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Angiotensin II-induced aortic aneurysms

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Abstract

Abdominal aortic aneurysms (AAAs) are permanent dilations of the aorta that pose a significant health problem. These dilations are relatively asymptomatic, but insidious, as rupture of an AAA is associated with a high degree of mortality due to excessive blood loss. The prevalence of AAAs increases with advancing age in both males and females, but is greater in males compared to females at all ages. Despite the common occurrence of this disease, we have limited understanding of the sequential biochemical and cellular events of AAA initiation and progression. In part, the lack of knowledge is due to the practical difficulties of studying this asymptomatic disease in humans. Recently, several mice models of AAAs have been developed that have provided novel insights into mechanism of AAA formation. The most widely used model of AAAs induces the disease during chronic subcutaneous infusion of angiotensin II. The purpose of this chapter is to introduce the background of this model, provide experimental details of its implementation, and discuss its relevance to the human disease.

Introduction

Studies on the chronic subcutaneous infusion of angiotensin II (AngII) into hyperlipidemic mice were initiated as an attempt to develop a model of hypertension-induced atherosclerosis.¹⁻³ These initial studies demonstrated that AngII infusion was a profound stimulus in the development of atherosclerosis. However, contrary to the original hypothesis of the studies, this marked atherogenic effect was due to direct inflammatory effects rather than elevations in blood pressure.³ During the processing

of the aortas from AngII-infused mice, the presence of large abdominal aortic aneurysms (AAAs) were noted in the suprarenal aorta.² The ability of AngII infusion to promote the development of AAAs in mice has now been duplicated by many laboratories.^{4–11}

The development of AngII-induced AAAs is a complex pathology that involves multiple cell types that initiate mechanisms at specific stages of AAA formation. Within days of AngII infusion, macrophages accumulate in the media of the AAA-prone region.¹² This leukocyte accumulation is associated with breaks in elastin fibers. Medial dissection, occurring early in the process, leads to aortic dilation with the integrity of the lumen being maintained by the adventitia. The medial dissection results in formation of an intramural thrombus. There is a profound infiltration of macrophages that presumably occurs in response to thrombus formation. The dissected region enters a phase in which leukocytes of the innate and acquired immune system infiltrate while the vessel remodels. The leukocytes that have been detected in AngII-induced aneurysm tissue include macrophages, T and B lymphocytes. The dissected region forms a 'neomedia' that becomes completely relined with endothelial cells. Prominent atherosclerotic regions form in the remodeled region at later stages in the progression of AAAs.¹²

This chapter will describe experimental design, methodology with discussion of the potential pitfalls of the AngII-induced model of AAA formation.

Methods

Mouse selection

The majority of published studies on AngII-induced AAAs have used mice that are hyperlipidemic either as a consequence of deficiency of either apolipoprotein E (ApoE) or low density lipoprotein (LDL) receptors. Studies using ApoE^{-/-} mice were either fed a normal diet or one supplemented with saturated fat.^{2,4} All the studies using LDL receptor^{-/-} mice have fed a diet enriched in saturated fat and cholesterol.^{1,13} No overt differences have been noted in the incidence, severity, or characteristics of AngII-infused AAAs between these two genetically targeted mice. To date, all the publications on AngII-induced AAAs in these genetically engineered hyperlipidemic backgrounds have used mice that have been backcrossed in a C57BL/6 background. Wild type C57BL/6 mice also develop AngII-induced AAAs, albeit at a much lower incidence.⁶ Interestingly, the low incidence of AAA formation did not increase when C57BL/6 mice were fed a high cholesterol diet that doubled serum cholesterol.¹⁴ Thus, at present it is unclear whether hyperlipidemia or pre-existing atherosclerosis accelerate AngII-induced AAA formation in genetically targeted mice.

There have been no studies that have demonstrated clearly an effect of age on the development of AngII-induced AAAs. Studies have used mice that range in age from 2.5 to 11 months of age at the initiation of AngII-infusion, with the typical start of AngII infusion at 10 weeks of age. Across this varied age range, the incidence of AngII-induced AAAs in published studies is fairly consistent (70–90 per cent

incidence in hyperlipidemic mice). One of the practical limits of using younger mice is related to their size. Implantation of the Alzet pump model most commonly used in these studies requires that the body weight of mice be at least 20 g to achieve effective drug delivery.

