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TNF- α and Shear Stress Induced Large Artery Adaptations

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Abstract

Background—TNF- α upregulation has been associated with both low and high shear induced arterial remodeling. To address this apparent paradox and to define the biology of TNF- α signaling in large arteries, we tested the hypotheses that differential temporal expression of TNF- α drive shear regulated arterial remodeling.

Materials and Methods—Both low and high shear environments in the same rabbit were surgically created for common carotid arteries (CCA). CCAs (n=60 total) were harvested after d0, d1, d3, d7, d14 and analyses included morphology, TNF- α and IL-10 mRNA quantitation. In separate experiments, animals received pegylated soluble TNF- α Type I receptor (PEG sTNF-RI) or vehicle via either short or long-term dosing to define the effect of TNF- α 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 upregulated TNF- α expression ~50 fold (p<0.001) at d1. Conversely, high shear induced delayed and sustained TNF- α 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- α signaling resulted in treatment induced changes in the remodeling of low or high shear arteries.

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

Keywords

TNF- α ; remodeling; restenosis; carotid artery

INTRODUCTION

Multiple lines of evidence link pro-inflammatory tumor necrosis factor- α (TNF- α) signaling to arterial wall injury and the resultant occlusive vascular pathologies. TNF- α , for instance, localizes early to areas of arterial injury in several species[1-3]. TNF- α 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- α attenuates the intimal

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hyperplastic response[5], likely mediated through the p55 receptor and nuclear factor-kappaB [6-8]. Recent work directly implicates TNF- α in occlusive arterial wall adaptations not only in response to direct injury, but also to lowered wall shear stress[8].

Conversely, TNF- α 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- α , outward arterial remodeling in response to increased local wall shear stress is abrogated[10]. Supporting this finding, the TNF- α inhibitors infliximab and etanercept attenuate collateral conductance in a rabbit hindlimb model of arteriogenesis[11].

This apparent paradox for the role of TNF- α signaling in vascular remodeling may be secondary to differences in temporal expression, anatomic localization and compartmentalization or receptor specificity[12]. These TNF- α signaling paradigms may also not apply to all vascular beds (e.g. large arteries versus arterioles). Additionally, TNF- α is processed from a 33 kD precursor molecule to a 26 kD membrane associated form, then it is cleaved by TNF- α converting enzyme to soluble 17 kD TNF- α [13]. Both membrane associated and soluble TNF- α are biologically active, and these varied forms may have evolved to provide differential biologic responses depending on the inciting circumstances. Finally, endogenous anti-inflammatory cytokines such as IL-10 may counter-regulate the final biologic activity of the inflammatory vascular cascades[14-16], though the exact role of IL-10 in arterial adaptations to shear perturbations remains undefined[17].

Delineation of cardiovascular roles of TNF- α signaling stands as an important task in view of the emergence of anti-TNF- α approaches in clinical medicine [18-23]. We thus sought to determine the temporal expression of pro- (TNF- α) 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- α inhibition on this remodeling response. We hypothesized that the expression of TNF- α and IL-10 are differentially regulated by wall shear, and that these differential expressions correlate with specific arterial wall adaptations. Additionally, we hypothesized that TNF- α 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 (3.0-3.5 kg) underwent unilateral distal carotid artery branch ligation to create defined regions of differential wall shear[24]. Briefly, rabbits were pre-medicated with ketamine (30 mg/kg, i.m.), intubated, and anesthetized with isoflurane. Intravenous heparin (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 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 utilized 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 N₂ for mRNA isolation or fixed in 10% buffered formalin for histologic 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 perturbation. Un-manipulated 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 at 50 mmHg via cannulation of the ascending aorta.

