

## Hemodynamic forces regulate mural macrophage infiltration in experimental aortic aneurysms

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### Abstract

Blood flow (BF) and wall shear stress (WSS) influence reactive oxygen species production and oxidative stress in abdominal aortic aneurysm (AAA) disease. To gain further insight into the mechanisms of hemodynamic influences on AAA inflammation, we examined aneurysm macrophage density, chemotaxis and survival under varying aortic flow conditions. Rat AAAs were created via porcine pancreatic elastase (PPE) infusion. In selected cohorts, AAA flow was increased via left common femoral arteriovenous fistula (AVF) creation (HF-AAA) or decreased by left common iliac ligation (LF-AAA). WSS was highest in HF-AAA ( $10.4 \pm 2.3$  dyn/cm<sup>2</sup> vs.  $2.4 \pm 0.4$  and  $0.5 \pm 0.2$  for NF- and LF-AAA, respectively,  $P < 0.001$ ) 7 days after PPE infusion, with reduced medial macrophage density and increased apoptosis. Adventitial macrophage density was not significantly influenced by flow. Monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) gene expression correlated with observed macrophage densities in the media and adventitia. Luminal flow conditions regulate AAA inflammation in part via influences on medial macrophage density. Hemodynamic forces may modulate AAA inflammation and diameter enlargement via direct regulation of intimal macrophage adhesion, transmural migration or survival.

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### Introduction

Abdominal aortic aneurysm (AAA) is an age-related transmural degenerative condition present in patients with classical atherosclerotic disease risk factors, including tobacco use, hypertension and male gender. Sentinel morphologic events in AAA disease include progressive medial smooth muscle cell (SMC) apoptosis, elastic lamellar dissolution and dysfunctional matrix remodeling and collagen neosynthesis in the setting of progressive expansion and ultimate aortic rupture. In animal models, and presumably in human disease, these events are initiated and sustained in the presence of a transmural monocytic inflammation (Krettek et al., 2003; Lopez-Candales et al., 1997; Pan et al., 2003).

The elaboration of pro-inflammatory cytokines and proteinases by infiltrative macrophages accelerates elastic lamellar degeneration and may promote SMC apoptosis in AAA disease (Boyle et al., 2001; Longo et al., 2002; Miller et al., 2002; Pyo et al., 2000). The importance of monocyte adhesion and migration in this pathogenetic process is underscored by the ability of CD-18 receptor inhibition to limit experimental aneurysm progression (Ricci et al., 1996). Aortic monocyte/macrophage endothelial adhesion, trans-intimal migration, medial localization and activation are promoted by regulatory proteins including monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Krishnaswamy et al., 1999; Sullivan et al., 2000; Willoughby et al., 2000).

Hemodynamic forces regulate vascular remodeling as well as monocyte adhesion and transmural migration (Nee-lamegham et al., 1998; Qian et al., 2002). High flow and resultant increased laminar shear stress promote endothelial cell (EC) retention and inhibit trans-intimal monocyte mi-

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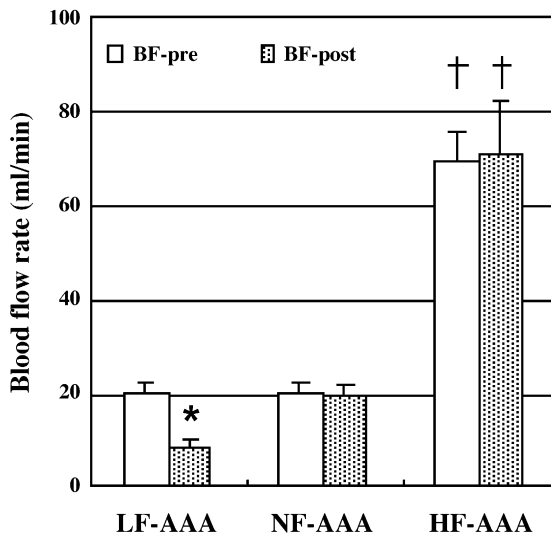


Fig. 1. Abdominal aortic blood flow before (BF-pre) or after (BF-post) PPE infusion (and iliac ligation) in low-flow (LF) group, and following PPE infusion alone in normal-flow (NF) and high-flow (HF) AAA groups; \* $P < 0.001$  between BF-pre and BF-post in LF-AAA; † $P < 0.001$  vs. LF-AAA and NF-AAA groups.

gration within adult arteries. Oscillatory and reduced antegrade shear stresses associated with resistive blood flow (BF) patterns increase EC apoptosis and intimal porosity (Masuda et al., 1999; Sho et al., 2001, 2002a,b). Shear conditions also regulate cytokine, chemokine and growth factor expression (Kosaki et al., 1998; Maus et al., 2002). Laminar unidirectional flow up-regulates expression of anti-inflammatory, anti-thrombotic and anti-adhesive gene products in cultured ECs (Wasserman et al., 2002). In contrast, oscillatory and reduced antegrade shear forces promote pro-inflammatory gene expression (Chen et al., 2003) and increase monocyte adherence to cultured bovine aortic ECs (Hsiai et al., 2001).