Gender plays a prominent role in the development of AngII-AAAs. As in the development of AAAs in humans, male mice are more prone to developing AngII-induced AAAs.¹⁵ Ovarian hormones influence the development of AngII-induced AAAs, as demonstrated by the attenuation of AAAs in male ApoE^{-/-} mice administered with 17beta estradiol.⁷ However, the reduction of endogenous estrogen by ovariectomy of female mice did not affect the incidence or severity of AAAs.¹⁵ In contrast, the reduction of male hormones by orchidectomy decreased the incidence and severity of AAAs to a level that was indistinguishable from females.¹⁵ Thus, although exogenous estrogen administration lessens AngII-induced AAA formation, it appears that androgens are the endogenous sex hormones that are responsible for the enhanced susceptibility of males. The mechanism underlying the androgen induced susceptibility for AngII-induced AAAs is undefined.

Preparation and implantation of Alzet pumps

All published studies on AngII-induced AAAs have used Alzet pumps to administer the octapeptide. Alzet pumps have a flexible reservoir that is surrounded by a salt sleeve and encased by a semi-permeable membrane. The salt sleeve promotes the flux of fluids across the membrane that compresses the drug-containing reservoir and displaces fluids at a highly controlled rate that is regulated by a flow moderator. Most studies have used the 2004 model which delivers fluids at a rate of approximately 2.5 μ l/h for 28 days. The flow rates vary with lot numbers and have sufficiently wide variance that the flow rate of a specific batch needs to be known in order to calculate peptide delivery rates.

Infusion rates of AngII that have generated AAAs have ranged from 500 to 2500 ng/kg/min.^{2,4,8} Most studies have used a rate of 1000 ng/kg/min that generates greater than 80 per cent AAAs in hyperlipidemic male mice. The calculation of the dose of AngII to be given to the mouse is determined by the required infusion rate and the weight of the mouse, taking into consideration the weight of the mouse over the duration of the 28-day infusion. Calculation of the AngII dose based on the body weight at the start of the study will lead to lower doses being administered at later stages of the infusion. Some investigators calculate the infusion rate based on the anticipated weight of the mice at the mid point of the infusion period. This calculation requires previous experience, since weight gain over the 28-day period can be altered by several variables including strain of mouse, diet, and gender. An additional consideration when calculating the infusion rate at study mid point is the effect of AngII infusion to decrease body weight, which has been observed under certain conditions (i.e. high fat-fed mice).

Thus, the infusion rate of AngII is based on the daily dose, weight of the mice, and the infusion rate of the specific batch of Alzet pumps. The first step in preparing the pumps is

the dissolution of AngII. AngII is commonly supplied as a lyophilised powder in glass bottles. However, AngII in solution has a high affinity for glass. Thus, AngII solutions must be dissolved in plastic vessels. Alzet pumps are filled as per the manufacturer's instructions. The 2004 model of Alzet pump requires a priming period of 40 hours prior to it extruding the solution from the reservoir. If an experimental design requires that peptide infusion starts immediately on implantation, the filled pumps will need to be incubated in sterile saline at 37°C prior to placement.

Most of the studies implant the pumps in the subcutaneous space. This is achieved by making a small cut in the skin in the back of the neck, using blunt dissection to form a pouch on the flank, placing the Alzet pump into the subcutaneous space, and closing the incision point with surgical glue or staples. The incision site should be monitored to ensure it retains its closure. Because female mice groom more extensively than males, they have a greater propensity to open the incision site.

Monitoring during AngII infusion

Mice should be visually inspected daily during the infusion of AngII. The majority of mice will have no overt adverse effects during the infusion of AngII. A small percentage of the mice will develop partial hind limb paralysis. Some mice will die during infusion due to ruptured AAAs. The incidence of mortality is based on several variables including the strain of mouse, gender, and dose. Necropsy should be performed on mice to determine the cause of death. The vast majority of the mice will have a large blood clot in the peritoneal cavity in the vicinity of the left kidney. A few mice will have clots in the upper thorax in the region of the ascending aorta.

During the infusion period, we acquire body weights on a weekly basis. As described above, the higher doses of AngII may lead to attenuation of body weight gain, or even loss. Blood pressure is also commonly monitored at intervals during the infusion of AngII. Systolic blood pressure is most commonly acquired using a computerized system in which blood flow is monitored during the inflation of a cuff on the tail.

Acquisition of AAAs

Most of the published studies have terminated mice after 28 days of AngII infusion. However, the incidence of AngII-induced AAAs is similar at 14 days of infusion.¹⁶ Infusions of AngII beyond 28 days can be achieved by replacing the Alzet pumps. The limited published studies show differing characteristics of AAAs with more protracted intervals of AngII infusion, although these have not been extensively characterised.¹²

At termination of studies, mice are perfused with saline to remove blood. The aorta can be dissected free either with, or without, perfusion with a fixative such as paraformaldehyde. Perfusion of the mouse with fixative at physiological pressures can better preserve the tissue. However, fixation ablates the immunoreactivity of

many antibodies. Thus, the decision of whether to use fixatives is based on the tissue characterization that will be performed.

