Direct Evidence for Cytokine Involvement in Neointimal Hyperplasia

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Background—Tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) are proximal inflammatory cytokines that stimulate expression of adhesion molecules and induce synthesis of other proinflammatory cytokines. In addition, TNF-α and IL-1 influence vascular smooth muscle cell migration and proliferation in vitro. In view of the inflammatory nature of neointimal hyperplasia (NIH), we tested the hypothesis that endogenous TNF-α and IL-1 modulate low shear stress–induced NIH.

Methods and Results—Mice underwent unilateral common carotid artery (CCA) ligation. Low shear stress in the patent ligated CCA has previously been shown to result in remodeling and NIH. Reverse transcriptase–polymerase chain reaction for TNF-α and IL-1α mRNA demonstrated both TNF-α and IL-1α mRNA in ligated CCAs, whereas normal and sham-operated CCAs had none. Mice lacking functional TNF-α (TNF−/−) developed 14-fold less neointimal area than WT controls (P<0.05). p80 IL-1 type I receptor knockout (IL-1RI−/−) mice tended to develop less (7-fold, P>0.05) neointimal area than WT controls. Furthermore, no IL-1α mRNA expression was detected in CCAs from TNF−/− mice; however, TNF-α mRNA expression was found in the IL-1RI−/− mice. Mice that overexpress membrane-bound TNF-α but produce no soluble TNF-α display an accentuated fibroproliferative response to low shear stress (P<0.05).

Conclusions—These results directly demonstrate that TNF-α and IL-1 modulate NIH induced by low shear stress. NIH can proceed by way of soluble TNF-α–independent mechanisms. Specific anti–TNF-α and anti–IL-1 therapies may lessen NIH. (Circulation. 2000;102:1697-1702.)

Key Words: remodeling ■ restenosis ■ atherosclerosis ■ carotid arteries

Myofibroblast migration into the intima followed by proliferation and matrix deposition (neointimal hyperplasia, NIH) is a central feature of early atherosclerosis, restenosis, and bypass graft failure.1-3 Most prior research focused on the biochemical mechanisms of NIH, although various mechanical forces on the arterial wall can also result in this fibroproliferative response.4 For example, intimal thickening and atherosclerosis tend to occur in areas of low wall shear stress, such as the carotid bifurcation.5 Complete signaling pathways from biomechanical fluid forces to changes in arterial cell biology have not been completely dissected.

Multiple lines of research suggest that NIH occurs by way of inflammation-dependent mechanisms.6 Circumstantial evidence links the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1) to these processes.7-11 TNF-α induces a substantial increase in expression of intercellular adhesion molecule-1 (ICAM-1) by human smooth muscle cells (SMCs) in vitro.3,12 Elevated TNF-α expression has been localized to areas of arterial injury.11,13,14 Similarly, increased levels of TNF-α precede migration of SMCs into the intima in the balloon-injured rat aorta by several days.13 TNF-α levels have been found to be elevated in atherosclerotic artery specimens.15 TNF-α–soluble receptor, a TNF-α antagonist, has been used to inhibit coronary artery neointima formation in cholesterol-fed rabbits that had previously undergone cardiac transplantation.16 However, information from this model, which relies primarily on dietary manipulation and transplant immunology, may not be relevant for other situations of arterial remodeling.

