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# TNF-$\alpha$ and Shear Stress-Induced Large Artery Adaptations

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***Background.*** **Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) up-regulation has been associated with both low and high shear-induced arterial remodeling. To address this apparent paradox and to define the biology of TNF-$\alpha$ signaling in large arteries, we tested the hypotheses that differential temporal expression of TNF-$\alpha$ drives shear-regulated arterial remodeling.**

***Materials and methods.*** **Both low- and high-shear environments in the same rabbit were surgically created for common carotid arteries. Common carotid arteries ($n$ = 60 total) were harvested after d0, d1, d3, d7, and d14 and analyses included morphology, TNF-$\alpha$, and IL-10 mRNA quantitation. In separate experiments, animals received pegylated soluble TNF-$\alpha$ Type 1 receptor (PEG sTNF-RI) or vehicle via either short- or long-term dosing to define the effect of TNF-$\alpha$ blockade.**

***Results.*** **The model yielded a 14-fold shear differential ($P < 0.001$) with medial thickening under low shear ($P = 0.025$), and evidence of outward remodeling with high shear ($P = 0.007$). Low shear immediately up-regulated TNF-$\alpha$ expression ~50 fold ($P < 0.001$) at d1. Conversely, high shear-induced delayed and sustained TNF-$\alpha$ expression (22-fold at d7, $P = 0.012$; 23-fold at d14, $P = 0.007$). Both low and high shear gradually induced IL-10 expression ($P = 0.002$ and $P = 0.004$, respectively). Neither short-term (5-day) nor long-term (14-day) blockage of TNF-$\alpha$ signaling resulted in treatment-induced changes in the remodeling of low- or high-shear arteries.**

***Conclusions.*** **Shear stress differentially and temporally regulates TNF-$\alpha$ expression in remodeling large arteries. However, TNF-$\alpha$ blockage did not substantially impact the final shear-induced morphology, suggesting that large arteries can remodel in response to flow perturbations independent of TNF-$\alpha$ signaling.**



***Key Words:*** **TNF-$\alpha$; remodeling; restenosis; carotid artery.**

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## INTRODUCTION

Multiple lines of evidence link proinflammatory tumor necrosis factor-$\alpha$ (TNF-$\alpha$) signaling to arterial wall injury and the resultant occlusive vascular pathologies. TNF-$\alpha$, for instance, localizes early to areas of arterial injury in several species [1–3]. TNF-$\alpha$ induces human smooth muscle cell (SMC) expression of leukocyte homing molecules such as ICAM-1 [4], stimulates SMC migration, and facilitates matrix degradation [1]. Lack of TNF-$\alpha$ attenuates the intimal hyperplastic response [5], likely mediated through the p55 receptor and nuclear factor-kappaB [6–8]. Recent work directly implicates TNF-$\alpha$ in occlusive arterial wall adaptations not only in response to direct injury, but also to lowered wall shear stress [8].

Conversely, TNF-$\alpha$ appears to also hold a role in lumen enlargement of small arteries and arterioles during exposure to high wall shear [9]. In mice lacking functional TNF-$\alpha$, outward arterial remodeling in response to increased local wall shear stress is abrogated [10]. Supporting this finding, the TNF-$\alpha$ inhibitors infliximab and etanercept attenuate collateral conductance in a rabbit hindlimb model of arteriogenesis [11].

This apparent paradox for the role of TNF-$\alpha$ signaling in vascular remodeling may be secondary to differences in temporal expression, anatomical localization, and compartmentalization or receptor specificity [12]. These TNF-$\alpha$ signaling paradigms may also not apply to all vascular beds (e.g., large arteries *versus* arterioles). Additionally, TNF-$\alpha$ is processed from a 33-kDa precursor molecule to a 26-kDa membrane-associated form; then it is cleaved by TNF-$\alpha$ converting enzyme to soluble 17-kDa TNF-$\alpha$ [13]. Both membrane-associated and soluble TNF-$\alpha$ are biologically active, and these varied forms may have evolved to provide differential biological responses depending on the inciting circumstances. Finally, endogenous anti-inflammatory cytokines such as IL-10 may counterregulate the final biological activity of the inflammatory vascular cascades



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[14–16], though the exact role of IL-10 in arterial adaptations to shear perturbations remains undefined [17].

Delineation of cardiovascular roles of TNF-$\alpha$ signaling stands as an important task in view of the emergence of anti-TNF-$\alpha$ approaches in clinical medicine [18–23]. We thus sought to determine the temporal expression of pro- (TNF-$\alpha$) and anti-inflammatory (IL-10) cytokines in a large artery in response to increased and reduced arterial wall shear stress and to define the impact of TNF-$\alpha$ inhibition on this remodeling response. We hypothesized that the expression of TNF-$\alpha$ and IL-10 is differentially regulated by wall shear and that these differential expressions correlate with specific arterial wall adaptations. Additionally, we hypothesized that TNF-$\alpha$ blockade abrogates large-artery outward adaptations in response to increased wall shear.

