

# Angiotensin II infusion induces site-specific intra-laminar hemorrhage in macrophage colony-stimulating factor-deficient mice

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## Abstract

Angiotensin II (AngII) infusion promotes macrophage infiltration into the aortic wall resulting in several forms of vascular pathology. To determine the causal role of macrophages in these vascular diseases, we used osteopetrotic (*op*) male mice in which a natural mutation ablates production of M-CSF and results in severe depletion of monocytes. AngII infusion into apoE<sup>-/-</sup> mice resulted in increased atherosclerosis that was attenuated in *op* mice. AngII infusion in *op* mice unexpectedly produced grossly discernable thickening of the proximal thoracic aorta characterized by intra-mural hematoma. This pathology was also observed in apoE<sup>+/+</sup> × *op* male mice, and therefore, independent of hyper-lipidemia. No perceptible structural properties of aortas from *op* mice could be discerned prior to AngII infusion. Regional effects in the contractile response to phenylephrine were noted in aortic rings with enhanced responsivity in the upper thoracic aortas of *op* mice compared to those from +/+ mice. No differences in contractile response were noted in aortic rings from the lower thorax. In conclusion, deficiency of M-CSF attenuated AngII-induced atherosclerosis but led to an unanticipated pathology of intra-laminar hemorrhage in the upper aortic regions.

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**Keywords:** Angiotensin; Aorta; Osteopetrosis; M-CSF; Hematoma

## 1. Introduction

Angiotensin II (AngII) infusion into mice promotes a number of vascular pathologies, including atherosclerosis and aneurysm formation [1–4]. A common feature of all forms of AngII-induced vascular pathology is macrophage infiltration that occurs in the intimal, medial, and adventitial layers of aortas [5,1,6]. The presence of macrophages in

these different locations is consistent with AngII promoting entry into aortic tissue by multiple mechanisms.

Macrophages are the major cell type present in lesions of hyper-cholesterolemia-induced atherosclerosis. To study the functional role of macrophages in hyper-cholesterolemia-induced atherosclerosis, previous studies have used mice with a natural frame shift mutation that results in ablation of macrophage-colony stimulating factor (M-CSF) [7]. Since M-CSF controls macrophage number and activity, these mice have a reduction in peripheral blood monocytes and some populations of tissue macrophages [8]. Mice are designated as osteopetrotic (*op*) since their osteoclast deficiency leads to increased bone density, cranial bone defects, and a lack of incisors. Introduction of the *op* mutation reduces hyper-cholesterolemia-induced atherosclerotic lesion size

**Abbreviations:** AngII, angiotensin II; AAAs, abdominal aortic aneurysms; M-CSF, macrophage-colony stimulating factor; PE, phenylephrine

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in both apoE  $-/-$  and LDL receptor  $-/-$  mice [9–11]. The effect of the *op* mutation may be a combination of M-CSF deficiency on both the number of circulating monocytes and macrophage function [12]. Macrophages are also the dominant cell type of the early aneurysmal response that forms in the abdominal aorta [6]. While monocytes are present in each form of AngII-induced vascular pathologies, the effect of a reduced number of this cell type is not known.

To determine the role of macrophages in AngII-induced vascular pathology, we initially used *op* mice that were crossed into an apoE  $-/-$  background. The *op* mice are available in a B6C3FE background, which necessitated the comparison to wild-type littermates. These studies demonstrated that the presence of the *op* mutation dramatically reduced the size of AngII-induced atherosclerotic lesions, in the same manner as previously shown for hyper-cholesterolemia-induced atherosclerosis. These studies also provided the unanticipated result that AngII infusion to *op* mice leads to intra-laminar hemorrhage that is localized to the upper thoracic aorta.