Morphology Measurements and Hemodynamic Calculations

Artery segments from perfusion fixed samples were embedded in paraffin and histologic cross-sections stained with Masson's trichrome and van Gieson's stains. Morphologic 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 (τ), and medial thickness were approximated using the following formulas.

$$D_L = \sqrt{D_V^2 - \frac{4(A_{EEL} - A_{IEL})}{\pi}} \quad \tau = \frac{32\mu Q}{\pi D_L^3} \quad MT = \frac{2(A_{EEL} - A_{IEL})}{P_{IEL} + P_{EEL}}$$

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

TNF- α 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 manufacture's protocol (Molecular Research Center, Cincinnati, OH). After treatment with DNase I (Ambion, Austin, TX), reverse transcription was complete using random hexamers (PE Applied Biosystem, Foster City, CA) to obtain a final cDNA concentration of 20 ng/ μ l. TaqMan RT-PCR for TNF- α (Table I) 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 25ul reaction volume (TaqMan Universal PCR Master Mix; PE Applied Biosystems, Foster City, CA). RT-PCR was simultaneously run for 18S 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 (subtraction of sham operated values).

TNF Inhibition Experiments

Pegylated soluble human TNF type I receptor (PEG sTNF-RI; Amgen), a 20 kd molecule containing a homodimer of human p55 covalently linked to a polyethylene glycol backbone [27], was used to inhibit soluble TNF- α binding to receptors. Molecular modification of these pegylated receptors, through deletion of 1.4 intracellular domains, served to reduce immunogenicity while having no impact on ligand binding[28]. Due to a conserved sequence homology, the compound has been demonstrated to abrogate the adaptive immune response across a range of species, including rabbit[27;29]. Porat *et al* confirmed that human p55 is able

to recognize and bind to rabbit TNF- α , and functionally block rabbit TNF- α bioactivity. In a rabbit septic shock model induced by *E. coli* infusion, recombinant human p55 significantly reduced TNF- α bioactivity in the serum and successfully rescued animal's life, with a survival rate of 100% for treated group versus 55.6% for saline control[29].

Based in part on the TNF- α expression results, two anti-TNF- α treatment strategies were utilized. First, an intense short-term course to block the early, acute effects of TNF- α , followed by examination of the morphology at 28 days. And second longer-term intervention (designed to explore the impact of prolonged TNF- α inhibition) was started a day before wall shear perturbation and continued over the entire 14-day experimental period, with a harvest time at 14 days. Wall shear was manipulated as described above. Animals were assigned via randomized block design, and subcutaneously dosed with either PEG sTNF-R1 (10mg/kg/day) or the same volume of PBS. At harvest, proximal carotid artery segments were perfusion fixed *in situ* with 4% formalin for morphologic analysis.

Immunohistochemical Analyses

Mouse anti-RAM-11 (Dako, Carpinteria, CA) monoclonal antibody was utilized for identification of macrophages within the remodeling arteries. Briefly, frozen sections (5 μ m) were fixed with 10% neutral buffered formalin. After blockage of non-specific binding (5% serum), sections were incubated with primary antibody at 4°C overnight followed by Texas Red conjugated rabbit anti mouse IgG H&L (abcam, Cambridgeshire, UK) at room temperature for one hour. Micrographs were taken using confocal microscopy. Formalin-fixed sections were rehydrated. Sections were incubated with biotinylated (Cat#21335, Pierce) rabbit anti-PEG sTNF-R1 (provided by Amgen, Inc) at 4°C overnight. ABC and DAB kits (Vector) were applied to visualize the specific staining. Counterstain was not performed.

Statistical Analysis

Data are presented as mean value $\pm$ standard error of the mean (SEM), and statistical statements made as appropriate based on Tukey multiple comparisons, one-way ANOVA, two-way repeated measures ANOVA, and Student's paired t-tests.

