Mechanisms of arteriogenesis: from cellular adhesion to therapeutic stimulation
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ANTI-TUMOR NECROSIS FACTOR-ALPHA THERAPIES ATTENUATE ADAPTIVE ARTERIOGENESIS

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CHAPTER 3

Abstract

**Background:** The specific antagonists of tumor necrosis factor-α (TNF-α) infliximab and etanercept are established therapeutic agents for inflammatory diseases such as rheumatoid arthritis or Crohn’s disease. Recent clinical trials investigated a potential benefit of anti-TNF-α therapy in patients with congestive heart failure. Unexpectedly, these studies revealed a worsening of the disease with TNF-α inhibition. Since TNF-α is known to be essential for the proliferation of pre-existing arteriolar connections towards functional collateral arteries (arteriogenesis), we tested *in vivo* the hypothesis that infliximab and etanercept hold anti-arteriogenic actions.

**Methods and Results:** Thirty-six rabbits underwent femoral artery occlusion and received infliximab (n=12), etanercept (n=12) or vehicle (n=12) according to clinical dosage regimes. After one week, conductance of the developing collateral vasculature was assessed with fluorescent microspheres and revealed a significant inhibition of arteriogenesis (collateral conductance [ml/min/100mmHg]: PBS: 52.39±8.14, infliximab: 35.15±7.67, etanercept: 33.31±10.10; p<0.001).

Immunohistochemical analysis showed reduced vascular smooth muscle cell proliferation and leukocyte accumulation around collateral arteries in the treated groups. Infliximab and etanercept were shown to bind to infiltrating TNF-α positive leukocytes, which are important mediators of arteriogenesis. Infliximab induced monocyte apoptosis, and both substances did not affect monocyte expression of the adhesion molecule Mac-1.

**Conclusion:** Leukocyte derived TNF-α serves as a pivotal modulator of arteriogenesis, which is attenuated with treatment with TNF-α inhibitors. Negative results in anti-TNF-α clinical trials for patients with chronic heart failure may be partially explained by the inhibition of collateral artery growth.
Introduction

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory 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, evokes pleiotropic immunomodulatory functions, including the upregulation of cellular adhesion molecules and secretion of chemokines such as IL-8 and MCP-1 by endothelial cells. Due to 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 as well as the soluble p75 TNF-α receptor fusion protein etanercept.

Whereas the pharmacological effects of TNF-α antagonists have been extensively studied in arthritis and Crohn's disease, little is known about the pharmacodynamic effects within the cardiovascular system. Levine and colleagues provided convincing data demonstrating increased serum levels of TNF-α in patients with advanced heart failure and following experimental and clinical studies further supported the "cytokine hypothesis" of heart failure.

Although several small pilot studies were initiated and demonstrated promising results for therapeutic TNF-α inhibition in patients with heart failure, these data could not be confirmed in large-scale randomized clinical trials. In the RECOVER and RENAISSANCE trials, etanercept treatment worsened the course of patients in a dose-dependent manner. The ATTACH trial, evaluating infliximab in 150 patients with moderate to severe heart failure, had to be aborted due to an increase in mortality and a higher hospitalization rate under high-dose infliximab treatment compared to placebo (7 deaths vs 0 deaths). In summary these unexpected (and so far unexplained) results raise concerns about the safety of TNF-α inhibitors, and questions paradigms detailing solely negative functions of TNF-α in chronic heart failure (CHF).

In previous studies we demonstrated that TNF-α plays an essential role during adaptive arteriogenesis. In contrast to angiogenesis (the sprouting of small caliber capillary networks), arteriogenesis results in the development of larger arterial blood vessels from small preexisting anastomoses, independent from ischemia. TNF-α localizes to infiltrating macrophages around proliferating arteries, and mice lacking functional TNF-α or the p55 TNF receptor show a significant reduction in collateral conductance compared to controls. Since coronary artery disease is the commonest cause of heart failure, and collateral artery growth is an important adaptive mechanism in these patients, we hypothesized that treatment with TNF-α inhibitors reduces arteriogenesis (and hence accelerates ischemia driven heart failure). We therefore tested the effects of infliximab and etanercept on arteriogenesis in a rabbit hindlimb model of arteriogenesis. Furthermore, we hypothesized that a potential effect might be due to changed patterns of cell adhesion molecule expression and/or an increased apoptosis rate of monocytes, critical mechanisms for adaptive arterial growth.
Material and Methods

Animal Model
This study conforms with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). After securing the appropriate institutional approvals, 36 New Zealand White rabbits underwent double unilateral femoral artery ligation as previously described\(^\text{19}\), leaving all collateral blood vessel stems intact. In this model, collateral arteries develop from preexisting anastomoses spanning from the arteria profunda femoris and the arteria circumflexa femoris to the arteria genualis and the proximal arteria saphena, the main supplying artery to the lower limb. Following standard clinical dosage regimes, the animals received a single bolus of infliximab (5 mg/kg, n=12) immediately after surgery, or a subcutaneous injection of etanercept (0.33mg/kg, n=12) after surgery and again at day three after the initial operation. A control group underwent femoral artery ligation only and received a single intravenous bolus of vehicle (1ml PBS, n=12).

