytes (arrows) are shown in red in a double-staining study with vascular smooth muscle actin (green) and nuclear staining (blue). Treatment with infliximab or etanercept resulted in significantly fewer CD11b-positive leukocytes around collateral arteries than treatment with PBS (P < 0.05).
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TNF-α ANTAGONISTS ATTENUATE ADAPTIVE ARTERIOGENESIS

Infliximab, respectively, on day 3 (P < 0.05) and 2.4% (SD 0.8) and 5.1% (SD 2.3) with PBS and infliximab, respectively, on day 5 (P < 0.05). With etanercept treatment, apoptosis did not differ significantly from controls: 2.7% (SD 2.0) and 2.9% (SD 2.1) on days 3 and 5, respectively.

Expression of the Mac-1 subunit CD11b, which is known to be an important adhesion molecule for infiltrating leukocytes in arteriogenesis (40), was measured in rabbit blood samples 3 and 5 days after femoral artery ligation. CD11b expression on monocytes did not show any significant differences between the treatment groups and was comparable to expression levels in healthy animals without femoral artery ligation: 76.9 (SD 26.6), 69.4 (SD 10.2), and 66.2 (SD 6.1) arbitrary fluorescence units with PBS, infliximab, and etanercept, respectively, on day 3 and 62.9 (SD 10.9), 70.8 (SD 2.2), and 58.8 (SD 15.0) arbitrary fluorescence units with PBS, infliximab, and etanercept, respectively, on day 5.

To detect potential differences in integrin expression in response to inflammatory stimuli with anti-TNF treatment, blood samples were incubated with LPS in vitro, and CD11b expression was compared with that of the unstimulated sample. No significant differences between the treatment groups were detected, with an increased expression of >50% in all groups: 61.4 (SD 21.3), 52.8 (SD 10.3), and (SD 16.0) percent increase in CD11b expression with PBS, infliximab, and etanercept, respectively, on day 3 and 63.1 (SD 21.4), 53.5 (SD 17.4), and 56.7 (SD 19.7) percent increase in CD11b expression with PBS, infliximab, and etanercept, respectively, on day 5 (Fig. 5).

DISCUSSION

We report that treatment with the TNF-α antagonists infliximab and etanercept significantly inhibits collateral artery growth in the rabbit hindlimb after femoral artery occlusion. Although a prior study demonstrated a reduced arteriogenic response to blood vessel occlusion in TNF-α and TNF-α p55 receptor knockout mice (19), we now directly implicate (in a different model) TNF-α as an essential mediator of collateral blood vessel growth. Reduction of collateral conductance correlated with fewer accumulating leukocytes around collateral arteries. This finding supports the inflammatory, monocyte-driven hypothesis of arteriogenesis (33).

Arteriogenesis, the proliferation of preexisting arterioles and small arteries to functional collateral arteries after occlusion of a large blood vessel, is an inflammatory process (6). Increased levels of shear stress in newly recruited collaterals lead to the upregulation of adhesion molecules (e.g., ICAM-1) and chemoattractant factors, resulting in a perivascular accumulation of leukocytes. Infiltrating monocytes/macrophages, in particular, have been shown to exhibit an important mediation function in arteriogenesis: Collateral artery growth is directly correlated to peripheral blood monocyte concentration (17), and chemotaxis or activation of monocytes via MCP-1 or transforming growth factor-β1 results in a significant increase in conductance of the developing collateral vasculature (18, 22, 41). Little is known about the mechanisms of action by which macrophages stimulate arterial growth, but the production of inflammatory cytokines (6), growth factors, and enzymes such as matrix metalloproteinases seems to be of functional importance (8). Furthermore, previous studies showed a strong immunohistochemical staining of monocytes for TNF-α (1). The modulatory function of TNF-α seems to differ significantly between capillary proliferation and arterial growth. Inhibition of TNF-α by intramuscular transfection of soluble TNF-α receptor 1 (p55) enhances angiogenesis in ischemic tissue (36). The role of TNF-α in arteriogenesis is controversial, most likely because of a significant dose dependency (6). A complete lack of functional TNF-α or its receptor p55 leads to a severe reduction of arteriogenesis in the peripheral circulation, whereas mutation of the p75 receptor molecule does not affect collateral growth (19). In patients with coronary artery disease, a direct relation has been observed between etanercept-treated and control animals. B: expression of the Mac-1 integrin subunit CD11b, which is a known mediator of monocyte adhesion in arteriogenesis, was comparable in all groups at both time points. C: increase of CD11b expression after stimulation of rabbit blood samples with LPS showed no significant differences in monocyte response to activating agents.

Fig. 5. Monocyte apoptosis and integrin expression. A: infliximab treatment resulted in increased apoptosis of circulating monocytes (P < 0.05) 3 and 5 days after initiation of treatment. Annexin V binding did not differ significantly between etanercept-treated and control animals. B: expression of the Mac-1 integrin subunit CD11b, which is a known mediator of monocyte adhesion in arteriogenesis, was comparable in all groups at both time points. C: increase of CD11b expression after stimulation of rabbit blood samples with LPS showed no significant differences in monocyte response to activating agents.
Studies of anti-TNF-α therapy in rheumatoid arthritis and Crohn’s disease demonstrated a reduction of specific pro-inflammatory chemokines and adhesion molecules on endothelial cells necessary for monocyte migration. Reduced expression of endothelial ICAM-1 (37) and MCP-1 (38) (the most potent single stimulator of arteriogenesis identified to date) with infliximab therapy suggests suppression of the proarteriogenic cascade. ICAM-1 is the counterpart of the Mac-1 integrin on the surface monocytes, whereas endothelial-leukocyte interaction seems to be a critical step in the induction of collateral growth (20).

Because TNF-α acts on endothelial cell activation, as well as on circulating cells, we also examined systemic activation of monocytes with TNF-α antagonist treatment. Interestingly, systemic Mac-1 expression on monocytes was recently shown to be influenced by local therapy with MCP-1 (40) in mice, and arteriogenic potency partly correlates with effects on monocyte integrin expression (16). However, neither Mac-1 expression on peripheral blood monocytes nor changes in integrin levels under inflammatory stimuli were influenced by TNF-α inhibitors in this study. Another mechanism modulating arteriogenesis is the survival time of monocytes/macrophages after migration (7). In patients with Crohn’s disease, treatment with infliximab significantly increased apoptosis of circulating monocytes (26), which might at least partly explain our findings. This infliximab-induced effect could be reproduced in this study for the rabbit species, showing increased levels of apoptotic monocytes, whereas endothelial-leukocyte interaction seems to be a critical step in the induction of collateral growth (20).

In this study, we demonstrate that TNF-α antagonists attenuate adaptive arteriogenesis in pathological conditions where arterial proliferation is an unwanted effect, e.g., the recurrence of arteriovenous malformations after transluminal occlusion or the growth of large “feeding arteries” in malignant tumors.
REFERENCES