## MATERIALS AND METHODS

### Rabbit Model of Common Carotid Artery High and Low Flow

This study was performed after securing appropriate institutional approval and conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Male New Zealand White rabbits (Harlan, Indianapolis, IN) (3.0–3.5 kg) underwent unilateral distal carotid artery branch ligation to create defined regions of differential wall shear [24]. Briefly, rabbits were premedicated with ketamine (Bedford Laboratories, Bedford, OH) (30 mg/kg, i.m.), intubated, and anesthetized with isoflurane. Intravenous heparin (Baster, Deerfield, IL) (1000 units) was administered, and bilateral common carotid arteries were exposed through a vertical midline cervical incision. Unilateral reduction in carotid artery flow was accomplished through placement of 8-0 silk suture (Ethicon Inc., Somerville, NJ) ligatures to completely occlude the internal carotid and three of the four primary branches of the external artery. As part of a separate protocol reported elsewhere [25], a portion of these animals also underwent vein interposition grafts in segments of the carotid artery remote from the artery used for this study. Mean blood flow rate through the arteries was recorded using an ultrasonic flow meter (2.0-mm probe, T106, Transonic Systems, Ithaca, NY) both before and after ligation of the distal arterial branches. At each specific time point after ligation (1, 3, 7, and 14 days), proximal common carotid arteries ($n = 5$ at each time point) were exposed, and bilateral flow measurements were performed. Harvested arteries were frozen in liquid $N_2$ for mRNA isolation or fixed in 10% neutral buffered formalin (Fisher, Suwannee, GA) for histological analyses.

Sham dissection of the arteries was performed in a separate experimental group ($n = 4$ at each time point) to distinguish the portion of the cytokine responses that were related to the surgical procedure rather than the flow pertubation. Unmanipulated arteries ($n = 4$) served as baseline (time zero).

*In vivo* morphology was assessed in a third experimental group harvested at baseline ($n = 6$) and 14 days ($n = 5$ for both high and low shear). Prior to surgical dissection, carotid artery segments were perfusion fixed with 2.5% glutaraldehyde (Sigma, St. Louis, MO) at 50 mm Hg via cannulation of the ascending aorta.

### Morphology Measurements and Hemodynamic Calculations

Artery segments from perfusion-fixed samples were embedded in paraffin and histological cross-sections were stained with Masson's trichrome and van Gieson's stains. Morphological analyses were completed using both *in vivo* external arterial diameter ($D_V$) and cross-sectional measurements (Axiovision version 3.1, Zeiss) on Masson and van Gieson's elastin-stained specimens. Specifically, *in vivo* lumen diameter ($D_L$), wall shear stress ($\tau$), and medial thickness were approximated using the following formulas:

$$D_L = \sqrt{D_V^2 - \frac{4(A_{EEL} - A_{IEL})}{\pi}}$$

$$\tau = \frac{32\mu Q}{\pi D_L^3}$$

$$MT = \frac{2(A_{EEL} - A_{IEL})}{P_{IEL} + P_{EEL}}$$

where $A_{EEL}$ and $A_{IEL}$ are the cross-area within the external elastic lamina (EEL) and internal elastic lamina (IEL), respectively; $P_{IEL}$ and $P_{EEL}$ are internal and external elastic lamina perimeter, respectively; $Q$ is the mean flow rate; $\mu$ is the viscosity of blood (0.035 Poise); and $MT$ represents medial thickness.

### TNF-$\alpha$ and IL-10 Expression Quantitation

Quantitative real-time two-step polymerase chain reaction (RT-PCR) was performed on paired arteries collected from all animals except the subset that underwent perfusion fixation at 14 days. Total RNA was isolated using TRI and BCP phase separation reagents according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). After treatment with DNase I (Ambion, Austin, TX), reverse transcription was complete using random hexamers (PE Applied Biosystems, Foster City, CA) to obtain a final cDNA concentration of 20 ng/$\mu$L. TaqMan RT-PCR for TNF-$\alpha$ (Table 1) and IL-10 was performed on a PE 7700 Sequence Detection System by using 200 nM forward primer, 200 nM reverse primer, 50 nM probe, and 20 ng cDNA per 25 $\mu$L reaction volume (TaqMan Universal PCR Master Mix, PE Applied Biosystems). RT-PCR was simultaneously run for $^{18}$S RNA on all individual samples as an internal control. Samples and controls were assayed in triplicate. The Comparative Ct Method was used for these experiments [26]. Individual value of $\Delta\Delta$Ct and 2 to the power of $\Delta\Delta$Ct was calculated for each sample. Cytokine mRNA is reported as fold induction over normal artery levels, with removal of induction from the surgical dissection (substraction of sham-operated values).

**TABLE 1**

**Quantitative RT-PCR Primers and Probes**

| Gene | Forward primer | Reverse primer | TaqMan probe |
|---|---|---|---|
| TNF-$\alpha$ | AGGAAGAGTCCCCAAACAACCT | GGCCCGAGAAGCTGATCTG | AGTCAACCCTGTGGCCCAGATGGTC |
| IL-10 | TGCGACAATGTCACCGATTT | TGCTGAAGGCGCTCTTCAC | ACTGCCTTGCTCTTGTTTTCACAGGG |