Hemodynamic perfusion measurements
Seven days after treatment, assessment of collateral conductance was performed in six animals per group as previously described\(^\text{19}\). In short, the animals were anesthetized with an intramuscular injection of ketamine (4-8mg/kg) and xylazine (8-9mg/kg) and heparinized (5000 Units). A pump-driven arterial shunt was established between the right carotid artery and the abdominal aorta. Differently labeled fluorescent microspheres (15µm, Molecular Probes, Leiden, The Netherlands) were injected at six different pressure levels into the shunt system. To avoid interference from neurogenic myogenic tonus, a continuous infusion of adenosine (1mg/kg/min) guaranteed maximal vasodilation. Total shunt flow as well as central and peripheral perfusion pressures were recorded on a computer system. A reference sample was withdrawn from the acutely occluded left femoral artery, and perfusion measurements of the left hindlimb served as an internal control. After enzymatic digestion of tissue samples, microspheres were counted in a flow-cytometer (Beckman-Coulter, Epics XL-MCL, Miami, USA). Conductance indices were calculated from the slope of the resulting flow/pressure relations\(^\text{19}\).

Immunohistology
Frozen tissue samples from the quadriceps muscle were cut in sections of 5µm, fixed in acetone and incubated over night with a specific anti-human antibody against TNF-\(\alpha\)\(^\text{15}\). 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, the IgG\(_1\) domain of both substances was conjugated with a FITC-like fluorochrome, using a fluorochrome antibody labeling kit (FluoroSpin 498, EMP Biotech., Berlin,
Germany). The labeled TNF-α-Antagonists were used like diagnostic antibodies on cryostat tissue sections from control animals in a double staining with TNF-α or CD11b. Vascular cell proliferation rates were quantified as percentage of Ki67 positive nuclei per total number of vascular smooth muscle cells per section, using an mouse anti-rat Ki67 antibody with cross reactivity to rabbit tissue (Clone MIB-5, Dako, Glastrup, Denmark) and a FITC-conjugated antibody against smooth muscle actin (Sigma, St. Louis, Missouri, USA). A Cy3-labeled anti-mouse IgG1 antibody was used as secondary agent (Amersham Biosciences, Upsalla, Sweden) for TNF-α, Ki67 and CD11b staining (Incubation 1h at room temperature). Nuclear staining was performed with Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA).

Negative controls for all immunological 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 a magnification of 200 with a minimum of 100μm between the sections.

Flow cytometric analysis of monocyte apoptosis, monocyte CD11b expression and TNF-α-inhibitor binding

To evaluate the apoptosis rate of circulating monocytes, EDTA blood samples of the treated animals were obtained at day three after the initial operation. Monocytes were identified by CD14 antigen expression (mouse anti-human CD14, PE conjugate, cross-reactive to rabbit, Dako, Glastrup, Denmark) 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 a FITC conjugated specific antibody (monoclonal mouse anti-rabbit CD11b, Clone 198, Research Diagnostics, Flanders, USA) in a double staining with CD14. To assess potential differences in response to leukocyte activating agents under infliximab or etanercept treatment, heparin blood samples were incubated for two hours with 100ng of LPS as a potent inducer of Mac-1 expression and again submitted to FACS-analysis.

Statistical analysis

Data are described as mean ± S.D. Differences between treatment groups were assessed using ANOVA with Bonferroni post hoc test for multiple comparisons.

Results

No animal suffered gangrene or gross impairment of hindlimb function due to the ligation of the femoral artery. Body weight of all animals remained stable during the treatment period.
Hemodynamic perfusion measurements
One week after unilateral femoral artery occlusion, PBS treated animals (control group) showed an approximately five-fold increase of collateral conductance compared to the acutely occluded contralateral hindlimb (10.5±3.15 ml/min/100mmHg after acute femoral artery ligation, 52.39±8.14 ml/min/100mmHg following one week treatment with vehicle). Infliximab as well as etanercept treated animals demonstrated a significant reduction of collateral conductance of more than 30% compared to control animals seven days after the initial operation (infliximab: 35.15±7.67 ml/min/100mmHg, etanercept: 33.31±10.10 ml/min/100mmHg, p<0.001 versus control), which was comparable between the two TNF-α antagonists (Figure 1).