An alternative mode to not fixing the tissue, but maintaining its patency, is to perfuse at a constant pressure of 100 mmHg through the heart with phosphate-buffered saline followed by warm (37°C) agarose (SeaPlaque GTG Agarose, low-melt, FMC BioProducts, Rockland, ME, USA) diluted in saline (3 per cent wt/vol) and colored with a green tissue dye. After the agarose solidifies, the abdominal aorta can be dissected free from the surrounding connective tissue.

Quantification of AAAs

The size and heterogeneity of AAAs provides some hurdles to the quantification of AAAs. There are three common approaches to the quantification of experimental AAAs, which are: (1) percentage incidence; (2) severity based on arbitrary comparisons; (3) measurements of the physical dimensions.

In addition to the quantification of AAAs following excision of the tissue, there are methods recently available for the detection and quantification of AAAs using noninvasive techniques in living animals. The only methodology that has been fully validated as an approach to date is the use of ultrasound.¹⁷ This is discussed later in this chapter.

Incidence of aneurysm formation

There is a lack of agreement as to what constitutes an AAA in humans. The criteria are based either on an absolute measurement of aortic width or one that is relative to a 'normal' segment.¹⁸ Similarly, there is also no uniform standard for the definition of an AAA in mouse models of the disease. However, all the initial studies on AngII-induced AAA have included the percent incidence as a quantitative parameter. The mean outer diameter of the suprarenal aortic region of male mice is approximately 0.9 mm. We have used the criterion of an expansion that is 50 per cent greater than normal as a definition of AAAs. This is based on absolute measurements since the involvement of AAA in the suprarenal region can be extensive and negate the ability to make comparisons with a 'normal' area.

Based on the acceptance of this specific criterion, the use of percentage incidence provides an easily defined mode of quantification. However, the use of a dichotomous variable is a relatively insensitive mode of statistical analysis. Therefore, unless a specific intervention produces dramatic differences on the development of AAA, large group sizes will be needed to achieve results with appropriate statistical power.

Severity indexes

We originally proposed a scheme for classifying the severity of aneurysm pathology based on the appearance of AAAs.⁷ This initial classification took into account the

