L-Selectin–Mediated Neutrophil Recruitment in Experimental Rodent Aneurysm Formation

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Background—This investigation tested the hypothesis that L-selectin is important in experimental abdominal aortic aneurysm (AAA) formation in rodents.

Methods and Results—Rat abdominal aortas were perfused with saline (control) or porcine pancreatic elastase and studied on postperfusion days 1, 2, 4, 7, and 14 (n=5 per treatment group per day). Neutrophil (polymorphonuclear leukocyte, PMN) and macrophage counts per high-powered field (HPF) were performed on fixed sections. L-selectin expression and protein levels in aortic tissue were determined by polymerase chain reaction and Western blot, respectively. Elastase-perfused aortic diameters were significantly increased compared with control aortas at all time points except day 1 (P<0.05). PMN counts significantly increased in elastase-perfused aortas compared with control aortas at days 1, 2, and 4, reaching maximum levels at day 7 (40.8 versus 0.3 PMNs/HPF, P=0.001). L-selectin mRNA expression in elastase-perfused aortas was 18 (P=0.018), 17 (P<0.001), and 8 times (P=0.02) times greater than control aortas at days 1, 2, and 4, respectively. Western blot demonstrated a significant 69% increase in L-selectin protein at day 7 in elastase- as compared with saline-perfused aortas (P=0.005). Subsequent experiments involved similar studies on postperfusion days 4, 7, and 14 of aortas from C57BL/6 wild-type (WT) mice (n=21) and L-selectin–knockout (LKO) mice (n=19). LKO mice had significantly smaller aortic diameters at day 14 as compared with WT mice (88% versus 123%, P=0.02). PMN counts were significantly greater in elastase-perfused WT mouse aortas as compared with LKO mouse aortas at day 4 after perfusion (12.8 versus 4.8 PMNs/HPF, P=0.02). Macrophage counts were significantly greater at all time points after perfusion in elastase-perfused WT mouse aortas compared with elastase-perfused LKO mouse aortas, with a maximum difference at day 7 after perfusion (13.3 versus 0.5 macrophages/HPF, P<0.001).

Conclusion—L-selectin–mediated neutrophil recruitment may be a critical early step in AAA formation. (Circulation. 2005;112:241-247.)

Key Words: aneurysm • aorta • cell adhesion molecules • leukocytes

Abdominal aortic aneurysms (AAAs) are an important illness with a high mortality rate. The 2000 National Vital Statistics Report on Deaths revealed AAAs to be the 10th leading cause of death in white men 65 to 74 years old,1 and the National Hospital Discharge Summary documented >36 000 open repairs of AAAs in the United States. The pathogenesis of AAAs involves a complex series of events, characterized by the degradation of elastin and collagen in the media and adventitia through a catalytic process that occurs after infiltration of inflammatory cells into the aortic wall.2–9 The macrophage, in particular, has been implicated as critical in the pathogenesis.10 The importance of the neutrophil (polymorphonuclear leukocyte, PMN) during AAA formation has also been established in humans11–13 and, more recently, in a mouse model of aneurysm formation.14 The mechanisms by which the neutrophil and macrophage are recruited into the aortic wall during aneurysm formation are important.15 In this regard, adhesion molecules, including the selectins, are relevant.

The selectins are a family of 3 adhesion molecules: E-selectin on the surface of activated endothelial cells, P-selectin on the surface of activated platelets and endothelial cells, and L-selectin, which is constitutively expressed on the surface of most leukocytes.16,17 The primary function of selectins is to promote leukocyte capture to sites of inflammation. Without selectins, inflammatory cell recruitment is...
significantly diminished.18–22 The role of selectins during AAA formation has not been studied. In the present investigation, we sought to evaluate the role of the selectins on inflammatory cell recruitment during experimental AAA formation.

Methods
Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass), male C57BL/6 (Jackson Laboratories, Bar Harbor, Maine), and L-selectin–knockout (LKO) mice18 (Dr John B. Lowe, Department of Pathology, University of Michigan Medical School) were studied. All rats weighed 200 to 250 g, and all mice weighed ~20 to 27 g and were 8 to 10 weeks old during the study. The experiments were approved by the University of Michigan Universal Committee on the Use and Care of Animals (8566 rats, 8593 mice).

Experimental AAA Formation
Rodents were anesthetized under 2% isoflurane inhalational anesthetic, a midline laparotomy was performed, and the abdominal aorta from just below the left renal vein to the bifurcation was isolated.19,23 The aortic diameter (AD) was measured with a Spot Insight Color Optical Camera (Diagnostic Instruments) attached to an operating microscope (Nikon) using Image Pro Express software (Media Cybernetics). The aorta was then perfused for 30 minutes in rats or 5 minutes in mice with porcine pancreatic elastase (specific activity 6.6 U/mg protein; E1250; Sigma Chemical). After aortic perfusion, AD measurements were obtained.