![Figure 1](image)

**Figure 1:** Collateral artery development under treatment with TNF-α inhibitors. Conductance of the collateral vascular system was derived from pressure/flow relations at six different pressure levels under maximum vasodilation with adenosin. Collateral conductance is significantly reduced after treatment infliximab or etanercept compared to PBS treated controls (P<0.001).

Immunohistology
To verify a cross-reactivity of infliximab and etanercept to the rabbit species, both substances were conjugated with a 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 (Figure 2). Staining with the modified infliximab and etanercept localized primarily to CD11b positive leukocytes in the peri-vascular tissue (Figure 2), which were also strongly positive for TNF-α. The proliferation index of vascular smooth muscle cells was significantly reduced after infliximab or etanercept application compared to the PBS-treated group (Figure 3): [%Ki67+...
Cells] PBS: 30.13±9.07, infliximab: 12.87±7.49, etanercept: 12.28±3.61, p<0.05.

Treatment with either TNF-α antagonist resulted in a significantly lower number of transmigrated CD11b positive leukocytes around collateral arteries compared to the PBS treated group (Figure 4). Whereas after solvent treatment with PBS, 44.61±6.16 CD11b positive leukocytes could be stained per mm² around the collateral artery, this number decreased to 28.11±10.96 after infliximab and to 26.96±11.54 after etanercept treatment (p<0.05 versus control). No differences in collateral blood vessel morphology were observed between the groups on H&E staining.

A

a) Nuclear staining  
b) TNF-α  
c) Infliximab-FITC  
d) Overlay a + b + c  
e) HE staining  
f) Etanercept-FITC Overlay

B

a) Nuclear staining  
b) CD11b  
c) Etanercept-FITC
Figure 2: Binding of infliximab and etanercept in rabbit tissue. To verify specific binding of infliximab and etanercept on rabbit tissue, both agents were conjugated with a FITC-like fluorochrome and used on histological sections of rabbit collateral arteries like a diagnostic antibody in double staining with TNF-α (A) or CD11b (B). Both TNF-α inhibitors bind to peri-vascular cells that are also strongly positive for TNF-α and represent infiltrating leukocytes, as verified by CD11b staining. Single filter images of double staining with vascular smooth muscle cells and endothelial cells do not reveal TNF-α expression on day seven after femoral artery ligation (b). Overlay images show limited binding of infliximab and etanercept to leukocytes positive for TNF-α (d, f).

Figure 3: Proliferation of vascular smooth muscle cells as percentage of Ki-67 positive nuclei of Hoechst 33342 positive nuclei (blue) at 400x magnification.
Immunofluorescent staining of the proliferation marker Ki-67 (red) in a double staining with α-smooth muscle actin as marker for vascular smooth muscle cells (green) revealed a significant reduction of proliferating cells under treatment with infliximab or etanercept (P = 0.05).

Figure 4: Accumulating leukocytes around collateral arteries after treatment with TNF-α inhibitors as CD11b positive cells per mm² at 200x magnification. (A) Transmigrated leukocytes (arrows) are shown in red in a double staining with vascular smooth muscle actin (green) and nuclear staining (blue). (B) Treatment with infliximab or etanercept resulted in a significantly lower number of CD11b positive leukocytes around collateral arteries when compared with PBS treated controls (P = 0.05).

Flow cytometric analysis of monocyte apoptosis and TNF-α-inhibitor binding
Fluorochrome-labeled infliximab and etanercept bound to monocytes and neutrophils from rabbit blood samples after activation with LPS in vitro and membrane permeabilization (data not shown).
Since 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 at day three after femoral artery ligation. Only infliximab treatment resulted in a significant increase of apoptotic cells compared to the control animals, expressed as percentage of all CD14+ monocytes.
Apoptosis under etanercept treatment did not differ significantly from controls (etanercept: 2.71%±1.97). Expression of the Mac-1 subunit CD11b, which is known to be an important adhesion molecule for infiltrating leukocytes in arteriogenesis\textsuperscript{20}, was measured in rabbit blood samples at day three 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 ([fluorescent units] PBS: 76.85±26.64, infliximab: 69.35±10.16, etanercept: 66.20±6.07). To detect potential differences of integrin expression in response to inflammatory stimuli under anti-TNF treatment, blood samples were incubated with LPS \textit{in vitro} and CD11b expression was compared relative to the un-stimulated sample. No significant differences between the treatment groups were detected, with an increased expression of more than 50% in all groups ([ratio stimulated / unstimulated] PBS: 61.43±21.34, infliximab: 52.83±10.32, etanercept 57.48 ± 15.97) (Figure 5).
Figure 5: Monocyte apoptosis and integrin expression. (A) Infliximab treatment resulted in increased apoptosis of circulating monocytes ($P<0.05$), whereas annexin-V binding under etanercept therapy did not differ significantly from 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. (C) Increase of CD11b expression after stimulation of rabbit blood samples with lipopolysaccharide showed no significant differences in monocyte response to activating agents.