We have previously demonstrated the regulatory influences of flow conditions on AAA oxidative stress, including macrophage heme oxygenase-1 expression and production of pro-inflammatory reactive oxygen species (Nakahashi et al., 2002). In subsequent experiments, we also linked luminal flow conditions to the density of endothelial and medial smooth muscle cells. To further

examine the relationship among flow, inflammation, cellularity and aortic structure, we examined the response of medial and adventitial macrophage chemotaxis and apoptosis to varying hemodynamic conditions within experimental aortic aneurysms.

## Materials and methods

Male Sprague–Dawley rats (250–350 g) were used in this experiment. All experimental procedures were performed using sterile technique and were approved by the Animal Care and Use Committee of Stanford University in accordance with the Guide for the Care and Use of Laboratory Animals. Following surgical procedures, rats were recovered in separate cages with free access to food and water.

### Experimental aneurysm creation

AAAs were created via porcine pancreatic elastase (PPE) infusion as previously described (Hoshina et al., 2003; Nakahashi et al., 2002). Briefly, following sodium pentobarbital (50 mg/kg ip) injection, PE-10 tubing was advanced from the right femoral artery retrograde into a 15-mm segment of the terminal aorta. Fifteen units of type I PPE (E-1250, Sigma) in 2 ml of physiological saline solution were infused over 2 h at constant physiologic pressure (100 mm Hg). Following infusion, the catheter and any residual elastase were withdrawn and inline aortic flow restored.

### Study groups

Three AAA groups were created: high flow (HF-AAA) via left femoral arteriovenous fistula (AVF) creation; low flow (LF-AAA) via left common iliac artery ligation; and normal flow (NF-AAA) without further surgical intervention. The use of these surgical adjuncts to modify AAA hemodynamic conditions has been previously described (Hoshina et al., 2003; Nakahashi et al., 2002). All AAA rats were sacrificed at 1, 5 and 7 days after PPE infusion ( $n = 8$  per time point per group). Additional rats without AAA or flow modifications were sacrificed to provide control aortic tissue.

Table 1  
AAA diameters as functions of time and wall shear stress

	AD (mm)			WSS (dyn/cm <sup>2</sup> )		
	LF-AAA	NF-AAA	HF-AAA	LF-AAA	NF-AAA	HF-AAA
Pre-OP	1.77 ± 0.05	1.69 ± 0.03	1.94 ± 0.06*†	21.61 ± 0.77	25.48 ± 1.80	56.90 ± 4.28*†
Post-OP	2.10 ± 0.07	2.03 ± 0.07	2.30 ± 0.09*†	5.37 ± 1.05	14.21 ± 1.65*	34.13 ± 4.26*†
Day-1	2.37 ± 0.09	2.19 ± 0.09	2.39 ± 0.14	3.05 ± 0.88	11.53 ± 2.29*	31.16 ± 5.52*†
Day-5	3.66 ± 0.07	3.34 ± 0.18	3.01 ± 0.09*	1.07 ± 0.15	3.11 ± 0.45*	15.97 ± 1.81*†
Day-7	4.92 ± 0.85	3.75 ± 0.21*	3.37 ± 0.23*†	0.49 ± 0.15	2.41 ± 0.43*	11.95 ± 3.23*†

AD: aortic outer diameter (mm); WSS: wall shear stress (dyn/cm<sup>2</sup>).

\* $P < 0.01$  vs. LF-AAA group.

† $P < 0.01$  vs. NF-AAA group.

### Physical measurements

Aortic flow was measured via ultrasonic flowmetry (Transonic Systems). Aortic external diameter was measured in situ before and after PPE infusion and immediately before sacrifice using electronic microcalipers. Measurements were consistently obtained at the point of maximum transverse diameter. Wall shear stress (WSS) in dynes per square centimeter was calculated as:  $WSS \text{ (dyn/cm}^2\text{)} = 4 \times \mu \times \text{BFR}/60\pi \times r^3$ , where  $\mu$  is the blood viscosity (0.035 poise) (Nakahashi et al., 2002; Sho et al., 2002a), BFR is blood flow rate (ml/min) and  $r$  is aortic radius (cm), assuming AAA

flow to be laminar and increasing on average 10% between days 7 and 14 following AVF creation (Dalman and Hoshina, 2002). Direct flow measurements in these models were not obtained at days 5 and 7 following PPE infusion due to excessive AAA friability and rupture risk.