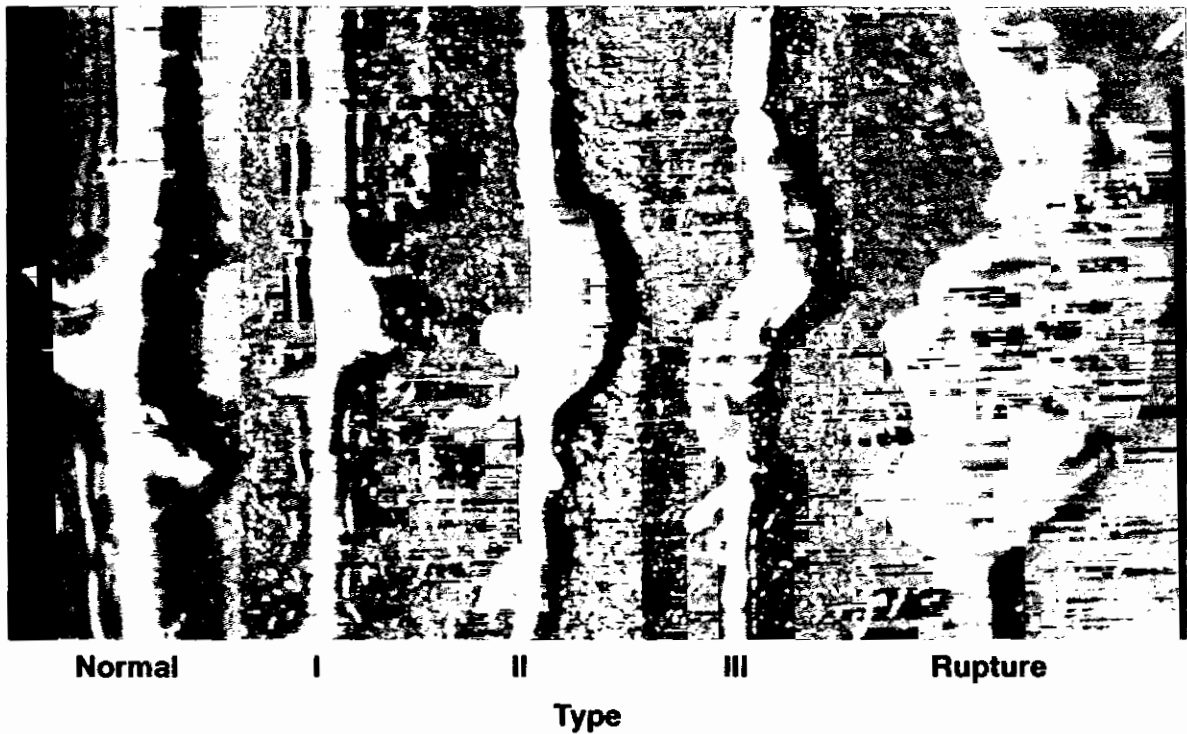


Figure 11.1 A suggested scale for quantification of the severity of AAAs that develop following infusion of AngII. Further description of these classes is provided in the text. For a color version of this figure, please see the images supplied on the accompanying CD

presence of thrombus.^{13,16} However, now that we have defined characteristics of AAAs at multiple stages following AngII infusion, we have realised that the presence of thrombus is more a function of time of AAA formation rather than severity. With several years of experience in assessing AngII-induced AAAs, we have modified this scheme. The descriptors of these types are:

- Type I: A discernable dilation that is 1.5 to 2 times the diameter of a normal abdominal aorta.
- Type II: A single large dilation that is more than 2 times the diameter of a normal abdominal aorta.
- Type III: Multiple dilations generally extending proximal to the suprarenal region.
- Rupture: The ultimate dire consequence of AAA formation. Ruptured AAAs are clearly evident by the appearance of clots in the retroperitoneum and death.

Examples of each type of AAA are provided in Figure 11.1.

Measurements of physical dimensions of AAAs

Unlike the dichotomous mode of defining AAA by incidence, continuous traits are a more powerful mode of generating statistical significance. Thus, there are a number of modes in which physical measurements can be performed to permit the use of

statistical analysis of continuous traits. There are several ways in which the physical dimensions of AAAs can be quantitated as described below.

Abdominal aortic weight

Abdominal aorta (defined as the region between the diaphragm and the ileal bifurcation) can be blotted and weighed. This has been used to determine the severity of AAA formation.¹⁵ While potentially useful, it should be noted that AAAs that have uniformly thinned walls may not have an increase in weight.

External aortic diameter

This is a common measurement in other mouse models of disease that involve elastase infusion or adventitial calcium chloride exposure.^{19,20} This is achieved by computer-assisted morphometric analysis of digital pictures of the aorta. These measurements are usually performed in dissected tissues. Although these measurements can be performed *in vivo*, the difficulty in defining the boundary between the adventitia and the surrounding tissue brings some subjectivity into the process.

Cross-sectional measurement

This is optimally performed on fixed aortas. Cross-sections of the aorta (2.5 μm in thickness) are made between the superior mesenteric and right renal arteries. A small portion of the right renal artery is left attached to the samples to facilitate orientation of the specimen. The tissue is dehydrated through a graded ethanol series, cleared with xylene, infiltrated with warm paraffin, embedded in paraffin blocks, cut at 5 μm thickness, and stained with hematoxylin & eosin. The lumen and adventitial circumferences at the maximal expanded portion of the suprarenal aorta are quantified by CSimple Imaging Systems (Compix, Mars, PA, USA), which are then used to calculate the luminal and outer diameters of the vessel. The wall thickness is calculated from the difference between the luminal and outer diameters.

Non-invasive ultrasound method

Recently, ultrasound machines have been developed that have the capabilities to detect blood vessels in mice. This advance permits the non-invasive detection of AAAs in mice.¹⁷ Thus, a single mouse can now be followed on a sequential basis.

At any interval during the infusion of AngII, each mouse is anaesthetised using 1.5 per cent isoflurane and laid supine on a platform with all legs taped to electrocardiogram electrodes for heart rate monitoring. Body temperature is monitored via a rectal thermometer and maintained at $\sim 37^{\circ}\text{C}$ using a heating pad and lamp. All hair is removed from the abdomen using a chemical hair remover (Nair; Carter-Horner, Mississauga, Ontario, Canada) to minimize ultrasound signal attenuation. To provide a coupling medium for the transducer, a pre-warmed ultrasound gel (Aquasonic 100; Parker Laboratories, Orange, NJ, USA) is spread over the abdominal wall. Imaging starts after waiting 1–2 min for the mouse to stabilise. The complete examination for each mouse lasts for about 5 min.

