The Development of an Animal Model of Complicated Atherosclerosis for Non-invasive Imaging

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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The goal of this thesis was to produce an animal model that develops atherosclerotic plaque featuring plaque neovascularization leading to intraplaque hemorrhage and is suitable for noninvasive imaging studies. Several strategies were tested for their effectiveness in producing such plaques in the rabbit aorta, including: a high cholesterol diet, vascular endothelial growth factor injections, therapeutic contrast ultrasound, and balloon catheter injury. It was found that a combination of the high cholesterol diet and balloon injury was able to achieve plaque neovascularization in a manner dependent on circulating plasma cholesterol levels. In addition, a contrast-enhanced magnetic resonance imaging technique implemented in the animal model was able to detect plaque neovascularization and monitor its change over time in a single group of animals. In conclusion, an animal model was created where plaque neovascularization occurs in a predictable fashion and can be studied with non-invasive magnetic resonance imaging.
Acknowledgements

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# Table of Contents

CHAPTER 1:........................................................................................................... 1

1.1 Cardiovascular Disease........................................................................................... 1
  1.1.1 Overview ........................................................................................................ 1
  1.1.2 Interventions .................................................................................................. 1
  1.1.3 Prevention of Clinical Events ......................................................................... 2

1.2 Structure and Function of Large Arterial Walls....................................................... 2

1.3 Pathobiology of Atherosclerosis............................................................................ 5
  1.3.1 Lipid Accumulation in the Vessel Wall .......................................................... 5
  1.3.2 Inflammation in the Vessel Wall .................................................................... 6
  1.3.3 Histological Classification of Lesions ............................................................ 7
  1.3.4 Plaque Destabilization ................................................................................... 7
    1.3.4.1 Large Necrotic Core ............................................................................... 8
    1.3.4.2 Thin Fibrous Cap .................................................................................. 8
    1.3.4.3 Macrophage Infiltration ....................................................................... 8
    1.3.4.4 Plaque Angiogenesis ........................................................................... 9
    1.3.4.5 Plaque Hemorrhage ........................................................................... 9

1.4 Plaque Angiogenesis and Intraplaque Hemorrhage as Imaging and Therapeutic Targets 10
  1.4.1 Overview ....................................................................................................... 10
  1.4.2 The Need for an Animal Model of Plaque Angiogenesis and Intraplaque Hemorrhage ................................................................. 12

1.5 Animal Models with Demonstrated Plaque Angiogenesis or Hemorrhage .............. 12
  1.5.1 Mice............................................................................................................... 12
  1.5.2 Swine............................................................................................................. 13
2.5.1 Dietary Cholesterol Modulates Plaque Formation ......................................... 46
2.5.2 Vascular Endothelial Growth Factor Accelerates Early Plaque Growth ............ 47
2.5.3 Therapeutic Contrast Ultrasound Regimen has no Effect on Plaque Growth ...... 48
2.5.4 Balloon Injury Produces Advanced Plaques ................................................. 50
2.5.5 Study Limitations ............................................................................................ 54
  2.5.5.1 Immunohistochemical Staining and Quantification ................................. 54
  2.5.5.2 Specimen Sampling .................................................................................... 54
  2.5.5.3 Animal Care ............................................................................................. 55
  2.5.5.4 Consistency of Interventions ...................................................................... 55
  2.5.5.5 Group Size .................................................................................................. 56
2.6 Chapter Summary ................................................................................................ 57
  2.6.1 Imaging of the Animal Model ........................................................................ 57
References .................................................................................................................. 61

CHAPTER 3: ............................................................................................................. 65

3.1 Introduction ........................................................................................................... 65
3.2 Materials and Methods.......................................................................................... 66
  3.2.1 Animal Model ............................................................................................... 66
  3.2.2 MR Imaging .................................................................................................... 66
  3.2.3 MR Image Analysis ....................................................................................... 67
    3.2.3.1 Comparison With Histology ................................................................. 67
    3.2.3.2 Comparison Over Time ......................................................................... 68
  3.2.4 Statistical Analysis ......................................................................................... 68
3.3 Results .................................................................................................................... 68
  3.3.1 Animal Model and Histological Analysis ...................................................... 68
3.3.2 MR Image Analysis ................................................................. 70
  3.3.2.1 Vessel Wall Enhancement Area Corresponds With Histological Measures ... 71
  3.3.2.2 Vessel Wall Enhancement Area Increases Over Time ......................... 72
3.4 Discussion ......................................................................................... 73
References ................................................................................................. 77

CHAPTER 4: ............................................................................................... 79

4.1 Thesis Summary ................................................................................... 79
4.2 Future Directions ................................................................................... 81
  4.2.1 Animal Model of Intraplaque Hemorrhage ........................................ 81
  4.2.2 Therapeutics for Intraplaque Hemorrhage ....................................... 82
  4.2.3 Imaging Plaque Neovascularization and Intraplaque Hemorrhage .......... 82
    4.2.3.1 Contrast-enhanced Magnetic Resonance Imaging ....................... 82
    4.2.3.2 Magnetic Resonance Imaging of Intraplaque Hemorrhage .......... 83
    4.2.3.3 Contrast Ultrasound Imaging .................................................... 83
4.3 Conclusion ............................................................................................ 83
References .................................................................................................. 85
List of Tables

Table 2.1. Relationships between plasma cholesterol levels and average intimal area.......... 45

Table 2.2. A summary of all the groups of animals used in the experiments....................... 59

Table 3.1. Pearson correlation coefficients of individual vessel wall enhancement area measures versus individual histological measures ................................................................. 71

Table 3.2. Pearson correlation coefficients of average vessel wall enhancement area measures versus average histological measures .............................................................................. 72
List of Figures

**Figure 1.1.** A cross-section of an artery that clearly illustrates three tissue layers. ..................... 3

**Figure 1.2.** An arterial section that shows prominent vasa vasora. ........................................ 5

**Figure 1.3.** An arterial section showing significant plaque burden with neovessels .............. 10

**Figure 1.4.** A magnetic resonance image obtained using the MRIPH sequence with its corresponding histological section ................................................................. 11

**Figure 2.1.** Representative histological sections from the group fed the high cholesterol diet for 20 weeks with no other interventions................................................................. 31

**Figure 2.2.** Bar graph comparing histological measures between groups of different cholesterol feeding and growth factor regimens ................................................................. 32

**Figure 2.3.** Section through the aortic arch of a rabbit that received the high cholesterol diet for 20 weeks with no other interventions in the arch................................................................. 33

**Figure 2.4.** Small artery from a rabbit on the high cholesterol diet for 20 weeks with no other interventions where neovessels have infiltrated the media and intima. .................................. 33

**Figure 2.5.** A section from the abdominal aorta of a rabbit fed the oxidized cholesterol diet.... 34

**Figure 2.6.** One of the plaques in the group that was given vascular endothelial growth factor in addition to the high cholesterol diet................................................................. 35

**Figure 2.7.** A section of an aortic arch from one of the rabbits that received vascular endothelial growth factor and endothelial denudation of the abdominal aorta................................. 36

**Figure 2.8.** Bar graph comparing histological measures of the groups on the high cholesterol diet for either 20 or 30 weeks and receiving different numbers of growth factor injections. ...... 37

**Figure 2.9.** Sections from the carotid artery and aortic arch from rabbits fed the high cholesterol diet for 30 weeks and given growth factor injections. .............................................. 38
Figure 2.10. Bar graph comparing histological measures between the groups that received weekly insonations and their non-ultrasound control groups.

Figure 2.11. A section from a rabbit fed the high cholesterol diet that developed advanced atheroma in response to endothelial denudation.

Figure 2.12. A section from a rabbit fed the high cholesterol diet that developed relatively moderate plaques in response to endothelial denudation.

Figure 2.13. Bar graph comparing histological measures of the 20-week (light bars) and 14-week balloon injury groups.

Figure 2.14. Scatterplots of the average intimal areas of the 20-week injury groups versus plasma cholesterol levels at various time points.

Figure 2.15. Scatterplots of the average intimal areas of the 14-week injury groups versus plasma cholesterol levels at various time points.

Figure 3.1. The method used to determine vessel wall enhancement area.

Figure 3.2. Representative histological sections of aortas from two different animals 10 weeks after endothelial injury.

Figure 3.3. Scatterplots with zero-intercept regression lines for each pair-wise comparison between all three histological measures.

Figure 3.4. Representative high-resolution, T1-weighted MR images of aortas from two different animals scanned prior to sacrifice at week 20.

Figure 3.5. Scatterplots with zero-intercept regression lines for MR vessel wall enhancement area versus histological measures.

Figure 3.6. Bar graph showing changes in average vessel wall enhancement area over time.
# List of Abbreviations and Symbols

## in Alphabetical Order

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>3-D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>apoE-/-</td>
<td>apolipoprotein e double knockout</td>
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<tr>
<td>DICOM</td>
<td>digital imaging and communications in medicine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ED</td>
<td>endothelial denudation</td>
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<tr>
<td>EPC</td>
<td>endothelial progenitor cell</td>
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<tr>
<td>Flt-1</td>
<td>FMS-like tyrosine kinase</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HC</td>
<td>high cholesterol</td>
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<tr>
<td>HIFU</td>
<td>high intensity focused ultrasound</td>
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<tr>
<td>IA</td>
<td>intimal area</td>
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<tr>
<td>IPH</td>
<td>intraplaque hemorrhage</td>
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<td>IVUS</td>
<td>intravascular ultrasound</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>LDLR</td>
<td>low density lipoprotein receptor</td>
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<td>MA</td>
<td>macrophage area</td>
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<td>MC</td>
<td>microvessel count</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<td>MI</td>
<td>mechanical index</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MRIPH</td>
<td>magnetic resonance intraplaque hemorrhage</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SPECIAL</td>
<td>SPECtral inversion at lipids</td>
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<tr>
<td>T1</td>
<td>spin-lattice relaxation time</td>
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<td>TE</td>
<td>echo time</td>
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<td>TR</td>
<td>repetition time</td>
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<td>US</td>
<td>ultrasound</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VEGFR-1</td>
<td>vascular endothelial growth factor receptor-1</td>
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<tr>
<td>VEGFR-2</td>
<td>vascular endothelial growth factor receptor-2</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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<tr>
<td>$\theta$</td>
<td>flip angle</td>
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CHAPTER 1:

Introduction

1.1 Cardiovascular Disease

1.1.1 Overview

Cardiovascular disease, which encompasses heart attack and stroke, remains among the top causes of death and disability worldwide and was responsible for more deaths in Canada in 2005 than any other disease category\(^1\). With costs measured in physician services, hospital expenditures, lost wages and decreased productivity, cardiovascular disease places an estimated $22.2 billion annual burden on Canada’s economy\(^2\). From a global perspective, the World Health Organization predicts that cardiovascular disease will become the leading cause of death in developing countries\(^3\). Damage to the heart and brain can arise from an occlusion in the arteries supplying these organs, with the underlying disease process being atherosclerosis. Atherosclerosis refers to plaque formation or the thickening of the arterial wall which can impinge on the blood-carrying capacity of the lumen, the centre of the vessel. The lumen may become obstructed by the gradual narrowing and occlusion of the lumen or by the formation of a blood clot, also known as thrombosis. In addition, a piece of the thrombus may break away and obstruct blood flow at a downstream location. Tissue damage occurs in the end organ due to ischemia, the shortage of blood supply that is necessary for the delivery of oxygen and other nutrients.

1.1.2 Interventions

A patient with compromised blood supply to the heart or brain may experience transient symptoms such as chest pain or dizziness, or may suffer a heart attack or stroke before he seeks
medical treatment. If the patient is not already receiving treatment, he may be prescribed drugs to inhibit clot formation or to systemically treat atherosclerosis by lowering blood cholesterol levels. Local, vessel-specific interventions include coronary artery bypass graft surgery, angioplasty and stenting in the vessel to keep it open, or endarterectomy to remove the plaque. In the coronary arteries, there may be multiple sites of plaque formation, suggesting that identifying the most at-risk sites is helpful for applying local interventions.

1.1.3 Prevention of Clinical Events

Before performing an invasive procedure, it is necessary to weigh the benefits gained against the risks and costs of such a procedure. In addition to physiological tests, medical imaging is used to diagnose patients who likely have carotid or coronary artery disease and is necessary for the assessment of the vessels in question prior to a localized intervention\textsuperscript{4,5}. Traditional imaging methods of determining plaque burden in a vessel involve measuring stenosis or narrowing of the lumen. This is readily accomplished using well-established procedures such as X-ray angiography, Doppler ultrasound and computed tomography angiography\textsuperscript{6,7}. While clinical data has shown that greater carotid stenosis presents a greater risk for future strokes\textsuperscript{8}, stenosis itself is not necessarily sufficient as a predictor of future clinical events. An ideal set of criteria would identify patients with vessel disease severe enough that acute intervention is necessary to prevent life-threatening clinical events. Plaque features that may help in identifying plaques at risk of causing thrombosis and subsequent clinical events will be introduced in a later section of this thesis.