### Tissue harvest

At second laparotomy, the infused aortic segment was identified, controlled and dissected free of surrounding retroperitoneal tissue. After measurements, rats were sacrificed via intentional anesthetic overdose. Selected rats from

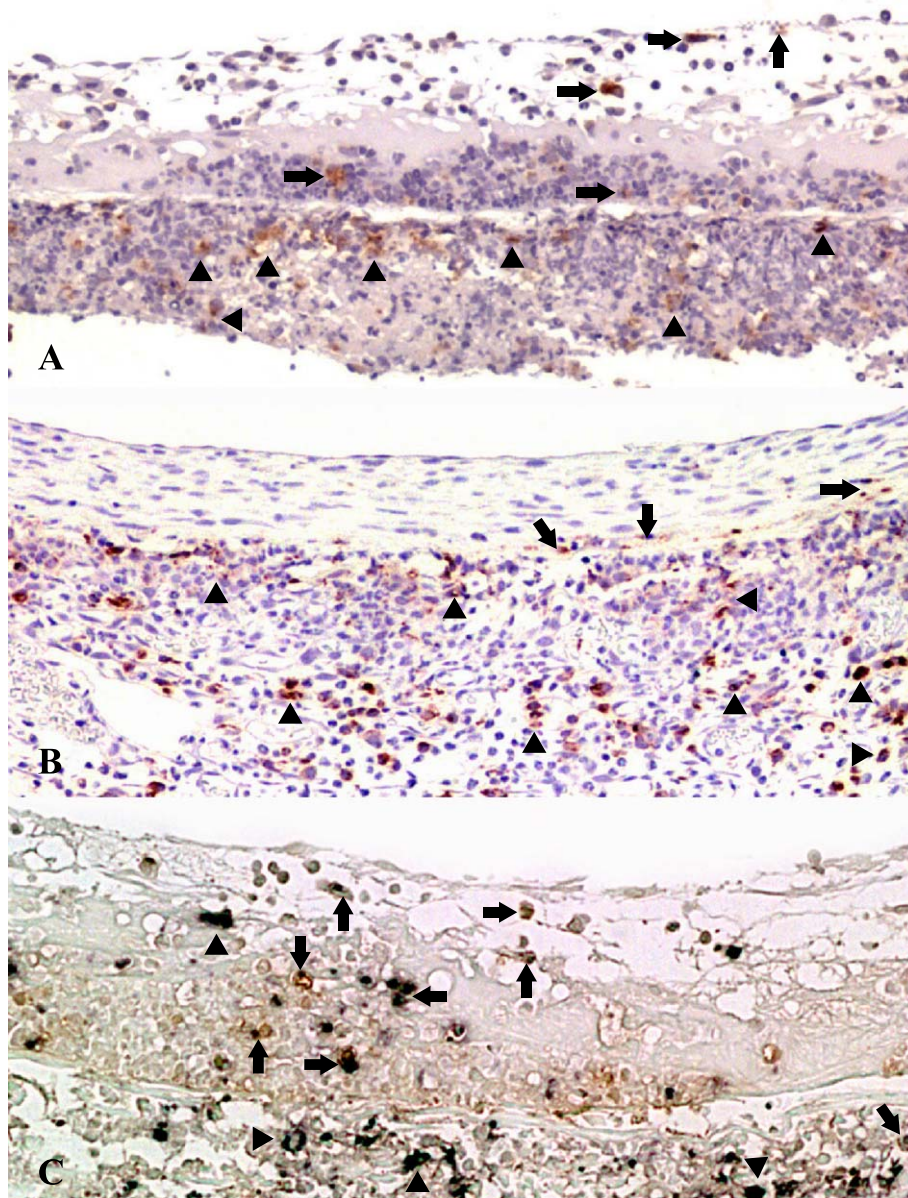


Fig. 2. Time-dependent AAA macrophage infiltration and apoptosis. (A) Post PPE infusion day 1; medial (arrows) and adventitial (arrowheads) macrophage infiltration. (B) Post PPE infusion day 7; relatively reduced medial (arrows) as compared to adventitial (arrowheads) macrophage infiltration. (C) Post PPE infusion day 1 double staining; medial and adventitial macrophages (arrowheads) and TUNEL + (apoptotic) macrophages (arrows) (original magnification  $\times 200$ ).

each cohort underwent pressure perfusion fixation with 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 30 min at 20°C at 100 mm Hg to obtain aortic tissue for morphologic analysis. AAAs destined for molecular analysis were frozen in liquid nitrogen and stored at -80°C.