Discussion
We report that treatment with the TNF-α antagonists infliximab or etanercept significantly inhibits collateral artery growth in the rabbit hindlimb after femoral artery occlusion. While a prior study demonstrated a reduced arteriogenic response to blood vessel occlusion in TNF-α and TNF-α p55 receptor knockout mice, 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 numbers of accumulating leukocytes around collateral arteries. This supports the inflammatory, monocyte driven hypothesis of arteriogenesis.

According to the unexpected results of the ATTACH and RENEWAL trials, two possible explanations for the worsening of CHF upon TNF-α antagonist treatment have been discussed. An intrinsic toxicity of the used substances versus an unknown deleterious effect of TNF-α inhibition in heart failure. The first theory refers to the cytotoxic effect of infliximab on cells with membrane-bound TNF-α, which could result in a complement mediated lysis of failing cardiomyocytes. The second explanation suggests that TNF-α has not just harmful effects in heart failure and that artificial inhibition might result in loss of a beneficial quality of inflammatory cytokines.

Arteriogenesis, the proliferation of pre-existing arterioles and small arteries to functional collateral arteries after occlusion of a large blood vessel, is an inflammatory process. Increased levels of shear stress in newly recruited
collaterals lead to the upregulation of adhesion molecules (e.g. ICAM-1) and chemotactic factors, resulting in a peri-vascular accumulation of leukocytes. Infiltrating monocytes/macrophages in particular have been shown to exhibit an important mediatory function in arteriogenesis: collateral artery growth is directly correlated to peripheral blood monocyte concentration and chemotraction and activation of monocytes via MCP-1 or TGF-β1 results in a significant increase in conductance of the developing collateral vasculature. However, little is known about the mechanisms of action by which macrophages stimulate arterial growth, but the production of inflammatory cytokines, growth factors and enzymes such as matrix-metalloproteinases seem to be of functional importance. Furthermore, previous studies have shown a strong immunohistochemical staining of monocytes for TNF-α. Knockout mice lacking functional TNF-α or the p55 TNF-α receptor show a severe reduction of arteriogenesis in the peripheral circulation, while mutation of the p75 receptor molecule does not effect collateral growth. These findings suggest an essential role of TNF-α in the inflammatory process of arteriogenesis, mediated via the p55 receptor.

Studies of anti-TNF-α therapy in rheumatoid 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 and MCP-1 (the most potent single stimulator of arteriogenesis identified to date) upon infliximab therapy suggests suppression of the pro-arteriogenic cascade. ICAM-1 is the counterpart of the Mac-1 integrin on the surface monocytes, while endothelial-leukocyte interaction seems to be a critical step in the induction of collateral growth.

Interestingly, a systemic Mac-1 expression on monocytes was recently shown to be influenced by local therapy with MCP-1 in mice, and arteriogenic potency partly correlates with effects on monocyte integrin expression. However, neither Mac-1 expression on peripheral blood monocytes, nor changes of integrin levels under inflammatory stimuli were influenced by TNF-α inhibiting treatment in this study. The demonstration of infliximab induced monocyte apoptosis in patients under treatment for chronic active Crohn’s disease could be reproduced in this study for the rabbit species, showing increased levels of apoptotic monocytes, as determined by annexin V staining. No such effect was seen for etanercept, making the stimulation of monocyte apoptosis unlikely to be the sole inhibiting mechanism for arteriogenesis. Both substances reduced the number of infiltrating leukocytes in the peri-vascular tissue of collateral arteries, though whether by direct inhibiting effects on leukocytes or via a reduced expression of chemotactic cytokines or adhesion molecules by vascular cells remains unknown. Prior histological studies as well as our current results localized TNF-α expression to infiltrating leukocytes and not to vascular tissue. Not so much the induction of arteriogenesis via increased shear stress and vascular response seems to be the primary target of TNF-α inhibitors, but more likely the secondary progression and stimulation of this process by monocyte infiltration. The selective binding of infliximab and etanercept to peri-vascular cells after in vitro incubation on histological sections supports this hypothesis.
Since coronary heart disease is the most frequent cause of congestive heart failure, the adaptive proliferation of collateral arteries supplying nutrient blood flow from outside the risk region to the endangered myocardium serves as important protective mechanism in patients with CHF. In this study we demonstrate, that TNF-α antagonists negatively affect adaptive arteriogenesis and thus reduce collateral blood flow to tissue at risk. Although this is probably only one of many functions of TNF-α in inflammatory cascade linked to cardiovascular disease, it might help to explain some of the discouraging results of inhibition of TNF-α in patients with heart failure. It also gives reason to speculate about a potential beneficial effect of inhibition of 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.

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