1.2 Structure and Function of Large Arterial Walls

Atherosclerotic lesions typically form in arteries of 1 mm diameter or larger. In a normal artery of this caliber, the vessel wall can be divided into three layers. Starting from the luminal surface
which encompasses flowing blood, these layers are the intima, media and adventitia (Fig. 1.1).

**Figure 1.1.** A hematoxylin and eosin (H&E) stained cross-section of an artery that clearly illustrates three tissue layers, with the vessel lumen indicated by the asterisk. A layer of endothelial cells (arrow) overlies the luminal side of the thickened intima. The media of this elastic artery is composed of alternating layers of elastin and smooth muscle cells and is separated from the intima by the internal elastic lamina. Since this tissue was fixed in a relaxed state, the wavy layers of elastin are conspicuous. The outer layer beyond the outermost layer of elastin is the adventitia and is composed mainly of fibrous tissue.

The intima of a large human vessel is made up of a continuous layer of endothelial cells sitting on a basement membrane. The endothelial cells are normally held together by intercellular connections known as tight junctions. Between this basement membrane and the internal elastic membrane (a porous elastic sheet) lies sparse connective tissue containing proteins and proteoglycans. As the blood-contacting layer of the vessel, the endothelium regulates vascular homeostasis in a multitude of ways. It serves as a barrier protecting the rest of the wall from the influx of unwanted cells and substances, maintains the balance between vasoconstriction and vasodilation, and keeps an anti-coagulant equilibrium.

The composition of the media varies depending on whether the vessel is an elastic artery or a muscular artery. Elastic arteries are the largest arteries (aorta, common carotid, pulmonary) and mainly serve as delivery conduits for blood. In contrast, muscular arteries (most other large
arteries) have the additional role of regulating blood pressure through vasoconstriction and vasodilation. The media of an elastic artery is composed of concentric layers of smooth muscle cells alternating with elastic layers which allow the vessel to stretch according to the demands of blood flow. The elastic layers are absent from muscular arteries as they are not designed to handle as large a volume of blood flow. Rather, the contiguous layers of smooth muscle cells act effectively to constrict or dilate in the regulation of blood flow. Finally, the outer boundary of the media is marked by the external elastic lamina.

The outermost layer of the large vessel wall adjoins surrounding connective tissue and is itself made up of collagen, elastin, fibroblasts, nerves and smaller blood vessels. While the inner layers rely on diffusion from the lumen for their supply of oxygen and other nutrients, the outer layers in a thick wall (ie. the adventitia and sometimes the outer layers of the media) have their own supply in the form of a network of small vessels known as the vasa vasorum (Fig. 1.2). The typical architecture of this network consists of primary vasa vasora which run parallel to the vessel and secondary vasa vasora which branch off of the primary vasa vasora and tend to follow a more circumferential pattern. The primary vasa vasora in turn originate from either the main lumen itself or from proximal branches of the main artery. As in any other capillary bed, the vasa vasorum has a venous side that drains into the corresponding vein.

While the above summarizes the structure and function of the normal, healthy arterial wall, significant alterations to all the vessel wall layers occur during the atherosclerotic disease process.
An H&E stained arterial section that shows prominent vasa vasora in the adventitia (arrows). Red blood cells in the lumens of these microvessels confirm their blood-carrying function.

1.3 Pathobiology of Atherosclerosis

The early stages of atherosclerosis are thought to proceed in a steady and linear manner. While the exact initiating event is still debatable, it is widely recognized that endothelial injury or dysfunction is likely the earliest detectable change before the accumulation of lipids take place in the arterial wall. This injury may result from differences in shear stress on endothelial cells lining the vessel lumen due to blood flow patterns.

1.3.1 Lipid Accumulation in the Vessel Wall

An endothelium that loses some of its barrier function may invite an increased influx of circulating cholesterol and other lipids into the vessel wall, upsetting the balance between influx and radially outward efflux of lipids. An increased influx of lipids (due to increased circulating cholesterol, endothelial dysfunction, or both) may lead to an increased lipid residence time in the wall, augmenting the chances of the lipids becoming oxidized or otherwise chemically modified (by acetylation, glycation, and immune complex formation). Of the various types of lipoproteins that carry hydrophobic cholesterol and triglycerides, low density lipoprotein (LDL) has received the most attention in the context of atherosclerosis.
Oxidized LDL exerts a myriad of pro-atherogenic effects, many of which contribute to increased retention of lipids in the vessel wall\textsuperscript{12,13} and invasion of inflammatory cells\textsuperscript{14}. In terms of the former, oxidized LDL promotes the aggregation of LDL particles, stimulates production of collagen by smooth muscle cells in the media, and bestows LDL particles with a greater affinity for the collagen matrix\textsuperscript{12,13}. With respect to inflammation, the particles themselves act as a chemoattractant for circulating monocytes while inducing expression of monocyte chemoattractant protein-1 (MCP-1) in endothelial cells\textsuperscript{14}.

1.3.2 Inflammation in the Vessel Wall

One of the major keys to understanding the progression of atherosclerotic disease is the role that inflammatory cells, most notably macrophages, play. Macrophages, whose circulating precursors are termed monocytes, are responsible for lipid accumulation in the vessel wall and for the destabilization of plaques in their later stages\textsuperscript{15,16,17}. As mentioned above, monocytes are recruited from the circulation by receptors expressed on dysfunctional endothelial cells and differentiate into macrophages. Within the vessel wall, macrophages phagocytose foreign substances and apoptotic cells. They express LDL receptor (LDLR) that facilitates phagocytosis of LDL and is downregulated when intracellular cholesterol levels are high. Macrophages also express scavenger receptors that are not downregulated and readily take up chemically modified LDL in an unregulated manner. This results in the gross accumulation of lipid droplets in the macrophages, known at this stage as foam cells. This early stage of plaque development has been termed the fatty streak.

As cholesterol accumulation continues, foam cells undergo apoptosis, leading to pools of extracellular cholesterol and cellular debris that form a thrombogenic necrotic core\textsuperscript{18}. Protecting the necrotic core from the bloodstream is a fibrous cap made up of smooth muscle cells and the matrix proteins they produce. The smooth muscle cells are thought to migrate from
the medial layer of the vessel and proliferate, contributing to plaque volume. This synthetic phenotype may be in part due to cytokines secreted by plaque macrophages. Macrophages are also associated with MCP-1 expression on neighbouring cells, thereby creating a positive feedback loop.

1.3.3 Histological Classification of Lesions

Up to this point in plaque progression, the process appears to be largely linear and is well-described by the American Heart Association-recommended classification developed by Stary et al. based on the examination of histological specimens. Under the scheme, different types of lesions are assigned roman numerals that ascend with increased lesion severity. Briefly, stages I-III of lesion progression are associated with lipid accumulation and are regarded as reversible. Type I lesions feature isolated foam cells and type II lesions, also known as fatty streaks, show multiple layers of foam cells. The extracellular accumulation of lipids classifies a lesion as type III.

Once a plaque forms a significant lipid core, it becomes a type IV lesion. It progresses to a type V lesion, or fibroatheroma, if the intimal cap overlying the lipid core becomes augmented by proliferating smooth muscle cells and collagen deposition. Under a decrease in lipid content, the plaque may become increasingly calcified (type VII) or fibrotic (type VIII). Overall calcification in the vascular tree is associated with age and severity of atherosclerosis, but calcification in a specific plaque may not necessarily render it more likely to cause a clinical event. A predominantly fibrous plaque is generally thought of as more stable and quiescent. Alternatively, either of the type IV or V lesions has the potential to progress to clinically significant plaques through various factors as described next.

1.3.4 Plaque Destabilization
Once a plaque has reached the atheroma or fibroatheroma stage with a fibrous cap overlying a necrotic lipid core, its progression may become more episodic in nature\textsuperscript{17}, with these complications being represented by the type VI stage. Erosion of the luminal surface of the plaque can lead to thrombosis and a subsequent plaque-enlarging healing response. A break in the fibrous cap can lead to not only to thrombosis but also hemorrhage into the plaque, which also contributes to plaque growth. It is estimated that over half of sudden coronary deaths associated with thrombus formation are due to fibrous cap disruption rather than erosion\textsuperscript{27}.

Several factors, supported by clinical evidence, contribute to plaque instability and their presence indicates a plaque more prone to fibrous cap fissure or rupture that could lead to occlusive thrombosis\textsuperscript{27,28,29}.

### 1.3.4.1 Large Necrotic Core

Necrotic core size has been associated with ruptured plaques in specimens from patients who died from acute coronary disease. In one study, the necrotic core made up more than 10\% of the plaque area in nearly 90\% of ruptured plaques\textsuperscript{27}. Composition of this lipid core is also important, with a higher free to esterified cholesterol ratio present in ruptured plaques\textsuperscript{30}. Possible sources of free cholesterol are degraded macrophages and red blood cell membranes, both of which are associated with later stages of plaque progression\textsuperscript{31}.

### 1.3.4.2 Thin Fibrous Cap

A thin fibrous cap acts is more likely to yield to mechanical forces and consequently fissure or outright rupture. Results from coronary artery histological specimens indicated that fibrous caps of <65μm thickness were present in >95\% of the ruptured plaque examined\textsuperscript{32}. The fibrous cap may be weakened by enzymes which break down extracellular matrix proteins\textsuperscript{33} or by the lack of smooth muscle cells\textsuperscript{33}.

### 1.3.4.3 Macrophage Infiltration
Macrophages elaborate enzymes called matrix metalloproteinases (MMPs) that degrade the fibrous cap, making it more susceptible to rupture\textsuperscript{17}. It has been found that ruptured plaques often have macrophage-rich regions located at the shoulders of the plaque\textsuperscript{28}. Macrophages are also a source of pro-angiogenic and pro-inflammatory factors, such that they encourage the growth of vessels into the plaque and the further infiltration of inflammatory cells\textsuperscript{17}.

**1.3.4.4 Plaque Angiogenesis**

Hypoxia within the thickened vessel wall stimulates small neovessel growth into the plaque\textsuperscript{34,35} (Fig. 1.3). These neovessels may originate from either the large vessel lumen or the vasa vasorum supplying the outer vessel layers, with the latter being 28 times more likely in atherosclerotic human coronaries\textsuperscript{36}. The neovessels often lack the tight junctions between endothelial cells and the pericytes that usually surround and support capillaries, thus rendering them more leaky and prone to rupture than normal capillaries\textsuperscript{37,38}. Macrophages are a major source of pro-angiogenic factors, most notably vascular endothelial growth factor (VEGF)\textsuperscript{17}. In turn, the neovessels serve as a conduit for macrophages and other inflammatory cells to enter the plaque\textsuperscript{39,34}.

**1.3.4.5 Plaque Hemorrhage**

When red blood cells enter the plaque--whether through frank hemorrhage (from the large vessel lumen) or intraplaque hemorrhage (from plaque neovessels)--they facilitate numerous processes that contribute to plaque instability. Red blood cell membranes contain high concentrations of cholesterol and increase the amount of free cholesterol in the plaque, leading to augmentation of the necrotic core\textsuperscript{31,39}. Red blood cell membranes also contain receptors which can bind a variety of chemokines that lead to inflammatory cell infiltration\textsuperscript{31,39}. Additionally, the hemoglobin content in red blood cells increases the amount of free iron in the plaque, leading to oxidation of
lipids. Oxidative stress leads to the elaboration of enzymes that can thin and weaken the fibrous cap\(^{40}\).

**Figure 1.3.** A hematoxylin blue stained arterial section showing significant plaque burden that has thickened the vessel wall beyond the oxygen delivery capabilities of the normal vasa vasorum. Endothelial cells are stained brown to allow the visualization of all microvessels. Some microvessel lumens are filled with an intravascular polymer that appears as black spots. A high density of neo vessels is present in all layers of the vessel wall, including the plaque itself.

### 1.4 Plaque Angiogenesis and Intraplaque Hemorrhage as Imaging and Therapeutic Targets

#### 1.4.1 Overview

The ability to monitor plaque angiogenesis and hemorrhage noninvasively through imaging has the potential to identify unstable plaques, to elucidate our understanding of the natural evolution of IPH, and to monitor the effects of treatments targeted towards plaque neovascularure and hemorrhage. Currently, a magnetic resonance imaging (MRI) sequence known as MRIPH that suppresses bright signal from fat and flowing blood can be used to detect signal from methemoglobin, a product of hemorrhage\(^{41}\) (Fig. 1.4). Evidence has already shown that in both symptomatic and asymptomatic carotid disease patients, the presence of IPH identified using this sequence is a sensitive predictor of clinical event recurrence\(^{42,43}\). Noninvasive imaging techniques aimed towards identifying plaques at risk of causing clinical events may influence the
decision to either locally intervene on the diseased artery or rely on medical therapy to treat the disease systemically.