*Quantitation of message for MCP-1 and GM-CSF*

Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR) was performed using the GeneAmp 7700 sequence detection system (Applied Biosystems) as previously described (Sho et al., 2002a). Briefly, high-quality total RNA was extracted using TRIzol reagent (GIBCO BRL) and was used to generate cDNA for oligo-dT oligodeoxynucleotide primer (T12-18) using the Superscript II reverse transcriptase system (Invitrogen). The following primers were designed using Primer Express software (Applied Biosystems) and synthesized: GM-CSF, 5'-tcaagaagctctgagcctcct-3' (forward), 5'-cggcctctggatggaggac-3' (reverse); MCP-1, 5'-tgcagttaatgcccaactca-3' (forward), 5'-gacacctgctgctggattc-3' (reverse). Equal amounts of cDNA were used in duplicate and amplified with the SYBR Green I Master Mix System (Applied Biosystems). PCR was performed as follows: thermal activation for 10 min at 95°C, and 40 cycles of PCR (melting for 15 s at 95°C and annealing/extension for 1 min at 60°C). A standard curve was constructed from dilution of total RNA (1:10, 1:100, 1:1000, 1:10000, 1:100000) from normal rat aorta. Amplification efficiencies were validated and normalized against β-actin. Correct PCR product size was confirmed by electrophoresis through 2% agarose gel with ethidium bromide.

*Cellular identification via immunostaining*

Mouse anti-rat ED-1 monoclonal antibody was obtained from Serotec Inc. GM-CSF rabbit IgG polyclonal and MCP-1 goat IgG polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. Biotinylated anti-mouse, rabbit and goat secondary antibodies and ABC kit were obtained from Vector Laboratories, Inc. Tissue sections were prepared for immunohistochemical staining as previously described (Sho et al., 2002a,b). Briefly, nonspecific binding was blocked with horse-serum (1:200 diluted in PBS) for 30 min at room temperature. After PBS rinse, sections were incubated with an antigen-specific primary antibody followed by biotinylated second antibody according to the manufacturer's protocol. The sections were counterstained with hematoxylin and examined via light microscopy.