IL-1 functions as a growth factor for fibroblasts, keratinocytes, lymphocytes, and SMCs and enhances activation of B and T cells,17,18 many of the cell types implicated in inflammation and NIH. More importantly, IL-1 is an activator of endothelial cell gene expression and is known to modulate the presence of cell adhesion molecules such as E-selectin on the endothelial surface.19 Finally, both TNF-α and IL-1 increase LDL binding to endothelium and smooth muscle6,20 and increase transcription of the LDL-receptor gene.21 Whether TNF-α and IL-1 have primary direct roles in vivo in regulating low shear stress–induced NIH or are involved secondarily remains uncertain.
In view of the inflammatory characteristics of NIH and the known central signaling roles played by the proinflammatory cytokines TNF-α and IL-1, we hypothesized that NIH proceeds by way of TNF-α– and IL-1–dependent mechanisms. Specifically, we hypothesized that low shear stress would induce TNF-α and IL-1 expression and NIH and that mice lacking either functional TNF-α or IL-1 would develop less neointima than wild-type controls. Moreover, we hypothesized that TNF-α modulation of NIH can proceed by way of soluble TNF-α–independent mechanisms and that overexpression of membrane-bound TNF-α will exacerbate the hyperplastic response to low shear stress.

Methods

Overall Experimental Strategy

The absence of TNF-α and IL-1 in the quiescent artery wall and their expression after induction of low shear stress were documented by use of the reverse transcriptase–polymerase chain reaction (RT-PCR). The consequences of lack of TNF-α and IL-1 were ascertained in transgenic mice expressing null forms of these cytokines or their receptors. The dependence of signaling on soluble TNF-α was investigated in mice that produce only the membrane-bound form of TNF-α. Finally, in an effort to better define signaling relationships between TNF-α and IL-1 in low shear stress–induced NIH, TNF-α and IL-1 mRNA expression was assayed in these transgenic mice.

Murine Model of Low Shear Stress–Induced NIH

A total of 24 TNF-null mice (TNF−/−), 22 IL-1 receptor I-null mice (IL-1RI−/−),, 23 I g A −/− mice, and 101 B6×129 wild-type (WT) mice were used to carry out the following experiments. Mice were male and 7 to 13 weeks of age. Each transgenic mouse strain used was on a B6×129 background, and all (TNF−/−, IL-1RI−/−, and IgA−/−) were maintained by brother-sister matings, as were B6×129 controls. Mice were anesthetized with inhaled methoxyflurane, then placed under anesthesia with 1.25% isoflurane, and IL-1RI−/− mice were euthanized 1 week after surgery.

For morphometric studies, CCAs were perfusion-fixed over 1 hour at 60°C (annealing), and 2 minutes at 72°C (extension); and a single cycle at 94°C for 2 minutes; a variable number of cycles with 1 minute at 94°C (denaturing), 1 minute at 60°C (annealing), and 2 minutes at 72°C (extension); and a final cycle at 72°C for 7 minutes. The PCR products were electrophoresed on a 2% agarose gel containing 0.4 μg/mL ethidium bromide.

TNF-α – and IL-1–Deficient Mice

To determine the effects of the absence of TNF-α and IL-1 in low shear stress–induced NIH, TNF−/− (n=14), IL-1RI−/− (n=8), and WT (n=43) mice underwent CCA harvest and morphometric analysis 4 weeks after ligation. The same tests in control WT mice were run concurrently with each cohort of transgenic mice.

Soluble TNF-α–Deficient Mice

Transgenic IgA−/− mice contain a site-directed deletion of the amino acids +1 through +12 in the gene coding for murine TNF-α. The resultant transgenic mouse possesses a mutation at the TNF-α–converting enzyme (TACE) cleavage site and is incapable of processing mTNF-α into the soluble form. Reinsertion of the mutated gene into a TNF-α–null mouse (tnf−/−) results in a transgenic mouse (tnf−/−×tgA−/−) that contains multiple copy numbers (~50) of the tgA−/− gene and overexpresses only mTNF-α.24

Immunohistochemical Analyses

Harvested CCAs were embedded in paraffin for immunohistochemical analysis 4 weeks after ligation. α-SMC actin antibody kits (Sigma) were used for identification of SMCs within the neointima and media.
Statistical Analysis

Differences in neointima formation in WT versus TNF−/−, IL-1RI−/−, and tgA86 mice were determined by Kruskal-Wallis ANOVA on ranks and then Dunn’s multiple comparison.