![Magnetic Resonance Image](image)

Figure 1.4. A magnetic resonance image (a) obtained using the MRIPH sequence on an *ex vivo* coronary artery specimen, with its corresponding H&E section (b). L indicates the location of the lumen on both panels while the asterisk on the micrograph denotes a dark pink region of hemorrhage that is contained within the vessel wall. Methemoglobin that forms following hemorrhage gives rise to the region of bright signal on the MRIPH image.

Currently, we can only speculate on the nature of intraplaque hemorrhage arising from plaque neovessels, such as whether extravasated red blood cells arise from neovessel leakage or rupture, and whether this process occurs continuously or in discrete events. In order to perform any longitudinal observations such as determining when hemorrhage occurs and whether there is a resulting change in plaque size, noninvasive imaging techniques that do not disturb the process itself are needed.

The source of intraplaque hemorrhage, plaque angiogenesis, is an attractive therapeutic and imaging target. It is hoped that the arrest of erythrocyte deposition and its ensuing atherogenic effects may lead to the stabilization of plaques. Recently, antiangiogenic therapy has been proposed as a strategy to either abolish or normalize the leaky neovasculature. Plaque neovessels form a potential drug delivery route into the plaque and express locally
elevated concentrations of receptors associated with inflammation, such as intercellular adhesion molecule and vascular cell adhesion molecule\textsuperscript{47}. Therefore, it may be possible to further enhance specificity of the drug to plaque neovessels by attaching a receptor-targeting moiety to the drug. In terms of imaging, techniques exploiting the accumulation of injected contrast agents within a neovessel-rich plaque could be used to monitor changes in plaque angiogenesis in response to treatment.

1.4.2 The Need for an Animal Model of Plaque Angiogenesis and Intraplaque Hemorrhage

The preceding section has identified several areas in which imaging could improve our knowledge and management of vulnerable plaques, including: the identification of plaques with neovasculature and IPH, the observation of to the natural history of IPH, and the monitoring of drug therapies that counter plaque angiogenesis or the effects of IPH. The ability to induce plaque angiogenesis and to control or predict its progression to IPH in an animal model rather than relying on human specimens would accelerate development of techniques for all of these applications. It is widely acknowledged that despite the potential uses for an animal model in which plaque angiogenesis leads to IPH, there currently is no model that reliably demonstrates this process\textsuperscript{38,44,46}.

1.5 Animal Models with Demonstrated Plaque Angiogenesis or Hemorrhage

1.5.1 Mice

The cholesterol-fed apolipoprotein E double knockout mouse (apoE\textsuperscript{-/-} mouse) is a common model used for the study of atherosclerosis. The mice are deficient in apolipoprotein E, which is a major protein found in triglyceride-rich lipid particles, namely chylomicrons and very low
density lipoproteins (VLDL). Without a functioning apoE to facilitate uptake of these particles, the transfer and catabolism of triglycerides to tissues and other lipid particles are impaired. This deficiency results in high levels of circulating chylomicron and VLDL remnants, which are highly atherogenic.

The cholesterol-fed version of this model tends to generate advanced lesions in the aorta and innominate arteries. Additionally, plaque neovessels are present in both arteries, and plaque hemorrhage has been shown in the innominate branch. Moulton et al have studied plaque angiogenesis and its inhibition in this model, showing that intimal capillaries appear in 13% of the advanced aortic lesions in mice aged 36-60 weeks that are fed 0.15% cholesterol from weeks 6 to 8. This proportion increases to 53% when the apoE-/- mice are bred with null collagen XVIII mice.

Virmani et al have concentrated on the innominate branch of the aortic arch, displaying advanced fibrofatty lesions in apoE-/- mice of 24 to 60 weeks fed a normal chow diet. Rates of intraplaque hemorrhage range from 17% to 75% in animals aged 30 to 60 weeks. However, this hemorrhage is not accompanied by plaque neovessels, suggesting that the hemorrhage originated from the main lumen.

The apoE-/- mouse model is simple, requires little intervention, and consistently forms advanced plaques in the innominate artery and aortic arch. However, murine vessels are very small (the innominate being about 1mm in diameter or less) and have far fewer and thinner arterial layers than in the human giving this model poor applicability for imaging studies and development of interventional techniques. Finally, although plaque angiogenesis and hemorrhage have been separately demonstrated in this model, intraplaque hemorrhage caused by plaque neovessels has not been observed.

1.5.2 Swine
Another animal model that has demonstrated intraplaque hemorrhage is the diabetic swine model. In a study by Gerrity et al\textsuperscript{52}, male Yorkshire swine (initially 8-12 weeks old and 15-20 kg) were injected with streptozotocin to induce diabetes by destroying the pancreatic beta cell population. While pigs on a high fat diet alone will develop coronary lesions with hemorrhage, this does not occur until 10 to 12 months of the diet. However, the addition of diabetes to the model accelerates complicated lesion progression and produces coronary lesions with hemorrhage and calcification as early as 20 weeks. It also results in hemorrhage and calcification in abdominal aorta lesions that, while not as severe as the coronary lesions, were also not present in the diet-only animals. Staining for neovessels was not performed, leaving open the possibility of the hemorrhage originating from neovessels. While the size of the animal makes the pig an excellent model for imaging and interventional work, the IPH observed was spontaneous without specific localization within the coronary tree.

Another way of generating plaques with neovessels is to perform coronary angioplasty, which likely causes damage to the vessel wall, particularly the endothelium. In pigs fed a normal diet and subjected to coronary angioplasty, microvessel appeared in the intima 28 days after injury, although these neovessels were few in number\textsuperscript{53}. As in the apoE\textsuperscript{-/-} mouse, intraplaque hemorrhage caused by plaque neovessels has yet to be demonstrated in a pig model.

1.5.3 Rabbits

The rabbit is a convenient model to work with because its major vessels are large enough to use for imaging and interventional research while the animals are small enough to maintain in higher numbers. Also important is their susceptibility to diet-induced atherosclerosis. In addition to feeding the New Zealand White rabbit a high cholesterol diet, some form of endothelial or deeper vessel wall injury is performed in the artery of interest (aorta, carotid, iliac, or femoral) to accelerate plaque formation. This damage renders the wall more vulnerable to the influx of
lipids and inflammatory cells and stimulates a proliferative response in the smooth muscle layer, leading to plaque growth in the injured wall. Methods of injury include the following: angioplasty balloon overdilatation\textsuperscript{54}, balloon inflation and withdrawal\textsuperscript{55}, and air desiccation\textsuperscript{54}. One model combines an initial air dessication of the femoral artery followed 4 weeks later by balloon overdilatation injury\textsuperscript{54}. At 4 weeks after the balloon angioplasty, plaques become neovascularized with an average maximal density of 58 microvessel cross-sections per mm\textsuperscript{2} of plaque area. This neovascularization is also accompanied by increased macrophage density. However, further details are not given as the study looked at several measures of plaque progression. A similar model also employs a double injury, this time using the withdrawal of an embolectomy balloon in the rabbit aorta twice, separated by 4 weeks\textsuperscript{56}. The data shown suggest extensive neovascularization in the combined intimal and medial area, with densities ranging mostly from 5 to 25 microvessel cross-sections per mm\textsuperscript{2} of plaque area. These methods show plaque angiogenesis and have the advantage of being able to induce focal lesions, but also involve invasive procedures that may restrict intra-arterial catheter access later on. Furthermore, no evidence of IPH has been demonstrated in the rabbit balloon injury model.

While the rabbit model remains a practical compromise in terms of size and develops plaques easily on a hypercholesterolemic diet, it may not be the most relevant to human physiology. The aorta is around the same size as a human coronary, but unlike the human coronary arteries and internal carotid artery, the rabbit aorta is an elastic artery and may develop lesions and respond to treatment in different ways. It has also been noted that the rabbit lipid profile is quite different from that of a human, with very low density lipoprotein being the major carrier of lipids\textsuperscript{57}. This would be especially important if testing out treatments that rely on alterations in lipid metabolism.

1.5.4 Summary of Animal Models
Although all the species presented above have yielded models that demonstrate either neovessels within the plaque area or hemorrhage within the plaque, an animal model showing plaque angiogenesis consistently leading to intraplaque hemorrhage does not exist. To meet the needs of the imaging and therapeutic applications described in the preceding section, a useful animal model of intraplaque hemorrhage should develop fatty lesions with extensive neovascularization on a consistent basis that either hemorrhages in a predictable manner or in response to an external stimulus. An ideal animal model, in addition to these characteristics, would possess a physiological and anatomic background similar to human in regards to vascular disease and require only minimally or noninvasive interventions.

1.6 Hypothesis and Overview of Thesis

1.6.1 Hypothesis

An animal model of atherosclerosis where plaque angiogenesis leads to intraplaque hemorrhage in a consistent and predictable manner can be created. Such a model will allow for the study of the progression of vulnerable plaques, including those containing intraplaque hemorrhage, with noninvasive imaging techniques.

1.6.2 Summary of Introduction

Disease of the vessel wall, known as atherosclerosis, is the most common underlying process that leads to death and disability from cardiovascular disease. Vessel wall thickening may lead to arterial stenosis, which is readily imaged with current techniques but does not predict all future clinical events. Plaque angiogenesis and subsequent deposition of red blood cells within the plaque are likely indicators of an unstable plaque that may rupture and cause an ischemic event. Plaque angiogenesis and hemorrhage make attractive targets for imaging and drug delivery, but new techniques are difficult to test due to a lack of animal models that demonstrate consistent
plaque angiogenesis leading to intraplaque hemorrhage. The work in this thesis aims to address this need for suitable animal model.

1.6.3 Thesis Structure

Chapter 1 of this thesis outlined the fundamentals of atherosclerosis, the motivation behind vessel wall imaging techniques, the need for the development of an animal model, and the current state of the art of such models. Chapter 2 will describe in detail the work done to develop a rabbit abdominal aorta model of plaque angiogenesis. The third chapter is a proof of concept study that demonstrates the feasibility of monitoring plaque angiogenesis in the rabbit model using MRI. The fourth and final chapter summarizes the first three chapters and elaborates upon the next steps for this line of research.
References


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CHAPTER 2:
Animal Model Development

2.1 Introduction

The goal of this thesis is to develop an animal model where plaque neovascularization leads to IPH. The rabbit aorta was chosen as the target vessel because it is comparable in size to human coronary and carotid arteries while the rabbit is small enough to house in numbers needed to test out each strategy for inducing plaque neovascularization and IPH. This chapter details the work done to create atherosclerotic plaques in the rabbit abdominal aorta that contain neovessels in the intimal and medial layers of the vessel wall. Strategies for inducing hemorrhage of these neovessels, the last step in producing true IPH, are beyond the scope of this chapter and are instead outlined in the final chapter.

The overall strategy for creating advanced plaques was to first induce baseline plaques with systemic interventions. Cholesterol feeding with both native and oxidized cholesterol was used to create lipid-rich lesions in the vessel walls of the rabbits while VEGF protein was administered systemically to produce conditions for encouraging plaque inflammation and angiogenesis. Superimposed on the systemic interventions were localized interventions where both reversible and irreversible damage were applied to the vessel wall to selectively accelerate plaque formation. Ultrasound (US) in conjunction with injected microbubbles was a noninvasive method of reversibly increasing vessel wall permeability to circulating cholesterol while inflation and withdrawal of a balloon catheter in the aorta was a direct physical means of causing vessel wall injury.

This chapter is structured such that the rationale and experimental methods are detailed for each intervention in order of least invasive to most invasive. Methods used for data
collection and analysis are then described. The results, including intergroup comparisons for each intervention are then provided in the same order, with significant findings and limitations of the study subsequently discussed.

2.2 Experimental Design and Interventions

2.2.1 High Cholesterol Diets

Rabbits are susceptible to diet-induced atherosclerosis and develop abnormally high levels of plasma cholesterol because of their inability to increase sterol excretion in response to high-cholesterol diets\(^1\). Saturated fats, such as peanut oil, are also used in atherogenic diets as they have been shown to increase lesion incidence and size\(^2\).

New Zealand White rabbits with initial weights of 3.1-3.5kg (Charles River Laboratories, Wilmington, USA) were housed in the Sunnybrook Comparative Research facility in accordance with the Sunnybrook Animal Care Committee guidelines or in the Toronto General Hospital Comparative Research facility in accordance with the University Health Network Animal Care Committee guidelines. All rabbit groups consisted of 4 subjects each and are summarized in Table 2.2 found at the end of this chapter. Rabbit were fed a high cholesterol (HC) diet containing 0.25% cholesterol and 6% peanut oil (TestDiet, Richmond, USA) for 20 weeks or longer, depending on the group. One additional group (Group 1b in Table 2.2) was fed a normal rabbit chow diet for 20 weeks (Ren’s Feed & Supplies Ltd, Oakville, Canada). To ensure that the diets were increasing plasma cholesterol levels, fasting blood samples were taken every 10 weeks and sent for testing (Idexx Laboratories, Mississauga, Canada). An enzymatic colourimetric assay for total plasma cholesterol using cholesterol esterase and cholesterol oxidase (CHOD-PAP, Roche Diagnostics, Laval, Canada) was performed on the samples.