Experimental Design
Male Sprague-Dawley rat aortas were perfused with either 1 mL isotonic saline (control) or 6 U/mL total of porcine pancreatic elastase in 1 mL isotonic saline. Saline-perfused and elastase-perfused aortas were measured at 1, 2, 4, 7, and 14 days after perfusion (n = 5 or 6 per treatment group per day). Segments of the infrarenal aorta were studied for gene expression and protein production with real-time polymerase chain reaction (PCR), Western blotting, and histology and immunohistochemistry studies.

Quantitative (Real-Time) PCR
mRNA gene levels were determined by quantitative PCR. mRNA was isolated from aortic segments by treatment with TRIZol reagent (Life Technologies) and reverse-transcribed by incubating with Oligo-(dT) primer and M-MLV Reverse Transcriptase (Life Technologies). Mouse aortas were perfused with elastase (0.322 U/mL) on day 0 and then measured and harvested on days 4, 7, and 14 after perfusion. Before aortic harvest, blood was drawn via heart puncture and analyzed as previously stated. Infrarenal aortic segments were used for histology and immunohistochemistry studies. AD increases were reported as a percent increase from baseline measurements, and an AAA was defined as a ≥100% increase in AD as compared with baseline.

Histology and Immunohistochemistry
Harvested aortas were fixed in fresh cold 4% paraformaldehyde for 16 to 24 hours followed by 70% ethanol. Segments were then paraffin-embedded, and 5-μm sections were mounted onto slides. Those prepared for immunohistochemical studies were treated for 10 minutes with H2O2 to block endogenous peroxidase activity and the appropriate diluted serum from the Vectastain ABC-AP Kit (Vector Laboratories) for at least 30 minutes at room temperature. Sections were subsequently incubated for 30 minutes at room temperature with primary antibodies, including rabbit anti-rat PMN antibody (Accurate Chemical & Scientific, Westbury, NY) for neutrophil staining, mouse anti-rat CD68 (Serotec, Raleigh, NC) for rat macrophage staining, and rat anti-mouse Mac-3 monoclonal antibody (BD Biosciences Pharmingen, San Diego, Calif) for mouse macrophage staining. Epitope unmasking was performed, when necessary, using Trizol in a Precision model pressure cooker (Cell Marque Corporation). Peroxidase-coupled species-appropriate secondary antibodies were added and visualized with an ECL chemiluminescence kit (Pierce) according to manufacturer’s instructions.

RESULTS
Elastase-Perfused Aortas Became Aneurysmal on Day 7
Rat ADs were significantly increased in elastase-perfused aortas as compared with saline-perfused aortas at days 2, 4, 7,
and 14 ($P<0.05$ for all time points, Figure 1A). Elastase-perfused rat aortas became aneurysmal on days 7 and 14, with increases of 263% and 434%, respectively. Furthermore, elastase-perfused ADs at days 7 and 14 were significantly different ($P<0.001$, ANOVA). A post hoc Tukey test documented significant differences between days 7 and 14 ($P<0.05$). Error bar represents ±SEM. Probability values were determined by unpaired $t$-test comparing elastase- and saline-perfused aortic diameters. B, Ratios of L-selectin mRNA in elastase-perfused aortas to L-selectin mRNA in saline-perfused aortas, after correcting for $\beta$-actin, are represented on y-axis as L-selectin mRNA. L-selectin mRNA levels in elastase-perfused aortas were 18 ($P=0.018$), 17 ($P<0.001$), and 8 times ($P=0.02$) greater than saline-perfused aortas at days 1, 2, and 4, respectively. Decreasing trend eventually led to undetectable levels of L-selectin mRNA expression in both groups at days 7 and 14. C, Western blot analysis of L-selectin protein levels in rat aortas. Elastase-perfused aortas were observed to have 1.7 times the amount of L-selectin as compared with saline-perfused aortas ($P=0.005$). L-selectin protein levels were corrected to total protein measurements. D, Representative Western blot documenting increased L-selectin protein levels at 7 d after perfusion in saline- (S) and elastase-perfused (E) rat aortas.

Elastase-Perfused Aortas Demonstrated a Significant Increase in L-Selectin Levels

Differences in E- and P-selectin mRNA levels could not be demonstrated by real-time PCR at any time point studied (data not shown). In contrast, aortic wall L-selectin mRNA levels in elastase-perfused rat aortas were 18 ($P=0.018$), 17 ($P<0.001$), and 8 times ($P=0.02$) times greater than saline-perfused rat aortas at days 1, 2, and 4, respectively (Figure 1B). L-selectin protein levels in elastase-perfused rat aortas were 1.7 times greater than saline-perfused rat aortas by day 7 ($P=0.005$; Figure 1C, D).