Results

TNF-α Expression Due to Low Shear Stress

TNF-α mRNA was evident by RT-PCR (nonquantitative) in the ligated CCAs of WT animals at 7 days after surgery (Figure 1A). No mRNA for TNF-α was found in the unligated CCAs at 1, 7, or 28 days or in the ligated CCAs at 1 and 28 days. No baseline expression of TNF-α was found in native CCAs before operation or in sham-operated animals (Figure 1C), and results were duplicated in an independent experiment.

IL-1α Expression Due to Low Shear Stress

IL-1α mRNA was found by RT-PCR (nonquantitative) in the ligated CCAs of WT mice at 4, 7, 10, and 14 days after ligation (Figure 1B). No IL-1 mRNA was noted in unligated CCAs or in the ligated CCAs 1 day after surgery. No baseline expression of IL-1α was found in native CCAs before operation or in sham-operated animals (Figure 1C), and the results were duplicated in an independent experiment.

Lack of Endogenous TNF-α and NIH

Intimal areas for TNF−/− mice were significantly less than for WT mice (Figures 2 and 3). The mean intimal area for TNF−/− mice was 0.002±0.001 mm², compared with 0.028±0.004 mm² (mean±SEM) for WT mice (P<0.05). This 14-fold decrease in neointima formation in knockout mice was confirmed by comparison of intima-to-media ratios (0.051±0.034 for TNF−/− versus 0.984±0.148 for WT, P<0.05).

Lack of Endogenous IL-1 and NIH

Intimal areas for IL-1RI−/− mice tended to be less than those of WT mice (Figures 2 and 3), although this trend failed to reach statistical significance by multiple-comparison methods. Compared solely with the WT results by the Mann-Whitney rank-sum test, the difference is highly significant (P=0.009). The mean intimal area for IL-1RI−/− mice was 0.004±0.002 mm², compared with 0.028±0.004 mm² (mean±SEM) for WT mice. The 7-fold decrease in neointima formation in the IL-1RI−/− animals was confirmed by comparison of intima-to-media ratios (0.115±0.057 for IL-1RI−/− versus 0.984±0.148 for WT).

Lack of Soluble TNF-α and NIH

tnf-α−/+×tgA86 mice developed 2.8-fold more neointima than corresponding tnf-α−/+ WT (0.081±0.017 versus 0.028±0.004 mm²; mean intimal area±SEM, P<0.05, Figures 2 and 3). Intima-to-media ratios confirmed more intima in the tnf-α−/+×tgA86 versus tnf-α−/+ animals (2.263±0.395 versus 0.984±0.148, P<0.05). Both the tnf-α−/+×tgA86 and WT (tnf-α−/++) mice contained similar amounts and distribution of α-SMC actin–positive cells within the neointima.

IL-1 Expression in TNF−/− Mice

No IL-1 mRNA was found in either the ligated or unligated CCAs of pooled TNF−/− mice 10 days after surgery (Figure

![Image](https://circ.ahajournals.org/doi/10.1161/CIRCULATIONAHA.115.021699)
time point at which IL-1 mRNA appeared to peak in the ligated carotid of WT animals. Results were duplicated in an independent experiment.

**Figure 1C.** Lack of biologically active TNF-α or IL-1 attenuates NIH, and overexpression of membrane-bound TNF-α exacerbates NIH in a murine carotid ligation model. Intimal areas for TNF−/− mice (n=24) were 14-fold less than for WT mice (n=101, *P<0.05), and there was a trend toward attenuation (7-fold less) for IL-1RI−/− mice (n=21). tnf−α−/−×tgA86 mice (n=14) lack soluble TNF-α. Their overexpression of membrane-bound TNF-α probably accounts for exacerbated NIH compared with WT controls (*P<0.05).