Since it is known that oxidized LDL is more atherogenic than native LDL, this study included one group of animals (Group 1c in Table 2.2) that was fed an oxidized cholesterol diet.
Previous work has shown that an oxidized cholesterol diet promotes early atherosclerotic lesion development in rabbits over a non-oxidized cholesterol diet with the same cholesterol concentration\textsuperscript{3}.

Cholesterol was oxidized by heating a thin layer of \( \geq 95\% \) pure cholesterol (Sigma-Aldrich, Oakville, Canada) in a 118\degree C oven for 48 hours. The cholesterol was then sent to the diet manufacturer (TestDiet, IN, USA) for incorporation into a custom diet. The diet was identical to the one fed to most of the animals, with the 0.25\% cholesterol replaced with the oxidized cholesterol. Due to supply issues, these rabbits were fed the HC diet during weeks 7 and 8.

\textbf{2.2.2 Vascular Endothelial Growth Factor}

VEGF modulates plaque growth and vessel wall angiogenesis in hypercholesterolemic animal models. VEGF given systemically in a low dose, whether through gene therapy or protein injection, has been associated with plaque growth in hypercholesterolemic animal models with deep vessel wall injury\textsuperscript{4,5,6,7}.

Recombinant human VEGF was reconstituted from lyophilized protein (R&D Systems) with distilled water to a concentration of 0.1 mg/ml, and then further diluted to a volume of 0.3ml for administration as an intramuscular injection at the proper dose.

To test the effect of different doses of VEGF in conjunction with the HC diet, 2 groups of 20 week hypercholesterolemic rabbits were given VEGF at weeks 5 and 10, one group at a dose of 2 \( \mu \)g/kg (Group 1d in Table 2.2) and the other at a dose of 4 \( \mu \)g/kg (Group 1e in Table 2.2). In addition, 3 groups of rabbits on the high cholesterol diet for 30 weeks were given 4 \( \mu \)g/kg of VEGF every 5 weeks (Groups 2a-c) starting at week 5 with each group given 2, 3 or 4 injections.

\textbf{2.2.3 Therapeutic Contrast Ultrasound}

Cavitation, or gas bubble formation in liquid due to local negative pressure, occurs in the
presence of ultrasound and can lead to bubble expansion followed by collapse. The collapse of bubbles causes microstreaming of the liquid, which can have important mechanical effects *in vitro* and *in vivo*. These effects are amplified with the addition of ultrasound contrast agents, lipid-coated microbubbles that provide cavitation nuclei and lower the ultrasound intensity needed to cause microbubble-related bioeffects while avoiding thermal effects from the ultrasound itself. Ultrasound with microbubbles has been used to facilitate the transfer of DNA fragments into cells, a phenomenon termed sonoporation. It is thought that pores are formed in the plasma membrane by mechanical forces associated with bubble collapse, and this increased permeability may render the endothelial lining of blood vessels (when microbubbles are injected intravenously) more vulnerable to the infiltration of cholesterol.

Rabbits were anaesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg). Ultrasound microbubble contrast agent (Definity, Lantheus Medical Imaging, Billerica, USA) was activated and slowly injected into a bag of 0.9% saline for continuous infusion. The infusion was set at a rate of about 37.5µl Definity/minute for either 10 minutes or 20 minutes. During the infusion, a Toshiba Aplio ultrasound system (Toshiba America Medical Systems, Tustin, USA) was used to insonate a cross-section of the abdominal aorta through the shaved skin of the left ventral torso. The probe position was drawn on the skin with a marker and every week the probe was placed at the marked location for insonation. The system was used in flash echo imaging mode such that every 5 seconds there was a burst of pulses (15 individual pulse sequences every 11.4 ms) at maximum acoustic power. In between bursts, the output mechanical index (MI) was kept to a minimum to allow for reperfusion of microbubbles in the region of interest. The acoustic focus was set at the level of the aorta, usually 2-4 cm from the transducer face.

One group of rabbits (Group 3a) on the HC diet was exposed to therapeutic ultrasound
for 10 minutes weekly with the PLT-604AT transducer (5.8MHz, 1.18-1.42 MI) for the duration of the 20 week diet. Another group of rabbits (Group 3b) on the HC diet with 4 µg/kg VEGF injections at weeks 5 and 10 was exposed to therapeutic ultrasound for 20 minutes weekly with the PLT-604AT transducer for the first 10 weeks and with the PVT-375BT (2.5 MHz, 1.1-1.34 MI) transducer for the following 10 weeks.

### 2.2.4 Balloon Injury

Endothelial damage with an embolectomy balloon catheter is a common way to accelerate atherosclerotic progression as it disturbs the normally anti-atherosclerotic functions of the healthy endothelium and allows for the influx and accumulation of circulating lipids. Two groups of 20-week hypercholesterolemic rabbits (Groups 4a and 4b) underwent balloon injury at week 10 after onset of the HC diet, with one group (Group 4b) receiving 4 µg/kg VEGF injections at weeks 5 and 9. In addition, two groups of 14-week rabbits (Groups 4c and 4d) underwent balloon injury at week 3, with one group (Group 4d) receiving 4 µg/kg VEGF injections at weeks 5 and 10.

Anaesthesia was induced with ketamine (50 mg/kg) and xylazine (5 mg/kg) and maintained using isoflurane (0.5-5 %). A right femoral cutdown was used to introduce and advance a 3 French Fogarty arterial embolectomy balloon catheter (Edwards Lifesciences, Irvine, USA) under fluoroscopic guidance to the level of renal branches of the aorta. The balloon was inflated with 0.15 ml of radioopaque contrast agent (Omnipaque, GE Healthcare, Waukesha, USA) and pulled back to the right iliac artery 3 times. The catheter was removed, the right femoral artery was ligated and the wound was sutured. For pain management and prevention of infection, all the animals received buprenorphine (0.02-0.05 mg/kg) daily for 3 days post-operatively and either Cefazolin (20 mg/kg daily for 3-5 days post-operatively) or Duplocillin (once the day before surgery and again 3 days later).
2.3 Data Analysis Methods

2.3.1 Specimen Processing

Prior to sacrifice at the appropriate endpoint (see Table 2.2), i.v. heparin was administered. Upon sacrifice, the thoracic aorta was cannulated to administer 45-90 ml of Microfil (FlowTech, Carver, USA), a radioopaque silicone compound, into the abdominal aorta. Upon the setting of the Microfil (approximately 30 minutes), the abdominal aorta including the iliac bifurcation and renal branches was extracted and put into 10% neutral buffered formalin. Following 24 to 48 hours of formalin fixation, the specimens were stored in 70% ethanol until processing. In the case of rabbits that had received therapeutic contrast ultrasound, the section that corresponded with the skin marking and therefore the focus of insonation was noted.

2.3.2 Immunohistochemistry

After fixation, the specimens were cut into blocks of 5 mm length and embedded in paraffin. Sections were taken from each block for hematoxylin and eosin (H&E), anti-CD31, and RAM11 staining. Endothelial cells were stained using monoclonal mouse anti-human CD31 antibody (Dako Canada) at a 1:25 dilution and visualized with diaminobenzidine. Macrophages were stained using monoclonal mouse anti-rabbit RAM11 (Dako Canada, Mississauga, Canada) at a 1:200 dilution and visualized with NovaRED (Vector Laboratories, Burlingame, USA). Hematoxylin blue was used as the counterstain for both the CD31 and RAM11 sections. Rabbit spleen was used as a positive control and an isotype negative control for each chromogen was performed for every block.

Each H&E slide was digitally captured at 2.5x magnification and plaque area was quantified by manually tracing the lumen and the intimal-medial boundary on ImageJ (NIH, Bethesda, USA). To visualize detail at the cellular level, the CD31 and RAM-11 slides were
digitized at 20x magnification on a ScanScope XT slide scanner (Aperio, Vista, USA). CD31-positive vessels within the vessel wall with periadventitial fat as the outer boundary were manually counted using Aperio ImageScope software. Vessels with a short axis diameter above 50 µm were excluded from the counts. RAM11-positive area was measured using the Positive Pixel Count algorithm in ImageScope, which identifies pixels within a given range of values for hue, saturation and intensity based on the HSI colour model. The range in which pixels were considered positive was kept constant for all slides, with positive values defined as hue from 0.9 to 1 and 0 to 0.1, saturation above 0.05 (out of 1), and intensity below 150 (out of 256 levels).

All the slide values from each aortic specimen were averaged, with the group average determined by the average intimal area, macrophage area, and microvessel count of each individual specimen within the group.

2.3.3 Statistical Analysis

To compare group averages against each other, Student’s t test for independent samples was performed. Equal variances were assumed unless the p-value from Levene’s test for unequal variances was 0.05 or less. In addition, the relationship between cholesterol levels in the rabbits that underwent balloon injury and the size of their plaques at the time of sacrifice was explored. The strength of the linear relationship between plasma cholesterol levels in the rabbits at the time they underwent endothelial denudation and average intimal area at the time of sacrifice was found using Pearson’s correlation coefficient. A p-value of 0.05 or less for either test was considered to be significant with all statistical analysis performed using SPSS (SPSS, Chicago, USA).

2.4 Results

2.4.1 High Cholesterol Diets
Most of the animals tolerated the diet well, but some rabbits displayed liver damage upon gross examination and jaundice owing to the stress placed on the liver to metabolize the large amounts of cholesterol. One rabbit whose tissues were examined by a pathologist had moderate liver fibrosis in addition to heart failure and pulmonary embolism likely secondary to atherosclerotic disease. In total, 5 rabbits died unexpectedly, possibly due to diet-related complications (see Table 2.2 for groups affected). Plasma cholesterol levels were measured to ensure effectiveness of the HC diet. These levels ranged from 0.3-0.7 mmol/L at week 10 and 0.4-1.0 mmol/L at week 20 for the normal diet group (Group 1b in Table 2.2) and from 10.9-42.5 mmol/L at week 10 and 13.3-91.3 mmol/L at week 20 for the HC diet groups (all groups except for Groups 1b and 1c).

Without any cholesterol enrichment, rabbits on the normal chow diet (Group 1b) did not develop any atherosclerotic plaques in the abdominal aorta. Rabbits on the HC diet for 20 weeks (Group 1a) displayed a few localized fatty deposits (Figs. 2.1a,c,e), especially near the renal branches (Figs. 2.1b,d,f) and RAM11 staining for macrophages showed variable plaque inflammation (Figs. 2.1 c-f). CD31 staining for endothelial cells found vasa vasora in the adventitia only, but in elevated numbers in the HC diet group over the normal diet group (Fig. 2.2). CD31 staining on one HC diet rabbit’s ascending aorta and aortic arch showed vasa vasora restricted to the adventitia and outer media (Fig. 2.3), as expected for thicker arterial walls.
Figure 2.1. Representative histological sections, stained with H&E (a-b) and RAM11 (c-f) from the group fed the high cholesterol diet for 20 weeks with no other interventions. Rectangular boxes correspond with panels directly below. (a,c,e) Section showing some fatty streak formation (lighter pink) with limited macrophage infiltration (brown colour). (b,d,f) Section showing more extensive plaque formation at one of the renal artery branches. Macrophage infiltration is more extensive within the plaque.
Figure 2.2. Histological measures of the following groups: (HC) High cholesterol diet for 20 weeks; (OxChol) Oxidized cholesterol diet for 20 weeks; (HC+2μg/kg VEGF) High cholesterol diet for 20 weeks with 2 μg/kg of VEGF given at weeks 5 and 10; (HC+4μg/kg VEGF) High cholesterol diet for 20 weeks with 4 μg/kg of VEGF given at weeks 5 and 10; and (Normal) Normal chow for 20 weeks. Each dot represents the average value per histological section for one rabbit, each bar represents the group average, and the error bars indicate the standard error of the group mean. The OxChol and VEGF groups were compared against the HC only group and normal diet group. Brackets indicate a significant difference between the groups on each end while (*) along the axis indicates a significant difference from the normal diet group (not shown in first two graphs).
Section through the aortic arch of a rabbit that received the high cholesterol diet for 20 weeks with no other interventions in the arch. (a) Endothelial cells lining the vasa vasorum are stained brown. Due to the increased thickness of this portion of the artery, the vasa vasorum is found in the outer half of the medial layer in addition to the adventitia. (b) Macrophages are stained brown and are located mostly in the luminal portion of the intima, with some positive staining in the media that corresponds with the area of the infiltrating microvessels. (*) denotes the lumen.