## 2. Methods

### 2.1. Mice

The *op* male mice (B6C3Fe *a/a-Csf1<sup>op</sup>*; stock number 000231; Jackson Laboratory) were bred to apoE  $-/-$  female mice (backcrossed 10 times in C57BL/6; Jackson Laboratory) to produce heterozygous *op/+*  $\times$  apoE  $+/-$  male and female mice. Parental lines of apoE  $-/-$  mice that were *op/+* were established and littermates were used for studies. For apoE  $+/+$  mice (C57BL/6 background), the *op* mice from Jackson were interbred and littermates used. Mice were housed in specific pathogen-free rooms. Since *op* mice lack incisors, all mice were fed a powdered laboratory diet (Harlan Teklad). All procedures were performed with the approval of the University of Kentucky, IACUC.

### 2.2. Genotyping of mice

The *op* mice have a single base-pair mutation in the M-CSF gene, which generates a stop codon 21 base-pairs (bp) downstream [7]. The M-CSF mutation was detected through enzyme digestion of PCR products. We designed PCR primers to flank the site of the mutation. *Op* screening used the following primers: 5'-GCTACCTAAAGAAGGC CTTTCT-3' and 5'-CTTGTCTGCTCCTCATAGTCC-3'. The PCR cycle was: 35 cycles: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 1 elongation cycle of 72 °C for 2 min. PCR products resulted in 158 bp fragment for the wild-type (*+/+*) and heterozygous (*op/+*), and 159 bp for homozygous (*op/op*) mouse. PCR product from the wild-type mouse contains an *MbolI* restriction site. Restriction digest with *MbolI* resulted in two fragments (136 and 22 bp) for the wild-type, while the *op* DNA is resistant to digestion (Fig. 1).

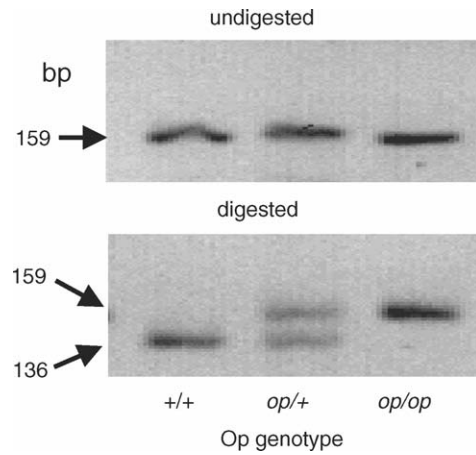


Fig. 1. The *op* mutation detected through DNA enzyme digestion. Shown are representative examples of electrophoresis of restriction enzyme digested DNA, detecting the wild-type allele at 136 bp, the intact *op* allele at 159 bp.

### 2.3. AngII infusion

AngII (1000 ng/kg min) or saline was infused via Alzet osmotic minipumps in 2- to 5-month-old *+/+*, *op/+*, and *op/op* mice in both apoE  $+/+$  and  $-/-$  background for 28 days (Model 2004). Alzet minipumps were implanted subcutaneously as described previously [2,1].

### 2.4. Complete blood cell counting

Ocular bleeding was performed and blood smears were stained with Hema III (Fisher). Complete blood cell counting was determined from 100 white blood cells per slide using a minimum of 2 slides per mouse.

### 2.5. Serum total cholesterol and lipoprotein distribution

Serum total cholesterol concentrations were measured by enzymatic colorimetric assay. Lipoprotein cholesterol distribution was determined by size exclusion chromatography as described previously [1].

### 2.6. Blood pressure measurements

Systolic blood pressure was measured in conscious mice by tail cuff using a Visitech BP-2000 platform as described previously [13].

### 2.7. Aortic harvesting

Aortas were harvested from mice as described previously [1,6], with the exception of male apoE  $+/+$  mice that were perfusion fixed (4% paraformaldehyde) at a pressure of 100 mmHg for morphological analysis of aortas. Sections (10  $\mu$ m) were collected serially from upper and lower thoracic aortas from these mice. Upper thorax was considered to be aortic segment 3 mm distal from the aortic arch and

lower thorax was considered the aortic segment 3 mm proximal from the last intercostal branch. The first 0.5 mm of the upper and lower thoracic aorta was used for quantifications of maximal and minimal medial thickness in sections 10  $\mu\text{m}$  apart using 4 sections per mouse as described previously [14]. The largest and smallest distance between internal and external elastic lamina was considered as maximal and minimal medial thickness, respectively.