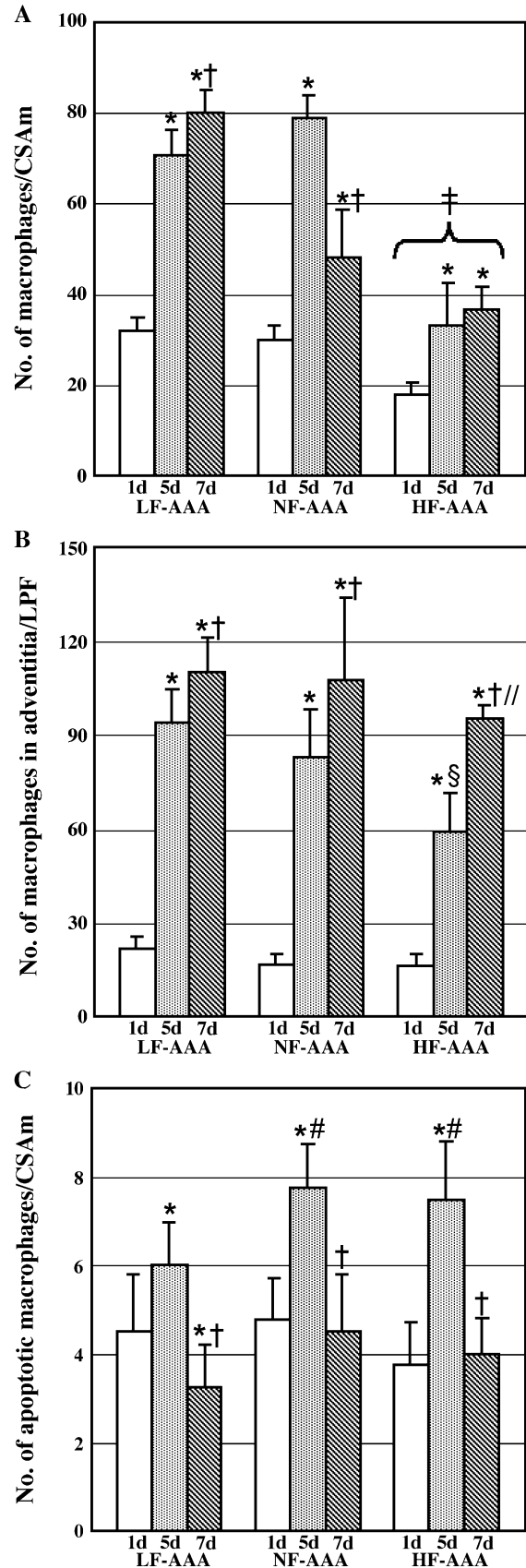


Fig. 3. Flow-dependent AAA macrophage infiltration and apoptosis. (A) Medial macrophage infiltration. (B) Adventitial macrophage infiltration. (C) Medial macrophage apoptosis. CSAm = cross-sectional area of media; LPF = low power field (×100). \**P* < 0.01 vs. day 1; †*P* < 0.01 compared to day-5; ‡*P* < 0.01 vs. LF-AAA and NF-AAA at each time point; §*P* < 0.01 vs. day 5 of LF-AAA and NF-AAA groups; ||*P* < 0.01 vs. day 7 of LF-AAA group; #*P* < 0.01 vs. day 5 of LF-AAA group.

Table 2  
Medial macrophage apoptosis index<sup>a</sup>

Day	LF-AAA	NF-AAA	HF-AAA
1	14.12 ± 3.73	15.95 ± 2.31	21.95 ± 8.54 <sup>‡, #</sup>
5	8.59 ± 1.99	9.84 ± 0.91*	23.36 ± 3.11 <sup>‡, #</sup>
7	4.09 ± 1.37*	10.04 ± 4.86* <sup>§</sup>	10.98 ± 1.81* <sup>‡, †</sup>

<sup>a</sup> TUNEL-positive macrophage / total macrophage × %.

\*  $P < 0.01$  vs. 1 day of PPE infusion.

<sup>†</sup>  $P < 0.01$  vs. 5 days of PPE infusion.

<sup>‡</sup>  $P < 0.01$  vs. LF-AAA group.

<sup>§</sup>  $P < 0.01$  vs. LF-AAA.

<sup>#</sup>  $P < 0.01$  vs. NF-AAA.

### Quantitation of AAA medial macrophage apoptosis

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining was used to detect characteristic DNA fragmentation in apoptotic cells as previously described (Hoshina et al., 2003). Briefly, tissue sections were prepared as above and incubated in 1% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. After PBS washing, DNA fragmentation was detected using an in situ apoptosis detection kit (Apop@Tag Plus Peroxidase, Intergen) following the manufacturer's protocol. Double staining was used to confirm cellular identity: sections were subsequently incubated with mouse anti-rat ED-1 monoclonal antibody for 1 h following required second antibody for 30 min and ABC method according to the manufacturer's protocol. Blue-gray color was developed by DAB kit (Vector Laboratories, Inc). TUNEL-positive nuclei stained a brown color and ED-1-positive staining was blue-gray.

### Medial and adventitial macrophage infiltration index

Medial macrophage infiltration was determined via cell counting throughout the entire media (two sections/rat) as ED-1-positive cells/cross section. Adventitial macrophage infiltration was determined via low power field (LPF, ×100) on the entire cross section. Eight LPF areas were selected on each entire cross section and ED-1-positive cell density was determined [ED-1 positive cells/LPF (two sections/case)]. All sections were counted twice and averaged to produce a final value.

### Medial macrophage apoptosis index

The medial macrophage apoptosis index was defined on medial cross section (two sections/case) as [TUNEL (+) + ED-1 (+)] cells/ED-1 (+) cells. All sections were counted twice and averaged for a final value.

### Statistical analysis

All data are reported as mean ± SD. Significance is determined via one-way ANOVA with the Bonferroni/Dunn correction for multiple comparisons.

## Results

Consistent with our previous observations using the variable flow AAA model (Hoshina et al., 2003; Nakahashi et al., 2002), AAA flow varied dramatically in response to either AVF creation or unilateral iliac ligation. Flow was not affected by the PPE infusion alone (Fig. 1, NF and HF AAA groups). AAA WSS was highest in the HF-AAA group, and decreased over time in all three groups in response to progressive aortic enlargement (Table 1). As previously reported, aortic WSS appeared to regulate AAA progression; 7 days after PPE infusion, LF-AAA was larger than NF-AAA and HF-AAA (4.9 ± 0.9 vs. 3.8 ± 0.2 and 3.4 ± 0.2 mm, respectively;  $P < 0.01$  vs. each other).