One of the histological sections from one of the rabbits on the HC diet (Group 1a) incidentally contained the cross-section of what is likely the inferior mesenteric artery, which runs parallel to the aorta. This section showed intimal and medial lipid deposits with macrophages and neovessels infiltrating the intima and media from the adventitia (Fig. 2.4).

Small artery, likely the inferior mesenteric, from a rabbit on the high cholesterol diet for 20 weeks with no other interventions. Microvessel (a,c) and macrophage (b,d) staining are shown in brown, with some microvessels containing black spots from intravascular polymer. (a,c) Without any mechanical injury, neovessels have infiltrated the media and intima of the vessel, through disrupted layers of elastin. (b,d) Macrophages colocalize with the neovessels, showing the tight relationship between inflammation and angiogenesis.
The oxidized cholesterol diet (Group 1c) was tolerated well by the rabbits and produced plasma cholesterol levels similar to those in the groups on the HC diet. Intimal area and macrophage area in the abdominal aorta were significantly elevated (Fig. 2.2) over the HC diet only group (Group 1a). Microvessel count was significantly higher than in the normal diet group (Group 1b) and slightly but not significantly higher than in the HC diet only group. Again, plaque formation occurred mainly near the renal branches. Cholesterol clefts, not seen in the HC diet group, were present in one of the abdominal aortic plaques. A few of the sections through this plaque also demonstrated a high density of microvessels at border of the adventitia and media, with a few microvessels invading the outer medial layers. In addition, one of the sections seems to indicate the beginnings of angiogenesis in the intima (Fig. 2.5).

![Figure 2.5. A CD31-stained section from the abdominal aorta of a rabbit fed the oxidized cholesterol diet. A few microvessels are seen in the outer layers of the media and spots of positive staining (arrows) in the intima may indicate the initial steps of angiogenesis in the plaque. (*) denotes the lumen.](image)

**2.4.2 Vascular Endothelial Growth Factor**

When 20-week rabbits were given 2 μg/kg of VEGF at weeks 5 and 10 (Group 1d), there was no significant difference compared with the HC-only group (Group 1a) with respect to any of the histological measures (Fig. 2.2) in the abdominal aorta. However, there was a non-significant increase in microvessel count in the 2 μg/kg VEGF group. Increasing the dose to 4 μg/kg (Group 1e) resulted in higher intimal area and macrophage area, though these differences were
not significant due to the large variability within this group. Although adventitial microvessels for both VEGF groups were significantly elevated over the normal diet group (Group 1b), there was no difference in the amount of adventitial microvessels between the two VEGF groups (Groups 1d and 1e).

Plaques that extended around most of the circumference of the aorta were seen in the 4 µg/kg VEGF group but not in the 2 µg/kg VEGF group (Fig. 2.6). One plaque showed abundant microvessels at the border of the media and adventitia of the thickest part of the plaque (Fig. 2.6c), similar to one of the plaques in the oxidized cholesterol group (Fig. 2.5).

![Figure 2.6](image)

**Figure 2.6.** One of the plaques in the group that was given 4 µg/kg of VEGF twice in addition to 20 weeks of the high cholesterol diet. The plaque is thicker and extends around a greater portion of the circumference than the plaques in the non-VEGF and the 2 µg/kg of VEGF group. Macrophages are stained red (b,d) and microvessels are stained brown (c). (c) A high density of microvessels (arrows) is seen on the medial-adventitial border of one of the thicker regions of the plaque, possibly marking the beginnings of medial neovascularization from the vasa vasorum.

In the uninjured ascending aortae and aortic arches of the 20-week HC group that underwent 2 injections of 4 µg/kg of VEGF and endothelial denudation of the abdominal aorta
(Group 3b), there is evidence of the angiogenic process beginning in the intima (Fig. 2.7), as opposed to being confined to the adventitia and media as in the HC diet only group (Fig. 2.3).

**Figure 2.7.** A CD31-stained section of an aortic arch from one of the rabbits that received 4 µg/kg of VEGF and endothelial denudation of the abdominal aorta. Even though the arch has not been injured, the outer half of the media contains vasa vasora (solid arrows) and brown spots (line arrow) in the intima potentially indicate the beginnings of angiogenesis.

The effects of extending the duration of the HC diet to 30 weeks in conjunction with VEGF were explored to investigate whether these noninvasive interventions could produce more advanced plaques. Out of the 4 rabbits that were given 4 µg/kg of VEGF at weeks 5 and 10, only 2 survived until the 30-week endpoint rendering it difficult to make inferences (Group 2a). In addition, only 3 of the 4 rabbits given VEGF at weeks 5, 10, 15 and 20 survived until the 30 week time point. When comparing each group (Groups 2a-c) against the 2-injection, 20-week rabbits (Group 1e), there were no significant differences in any of the histological measures, though there was an increasing trend in microvessel count with additional VEGF injections (Fig. 2.8). In terms of plaque neovascularization, none of the abdominal aortas examined in these groups exhibited intimal neovessels.

The right common carotid artery and the aortic arch of the rabbits in the 30-week groups were also subjected to immunohistochemical staining. While the same arteries in 20-week rabbits demonstrate little or no intimal neovascularization (Fig. 2.3), there were carotids and arches in all 3 of the 30-week groups containing complete intimal neovessels (Fig. 2.9).
Figure 2.8. Histological measures of the groups on the high cholesterol diet for either 20 or 30 weeks and receiving different numbers of VEGF injections at a dose of 4 μg/kg each (Groups 1e and 2a-c). All groups were on the high cholesterol diet for 30 weeks with the exception of the HC+2xVEGF group that was on the diet for 20 weeks. The other 3 groups were given 4 μg/kg of VEGF every 5 weeks starting at week 5 with either 2, 3 or 4 injections. Not all groups have data for all 4 rabbits due to premature deaths. Each dot represents the average value per histological section for one rabbit, each bar represents the group average, and the error bars indicate the standard error of the group mean.
Figure 2.9. Sections from the right common carotid artery (a-d) and aortic arch (e-f) from rabbits fed the high cholesterol diet for 30 weeks and given 4 µg/kg of VEGF at 3 time points. CD31-stained sections (a,c,e) show extensive neovascularization colocalized with intense macrophage staining (b,d,f). The adventitia is also positive for macrophages in both vessels. (*) denotes adventitial side of the vessel wall.
2.4.3 Therapeutic Contrast Ultrasound

The histological sections corresponding to the insonated locations in either the HC+US (Group 3a with Group 1a as non-US control) or the HC+VEGF+US group (Group 3b with Group 1e as non-US control) did not show any consistent local differences in any of the measures. None of the histological measures differed significantly between the two therapeutic ultrasound groups and their non-ultrasound controls (Fig. 2.10).

2.4.4 Balloon Injury

Injured abdominal aortas, regardless of VEGF administration, displayed circumferentially thickened intimas with consistently large plaques (Figs. 2.11 and 2.12). The intimas were composed of both fibrous and fatty regions, sometimes with a fibromuscular layer overlying a lipid-rich region. The fatty regions were predominantly composed of foam cells and occasionally cholesterol clefts, without evidence of extracellular lipid cores. In some of the rabbits (see Groups 4a-d in Table 2.2 for rates of occurrence), several of the histological sections contained portions of media with disrupted elastin layers and heavy infiltration of lipids between the elastin layers. These areas often coincided with macrophage and neovessel infiltration in the media and intima, as seen in the inferior mesenteric artery in one of the HC rabbits (Fig. 2.4). In addition to these lipid-rich regions, RAM11 staining sometimes demonstrated the presence of macrophages in the adventitia that was not seen in any of the non-injured abdominal aortas.

Whether or not the animals received VEGF injections before balloon injury in the 20-week groups (Groups 4a and 4b) did not make a significant difference in terms of intimal area, macrophage area, or microvessel counts, although all measures were higher in the non-VEGF control group (Fig. 2.13). Similarly, there were no significant differences in any of the histological measures between the 14-week injury groups (Groups 4c and 4d). The group that received VEGF injections after injury showed increased intimal area, but lower macrophage area
and microvessel counts. Intimal and macrophage area were only available for 3 of the rabbits in the VEGF group because the aorta of one of the animals (which died unexpectedly) was not injected with Microfil and fixed in a dilated state.

Figure 2.10. Histological measures of the groups that received weekly insonations (Groups 3a-b) and their non-ultrasound control groups (Groups 1a and e). All groups were on the high cholesterol diet for 20 weeks, with the ultrasound groups (US) being insonated every week during the diet. Group names with 2xVEGF indicate 4 μg/kg of VEGF administered at weeks 5 and 10. 10minUS indicates that each weekly insonation lasted for 10 minutes and 20minUS indicates 20 minute insonations. Each dot represents the average value per histological section for one rabbit, each bar represents the group average, and the error bars indicate the standard error of the group mean.
Figure 2.11. A section from a rabbit fed the high cholesterol diet that developed advanced atheroma in response to endothelial denudation. The H&E section (a-b) shows red blood cells within the intima and discontinuous elastic layers. High-magnification images (b,d,f) correspond to the rectangle shown in (a). The CD31-stained section (c-d) shows extensive neovascularization that extends around half of the circumference within the intima. The vessels are concentrated mainly in lipid-rich regions. The RAM11-stained section (e-f) shows widespread macrophage infiltration throughout the whole vessel wall.
Figure 2.12. A section from a rabbit fed the high cholesterol diet that developed relatively moderate plaques in response to endothelial denudation. The H&E section (a-b) shows more isolated elastin disruption. High-magnification images correspond to the rectangle shown in (a). The CD31-stained section (c-d) shows vasa vasora confined to the adventitia of the aorta. The RAM11-stained section (e-f) shows macrophage infiltration limited to discrete areas in the intima and media.
Figure 2.13. Histological measures of the 20-week (light bars) and 14-week balloon injury groups (dark bars). The 20-week groups underwent endothelial denudation (ED) with a balloon catheter at week 10 of the high cholesterol diet, with one group receiving 4 μg/kg of VEGF at weeks 5 and 9. The 14-week groups underwent endothelial denudation at week 3 of the high cholesterol diet, with one group receiving 4 μg/kg of VEGF at weeks 5 and 10. Each dot represents the average value per histological section for one rabbit, each bar represents the group average, and the error bars indicate the standard error of the group mean. Bracket indicates a significant difference between the two groups on either end. All other differences were non-significant.

Looking at the intragroup variation in plaque growth in response to balloon injury, there is evidence of a relationship between plasma cholesterol in the rabbits at the time of and in the weeks following balloon injury and plaque size and angiogenesis at the time of sacrifice.
Pearson’s correlation coefficient was found for the relationships between intimal area and cholesterol levels at various time points within each group of four rabbits (Figs. 2.14 and 2.15), with results summarized in Table 2.1.

**Figure 2.14.** Scatterplots of the average intimal areas of the 20-week groups (Groups 4a-b) versus plasma cholesterol levels at various time points. The group with VEGF (dots) exhibited a strong positive relationship between intimal area and plasma cholesterol at the time of endothelial denudation (a) that weakened when intimal area was compared with cholesterol at week 20 (b). By contrast, the group without VEGF (circles) demonstrated only a weak positive relationship between intimal area and plasma cholesterol at the time of endothelial denudation (a) and at week 15 (b).

**Figure 2.15.** Scatterplots of the average intimal areas of the 14-week groups (Groups 4c-d) versus plasma cholesterol levels at various time points. There were strong positive relationships between intimal area and cholesterol level at both the time of balloon injury (a) and at week 14 (b) for both the VEGF (dots) and non-VEGF (circles) groups. The relationships are stronger at the 14-week time point. One data point is missing from the VEGF group because premature death prevented one specimen from being fixed in the usual manner.

For the 20-week group that underwent balloon injury at week 10 and received VEGF
(Group 4b), intimal area significantly correlated with cholesterol levels at the time of injury, but not 10 weeks later at the time of sacrifice. Its non-VEGF control group (Group 4a) did not show any significant correlations, likely owing to the tight range in both cholesterol level and intimal area.

For the 14-week group that underwent balloon injury at week 3, the correlations between intimal area and cholesterol level at the time of balloon injury were not significant. However, intimal area in the VEGF group (Group 4d) correlated significantly with cholesterol 10 weeks later at the time of sacrifice. Intimal area in the non-VEGF control group (Group 4c) correlated more strongly with cholesterol at the time of sacrifice than at the time of balloon injury, but this correlation was still not significant.

**Table 2.1.** Correlations between plasma cholesterol levels and average intimal area at different time points for each balloon injury group.