### 2.8. Vascular pathology

Aneurysm occurrence was defined as a 50% increase in external diameter of the abdominal aorta. Atherosclerosis was quantified on the aortic intimal surface in the arch and thorax as described previously [14]. The abdominal segment was excluded because of the occurrence of abdominal aortic aneurysms (AAAs).

### 2.9. Histology and immunocytochemistry

Ten micrometer cross-sections were collected serially from aortic arches and upper thoracic segments of Ang-II infused male mice. Histological stainings were performed using Movat's pentachrome, and hematoxylin and eosin, as described previously [1,15]. Immunocytochemistry for macrophages was performed on several sections of aortas per mouse using a rabbit antimouse antibody (Accurate Chemical Co.) diluted 1:1000 and biotinylated goat antirabbit IgG secondary antibody. PECAM immunostaining was performed using a rat antimouse monoclonal antibody (Becton Dickinson). Peroxidase-based ABC system and the red chromagen, AEC, were used to detect the antigen–antibody reaction [16]. Controls included nonimmune sera.

### 2.10. Contractile function

Aortas from six male wild-type and four *op*  $\times$  apoE $^{-/-}$  male mice were removed and adventitia was carefully dissected free. Measurement of contractile activity was performed using aortic rings as described previously [17,18]. Upper and lower thoracic segments (3 mm) were mounted by passing two tungsten wires through the arterial lumen and bathed in wells filled with Krebs Henseleit solution. Tension (1 gm) was maintained continuously and recorded on a Micro-Med instrument. After 30 min for equilibration, tissues were incubated with agonists.

### 2.11. Statistics

Mean and S.E.M. were calculated for each parameter. Data were analyzed with SigmaStat by parametric or nonparametric analysis as appropriate. One- or two-way ANOVA were used depending on the number of experimental variables. Significant interactions were analyzed using a Tukey post hoc test for blood pressure and medial quantifications, Holm–Sidak test for monocytes and cholesterol data, and

Dunn's test for atherosclerosis data. Comparisons of the incidence of aneurysms was performed with logistic regression and contractility experiments were analyzed with linear regression using SAS.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Detection and phenotype of *op* mice

Fig. 1 shows a representative agarose gel of the restriction enzyme digest of DNA from +/+, *op*/+, and *op/op* mice. The undigested products from +/+, *op*/+, and *op/op* mice had lengths of 158, 158, and 159 bp, respectively, that were indistinguishable by gel electrophoresis. Upon restriction enzyme digestion, the *op* fragment remained at 159 bp, while the product for wild-type M-CSF gene was reduced to 136 bp.

As reported previously, *op* mice had decreased body weight, lack of teeth, domed skull, and short kinked tails. Moreover, *op* mice had decreased fertility and increased mortality. As expected, *op* mice had a reduction in numbers of monocytes in the blood to less than 23–27% of controls. Serum cholesterol concentrations in +/+ versus *op*– mice were not significantly different between saline and AngII-infused mice. Systolic blood pressure was not different among groups during saline infusion. AngII infusion significantly increased systolic blood pressure in all three genotypes, with no significant differences among groups (Table 1).

### 3.2. AngII-induced vascular pathology

Atherosclerotic lesion size was quantified on the aortic intimal surface of the arch and thoracic region. Lesions were either absent or extremely small in mice infused with saline for 28 days. The abdominal segment of AngII-infused mice was excluded because of the occurrence of a few abdominal aneurysms. AngII-infused *op/op* mice had significantly smaller lesions than their wild-type littermate controls (Fig. 2).

Abdominal aortic aneurysms were only detected in a small number of apoE $^{-/-}$  mice, unlike our previous studies [1,13]. The incidence of AAAs in apoE $^{-/-}$  mice was 14, 13, and 50% in +/+, *op*/+, and *op/op* groups, respectively ( $P > 0.05$ ).