L-Selectin Expression and Neutrophil Infiltration Precede AAA Formation

PMNs were significantly increased in elastase-perfused rat aortas compared with saline-perfused rat aortas at days 1, 2, 4, and 14, reaching maximum levels at day 7 (40.8 versus 0.3 PMNs/HPF, $P=0.001$; Table 1). PMNs peaked on day 7, coinciding with the onset of an aneurysm phenotype. Macrophage counts in the aortic wall followed a trend similar to the PMNs, with macrophages/HPF steadily rising to a peak at day 7 (17.5 macrophages/HPF in elastase-perfused rat aortas versus 1.2 macrophages/HPF in saline-perfused rat aortas, $P<0.001$; Table 1).

L-Selectin Deficiency Suppressed Experimental AAA Formation

Because increased L-selectin levels in aneurysmal aortas could be secondary to increased numbers of PMNs in the aortic wall and therefore serve only as an inflammatory marker, LKO mice were studied to more accurately char-
characterize the role of L-selectin in AAA formation. Elastase perfusion of LKO mouse aortas resulted in significantly smaller ADs at day 14 as compared with wild-type (WT) mouse aortas (88% versus 123%, P=0.02; Figure 2). In addition, the AAA phenotype incidence was reduced in LKO mice versus WT mice (38% versus 67%). These observations appear to be associated with a reduced neutrophil infiltration into the aortic wall, with fewer PMNs at day 4 after elastase perfusion in LKO mice as compared with WT mice (4.8 PMNs/HPF versus 12.8 PMNs/HPF, P=0.02; Table 2). In addition, there were significantly fewer macrophages in LKO mouse aortas compared with WT mouse aortas at all time points (P<0.002; Table 2). PMN counts in WT mice peaked on day 4, preceding macrophages, which peaked on day 7 (Table 2, Figure 3). Of note, PMN counts were significantly greater in LKO mouse aortas as compared with WT mouse aortas at day 14 only (2.73 PMNs/HPF versus 0.96 PMNs/HPF, P=0.02; Table 2). This observation may be a secondary effect, however, because macrophage counts were elevated in WT mouse aortas and could potentially have decreased PMN counts at day 14 in the WT mouse aortas via phagocytosis. Circulating levels of neutrophils and monocytes were not different between WT and LKO mice (data not shown).

**Discussion**

This investigation is the first to document a convincing association between L-selectin expression and AAA formation in 2 rodent models. L-selectin, by real-time PCR and

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MPs indicates macrophages. PMN and macrophage counts in the aortic wall of elastase-perfused rats peaked at day 7. There was a significant difference in PMN and macrophage counts in time points studied among elastase-perfused rats (P<0.001 PMN, P=0.004 MP, ANOVA). Post hoc Tukey test indicated significant difference between PMN counts in 7-d elastase-perfused aortas vs every other time point except day 1 and MP counts in 7-d elastase-perfused aortas vs day 1 and 2. Data represented as cells/HPF±SEM. Unpaired t test compared saline-perfused to elastase-perfused aortas.

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Abbreviations as in text and Table 1. Counts in aortic wall of WT mice peaked at days 4 and 7, respectively. There was a significant difference in PMN and macrophage counts in time points studied among WT mice (P<0.001, for PMN and MP, ANOVA). Post hoc Tukey test indicated a significant difference between PMN counts at day 4 vs all other time points and in macrophage counts at day 7 vs all other time points. Data represented as cells/HPF±SEM. Unpaired t test compared WT and LKO mice.
protein analysis of the aortic wall, was the only member of this adhesion molecule family to be increased during rat experimental AAA formation. Furthermore, increases in L-selectin correlated with increases in neutrophil and macrophage counts in the aortic wall.

Although L-selectin is rapidly cleaved from the surface before extravasation across the vessel wall, in vitro studies have documented increased L-selectin mRNA levels and surface expression during activation. Therefore, further studies elucidating the specific role of L-selectin after elastase perfusion of WT and LKO mouse aortas demonstrated that L-selectin deficiency results in decreased aneurysm size and incidence. In addition, peak neutrophil and macrophage counts in the aortic wall of WT mice at 4 and 7 days after perfusion, respectively, did not occur in the LKO mice.