RESULTS

Hemodynamic Environments and Morphological Adaptations

Detailed descriptions of many of the biomechanical, morphologic, and proliferative aspects of this model are reported elsewhere[24]. Distal branch ligation resulted in an immediate 90% decrease ($p<0.001$) of flow of the ipsilateral arteries and a 36% compensatory flow increase ($p=0.01$) in the contralateral arteries. An approximate 15-fold difference in mean flow rate was consequently observed through the 14-day perfusion period (Figure 1) and 14-fold (13.38 ± 1.4 vs 0.97 ± 0.23 dynes/cm², $p<0.001$) initial difference in wall shear was calculated. Examination of perfusion fixed, vehicle treated control arteries demonstrated a reduction in lumen diameter ($p=0.007$) and an increase in media thickness ($p=0.025$) following fourteen-day exposure to a low shear environment. Histologic evaluation revealed no evidence of intimal thickening. Geometric measurements such as lumen diameter, medial thickness, medial area, and the external elastic lamina length were the same between the un-manipulated left and right arteries, confirming no baseline differences between these vessels.

TNF- α and IL-10 mRNA Expression Quantitation

After subtraction of sham operated values to isolate the effects of wall shear, TNF- α mRNA in low wall shear arteries was rapidly induced almost 50-fold beyond sham operated arteries (48.5 ± 17.3 , Figure 2). Low shear overall significantly upregulated TNF- α mRNA ($p=0.005$ by

two way ANOVA), with the greatest induction one day after placement of the low shear environment ($p < 0.001$ by Tukey multiple comparison). High shear, in contrast, yielded delayed induction of TNF- α mRNA, with levels not significantly elevated until 7 ($p = 0.012$) and 14 ($p = 0.007$) days after an acute wall shear stress increase.

In contrast, alterations in wall shear led to a delayed upregulation of IL-10 expression ($p = 0.002$ and $p = 0.004$ for low and high shear, respectively), with the greatest induction at 14 days. No significant difference in the IL-10 response was identified as a function of shear.

Immunohistochemical Analyses

No RAM-11 positive cells were found either on the surface of the lumen or within the media for any time point or shear situation. Sparse RAM-11 positively stained cells were identified in the adventitia, again without notable temporal or shear dependence.

Anti-TNF- α Interventions

None of the PEG sTNF-R1 treated animals exhibited clinically detectable side effects or complications as measured by body temperature, change in weight, and feeding. However, small superficial skin ulcers associated with the neck incision occurred in two long-term PEG sTNF-R1 treated animals, and these were present at the time of harvest.

Compared to the vehicle controls, early short-term blockage of TNF- α signaling did not significantly change the pattern of arterial wall response at 28-days to the shear manipulations (Figure 3). For both PEG sTNF-R1 and vehicle treated arteries, increased wall shear resulted in significant dilation, while decreased wall shear caused negative remodeling, as reflected by distinct changes in lumen diameter, medial thickness, and EEL length between low shear and high shear arteries. In addition to the similar response pattern, the extent to which the arteries adapted to changes in wall shear was not significantly different between treated and vehicle control groups for both low and high shear arteries.

Under the long-term treatment conditions, vehicle treated arteries remodeled similar to controls. Negative adaptations were not observed under the conditions of this experiment, probably related to the shorter time frame (14 days) of vessel exposure to the altered wall shear.

PEG sTNF-R1 localized to the perivascular cells of small arterioles, venules and capillaries within the adventitia. Large artery endothelium, medial smooth muscle cells and most adventitial fibroblasts were negative for PEG sTNF-R1 staining, which co-localizes with endogenous TNF- α (Figure 4). No substantial difference in the number of PEG sTNF-R1 positive vessels was observed between low and high shear arteries.