Unexpectedly, *op* mice infused with AngII had increased aortic thickening and grossly visible intra-mural hematoma that extended from the upper thorax to the aortic arch (Fig. 3). The incidence of this pathology in apoE $^{-/-}$  mice was 7% in +/+ mice, 6% in *op*/+ and 75% in *op/op* mice ( $P < 0.05$  +/+ and *op*/+ versus *op/op*). Since hyper-lipidemia promotes the development of AngII-induced AAAs [19], AngII was infused into apoE wild-type male mice. In contrast to the hyper-lipidemia promoting effects of AngII-induced AAAs [19], the incidence of the gross thickening and intra-mural hemorrhage was similar in *op* normolipidemic mice (60%).

Table 1  
Effects of M-CSF deficiency on apoE<sup>-/-</sup> mice infused with saline or AngII

Infusion	op genotype	Monocyte count (per 100 WBC)	Serum cholesterol (mg/dl)	Blood pressure (mmHg)
Saline	+/+	9.7 ± 0.9* (4)	458 ± 31 (4)	133 ± 9 (4)
	op/+	6.0 ± 0.7* (4)	435 ± 71 (4)	123 ± 5 (4)
	op/op	2.7 ± 0.5 (4)	502 ± 34 (4)	126 ± 12 (4)
AngII	+/+	8.4 ± 0.2* (7)	344 ± 16 (14)	168 ± 4† (14)
	op/+	5.8 ± 0.5* (9)	387 ± 17 (15)	147 ± 7† (15)
	op/op	1.7 ± 0.6 (3)	491 ± 87 (4)	168 ± 12† (4)

Values are presented as means ± S.E.M. (n=4 for saline-infused groups and n=14, 15, and 4, for +/+, op/+, and op/op male mice infused with AngII, respectively). Blood pressure data represent the mean ± S.E.M. per group at 4 weeks after AngII or saline infusion. The number in parenthesis denotes the number of individual mouse measurements.

\* P < 0.05 vs. op/op for comparisons of AngII groups.

† P < 0.05 for comparisons of saline vs. AngII infusion.

One of the normolipidemic mice died of hemothorax caused by aortic arch rupture.

Histological examination of AngII-infused aortas from wild-type mice had no obvious pathology (Fig. 4A, C and E); while op male mice had circumferential intra-laminar hemorrhage in the upper thoracic aortas (Fig. 4B). Widespread hemorrhage was noted within the medial elastin fibers with increased space between lamellae, breaks in elastin fibers, and a small number of nuclei in these regions (Fig. 4D and F, arrow). The presence of the hemorrhage was predominantly in the outer margins of the aorta and the external elastic lamina was intact. This was confirmed in Movat's stained sections (data not shown). Large hematomas were localized in some areas of the arch and upper thorax of op male mice in the outer laminar units (Fig. 4D). Increased collagen deposition was noted in the adventitia of op mice (Fig. 4B). Immunocytochemical examinations demonstrated lack of macrophages in the areas of circumferential hemorrhage and presence of macrophages at the edge of the large hematoma of the op mice (Fig. 4G and H). PECAM immunostaining was readily observable in the intima and adventitia of all mice, but was never detected in media of either strain of mice subjected to saline or AngII infusion (data not shown).

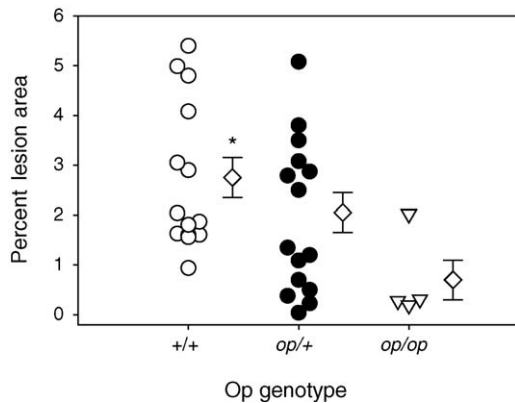


Fig. 2. AngII-induced atherosclerosis in apoE<sup>-/-</sup> mice. Percent of the intimal area of the arch and thoracic aorta covered by grossly discernable lesions was quantified in apoE<sup>-/-</sup> male mice. Each symbol represents values from individual mice, diamonds represent means, and bars are S.E.M. \*P < 0.05 for op/op vs. the indicated genotype.