L-selectin deficiency most likely functions to suppress AAA formation through impaired neutrophil and macrophage recruitment because neutrophils and macrophages have been documented as key participants in AAA pathogenesis and L-selectin mediates neutrophil and macrophage rolling to sites of inflammation. Impaired macrophage recruitment may not be solely the result of L-selectin deficiency but could also be attributed to other mechanisms, including diminished recruitment of neutrophils, which produce proteolytic enzymes, and reactive oxygen species, which potentially stimulate the secretion of leukocyte chemotactic cytokines. Furthermore, because the neutrophil appeared in the aortic wall before the macrophage in the WT mice, a decrease in neutrophils in the aortic wall of LKO mice most likely resulted from a lack of L-selectin. Unlike other studies, which have used WT bone marrow to rescue a phenotype in KO mice deficient in enzymes produced by bone marrow–derived cells and mesenchymal cells, bone marrow transplantation was not required in this study because L-selectin is found only on bone marrow–derived white blood cells. Therefore, because the cell origin of L-selectin was known, it was not necessary to perform bone marrow transplantation. Finally, the roles of E- and P-selectin during this disease process cannot be ruled out on the basis of mRNA expression data from the initial rat experiments; rather, they need to be further analyzed via the respective KO mice.

AAA pathogenesis is viewed as a multifactorial process. Traditional concepts of this disease process implicate an initial injury to the aortic wall, followed by degradation of extracellular matrix proteins, which then serves as a catalyst for inflammation. After injury, increased cytokine production promotes further inflammatory cell recruitment and secretion of proteolytic enzymes, such as matrix metalloproteinases, and reactive oxygen species by inflammatory and mesenchymal cells. This culminates in extracellular matrix degradation, cell damage, and a proinflammatory environment that leads to aortic wall weakening and eventual AAA formation.

Most studies investigating AAA disease have focused on events taking place in the aortic wall during AAA formation. A study by Ricci et al was the first to focus on events initiating inflammatory cell recruitment. Antibody blockade of CD18, a subunit of the integrin adhesion molecules found on leukocytes that promote firm adhesion to the endothelial surface, decreases AAA size and macrophage recruitment after elastase perfusion. To date, the present investigation is the only one to have examined the role of selectins during AAA pathogenesis.

Limitations of the present investigation include the observation that only a partial reversal of the aneurysm phenotype occurred in the LKO mice. Clearly, other
adhesion molecules, such as integrins, which were still present, also promote inflammatory cell recruitment and may play an important role.\textsuperscript{32} In addition, it is difficult to determine the relevance of these observations to human AAA formation because many have criticized the elastase-perfusion aortic aneurysm model as an acute inflammatory aneurysm model. Furthermore, the importance of L-selectin during human AAA pathogenesis cannot be adequately assessed because L-selectin plays a functional role before AAA formation, as demonstrated by the early recruitment of inflammatory cells seen in the present study. The ability to analyze human aortic tissue before it becomes aneurysmal and to accurately predict which patients develop aneurysms is therefore not possible. Finally, the presence of L-selectin expression in human aortic aneurysms would provide no direct evidence as to the mechanistic importance of L-selectin during human AAA pathogenesis because L-selectin, acting as an inflammatory marker, would be expected to be elevated.

Despite these limitations, L-selectin is required for neutrophil recruitment during the initial stages of aneurysm formation. Previous studies documenting the attenuation of ischae-reperfusion injury during myocardial infarction\textsuperscript{33} and thrombus resolution\textsuperscript{34} through selectin blockade provide support for a potential L-selectin-targeted novel treatment to prevent AAA development.

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**Disclosure**

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**References**

CLINICAL PERSPECTIVE

Significant progress has been made in the understanding of the pathogenesis of abdominal aortic aneurysms (AAAs). The majority of this work has come from studies of patients with end-stage aneurysm disease before either open or endovascular repair. Although critical enzymes and cytokines have been identified, the paradigm by which aneurysms are initiated has not been well established. It is known that akin to atherosclerosis, aneurysm development involves the recruitment of various leukocytes. Whereas the infiltration of lymphocytes and macrophages has been studied extensively during aortic aneurysm pathogenesis, few studies have addressed the role of the neutrophil in aneurysm development. On the basis of earlier experiments, we hypothesized that the neutrophil plays a critical role during the early stages of aneurysm development. The study by Eliason et al confirms that the neutrophil is required for aneurysm formation in the mouse elastase perfusion model of AAA formation. This appears to be independent of chemokine or gelatinase secretion, suggesting that some other enzyme in the neutrophil is critical in aneurysm formation. Hannawa et al performed additional experiments using the elastase-perfusion model in rats and documented that L-selectin, found only on bone marrow–derived cells, is upregulated early in concordance with neutrophil migration into the aneurysm wall. To prove that the presence of L-selectin in the aortic wall was not merely a marker of inflammation, L-selectin–knockout mice were subjected to elastase perfusion of the aorta and found to be protected from aneurysm formation. Studies such as these are beginning to shed light on early events during aortic aneurysm formation. This understanding is critical in that there are no medical therapies aimed at inhibiting aneurysm initiation and growth. Because diseases of the aorta are the 14th leading cause of death in the United States, and both the incidence and the prevalence of aortic aneurysms is increasing, it is hoped that understanding the pathogenesis of AAAs, especially the initiating events, will become a more widely recognized public health concern.
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