Inflammation as defined by aortic macrophage infiltration was present beginning 1 day after PPE infusion in all three groups (NF-AAA, Figs. 2A, B) and increased over the next several days in concert with progressive aneurysm enlargement. Similar to aneurysm diameter, medial macrophage

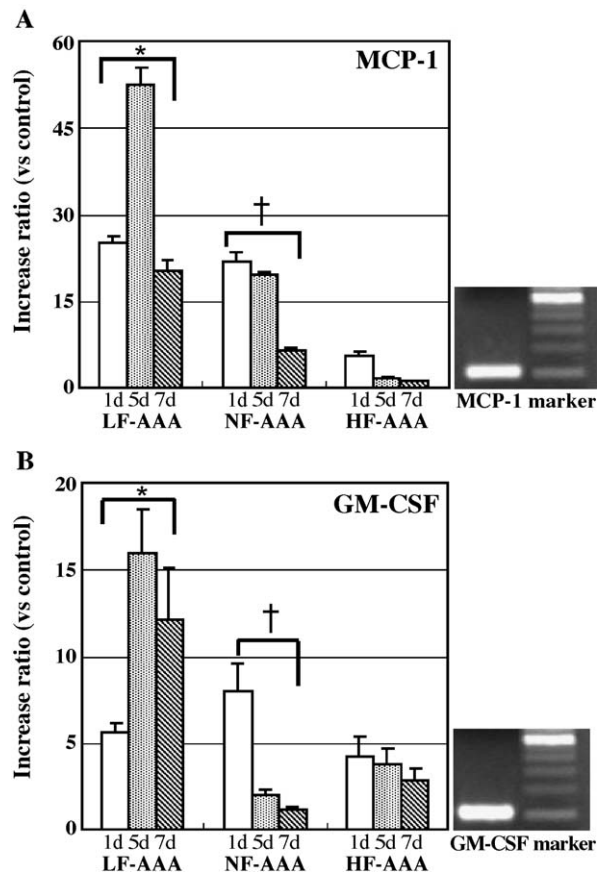


Fig. 4. Flow-dependent AAA MCP-1 and GM-CSF gene expression. (A) Reduced flow/wall shear stress (WSS) up-regulated AAA MCP-1 gene expression while high flow/WSS down-regulated AAA MCP-1 expression compared to normal flow-AAA. (B) Reduced flow/WSS up-regulated AAA GM-CSF gene expression as vs. normal flow-AAA and high flow-AAA. \* $P < 0.0001$  vs. NF-AAA and HF-AAA; <sup>†</sup> $P < 0.0001$  compared to HF-AAA. Specific PCR product bands (101 bp for MCP-1, 101 bp for GM-CSF) were detected in 2% agarose gel after 40 cycles of real time RT-PCR.