A newly developed ultrasound biomicroscope with built in software analysis (Vevo 660; VisualSonics, Toronto, Ontario, Canada) can be used. The single-crystal mechanical transducer has a central frequency of 40 MHz, a focal length of 6 mm, and a frame rate of 30 Hz. The maximum field of view of two-dimensional imaging is obtained in a $20 \times 20 \mu\text{m}$ area of focal plane with spatial resolution of $68 \mu\text{m}$ (lateral) by 39 mm (axial). A longitudinal image of the abdominal aorta between the diaphragm and ~ 3 mm below the level of the left renal artery is acquired. In addition, transversal images of the suprarenal aorta, at the level of the suprarenal gland, and the infrarenal aorta, ~ 1 mm below the left renal artery, are taken. Doppler signals measuring the cardiac cycle obtained from the position in the middle of the assumed vessel are used to validate that the concerned image is the abdominal aorta. The obtained images are stored digitally on a built in hard drive for off-line analysis. The abdominal aortic measurements are taken off-line with a built in software from both the longitudinal and transversal images.

Characterisation of AAAs

The cellular and chemical composition of AngII-induced AAAs are complex and heterogeneous. These can be characterised by techniques such as histology, immunocytochemistry and *in situ* hybridisation. Using these techniques, some of the most prominent features can be described including integrity of extracellular matrix, cellular composition, and expression of protein involved in the disease process. Although these are largely routine techniques, the application of immunocytochemical techniques can provide some challenges in non-specific staining, presumably due to the high abundance of extracellular matrix elements.

Since extracellular proteases are thought to be involved in the development of AAAs, their characterisation is common, particularly for the matrix metalloproteinases (MMP). This is commonly performed on extracts of aortic tissues that are subjected to gel zymography.^{4,8,10} This process requires the electrophoresis of the tissue extract in polyacrylamide gels that are impregnated with a MMP substrate. The most common substrate is gelatin, that is acted on by MMP-2 and -9. This process permits detection of these specific MMPs and relative presence of latent versus activated enzyme.

Discussion

AngII-induced AAAs have been used in many studies to define mechanisms of the disease. The extrapolation of findings require that the model is recapitulating the human disease. The extent to which AngII-induced AAAs in mice mimick the human disease is largely unknown. This is primarily due to a dearth of information on the mechanisms of the human disease. One similarity in the mouse model is the increased proclivity of males to develop the disease as in humans. Some pathological characteristics of the human disease are reproduced in this mouse model. These include fragmentation of elastic fibers, proteolytic destruction of medial connective tissue, regions of intact media, inflammation, and atherosclerosis.²¹ All these characteristics have been described in AngII-induced AAAs.¹² However, the location of the AAAs is an overt difference between the human and mouse disease. In humans, AAAs occur in the infrarenal area; while in AngII-infused mice, they have been uniformly localized to the suprarenal aortic region. This may represent a difference in the stress patterns in the aorta of the biped human versus the quadruped mouse. Interestingly, this location is also where AAAs occur in mice that are hyperlipidemic, endothelial nitric oxide synthase deficient, or smooth muscle specific deficient in LDL receptor-related protein.^{22–25}

The early publications on this model used aged hyperlipidemic mice that had extensive atherosclerotic lesions. However, many of the recent studies have used younger mice in which atherosclerotic lesions are sparse, especially at the site of AAA formation. The lack of a required role of atherosclerosis in this process can be gleaned from studies that show AngII-induced AAAs can occur in normolipidemic mice.⁶ However, the incidence of AAAs is much less in normolipidemic mice. Feeding wild type mice a high cholesterol diet did not affect the incidence of AngII-induced AAAs.¹⁴ However, cholesterol supplementation of diets produces only minor increases in plasma cholesterol concentrations. Thus, hypercholesterolemia may facilitate the development of AngII-induced AAAs through promotion of atherosclerosis or vascular inflammation. Hypercholesterolemia may also enhance the development of AngII-induced AAAs through enhancing the cellular responses to AngII. For example, smooth muscle cells are presumed to be pivotal to the development of AAAs and AT1 receptor expression in this cell type is enhanced by increasing cellular cholesterol.²⁶

Overall, the infusion of AngII into hyperlipidemic mice is a highly reproducible model for the production of AAAs. Although the model does not recapitulate all aspects of the human disease, many facets are present in both human and mouse aneurysmal tissue. Therefore, it provides a mode of defining mechanisms involved in the initiation and propagation of AAAs.

Acknowledgments

Work in the Cassis and Daugherty laboratories is supported by the National Institutes of Health (HL62846, HL73085 and HL70239). We appreciate the editorial assistance of Debra Rateri.

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