Discussion

Inflammatory mechanisms dominate contemporary theories of the pathophysiology of occlusive arterial vascular disease[8;30], and these lesions tend to occur at areas of decreased wall shear stress[31]. Broad anti-inflammatory strategies have been advocated to attenuate occlusive pathologies[32]. Conversely, pro-inflammatory messengers such as TNF- α and monocytes co-localize to areas of active positive, outward remodeling, which usually occurs in the setting of increased wall shear stress[9;33;34]. Lack of these inflammatory signaling pathways abrogates outward arterial remodeling in response to increased local wall shear[10; 35]. Pro-inflammatory approaches have been offered to promote arteriogenesis, or compensatory outward remodeling[36-39]. However, a troublesome side effect of arteriogenic therapies may be acceleration of occlusive inflammatory vascular processes such as atherosclerosis[40]. Conversely, anti-inflammatory strategies may attenuate the hosts' endogenous response to vascular occlusions, that is, recruitment of collateral vessels.[11]

Detailed knowledge regarding the inflammatory mediators of the variety of hemodynamically driven arterial wall adaptations is critical to understand appropriate vascular therapies. The dynamics of these mediators (and the impact of their blockade) in larger conductance arteries has not been previously defined.

Thus, we determined the temporal expression of TNF- α and IL-10 in response to increased and decreased arterial wall shear stress in larger arteries. These two cytokines studied are associated with pharmacologic agents that have progressed to clinical trials[18;41-43]. The model employed offers immediate and distinct flow environments, and clinically relevant changes in flow along with the statistical power of high and low flow within the same animal. Consistent with prior experimental systems and as shown in Figure 3, high flow and low flow arteries remodeled in opposite directions. These hemodynamics, combined with the marked changes in arterial morphology, provide a useful *in vivo* tool for determining the molecular mediators and time course of large artery remodeling.

The current results confirm that TNF- α expression increases in both low[8] and high[9] shear stress settings. However, a novel revelation is that low shear stress acutely upregulates TNF- α dramatically within the first day after flow reduction, while the sustained TNF- α induction in the high wall shear stress setting does not occur until approximately a week later. While there appears to be an immediate reduction in basal TNF- α expression in the setting of high shear, this finding is probably of limited biologic significance since baseline TNF- α activity is essentially zero. We do not provide evidence that this differential temporal expression stands as the sole mechanism for regulation, and multiple other factors such as anatomic localization and compartmentalization, receptor specificity[12], or membrane vs soluble TNF- α [44] signaling have not been excluded. However, these differential temporal windows offer strategies for appropriately timed pro- or anti- TNF- α therapies to modulate arterial adaptations.

The short-term anti-TNF- α regimen demonstrated that blockage of the early acute TNF- α signaling results in limited impact on the subsequent remodeling. Under the conditions of long-term TNF- α blockade, even the vehicle control group did not demonstrate significant remodeling, suggesting that for this model, at least 28 days of flow perturbation is necessary to drive measurable morphologic changes. The data are included to support this concept, and to confirm no drastic large artery changes with longer-term anti-TNF- α therapy.

PEG sTNF-RI was not detected via immunologic staining in the intima and media, suggesting that TNF- α detected in the mRNA assay does not accumulate in either endothelium or medial smooth muscle cells. Noteworthy is the localization of PEG sTNF-RI (thus TNF- α protein) to small arterioles in the adventitia. This distribution feature supports a role for TNF- α in smaller vessel arteriogenesis, which is consistent with our previous observations and others[9;10].

IL-10 is expressed in human occlusive vascular lesions and is associated with decreased signs of inflammation. For example, it promotes synthesis of functional TNF- α inhibitors[45;46] and IL-1[47]. Several lines of evidence suggest a protective role for IL-10 with regards to occlusive vascular disease[16;48;49], and IL-10 has been proposed as an “immunologic scalpel” for atherosclerosis[50], though the exact role of IL-10 in low shear stress arterial wall adaptations remains unclear[17]. Both low and high shear stress induced IL-10 expression. Notably intimal hyperplasia does not develop in the rabbit low shear stress model (even with complete carotid occlusion, data not shown). Future research into other systems in which the blood vessel responds via development of intimal hyperplasia may decipher whether the IL-10 is playing a protective role under the conditions of the current experiment.