### 3.3. Regional structural and functional characteristics of aorta in op mice

To determine whether there were any discernable structural changes in the aortas of op mice that would predispose to the AngII-induced pathology described above, upper and

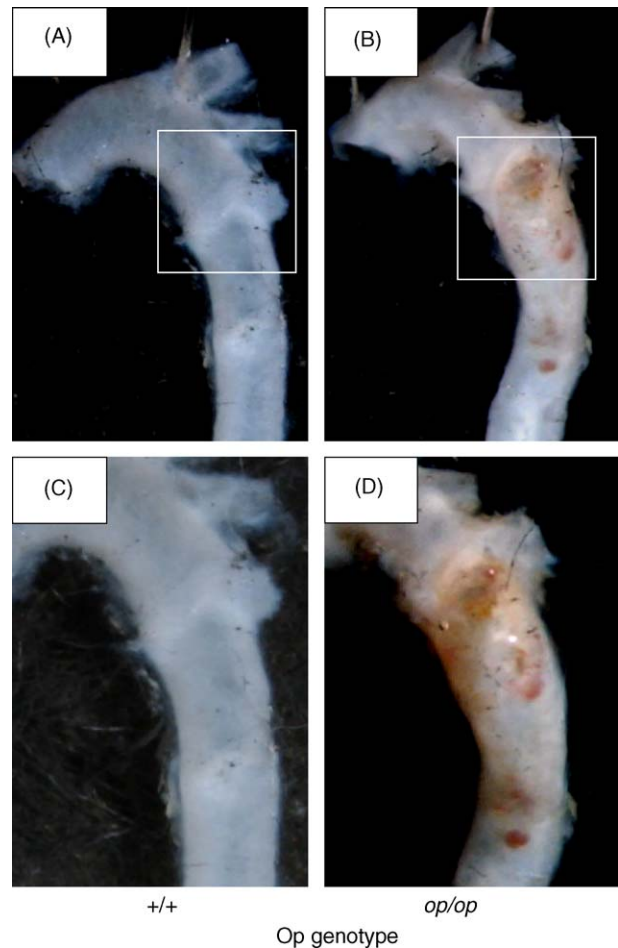


Fig. 3. AngII-induced hematoma formation in upper thoracic aortas of apoE<sup>+/+</sup> male mice. Representative examples of aortas from mice infused with AngII for 28 days. Aortas from wild-type (A and C) and op (B and D) mice are shown at low and high magnifications, respectively.