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol at surgery</th>
<th>Cholesterol at sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>20wksHC+ED†</td>
<td>( R^2 = -0.028 )</td>
<td>( R^2 = 0.215 )</td>
</tr>
<tr>
<td>20wksHC+ED+VEGF</td>
<td>( R^2 = 0.985^* )</td>
<td>( R^2 = 0.160 )</td>
</tr>
<tr>
<td>14wksHC+ED</td>
<td>( R^2 = 0.805 )</td>
<td>( R^2 = 0.997^{**} )</td>
</tr>
<tr>
<td>14wksHC+ED+VEGF</td>
<td>( R^2 = 0.742 )</td>
<td>( R^2 = 0.990 )</td>
</tr>
</tbody>
</table>

†cholesterol measured at 5 weeks after surgery as opposed to at time of sacrifice
*significant, \( p<0.05 \)
**highly significant, \( p<0.01 \)

Cholesterol at the time of injury was also related to the eventual development of intimal neovessels. Looking at both groups of 20-week injury rabbits, only rabbits with a cholesterol level of 26.7 mmol/L and above at the time of injury went on to demonstrate at least one location along the abdominal aorta with lipid and neovessel infiltration of the intima. Conversely, rabbits with a cholesterol level of 19.0 mmol/L and below at the time of injury did not show any intimal neovascularization.

In the case of both groups of 14-week injury rabbits, the minimum cholesterol level at
the time of injury that resulted in neovessel infiltration of the media of the abdominal aorta was 6.1 mmol/L. However, one of the rabbits only demonstrated a small amount of medial but not intimal neovascularization with the same cholesterol level at the time of injury.

2.5 Discussion

2.5.1 Dietary Cholesterol Modulates Plaque Formation

The addition of cholesterol to the rabbit diet was necessary for the generation of atherosclerotic plaques, since rabbits on the normal diet did not develop any plaques in the vessels examined. The HC diet generated small, lipid-rich plaques on its own in the abdominal aorta, but did not result in any advanced plaques within 20 weeks. The HC diet used here was of a low percentage of cholesterol, compared to diets of up to 2% cholesterol used either in shorter studies or in a pulsed fashion\(^9,10\). Longer periods of time have been used in previous studies to generate larger and more advanced plaques with lipid-rich necrotic cores and calcification in the aorta\(^11,12\).

Longer studies and higher percentages of cholesterol and fat may cause liver damage which could contribute to unexpected deaths. Keeping the duration of our HC diet under 20 weeks may prevent most diet-related deaths. An alternative would be to lower the proportions of cholesterol and peanut oil in the diet; for example, a 0.15% cholesterol and 6% peanut oil diet results in a stable, physiologically relevant cholesterol level in rabbits\(^1\). On the other hand, switching the diet to normal chow after complicated atherosclerosis is achieved could result in plaque regression\(^12,9\) which would confound the effects of subsequent interventions.

Oxidizing the cholesterol before adding it to the diet had the effect of accelerating plaque progression, suggesting that its levels were increased in the circulation allowing it to exert its proatherogenic effects. Macrophage area exceeded that of the non-injury VEGF groups despite comparable intimal areas, suggesting that the oxidized cholesterol serves as a constant inflammatory stimulus. Administering oxidized cholesterol in the diet may therefore allow for
the amount of cholesterol in the diet to be reduced to a more realistic level while still producing the same amount of plaque formation.

### 2.5.2 Vascular Endothelial Growth Factor Accelerates Early Plaque Growth

The addition of systemic injections of VEGF to the HC regimen was meant to further stimulate plaque progression and create an environment conducive to plaque angiogenesis. Since the 4 µg/kg VEGF group (Group 1e) demonstrated a greater increase in all the histological measures over the HC diet control group (Group 1a) than the 2 µg/kg VEGF group (Group 1d) did, the results suggest a dose-dependent effect exerted by the VEGF on both inflammation and adventitial neovascularization. Based on previous work\textsuperscript{13,14}, the 2 µg/kg VEGF group was expected to show an increase in these measures, but the longer interval between VEGF injections and sacrifice may have diminished any early gains in plaque progression over the control group.

Despite an increase in the histological measures with the higher dose of VEGF, it was recognized that thicker plaques would be needed to create the hypoxic stimulus needed for plaque angiogenesis. Therefore, a longer HC diet was used to develop larger plaques to investigate the effects of VEGF in more advanced vessel wall disease. However, the combination of additional VEGF injections and a longer HC diet (Groups 2a-c) did not produce any plaques in the abdominal aorta that featured plaque neovascularization. There was also little evidence to suggest that this approach produced larger or more complicated plaques. Given the underwhelming results, extending the duration of the diet is not an effective enough strategy to justify the number of diet-related deaths.

An incidental finding showed that the carotid arteries and aortic arches from the 30-week rabbits occasionally demonstrated intimal angiogenesis. However, these arteries would not be ideal for imaging or performing interventions as the carotid is small for these applications (~2mm) and the arch is difficult to access with ultrasound and can be obscured by breathing
artifacts in MRI. However, the key to the appearance of more advanced plaques in these areas is likely related to turbulent and stagnant blood flow, as opposed to steady, laminar flow. Endothelial cell function is disrupted in the face of oscillatory or low shear stress, leading to greater permeability to cholesterol and increased inflammatory cell adhesion and infiltration. It is therefore expected that some form of endothelial injury, such as therapeutic ultrasound or a balloon catheter, would accelerate plaque formation.

As for the effects of VEGF, it has been proposed in previous work that low-dose, systemic administration of VEGF in mice and rabbits stimulates the production of monocytes in the bone marrow and their subsequent mobilization into the circulation. Consequently, more monocytes would be available to migrate into the vessel wall and become macrophages. At the same time, it is thought that endothelial progenitor cells (EPCs) increase in the circulation in response to VEGF, which may affect reendothelialization after injury to the vessel wall, a situation that is relevant to the balloon injury groups.

Aside from the injections of exogenous VEGF, the HC diet may have contributed to increased endogenous VEGF levels. In one study, serum VEGF concentrations were elevated in rabbits fed a high cholesterol diet as opposed to a normal diet (239.91 pg/ml on a 1% cholesterol diet after 4 weeks vs. 46.17 pg/ml). While details are not known about the duration of this effect or its cholesterol level dependence, this may be a mechanism through which cholesterol contributes to inflammation in the vessel wall.

2.5.3 **Therapeutic Contrast Ultrasound Regimen has no Effect on Plaque Growth**

As mentioned above, endothelial injury can accelerate atherosclerotic processes in the vessel wall. A noninvasive method of potentially inducing endothelial injury is therapeutic ultrasound. However, the weekly insonations of the abdominal aorta in this study did not have any
significant effects on the vessel wall that were measured. There are several potential factors for this apparent lack of effect related to both the insonation regimen and physiological limitations.

Microvascular damage has been previously documented following the use of microbubbles with both clinical and experimental ultrasound systems. Observations of petechial hemorrhage, leakage of albumin-bound dye, and extravasation of polymer microspheres\textsuperscript{16,17} all indicate that microbubble-potentiated ultrasound can cause increased vascular permeability and capillary rupture even at powers attainable with clinical ultrasound systems. However, ultrasound with microbubbles exerts more subtle effects on larger vessels, such as the rabbit auricular artery that is about 1 mm in diameter. At lower ultrasound frequencies (1.13 MHz and 2.8 MHz) but peak negative pressures similar to those used in this thesis, ultrasound with another clinical ultrasound contrast agent has been shown to cause reversible endothelial cell damage in the auricular artery\textsuperscript{18} as well as microscopically visible endothelial damage in the auricular vein\textsuperscript{19}. However, the rabbit aorta is 4 mm in diameter with several times the flow velocity of the auricular vessels, which could mitigate any microbubble-potentiated damage.

Besides endothelial damage, ultrasound with microbubbles at lower pressures has been applied in the field of gene therapy where ultrasound exposure induces sonoporation, the ultrasound-mediated uptake of particles in cells. Uptake of particles larger than typical LDL particles has been demonstrated\textsuperscript{20} and the hydrophilic surface of the temporary pores\textsuperscript{21} should not impede the passage of these particles through the cell membrane. However, this effect is transient and increased uptake of LDL should cease within minutes of the end of insonation\textsuperscript{22}. Even if LDL uptake is increased, native LDL normally washes through the vessel wall with a balance between influx from the endothelium and efflux through the adventitia\textsuperscript{23}. If the balance is upset with a greater tendency towards influx of LDL, a greater residence time for LDL in the vessel wall may result in its oxidation, which is the initiating step in atheroma formation. It is
possible that the weekly insonation regimes in these experiments were not be enough to upset this balance and create any lasting effects.

### 2.5.4 Balloon Injury Produces Advanced Plaques

Mechanical endothelial denudation with a balloon catheter, as expected, proved to be a more severe method of injuring the endothelium. One limitation of this technique is that it damages the endothelium to an unrealistic degree such that the transport of contrast agents or drugs across this endothelium may not resemble that in a native human plaque. As a more invasive procedure however, it was effective in consistently promoting plaque growth along the entire injured stretch of aorta. Both of the 20-week balloon injury groups contained rabbits that developed aortic plaque angiogenesis (Groups 4a and 4b). Intimal neovessels were consistently accompanied by medial neovessels, entailing their origins from the vasa vasorum in the adventitia. The plaque neovessels were usually associated with lipid-rich plaques and disrupted elastin layers, similar to human plaques and rabbit plaques generated by the HC diet alone (Figs 2.3 and 2.9). These similarities suggest that the injury model generates biologically and mechanically relevant plaques. The presence of neovessels in these less mechanically stable areas likely renders them more vulnerable to leakage and rupture and the fact that this environment is recreated in the injury-induced plaques may be an important characteristic.

In the 20-week rabbits, all the histological measures were higher in the non-VEGF control group (Group 4a) than in the VEGF group (Group 4b). In the 14-week groups (Groups 4c and 4d), the only measure that was higher in the VEGF group was intimal area, while macrophage area and microvessel count were lower in the VEGF group. However, none of these differences were statistically significant. The discrepancy between results from previous studies involving the administration of VEGF in conjunction with vessel wall injury and the lack of any significant effects in this study may be explained by the dose and route of administration. The
single intramuscular injection of VEGF protein would have remained at a serum concentration of 250 pg/ml (endogenous level in the hypercholesterolemic rabbit) or higher for less than 40 minutes, assuming a circulation half-life of 3 minutes, a rabbit blood volume of 60 ml/kg and a hematocrit of 40%. On the other hand, serum levels of VEGF after coronary bypass surgery have been shown to remain elevated up to 6 days after the procedure as part of a repair response. Percutaneous transluminal angioplasty followed by bare metal stent placement, which may cause deep vessel wall injury, increases serum VEGF levels for up to 3 months.

Therefore, the short-lived injection of VEGF in our experiments likely did not elicit a differential response when given one week before or two weeks after endothelial denudation.

When exogenous VEGF is introduced into a model of arterial injury in a sustained manner, there is a myriad of factors that affect subsequent changes in the damaged vessel wall. As mentioned, stimulation of bone marrow cells, specifically monocytes and EPCs, through the VEGFR-1 receptor can increase circulating levels of these cells. In an injury model where the damage is confined mostly to the endothelium, reendothelialization by EPCs likely exerts more of an effect than infiltration of monocytes while the reverse may be true in an injury model where the damage extends to the outer layers of the vessel.

However, the importance of the increased mobilization of bone marrow cells has been called into question by a recent study. This study in normocholesterolemic mice showed that abolishing Flt-1 activity (the murine analog of VEGFR-1) had no influence on intimal proliferation following vascular injury while the elimination of all VEGF signaling showed a significant decrease in intimal thickness. It has also been proposed that VEGF acts on the VEGFR-2 receptors on smooth muscle cells in the vessel wall to increase monocyte chemoattractant protein-1 (MCP-1) production. Therefore, damage to the outer vessel wall (which likely occurs in our balloon injury model) could induce local VEGF expression and
kickstart a positive feedback cycle where VEGF causes an increase in MCP-1, leading to the infiltration of VEGF-secreting macrophages.

The route of administration of VEGF also plays a major role in determining the relative importance of its various actions. Local effects on endothelial cells are mediated through the VEGFR-2 receptor and include more direct pro-angiogenic effects such as increased endothelial permeability, induction of vasodilation, and extracellular matrix degradation\(^\text{33}\). Local delivery of the VEGF gene or protein has been shown in various studies to decrease\(^\text{34,35}\) or increase\(^\text{36}\) neointimal thickening, perhaps reflecting accelerated reendothelialization from uninjured neighbouring endothelium balanced with a local inflammatory effect. It may be that delivery of VEGF to all layers of the vessel wall rather than the endothelium only is important for eliciting a predominantly atherogenic response. In any case, it is apparent that inflammation is the key mediator of the proatherogenic effects of VEGF.