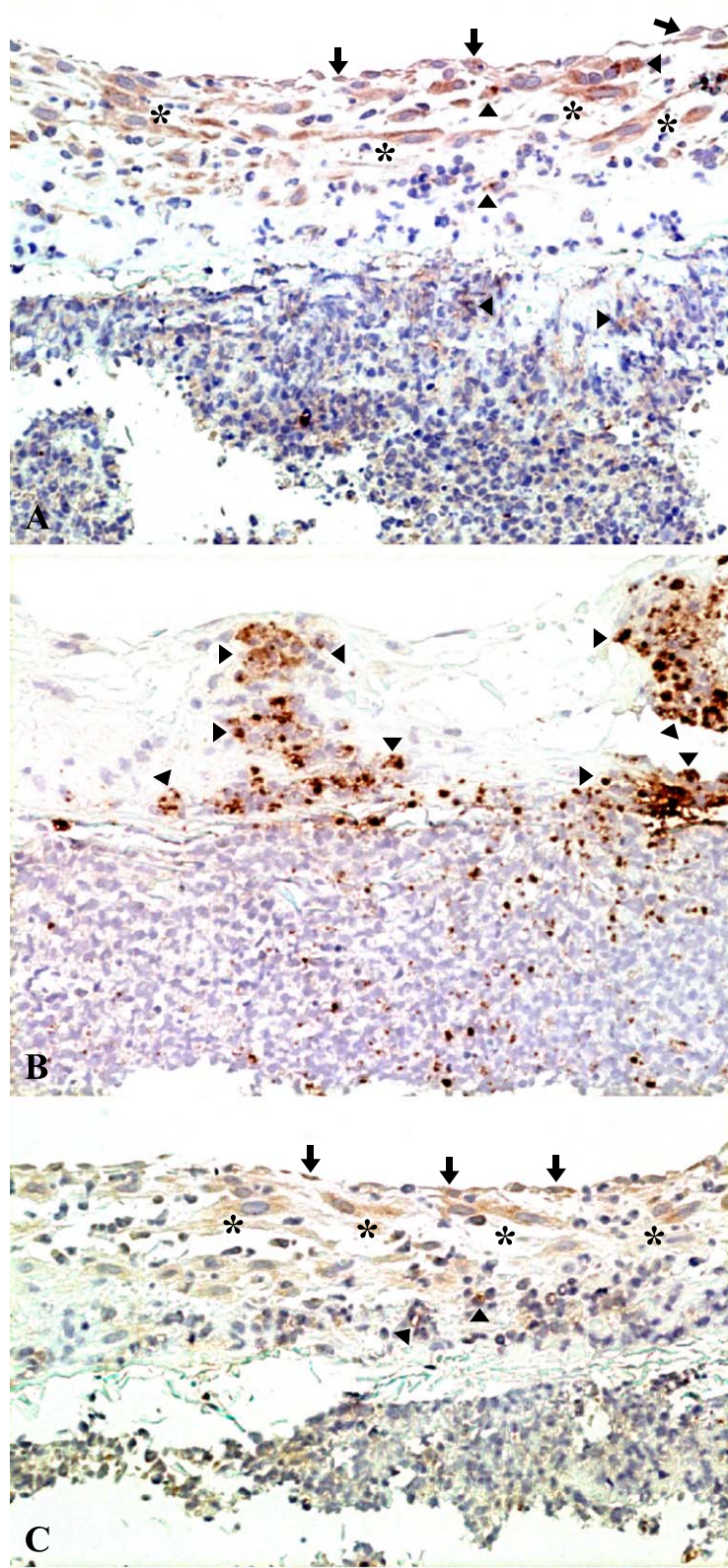


Fig. 5. MCP-1 and GM-CSF localization via immunostaining. (A) MCP-1 protein was detected in endothelial cells (arrows), smooth muscle cells (asterisks) and macrophages (arrowheads). (B and C) GM-CSF protein was detected in the endothelial cells (arrows), smooth muscle cells (asterisks) and macrophages (arrowheads) (original magnification  $\times 200$ ).

infiltration varied inversely in response to WSS (Fig. 3A). Higher aortic flow and WSS also were associated with increased medial macrophage apoptosis and a higher apoptosis index (Figs. 2C and 3C, Table 2). Adventitial macrophage infiltration also varied with luminal flow conditions but to a lesser degree generally than that noted in the media. Although differences were not apparent within the first day after PPE infusion among the three flow groups, by the fifth day, HF adventitial macrophage infiltration was significantly less than that noted in NF or LF-AAA (Fig. 3B).

We measured AAA MCP-1 and GM-CSF mRNA levels at representative time points following PPE infusion to gain insight into the mechanism(s) of flow and shear-stress-mediated macrophage density modulation under these conditions. AAA MCP-1 and GM-CSF mRNA expression were increased at all time points compared to control aorta regardless of flow status. Within the variable flow models, both MCP-1 and GM-CSF were most highly expressed in LF-AAA. In addition, MCP-1 expression decreased from LF to NF to HF-AAA in a dose response fashion (Figs. 4A, B). Immunostaining localized MCP-1 expression primarily to macrophages and medial smooth muscle cells. Less expression was noted on endothelial cells. GM-CSF was expressed in endothelial cell, medial smooth muscle cells and macrophages; strong macrophage expression was noted at all time points (Fig. 5).

## Discussion

Although flow conditions clearly influence intramural vascular inflammation, the mechanisms underlying these influences remain incompletely understood. In this experiment using macrophage density as a surrogate marker for mural inflammation, we show that medial AAA macrophage concentrations vary inversely as functions of luminal flow volume and WSS. Macrophage apoptosis and mural pro-inflammatory chemokine and growth factor expression correspond generally to these histologic observations. These findings are especially significant in the light of the previously recognized ability of flow and WSS to modulate experimental AAA morphology and progression.

Both MCP-1 and GM-CSF expression in experimental aneurysms correlated with observed macrophage densities and aneurysm diameters. Both regulatory peptides play important roles in degenerative human vascular diseases such as AAA. MCP-1, which stimulates macrophage recruitment and migration into inflamed tissues, was recently promoted as a clinically relevant biomarker for systemic vascular disease (Lemos et al., 2003). GM-CSF regulates hemopoietic cell proliferation, differentiation and migration, potentially accelerating chronic cardiovascular diseases by stimulating bone marrow release of pluripotent monocyte precursor cells (Ujihara et al., 2001; Woldbaek et al., 2002).