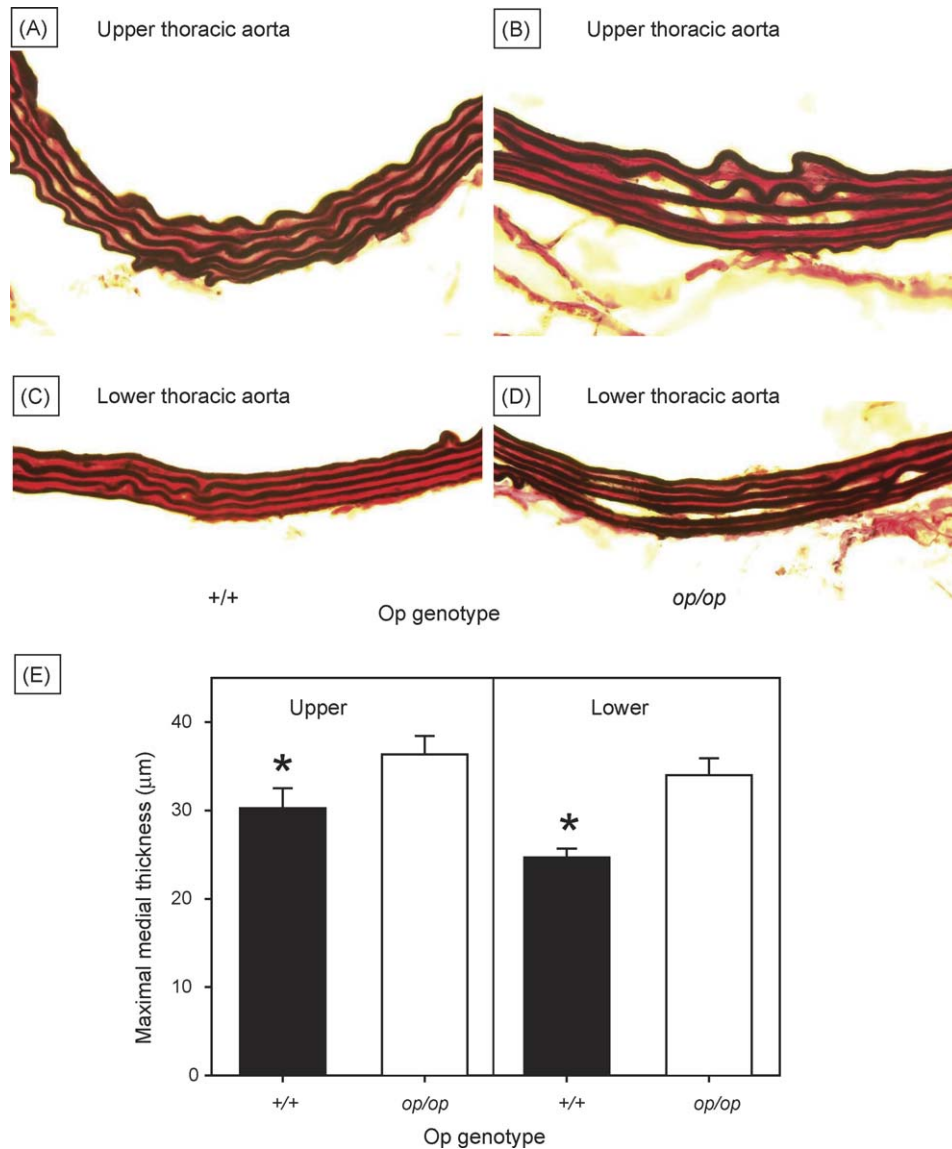


Fig. 5. Medial structure of upper and lower thoracic aorta of wild-type and *op* × apoE<sup>+/+</sup> mice. Shown are representative examples of Movat's staining of upper and lower thoracic segments of wild-type and *op* male mice with regions of increased medial thickness in *op* mice (B and D) and normal aorta in wild-type mice (A and C). Maximal medial thickness of upper (E) and lower thoracic aortas from wild-type and *op* mice. \**P* < 0.05.

lower thoracic tissue sections from perfusion fixed aortas from +/+ and *op/op* male mice were compared. Aortas from both groups had grossly similar appearance with similar numbers and structure of elastin fibers (Fig. 5). A characteristic of the *op* mice was thickening of the medial layers that were confined to the antero-lateral area. This led to an increased maximal medial thickness by 20–37% in these mice (Fig. 5B, D and E). However, these structures were observed in both the lower and upper thoracic region. Therefore, we were not able to discern any structural change in the aorta of *op* mice that would predispose to the intra-laminar hemorrhage in the upper thoracic region.

To determine any discernable functional differences in aortic areas that are either prone or resistant to AngII-induced

intra-laminar hemorrhage, contractile activity of aortic rings from wild-type and *op* mice were studied. AngII had minimal contractile activity on any region of the thoracic mouse aorta, as described by others [18]. Incubation with phenylephrine caused contractions of rings from the lower thoracic aorta that were equivalent in rings from wild-type and *op* mice (Fig. 6A). In contrast, the aortic rings from the upper thoracic region of *op* mice were considerably more responsive to PE than tissue from wild-types (Fig. 6B).