Although the VEGF injections did not increase vessel wall angiogenesis in the injury model, an important relationship between plasma cholesterol level and plaque growth was observed. It was found that within the group of 20-week rabbits (Groups 4a and 4b) that received VEGF and underwent balloon injury at week 10, there was a significant positive correlation between blood cholesterol level at the time of the balloon injury and average intimal area upon sacrifice. Though not significant, this trend was also observed in the 14-week injury groups (Groups 4c and 4d). Among these groups however, intimal area was more strongly correlated with plasma cholesterol level at the time of sacrifice. It is possible that circulating cholesterol levels at the time of endothelial denudation and for the following few weeks while the endothelium is regenerating\(^\text{37}\) are the most indicative of the eventual evolution of the plaque. The vessel wall would be most vulnerable to lipid and inflammatory cell infiltration while the endothelium is undergoing repair. The stronger association for the 14-week groups with
cholesterol levels at the time of sacrifice could be explained by the low cholesterol levels at the
time of balloon injury, a relatively early week 3. At this point, the cholesterol levels are still
rising, as opposed to the cholesterol levels at the time of injury of the 20-week rabbits (week 10).
If the vessel wall is vulnerable for several weeks after injury, then the amount of increase in the
cholesterol levels in those weeks also plays an important role.

Cholesterol levels, possibly due to their association with plaque size, seemed to also
indicate whether the plaque would go on to develop plaque neovessels 10 weeks after injury.
Based on cholesterol levels at the time of injury, a threshold level can be established above
which the rabbit goes on to develop abdominal aorta plaques that contain intimal neovessels.
This threshold value is dependent on the time of injury relative to the initiation of the high
cholesterol diet. Below this threshold, neovascularization of the media and intima is minimal or
nonexistent. It is not known whether the plaques would eventually go on to develop plaque
neovessels give sufficient time. However, the results suggest that monitoring the blood
cholesterol of rabbits on an HC diet could be useful tool for determining the optimal time point
at which to perform balloon injury on a rabbit to produce plaque angiogenesis within a known
time frame.

Previous single injury models in the rabbit aorta have shown limited plaque
neovascularization due to a shorter time period between endothelial denudation and sacrifice as
well as a shorter cholesterol feeding period prior to injury\textsuperscript{38,39}. Other models have demonstrated
plaque neovascularization of an extent similar to that achieved in this study, but have required a
second balloon injury\textsuperscript{40,41} or a prolonged cholesterol feeding period\textsuperscript{42}. The 10 weeks of
cholesterol feeding prior to balloon injury in this model seem to play an important role in the
eventual development of plaque neovessels. Given the results from the oxidized cholesterol
group, it may be possible to achieve similar plaques with a lower and more realistic amount of
circulating cholesterol if that cholesterol has an elevated oxidized fraction. The oxidized cholesterol may increase the affinity of cholesterol for the vessel wall and lower the amount of cholesterol influx needed to cause sufficient retention in the wall. Also, it may provide the chronic inflammatory stimulus post-injury that was not seen with the VEGF injections.

2.5.5 Study Limitations

As in any experimental study, several limitations yielded sources of error in the data collected. Some of these limitations are outlined below.

2.5.5.1 Immunohistochemical Staining and Quantification

Immunohistochemical staining of tissue is very difficult to standardize in terms of final stain intensity and sensitivity. The amount of time between sacrifice and immersion of the specimen in fixative, the amount of time the specimen spends in the fixative, the concentrations of reagents used, the stability of the chromogen used, and the ambient temperature during antibody incubation are all examples of factors that can affect the outcome of the staining protocol from one batch to another. While stain intensity may not greatly impact manual vessel counting, macrophage area is determined based on colour and intensity and an abnormally strong or weak stain could have easily affected the number of pixels counted as positive.

While manual vessel counting was not adversely affected by small variations in stain intensity, the subjective nature of the process was a likely source of error. Microvessels in longitudinal profile that appeared distinct on the slice may have in fact been parts of the same vessel segment, artificially elevating the microvessel counts. As well, microvessel detection likely increases over time as the observer becomes more experienced in identifying positively stained microvessels.

2.5.5.2 Specimen Sampling

Average histological values for each rabbit were calculated to provide a single value that
describes the severity of vessel wall disease in the abdominal aorta. However, vessel wall thickness and composition was not necessarily uniform along the whole length of the abdominal aorta, so average values would have depended on the sampling along the length of the aorta. Since it was difficult to mark a 5 mm specimen block without interfering with the visibility of vessels in the adventitia, blocks were embedded with one of either end down, meaning that sections were not likely evenly spaced along the aorta. In fact, two sections from adjacent blocks may end up being almost identical because of this. Another way in which sampling may skew the results applies mainly to the non-injury groups. In these groups, plaques tended to form mostly near the renal branches and so an increased numbers of sample near that area would skew the average intimal area higher.

2.5.5.3 Animal Care

In vivo work is difficult to standardize due to difference in behavior between rabbits. Since all the rabbits in this study came from the same supplier, they are likely genetically similar to each other. However, individual rabbit behaviour is difficult to regulate and the availability of food ad libitum as opposed to a fixed daily ration resulted in different amounts of food being ingested between rabbits. Eating habits depended on the rabbits’ overall health and mien, which in turn were affected by environmental variables and interaction with animal care staff. The variability in plasma cholesterol levels clearly reflected this range in behavior, as well as any underlying physiological differences.

In the injury groups, a few rabbits tended to chew at the surgical wounds or toes on their right hind limbs, necessitating further antibiotic treatment and likely affecting levels of circulating monocytes. Also, the analgesic buprenorphine sometimes has the side effect of reducing appetite and some rabbits had to be coaxed back onto the diet.

2.5.5.4 Consistency of Interventions
Although shipments of the high cholesterol diet would have been consistent upon arrival, the long term storage of the food without refrigeration may have resulted in nutrient loss over time and variability in the nutrients consumed by different groups of rabbits. Also, differing degrees of oxidation may have occurred in the lipids, with oxidized fats and cholesterol potentially being more proatherogenic.\textsuperscript{43}

The effects of balloon-mediated endothelial denudation on intimal hyperplasia, medial necrosis, elastic lamina disruption, and vessel constriction in a healthy rabbit aorta are mediated by balloon catheter pressure and vessel diameter.\textsuperscript{44} Balloon pressure is not likely an issue here with a constant volume of liquid used to inflate the balloons, but vessel diameter certainly varies between rabbits and along the length of the aorta. However, plasma cholesterol level may be the more dominant factor in eventual plaque development than small discrepancies in degree of wall injury.

In addition to the power-related limitations mentioned previously, other factors contributed to the inconsistent delivery of ultrasound to the target location. The ultrasound beam had to travel through the ribcage and abdominal cavity to reach the abdominal aorta and tissue attenuation of the ultrasound beam likely varied drastically from week to week. Compounded with this problem was the fact that the insonation location was marked on the skin, which can shift and stretch easily in relation to the organs. Also, the drip rate of the Definity and saline mixture was not easily controlled and often there would be some mixture leftover.

\textbf{2.5.5.5 Group Size}

Due to the large standard error in the group averages relative to the differences between the groups, a much larger number of animals per group would have been needed to show the differences as statistically significant. The solution does not lie in taking more samples along the aorta as these samples would be correlated with one another. A more acceptable group size
would have been around 8-10 animals per group. However, a small group size was necessary to test the many different parameters while adhering to housing and budget constraints.

2.6 Chapter Summary

Several approaches were explored for the development of an animal model of plaque angiogenesis. Cholesterol feeding was fundamental in plaque formation while oxidation of the cholesterol prior to feeding accelerated plaque growth. In terms of endothelial injury, the therapeutic ultrasound regimen used in this study did not result in any observable differences while balloon injury was effective in generating large, advanced plaques that sometimes exhibited intimal neovascularization. Low dose, systemic injections of VEGF increased plaque growth, inflammation, and adventitial angiogenesis in a manner similar to the oxidized cholesterol, but failed to exert any significant effects when combined with the injury model.

In summary, the injury model with 10 weeks of cholesterol feeding prior to endothelial denudation yields advanced plaques at 10 weeks after injury, with plaque size and neovascularization occurring in a predictable, plasma cholesterol-dependent manner. A working model of plaque neovascularization would consist of placing the rabbits on the 0.25% cholesterol and 6% peanut oil diet starting 10 weeks prior to balloon injury as described above. If the plasma cholesterol level measured at week 10 prior to scheduled balloon injury in a rabbit is less than 20 mmol/L, then the procedure should be delayed until this level is reached. Waiting for plasma cholesterol to reach this level increases the likelihood of producing intimal neovascularization in the abdominal aorta 10 weeks after balloon injury.

2.6.1 Imaging of the Animal Model

One of the proposed applications of the animal model was to provide a platform on which diagnostic imaging techniques could be developed and tested. Not only would an animal model of plaque neovascularization and IPH facilitate this type of development, but noninvasive
imaging techniques could then be used to observe plaque progression in the model in response to IPH and IPH-targeted therapeutics. One such noninvasive imaging modality is MRI, as mentioned in the previous chapter. The intravascular injection of an MR contrast agent can highlight regions of dense neovascularization, as demonstrated by data collected from one of the balloon injury groups. The next chapter shows that MRI can indeed monitor plaque progression in this animal model.
Table 2.2. A summary of all the groups of animals used in the experiments, including group numbers, abbreviated names, regimens, and results. Each group was composed of 4 rabbits. (*) denotes a statistically significant difference; all other trends in the results described were not statistically significant.

<table>
<thead>
<tr>
<th>Group number</th>
<th>Group abbreviation</th>
<th>HC diet?</th>
<th>Duration (weeks)</th>
<th>Group description</th>
<th>Results for the abdominal aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IA = intimal area</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MA = macrophage area</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MC = microvessel count</td>
</tr>
<tr>
<td>Dietary cholesterol and VEGF groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>HC</td>
<td>Y</td>
<td>20</td>
<td>Control group with high cholesterol diet only</td>
<td>Fatty streak formation</td>
</tr>
<tr>
<td>1b</td>
<td>Normal</td>
<td>N</td>
<td>20</td>
<td>Control group with normal rabbit diet</td>
<td>No plaque formation</td>
</tr>
<tr>
<td>1c</td>
<td>OxChol</td>
<td>See description</td>
<td>20</td>
<td>Oxidized cholesterol diet</td>
<td>IA*, MA*, and MC increased over HC control group</td>
</tr>
<tr>
<td>1d</td>
<td>HC+2 μg/kg VEGF</td>
<td>Y</td>
<td>20</td>
<td>Intramuscular 2 μg/kg VEGF at weeks 5 and 10.</td>
<td>MC increased over HC control group</td>
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<tr>
<td>1e</td>
<td>HC+4 μg/kg VEGF or HC+2×VEGF</td>
<td>Y</td>
<td>20</td>
<td>Intramuscular 4 μg/kg VEGF at weeks 5 and 10.</td>
<td>IA, MA, MC increased over HC control group</td>
</tr>
<tr>
<td>Extended diet and VEGF groups</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2a</td>
<td>HC30wks+2×VEGF</td>
<td>Y</td>
<td>30</td>
<td>Intramuscular 4 μg/kg VEGF at weeks 5 and 10.</td>
<td>2 animals died prematurely; inconclusive</td>
</tr>
<tr>
<td>2b</td>
<td>HC30wks+3×VEGF</td>
<td>Y</td>
<td>30</td>
<td>Intramuscular 4 μg/kg VEGF at weeks 5, 10 and 15.</td>
<td>1 animal died prematurely; IA, MA, MC increased over HC+2×VEGF group</td>
</tr>
<tr>
<td>2c</td>
<td>HC30wks+4×VEGF</td>
<td>Y</td>
<td>30</td>
<td>Intramuscular 4 μg/kg VEGF at weeks 5, 10, 15 and 20.</td>
<td>MC increased over HC+2×VEGF group</td>
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</tbody>
</table>
### Therapeutic contrast ultrasound and VEGF groups

<table>
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<tr>
<th>3a</th>
<th>HC+10minUS</th>
<th>Y</th>
<th>20</th>
<th>10 minutes of ultrasound every week</th>
<th>Same as HC group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3b</td>
<td>HC+20minUS+2×VEGF</td>
<td>Y</td>
<td>20</td>
<td>20 minutes of ultrasound every week; intramuscular 4 μg/kg VEGF at weeks 5 and 10.</td>
<td>1 animal died following induction of anaesthesia; results same as HC+2×VEGF group</td>
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</tbody>
</table>

### Endothelial denudation and VEGF groups

<table>
<thead>
<tr>
<th>4a</th>
<th>HC20wks+ED</th>
<th>Y</th>
<th>20</th>
<th>Endothelial denudation at week 10</th>
<th>Large plaques; IA, MA, MC increased over all non-injury groups; 4/4 with intimal neovascularization</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b</td>
<td>HC20wks+2×VEGF+ED</td>
<td>Y</td>
<td>20</td>
<td>Endothelial denudation at week 10; intramuscular 4 μg/kg VEGF at weeks 5 and 9.</td>
<td>Same as HC20wks+ED group; 2/4 with intimal neovascularization</td>
</tr>
<tr>
<td>4c</td>
<td>HC14wks+ED</td>
<td>Y</td>
<td>14</td>
<td>Endothelial denudation at week 3</td>
<td>Large plaques; IA, MA, MC* smaller than HC20wks+ED group; 2/4 with intimal neovascularization</td>
</tr>
<tr>
<td>4d</td>
<td>HC14wks+2×VEGF+ED</td>
<td>Y</td>
<td>14</td>
<td>Endothelial denudation at week 3; intramuscular 4 μg/kg VEGF at weeks 5 and 10.</td>
<td>1 animal died during week 14 imaging; IA increased, MA decreased, MC same as HC14wks+ED group; 2/4 with intimal neovascularization</td>
</tr>
</tbody>
</table>
References


CHAPTER 3: Contrast-enhanced Magnetic Resonance Imaging of the Animal Model

Adapted from:
Serial, Contrast-Enhanced Vessel Wall MRI with Gadofosveset Monitors Plaque Neovascularization Over Time
Stephanie E.G. Chiu, General Leung, James Q. Zhan, and Alan R. Moody
Submitted to the Journal of Vascular and Interventional Radiology (March, 2010)

3.1 Introduction

Atherosclerosis, the most common underlying cause of heart disease and stroke, refers to the formation of lipid-enriched lesions within arterial walls. The accumulation of lipids, proliferation of cells, and production of fibrous tissue can cause narrowing of a vessel lumen, though most deaths from acute coronary disease are a result of vulnerable plaque rupture rather than severe narrowing\(^1\). Plaque neovascularization, a destabilizing factor, has recently developed into an attractive therapeutic target\(^2,^3\). Arising from the vasa vasorum in the outer vessel wall, these neovessels are a possible source of intraplaque hemorrhage and conduit for inflammatory cells. Their presence has been associated with proatherogenic contributors\(^4,^5\), including lipid accumulation, oxidative stress, and macrophage infiltration.