The intact endothelium is also an important player in the inflammatory response. Injured endothelial and vascular

smooth muscle cells synthesize and secrete chemokines such as MCP-1 that attract mononuclear cells to the arterial wall. Stabilization of the intimal layer by shear-stimulated endothelial cell proliferation and migration limits monocyte adhesion and transmural migration and reduces pro-inflammatory gene expression. Using these models, we have previously demonstrated that elevated antegrade WSS increases EC and medial SMC density in experimental AAA (Hoshina et al., 2003). This pro-proliferative response of intrinsic vascular cells suggests that, in addition to influences on chemokine and growth factor expression, increased antegrade and laminar flow reduces monocyte adhesion/macrophage infiltration via intimal regeneration and improved barrier function viability.

EC MCP-1 expression is modulated in part through production and release of nitric oxide (NO) (Masu et al., 2002; Wung et al., 2001). One mechanism by which increased flow and antegrade laminar WSS may reduce experimental AAA progression may be through increased eNOS activity and NO production in response to temporal shear stress gradients, NO-mediated decrease in MCP-1 expression and reduced intimal monocyte binding (Hsiai et al., 2001). This possibility is supported by our previous observation demonstrating increased eNOS expression in high-flow AAA (Nakahashi et al., 2002) and related work demonstrating increased aortic NO release following femoral AVF creation (Guzman et al., 1997). Following release, low-molecular-weight chemokines such as MCP-1 are capable of establishing trans-intimal chemotactic gradients that modulate monocyte extravasation and transmural migration (Berk et al., 2001). These gradients may be disrupted or reduced by high-flow-induced EC NO generation.

GM-CSF, a member of the hematopoietic growth factor family, is produced and released by monocytes, fibroblasts, endothelial cells and smooth muscle cells. It plays an integral role in stimulating granulocyte/macrophage progenitor cell proliferation and differentiation (Ujihara et al., 2001). GM-CSF also prolongs survival, significantly reducing or delaying apoptosis experienced by circulating and adherent blood monocytes and resident tissue macrophages (Kosaki et al., 1998). Infiltrative macrophages in inflammatory foci typically undergo rapid apoptosis unless provided with specific growth or survival stimuli. GM-CSF may inhibit macrophage apoptosis via regulation of anti-apoptotic gene transcription (Gardai et al., 2003). MCP-1 release, required for monocyte recruitment, has little or no effect on macrophage survival. Our morphologic observations suggest that variable aortic flow conditions may influence macrophage apoptosis, AAA inflammation and progression via influences on GM-CSF expression and release. GM-CSF also induces sustained macrophage superoxide release in vitro (Quentmeir et al., 2003).

Recent experimental data suggest that the DNA-binding protein nuclear factor-kappa beta (NF- $\kappa$ B) plays a significant role in AAA pathogenesis (Tham et al., 2002). Following cytoplasmic activation and release, NF- $\kappa$ B regulates tran-

scription and expression of many pro-inflammatory cytokines, chemokines, inducible enzymes and adhesion molecules. Recent reports indicate that NF- $\kappa$ B activation regulates MCP-1 expression (Denk et al., 2001; Zoja et al., 2002) in part via influences of flow conditions and wall shear stress (Bao et al., 1999). Like MCP-1, heme oxygenase-1 (HO) expression is also regulated by shear and strain forces (Tulis et al., 2001). This stress response protein plays an important role in cellular and tissue defense against oxidative stress, catalyzing carbon monoxide (CO), bilirubin and biliverdin formation as byproducts of heme metabolism. We have shown that increased luminal flow in this model increases AAA macrophage HO-1 expression and reduces oxidative stress (Nakahashi et al., 2002). Interestingly, CO has been recently shown to modulate NF- $\kappa$ B regulation of GM-CSF expression via overexpression of HO-1 (Sarady et al., 2002; Song et al., 2003). To follow-up on these observations, we are currently examining the role that flow and shear forces play in regulating NF- $\kappa$ B activation and nuclear translocation in AAA disease.

In summary, hemodynamic conditions exert considerable influence on transluminal macrophage density gradients in experimental AAA. These findings suggest that hemodynamic influences on endothelium stability and permeability or production of endothelial-derived diffusible cytokines and chemokines may modulate transmural aortic inflammation and aneurysm progression. These investigations provide additional insights into the mechanisms of hemodynamic influences on aneurysm pathogenesis.

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