#### 4. Discussion

AngII infusion into mice promotes the infiltration of macrophages into all the major regions of arteries; intima,

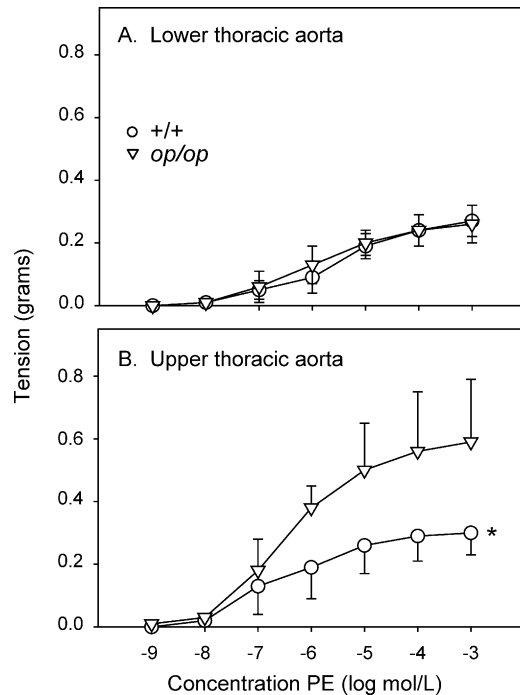


Fig. 6. Regional differences in contractility response to phenylephrine (PE). Contractility response of aortic rings to PE in tissue from the lower (A) and upper (B) thoracic regions of wild-type and *op* × apoE<sup>-/-</sup> mice. Symbols represent means and bars are S.E.M. of wild-type (*n* = 6) and *op* (*n* = 4) mice. \**P* < 0.05 for comparison of genotypes.

media, and adventitia. The infusion of AngII into hyper-lipidemic mice leads to several distinct vascular pathologies including augmentation of atherosclerosis and development of aneurysms.

The presence of the *op* mutation has been shown previously to decrease the size of hyper-cholesterolemia-induced atherosclerotic lesions in both LDL receptor <sup>-/-</sup> and apoE<sup>-/-</sup> mice [9–11]. The atherosclerotic lesions in these models predominantly contain lipid-laden macrophages. Therefore, the profound reduction in lesion size in *op* mice may be attributable to the marked reduction in circulating monocytes, combined with a decreased ability to recruit them to the site of lesion formation [20]. AngII infusion has the potential to promote atherosclerosis through mechanisms distinct from those induced by hyper-cholesterolemia. However, both stimuli produce lesions in which the area is predominantly filled with lipid-laden macrophages [21,1]. Also, hyper-cholesterolemia promotes the development of lesions through angiotensin peptide based mechanisms [22]. Therefore, it seems likely that AngII infusion augments the same mechanisms of atherosclerosis as hyper-cholesterolemia. In agreement with this premise, the *op* mutation also virtually abolished the development of AngII-induced atherosclerosis, even in the presence of increased plasma cholesterol concentrations.

It has been shown previously that infusion of AngII into apoE<sup>-/-</sup> and LDL receptor <sup>-/-</sup> mice leads to the development of AAAs [13,1,2,19], although a failure to observe this

pathology has also been reported in apoE<sup>-/-</sup> mice [3]. The pathology of AAAs is more heterogenous and complex than that of atherosclerotic lesions. Many cell types are present at different stages of the disease, but macrophages are the earliest cell type to infiltrate the aneurysmal area and are present throughout the evolution of the disease [6]. AngII was infused into *op* × apoE<sup>-/-</sup> mice to determine whether the presence of macrophages was an active component of the aneurysmal disease process. However, there was an unexpectedly low incidence of AngII-induced AAAs in this study. A major difference in the mice used in this study was the genetic background. It is well known that genetic background has a dramatic effect on the development of atherosclerosis, although there is no information in AAA models [23]. Others have bred *op* mice into the C57BL/6 background that is used in previous studies of hyper-cholesterolemia-induced lesion formation [10], however, the available *op* mice have a strain background of B6C3Fe. Further studies will be needed to determine the role of strain background on AAA formation.

