In Vivo Blockade of Tumor Necrosis Factor-α Accelerates Functional Endothelial Recovery After Balloon Angioplasty

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Background—Tumor necrosis factor-α (TNF) is expressed locally in arteries at sites of balloon injury. In vitro studies have shown that TNF inhibits cell cycle progression and induces apoptosis in endothelial cells. Accordingly, we performed a series of experiments to test the hypothesis that inhibiting TNF could accelerate endothelial recovery after angioplasty.

Methods and Results—TNF soluble receptor (TNFsr) has been shown to neutralize the actions of TNF in vitro and in vivo. Sprague-Dawley rats received TNFsr versus control IgG through an intraperitoneal injection. De-endothelializing balloon injury was then performed, and animals were killed after 1 week to evaluate re-endothelialization (Evans blue dye staining) and after 2 weeks to evaluate re-endothelialization and endothelial function. At both time points, blockade of TNF using TNFsr resulted in an increase in re-endothelialization, as measured as absolute area and percent area re-endothelialized. TNFsr also accelerated functional endothelial recovery, which manifest as an increase in nitric oxide production. Neointimal thickening was also shown inhibited.

Conclusions—In vivo blockade of TNF accelerates functional endothelial recovery after barotraumatic de-endothelializing injury. These findings suggest that locally expressed TNF acts to inhibit functional endothelial recovery after angioplasty and that transient blockade of TNF may improve the long-term success of angioplasty. (Circulation. 2001;104:1754-1756.)

Key Words: endothelium ■ angioplasty ■ restenosis ■ tumor necrosis factor

In postnatal mammals, the inner monolayer of cells that line the walls of arteries is composed almost exclusively of differentiated, nonproliferating endothelial cells. These mature endothelial cells can undergo phenotypic modulation and re-enter the cell cycle in response to certain physiological and pathological stimuli. An extreme example of pathological endothelial stimulation occurs during balloon angioplasty, in which the endothelial monolayer is mechanically abraded and largely removed. After such an injury, the normally quiescent endothelial cells adjacent to the injured area must begin proliferating and migrating to “heal” the wounded area of the vessel.1,2 Simultaneously, balloon injury induces the local expression of a cascade of growth factors and cytokines, including tumor necrosis factor-α (TNF).3,4 The potential contribution of TNF to arterial lesion formation is further suggested by the demonstration, in our laboratory and others, that TNF can induce the apoptosis of endothelial cells.3 Others have shown that TNF can also stimulate proliferation6 or induce apoptosis7 in vascular smooth muscle cells.

Given these documented potential effects of TNF, which is expressed locally at sites of balloon injury, we performed a series of experiments to test the hypothesis that in vivo blockade of TNF would accelerate endothelial recovery after angioplasty.

Methods

To test this hypothesis, we used the well-characterized rat carotid injury model.

Balloon Injury

All rats underwent balloon denudation of the carotid artery, as previously described.5

TNF Soluble Receptor Treatment

Male Sprague-Dawley rats (Charles River Labs, Wilmington, Mass) were divided into 2 groups. The treatment group consisted of 16 animals receiving intraperitoneal injections of 2.5 mg/kg TNF soluble receptor (TNFsr; generously supplied by Dr Michael B. Widmer, Immunex, Seattle, Wash), with the first dose administered before balloon injury (day 0) and subsequent doses administered every third day (days 3, 9, 12, etc) The control group consisted of 15 animals that received intraperitoneal injections of human IgG (Sigma) in an equivalent dose.

The TNFsr used was a recombinant fusion protein consisting of 2 ligand binding regions from the human p80 (TNFR2) receptor linked to the Fc region of human IgG1 (human IgG was therefore used as the control). This soluble receptor molecule has previously been shown to neutralize human, rodent, and rabbit TNF in vitro and in vivo.6 The dosage used (2.5 mg/kg IP every 3 days) was chosen on the basis of previously published studies and a pilot series performed in our laboratory.

Evaluation of Re-Endothelialization

Re-endothelialization was assessed by staining with Evans blue dye (0.5 mL of 0.5% Evans blue dye; Sigma Chemical Co.).8 To verify
that the Evans blue stain accurately depicted the presence or absence of endothelium, sections of completely or partially re-endothelialized carotid arteries (based on Evans blue appearance) were stained with antibodies to CD31, BS1 lectin, and factor VIII.

### Evaluation of Recovery of Endothelial Function
To determine if functional recovery of the endothelium was accelerated by treatment with TNFsr, the production of nitric oxide by excised arterial segments was measured using the Greiss reaction.8

### Evaluation of Intimal Hyperplasia
Neointimal thickening was evaluated by measuring the total area of neointima in longitudinal sections of elastic-trichrome–stained arteries. The area of the media was also measured, and the intima/media ratio was calculated.

### Data Analysis
Data are presented as mean ± SEM. Student’s t test was used to evaluate the differences between the 2 experimental groups. Statistical significance was assigned when \( P < 0.05 \).