**Figure 2.** Lack of biologically active TNF-α or IL-1 attenuates NIH, and overexpression of membrane-bound TNF-α exacerbates NIH in a murine carotid ligation model. Intimal areas for TNF−/− mice (n=24) were 14-fold less than for WT mice (n=101, *P<0.05), and there was a trend toward attenuation (7-fold less) for IL-1RI−/− mice (n=21). tnf−α−/−×tgA86 mice (n=14) lack soluble TNF-α. Their overexpression of membrane-bound TNF-α probably accounts for exacerbated NIH compared with WT controls (*P<0.05).

1C), the time point at which IL-1 mRNA appeared to peak in the ligated carotid of WT animals. Results were duplicated in an independent experiment.

**TNF-α Expression in IL-1RI−/− Mice**

mRNA for TNF-α was detected in the ligated CCAs of IL-1RI−/− mice 7 days after surgery, the time point at which TNF-α was present in the ligated CCAs of WT animals (Figure 1C). Results were duplicated in an independent experiment.

**Immunohistochemical Analysis**

WT controls exhibited a large amount of neointima containing α-SMC actin–positive cells. Analysis of the ligated CCAs in TNF−/− animals revealed α-SMC actin–positive cells within the small amount of neointima formed. Of the 8 IL-1RI−/− mice that survived the initial surgical ligation, only 1 carotid showed evidence of NIH (Figure 3). α-SMC actin staining of this carotid demonstrated the presence of myofibroblasts.

**Discussion**

Although both in vitro and in vivo observations correlate inflammation with the arterial wall response to “injury” (chemical or mechanical), few directly address whether the fibroproliferative response occurs by way of inflammatory pathways or whether the inflammation is secondary to some other driving mechanism. Arguments that atherosclerosis and NIH are inflammatory phenomena suggest an important role for the cytokines TNF-α and IL-1 in this process. A recently described murine model of NIH, coupled with the development of transgenic mice lacking functional TNF-α and IL-1, allowed us to more clearly identify the role of TNF-α and IL-1 in low shear stress–induced NIH. Our results of experiments in the intact animal demonstrate that lowered shear stress induces local arterial wall expression of both TNF-α and IL-1 and that without biologically active TNF-α or IL-1 signaling pathways, NIH is greatly attenuated. Lack of IL-1α mRNA expression in TNF−/− animals suggests that IL-1α induction in response to low shear stress may occur by way of TNF-α–dependent mechanisms. However, more detailed time course experiments (IL-1α message appeared before TNF-α message in the RT-PCR experiments) and investigation of IL-1β will be necessary to verify this relationship that has been noted in prior in vitro experiments.

Transgenic mice lacking functional TNF-α and IL-1 have added much to the knowledge of the properties of both TNF-α and IL-1 and their receptors. Both TNF−/− and IL-1RI−/− mice are phenotypically normal. The TNF−/− mice cannot produce biologically active TNF-α and do not die when exposed to lipopolysaccharide (LPS)–induced sepsis, and the IL-1RI−/− mice failed to respond to IL-1 in a number of assays. IL-1RI−/− mice do not exhibit an upregulation of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), or other endothelial cell adhesion molecules on stimulation with IL-1 but do express these molecules on stimulation with LPS.
TNF-α exists in 2 biologically active forms: a soluble form and a membrane-bound form. Both are capable of signaling through the TNF type 1 and 2 receptors. TNF-α is produced in the cell in a 33-kDa form. It is then modified to a 26-kDa form and inserted into the cell membrane, resulting in membrane-bound TNF-α. Membrane-bound TNF-α can then be cleaved by TACE into a 17-kDa soluble form. Transgenic *tgA* mice contain a site-directed deletion, leading to TACE resistance. Reinsertion of the mutated gene into a TNF-α-null mouse (tnf-α–/–) results in a transgenic mouse (tnf-α–/– × *tgA*) that contains multiple copy numbers (~50) of the *tgA* gene and thus overexpresses mTNF-α. The exacerbated fibroproliferative response observed in these mice may be due to this overexpression of this proinflammatory cytokine.