The current work has clear experimental and model limitations that give reason for interpretive caution. Absolute confirmation that the decoy effectively abrogates a biologically significant amount of TNF- α in rabbits is not directly addressed by our results, but this has been defined

by others[29]. Despite systemically high dosages, PEG sTNF-R1 binding to the rabbit TNF- α may have been biologically incomplete. Possibly surgery itself drives systemic (non artery wall source) TNF- α levels that overwhelm the pharmacologic agent, however our dosage (10mg/kg) was twice as much as the effective dosage (5mg/kg) for the treatment of rabbit sepsis [29]. The short-term dosing regimen opens the possibility that a “catch up” phenomenon may be taking place[51;52]. The current findings—defined shear and time dependent cytokine expression patterns whose roles are not clearly delineated by direct *in vivo* blockade, probably point to a sophisticated biology for TNF- α and its pharmacologic blockage.

In summary, low shear uniquely upregulates large artery wall TNF- α acutely (associated with negative remodeling), while high shear results in delayed and sustained expression as the artery outwardly remodels. Both low and high shear perturbations gradually induce IL-10 mRNA independent of wall shear. For small arteries and arterioles, these temporal pro- and anti-inflammatory cytokine dynamics stand as potential mechanisms for differential modulation of arterial wall adaptations to altered shear stress, and offer strategies for appropriately timed pro- or anti-inflammatory therapies to modify arterial wall adaptations. However, anti-TNF- α approaches such as those tested here appear to hold no substantial detrimental effects on flow induced large artery outward remodeling, nor do they abrogate the negative large artery remodeling associated with low wall shear.

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Figure 1. Common carotid artery flow rates over time for the surgically created distinct flow environments (n=5 per group).

Figure 2.

Fold induction of carotid mRNA expression after subtraction of effects due to surgical manipulation (n=5 per group). Low shear significantly induces TNF- α acutely (day 1), while the upregulation is relatively delayed (day 7-14) but sustained in arteries exposed to high wall shear. The high shear TNF- α pattern is similar to that of IL-10, which was overall not shear dependent.

Figure 3.

Summary of morphometry for short-term (n=8 per group; 28 day harvest) and long-term (n=5-6 per group; 14 day harvest) PEG sTNF-RI treated arteries. Un-manipulated left (LCCA) and right (RCCA) common carotid arteries are included to demonstrate the geometric similarity between LCCA and RCCA. At 28 days arteries had distinctly remodeled under differential wall shear force. Blocking just the early spike of TNF- α signal exerted no significant impact on the arterial wall remodeling (short term), nor did longer term TNF- α blockade. At 14 days, even the long-term vehicle control group did not demonstrate significant remodeling—the long-term data are included to confirm no dramatic large artery changes with longer-term anti-

TNF- α therapy. * $p < 0.05$, high shear arteries vs low shear arteries in lumen diameter, medial thickness, and EEL length for both treated and vehicle groups.

Figure 4. Immunohistochemistry assay for PEG sTNF-RI in the cross sections of a high shear long-term treated artery. PEG sTNF-RI was not detected in either the luminal surface/endothelium or medial smooth muscle cells (A). Arrowheads and arrows indicate internal and external elastic lamina respectively. PEG sTNF-RI (and therefore TNF- α protein) localizes to the endothelium and perivascular cells of small vessels within the adventitia (B).

Table 1

Cytokine primers and probes for quantitative real-time two-step polymerase chain reaction

Gene	Forward Primer	Reverse Primer	TaqMan Probe
TNF- α	AGGAAGAGTCCCCAA ACAACCT	GGCCCGAGAAGCTG ATCTG	AGTCAACCCTGTGGCC CAGATGGTC
IL-10	TGCGACAATGTCACC GATTT	TGCTGAAGGCGCTCT TCAC	ACTGCCTTGCTCTTGTT TTCACAGGG