### Results

#### Blockade of TNF Accelerates Endothelial Recovery After Balloon Injury
At both 1 and 2 weeks after balloon injury, the arteries of animals treated with TNFsr showed a greater area of recovered endothelium, as evidenced by Evans blue staining (Figures 1 and 2). Quantification of Re-endothelialization, as either the absolute area re-endothelialized (Figures 2A and 2C; 1 week: IgG 5.98 ± 0.61 mm\(^2\) versus TNFsr 8.34 ± 0.48 mm\(^2\), \( P < 0.02 \); 2 weeks: IgG 6.98 ± 0.45 mm\(^2\) versus TNFsr 8.72 ± 0.42 mm\(^2\), \( P < 0.03 \)) or the percentage of the originally injured area (Figures 2B and 2D; 1 week: IgG 44.8 ± 3.7% versus TNFsr 67.5 ± 3.9%, \( P < 0.03 \); 2 weeks: IgG 56.9 ± 3.7% versus TNFsr 72.2 ± 3.9%, \( P < 0.02 \)) revealed a significant acceleration of endothelial recovery in the TNFsr treatment group compared with the control group.

### Functional Endothelial Recovery Is Enhanced by TNF Blockade
Nitric oxide production by TNFsr-treated arteries was significantly greater than that in placebo-treated arteries (Figure 2E; IgG 0.008 ± 0.0003 × 10\(^{-4}\) mmol·L\(^{-1}\)·mm\(^{-2}\) versus TNFsr 0.012 ± 0.0003 × 10\(^{-4}\) mmol·L\(^{-1}\)·mm\(^{-2}\), \( P < 0.001 \)), indicating that the acceleration of anatomic endothelial recovery demonstrated above was accompanied by a similar improvement in endothelial function.
Acceleration of Re-Endothelialization by TNF Blockade Is Accompanied by Inhibition of Neointimal Formation

The intima/media ratio was significantly reduced in the TNFsr group (Figure 2F; IgG 1.175±0.08 versus TNFsr 0.906±0.06, P<0.01).

Discussion

The findings of this investigation have 2 immediate implications. First, with the clinical availability of TNF receptor antagonists, a possible means of inhibiting restenosis after angioplasty could be considered. For example, the drug used in the studies detailed above (Enbrel) is Food and Drug Administration–approved to treat rheumatoid arthritis and, therefore, has a well established safety profile. Pilot clinical studies of this approved drug could be considered.

Second, although these findings imply that TNF exerts a significant deleterious effect on endothelial recovery after angioplasty, a distinct mechanism by which the ligation of one or both of the TNF receptors results in delayed re-endothelialization is not yet defined. Clarification of this mechanism could present other, more discrete, therapeutic possibilities.

TNF is one member of a family of ligands that activate a corresponding family of type I transmembrane proteins and share a conserved cysteine-rich amino acid motif in the extracellular ligand binding domain.10 TNF was first described as a factor induced by endotoxin that was capable of causing hemorrhagic tumor necrosis, but initial expectations that TNF could be used to treat cancer were not fulfilled because of toxicity. Subsequently, a variety of actions of TNF have been identified, including the induction of apoptosis in a variety of cell types, bone resorption, T cell proliferation, insulin resistance, and protection against infection.10

TNF is expressed by vascular smooth muscle cells after balloon injury.3,4 We previously demonstrated that TNF is capable of inducing growth arrest and apoptosis of endothelial cells in vitro, providing one clear possible mechanism of its adverse effect on endothelial recovery after angioplasty.11 TNF is also capable of exerting widely ranging “activating” influences on endothelial cells, including activating mitogen-activated protein (MAP) kinase cascades, contributing to angiogenesis, inducing growth factor synthesis, modifying adhesion molecule expression regulation of nitric oxide synthesis, and inducing apoptosis.12–14 The variety of TNF effects on endothelial cells may be mediated in part by the activation of the two TNF receptors (TNFR1, also referred to as p55 or p60, and TNFR2, also referred to as p75 or p80) at different TNF concentrations. The soluble TNF receptor used in the studies detailed in the present article would abrogate TNF effects at both known receptors by making TNF less available for the ligation of either receptor. Ongoing studies in mice lacking only one of the TNF receptors may clarify the role of individual receptor subtypes in endothelial recovery.

Although TNF has long been considered a potential factor in atherogenesis,15 recently attention has focused on the potential role of TNF in the pathophysiology of heart failure.16 The present findings suggest that TNF may also negatively regulate the recovery of the endothelium after angioplasty. Data from clinical trials of intracoronary brachytherapy for the prevention of stent restenosis, suggesting that the inhibition of re-endothelialization, possibly mediated by radiation induced endothelial cell apoptosis,17 was associated with a marked increase in stent thrombosis18 has highlighted the importance of endothelial recovery in optimizing clinical outcome after percutaneous coronary intervention. The present data suggest that inhibiting TNF effects after angioplasty may represent a strategy that could be used independently for restenosis prevention or to complement other therapies, such as those targeting smooth muscle cell proliferation.

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References

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