Potential limitations to our studies exist. The transgenic mice may compensate for defective signaling pathways by altering transcription and translation of related proteins. These proteins could have a secondary effect on low shear stress–induced signaling. Also, although the arteries were followed for NIH well past the time point at which it usually initiates in this model, we cannot rule out an extreme delay in the formation of an equivalent amount of neointima in the mice lacking proinflammatory cytokines. Working in murine models poses limitations on traditional molecular techniques. For instance, recovery of sufficient RNA for Northern analysis, even with pooled samples, is impractical. Finally, differences in platelet function may exist among the various mouse genotypes studies. We have found murine platelet studies difficult to reproducibly complete.

TNF-α signaling probably potentiates NIH due to low shear stress by multiple mechanisms. TNF-α induces expression of a host of biologically active molecules that may accentuate the fibroproliferative response to injury: ICAM-1 and other cell adhesion molecules, prostanoids, and other cytokines. At the cellular level, TNF-α expression by medial SMCs precedes their proliferation in a rabbit balloon-injury model, suggesting that TNF-α may contribute to their activation, migration, and proliferation. This may occur by way of mitogen-activated protein kinase mechanisms. Inflammatory cytokines can activate nuclear factor-κB in vitro, and nuclei of human atheroma intimal SMCs contain nuclear Rel family members p65 or p50 protein, whereas normal artery sections do not. More mediators of TNF-α signaling are being identified by molecular techniques, such as differential display. Finally, TNF-α stabilizes IL-1 mRNA, possibly accentuating IL-1–driven mechanisms of NIH.

The mechanisms by which IL-1 influences neointimal formation most likely occur at several levels. First, IL-1 may have effects at the level of cellular adhesion. In one study, E-selectin mRNA was demonstrated to be significantly elevated in the kidney, lung, and heart of normal mice after treatment with exogenous IL-1. Because mice lacking the functional p80 type I receptor do not upregulate E-selectin or VCAM-1, leukocyte rolling, firm adhesion, and diapedesis may not occur. In vitro experiments using prestimulated SMCs and normal endothelial cells showed a reduced migration of peripheral blood mononuclear cells into the subendothelial space after treatment with antibodies to IL-1. Inflammatory cells critical to NIH (such as macrophages and T helper cells) may be unable to localize to the site of injury and initiate a full inflammatory response. In addition, IL-1 is a growth factor for several cell lines and is known to enhance activation of both T and B cells. The role of T helper cells in inflammation and atherosclerosis is well defined. In the absence of IL-1, T cells may remain unactivated or only partially activated and may not be capable of contributing to and propagating the inflammatory response and subsequent NIH. IL-1 can act as a mitogen for vascular SMCs and sustain arterial wall inflammation by inducing IL-1 gene expression.

In our experiments, the IL-1RI−/− CCAs had few α-SMC actin–positive cells within the small amount of neointima (if any) present. IL-1 receptor antagonist, a member of the IL-1 family and specific for both IL-1α and IL-1β, has been shown to reduce rat vascular SMC proliferation in vitro. As additional support of a role for proinflammatory cytokines in NIH, both exogenous TNF-α and IL-1 induced neointima formation in normal porcine coronary arteries. Our data do not define whether the low shear stress–induced TNF-α and IL-1 signalings are confined to the artery wall or more systemic in scope.

In summary, these experiments in the intact animal demonstrate that TNF-α and IL-1 modulate low shear stress–induced NIH, thus providing a direct proinflammatory cytokine signaling link between biomechanical forces to a blood vessel wall and the remodeling response of the artery. The mechanical force of lowered shear stress induces local arterial wall expression of both cytokines. These signaling pathways can proceed by way of soluble TNF-α–independent mechanisms. Without biologically active TNF-α or IL-1 signaling pathways, NIH is greatly attenuated, and it is exacerbated when mTNF-α is overexpressed. Specific anti–TNF-α and anti–IL-1 therapies may lessen NIH.

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References

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