Unexpectedly, we found that AngII infusion into *op* mice led to aortic thickening and hematoma formation that was localized to the upper thoracic and arch regions of the aorta. Histological examination of the tissues from this region demonstrated the presence of intra-laminar hemorrhage that was predominantly in the outer layer of the artery as defined by the presence of erythrocytes within the medial layers. Adventitial inflammation and thickening were also observed, as noted by others [5]. The production of intra-laminar hematoma did not require the presence of hyper-cholesterolemia, since *op* mice that were apoE<sup>+/+</sup> also developed this pathology with the same incidence as apoE<sup>-/-</sup> mice. Macrophages were present in the margins of the intra-laminar hematoma of *op* male mice. This macrophage accumulation was not as profound as that observed in the arterial thrombus formed in the abdominal region of AngII-infused mice that are wild-type for M-CSF, probably, due to the diminished number of circulating monocytes in *op* mice.

The basis for the localization of the intra-laminar hemorrhage to the outer margins of the upper aorta remains to be further defined. It is assumed that the small number of laminar units of the mouse aortas precludes the development of vasa vasorum in the medial region [24]. Thus, the presence of the blood cells in the intra-laminar space is probably not attributable to rupture of vasa vasorum within the aortic media. Moreover, external elastic lamina was grossly intact, so we were unable to provide evidence that the red blood cells originated from rupture of vasa vasorum in the adventitial layer. Since AngII has angiogenic properties [25], we examined the possibility that aortas of *op* mice form abnormal medial neovessels with a propensity to rupture during AngII infusion. However, we were unable to detect the presence of neovessels, as defined by PECAM-1 immunostaining, in the media under either basal conditions or following AngII infusion (data not shown). We were also unable to detect any site-specific structural changes or decrease in the integrity of elastin fibers in *op* mice prior to AngII infusion that would

serve as an entry point for red blood cells. The antero-lateral area of *op* mouse aortas displayed a slight, but significant, hypertrophy. However, this was observed throughout the length of thoracic aortas. Previous studies have demonstrated that AngII infusion leads to a predominance of inflammatory response in the adventitia [5], although this does not explain the presence of the erythrocytes in the outer margins of the aorta. Finally, there was no difference in the AngII-induced blood pressure increase that could account for the pathology in *op* mice.

Although we were not able to demonstrate any structural differences between the upper and lower thoracic aorta, studies on aortic rings demonstrated functional difference between these regions. Contractility experiments have been used previously to demonstrate functional differences for specific aortic regions for a number of substances including, TGF-beta [26], norepinephrine [27], and AngII [18]. These differing responses throughout the aorta are probably attributable to the regional difference in the embryological origin of smooth muscle cells [28]. In the present study, we examined the contractility response of aortic segments within the thoracic aorta. The ability of PE to contract aortic rings was greatly enhanced in the upper thoracic aorta of *op* mice compared to the same region in the control mice. There were no differences in the contractile response to phenylephrine in the lower thoracic aorta of either *op* or control mice. While these responses do not determine any overt rationale for the mechanisms of the intra-laminar hemorrhage, they provide a description that differences exist in these two regions of the thoracic aorta that have differing susceptibility to AngII-induced pathology. This pathology might reflect not only the effect of monocyte deficiency, but the effect of M-CSF deficiency on the vessel wall integrity. M-CSF is not only effective on macrophages, but also has multiple functions on smooth muscle cells and is involved in macrophage-endothelial cell interactions [29,30].

In conclusion, we demonstrated that M-CSF deficiency reduced AngII-induced atherosclerosis, but predisposes male mice to intra-laminar degradation and hematoma formation localized in the upper thoracic aorta.

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