Both extravascular and intravascular contrast agents have been applied towards the visualization of plaque neovascularization. Dynamic contrast-enhanced magnetic resonance imaging MRI with an extravascular contrast agent yields parameters that associate with neovascularization in human carotid arteries\(^6\). Intravascular ultrasound microbubble contrast
agents have also been used to visualize regions of plaque neovascularization in patients. Recently, an albumin-binding gadolinium complex, gadofosveset (Bayer Schering Pharma, Berlin, Germany), has been approved for clinical use. Unlike its MRI contrast agent predecessors, gadofosveset mostly stays within the vasculature and has a 16.3 hour elimination half-life in normal volunteers. Albumin-binding, intravascular agents demonstrate potential for longer image acquisition times and simple quantification of plaque neovascularization.

We aim to show that gadofosveset-enhanced MRI can provide a measure in vivo that correlates with histologically measured plaque neovascularization, and that this same measure can be used to noninvasively track changes in plaque neovascularization over time.

### 3.2 Materials and Methods

#### 3.2.1 Animal Model

The rabbits imaged in this study were the 4 rabbits in Chapter 2 that were on the HC diet for 20 weeks, were given 4 µg/kg of VEGF at weeks 5 and 9 and underwent balloon injury at week 10 (Group 4b in Table 2.2). Histological processing and analysis were performed as described in Chapter 2. Average measures along each abdominal aorta were found for intimal area, microvessel count, and macrophage area using slides corresponding to the MR imaging volume at week 20.

#### 3.2.2 MR Imaging

The animals were imaged prior to endothelial denudation at week 9 and afterwards at weeks 15 and 20 using a GE 3.0T EXCITE MR system (GE Healthcare, Waukesha, WI) and a 13 cm custom, receive-only surface coil. The sequence used was a 3-D, axial, high-resolution (375 µm × 375 µm in-plane and 1.6-2.0 mm slice thickness), T1-weighted, fast spoiled gradient-echo sequence (TR/TE/θ= 7.5/3.5/15, FOV=120 mm, acquisition matrix=320 × 320, 52 slices). The
sequence used SPECIAL (SPECtral Inversion At Lipids) and included a low $b$ value diffusion pulse to attenuate the signal from through-plane blood flow. At all imaging time points, the abdominal aorta was imaged after an ear vein injection of gadofosveset (0.2 ml/kg, Bayer Schering Pharma, Berlin, Germany). The acquisition time of the sequence was 12 minutes and the scan was started 3 minutes after contrast injection to avoid any first-pass effects.

3.2.3 MR Image Analysis

3.2.3.1 Comparison With Histology

Using the left renal branch as a landmark and an estimated average shrinkage factor to convert $in vivo$ vessel length to fixed specimen length, MR slices from week 20 corresponding closest to the centre of each specimen block were selected for analysis. Post-contrast DICOM images were interpolated linearly to a resolution of 58.5 μm × 58.5 μm and placed in random order for ROI (region of interest) analysis using MATLAB (The MathWorks, Natick, MA).

Two blinded observers (S.E.G.C. and G.L) drew two ROIs for each slice to find vessel wall enhancement area (Fig. 3.1). Isolated, high intensity spots that appeared to have 100% blood volume were excluded as they were considered likely to be adjacent arteries.

![Figure 3.1](image)

**Figure 3.1.** An example of the method used to determine vessel wall enhancement area for each MRI slice. Scale bar represents 2 mm. (a) The original image. (b) Regions of interests (ROI) were drawn by each user. The red ROI corresponds to the outer contour of the bright ring that surrounds the dark lumen of the aorta and the green ROI corresponds to the outmost extent of the vessel wall. (c) A threshold was set by the user with the isointense contour of the threshold value (cyan) overlaid on the image to provide visual feedback to the user. (d) Pixels outside of the red ROI and inside of the green ROI with intensity values above the threshold value were included in the vessel wall enhancement area.
3.2.3.2 Comparison Over Time

Average vessel wall enhancement area for each rabbit was also found for MR images acquired at weeks 9, 15 and 20, ensuring that for each animal the slices analyzed at all three time points covered the same portion of aorta.

3.2.4 Statistical Analysis

The strength of the linear relationship between vessel wall enhancement area and each of the histological measures was found using Pearson’s correlation coefficient \((n = 51\) for the individual measures). Pearson’s correlation coefficient was also found for the relationships between each of the histological measures based on all histological sections available \((n = 67)\).

The intraclass correlation coefficient between the two observers was found for all the week 20 vessel wall enhancement area measures using a 2 way random model with measures of absolute agreement. Finally, paired \(t\) tests were done on the group means of the MR measures from each time point to test for significant differences. In this study, a \(p\)-value of 0.05 or less was considered significant with all statistical tests performed using SPSS (SPSS, Chicago, IL).

3.3 Results

3.3.1 Animal Model and Histological Analysis

Two of the animals developed large plaques in the abdominal aorta with neointimal vessels, while the other two had plaques with little or no intimal neovascularization (Fig. 3.2). To investigate the interdependence of the histological measures, Pearson’s correlation coefficient was found for each of the following pairs: intimal area vs. macrophage area, intimal area vs. microvessel count, and macrophage area vs. microvessel count (Fig. 3.3). While intimal area was moderately associated with macrophage area \((R^2 = 0.666)\) and microvessel count \((R^2 = 0.586)\), macrophage area and microvessel count shared a strong association \((R^2 = 0.805)\).
Relationships between all the histological measures were positive and significant ($p < 0.001$).

**Figure 3.2.** Representative histological sections of aortas from two different animals 10 weeks after endothelial injury. Whole aortic cross-sections stained with hematoxylin and eosin (a+b) are shown with overlying rectangles indicating corresponding high-magnification close-ups of CD31-stained sections (c+d). Microvessels are indicated by CD31-positive endothelial cells (stained dark brown), with some microvessels containing an intravascular silicone compound injected after sacrifice (black spots). (a+c) An artery that developed moderate-sized plaques with vasa vasorum confined to the adventitia. (b+d) An artery that developed large plaque with neovessels infiltrating the inner layers of the arterial wall. Scale bars denote 500 µm (a+b) and 200 µm (c+d).
Figure 3.3. Scatterplots with zero-intercept regression lines for each pair-wise comparison between all three histological measures: intimal area, macrophage area, and microvessel count. Data represents all the histological sections in all rabbits that were compared with MR vessel wall enhancement area at week 20 \((n=51)\). Pearson’s correlation coefficients for comparisons were (a) \(R^2 = 0.666\), (b) \(R^2 = 0.586\), and (c) \(R^2 = 0.805\), with all p values less than 0.001.

3.3.2 MR Image Analysis

Post-contrast images contain a ring of high signal intensity corresponding to the adluminal surface of the aorta (Fig. 3.4a). Some of the slices acquired from the two animals with neovessel-rich plaques show surrounding regions that are enhanced but still hypointense to this bright ring (Fig. 3.4b).

Figure 3.4. Representative high-resolution, T1-weighted MR images of aortas from two different animals scanned prior to sacrifice at week 20. (a) Image from an aorta showing no plaque neovascularization. A bright ring (solid arrow) surrounding the dark lumen indicates the adluminal surface. (b) Image from an aorta showing extensive plaque neovascularization. Surrounding the adluminal bright ring (solid arrow) are additional enhancing regions (line arrows). Scale bar represents 2 mm.
3.3.2.1 Vessel Wall Enhancement Area Corresponds With Histological Measures

The Pearson correlation coefficients for the comparisons between individual vessel wall enhancement area measures and corresponding histology values are shown in Table 3.1.

Table 3.1. Pearson correlation coefficients of individual vessel wall enhancement area measures versus individual histological measures

<table>
<thead>
<tr>
<th>Observer</th>
<th>Microvessel count†</th>
<th>Intimal area</th>
<th>Macrophage area†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$ value</td>
<td>$p$-value</td>
<td>$R^2$ value</td>
</tr>
<tr>
<td>Observer 1</td>
<td>0.285*</td>
<td>0.043</td>
<td>0.249</td>
</tr>
<tr>
<td>Observer 2</td>
<td>0.376**</td>
<td>0.007</td>
<td>0.273</td>
</tr>
</tbody>
</table>

†logarithmic transformation performed before correlation to normalize the distribution
*significant, $p<0.05$
**highly significant, $p<0.01$

Average vessel wall enhancement area for all animals plotted against each of the histological measures is shown for Observer 1 (Fig. 3.5). Averaging the values strengthened the associations between the MRI and histological measures but raised the $p$-values due to the reduced number of data points (Table 3.2). The intraclass correlation coefficient for the vessel wall enhancement areas for all 51 slices between the two observers was 0.707 ($p < 0.001$).

Figure 3.5. Scatterplots with zero-intercept regression lines for MR vessel wall enhancement area, measured by Observer 1, versus (a) microvessel count, (b) intimal area, and (c) macrophage area. Each point represents the average of the values taken from one animal. Pearson’s correlation coefficients for each comparison were (a) $R^2 = 0.864$, $p = 0.136$, (b) $R^2 = 0.849$, $p = 0.151$, and (c) $R^2 = 0.997$, $p = 0.003$. 
### Table 3.2. Pearson correlation coefficients of average vessel wall enhancement area measures versus average histological measures

<table>
<thead>
<tr>
<th></th>
<th>Microvessel count</th>
<th>Intimal area</th>
<th>Macrophage area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$ value</td>
<td>$p$-value</td>
<td>$R^2$ value</td>
</tr>
<tr>
<td>Observer 1</td>
<td>0.864</td>
<td>0.136</td>
<td>0.849</td>
</tr>
<tr>
<td>Observer 2</td>
<td>0.908</td>
<td>0.092</td>
<td>0.968*</td>
</tr>
</tbody>
</table>

* $p<0.05$
** $p<0.01$

### 3.3.2.2 Vessel Wall Enhancement Area Increases Over Time

Both observers noted an increasing trend over time in vessel wall enhancement area, with group averages and individual animal values shown for Observer 1 (Fig. 3.6). The paired $t$-test results indicated a significant difference in the group average between weeks 15 and 20 for Observer 1 ($p = 0.05$), but not for Observer 2 ($p = 0.075$), and not between weeks 9 and 15 for either observer.

![Figure 3.6](image_url)

**Figure 3.6.** Changes in average vessel wall enhancement area as measured by Observer 1 over time, with data shown at week 9 (before endothelial denudation), week 15 (5 weeks after endothelial denudation), and week 20 (time of sacrifice). Averages for each animal are plotted, along with the group average and its standard error for each time point. According to paired $t$ tests, there was a significant increase in the group mean from week 15 to week 20 ($p = 0.05$) for Observer 1.
3.4 Discussion

The rabbit aorta is of a size appropriate for clinical MRI field strengths and has been the model of choice in preclinical MRI studies of plaque neovascularure. Despite the variability in plaque development between animals in this study, the association between macrophage area and microvessel count is very strong, reflecting the close relationship between inflammation and angiogenesis. A downside to this close correlation is the fact that inflammation and angiogenesis together will affect vascular permeability and vascular volume fraction, with both properties contributing to the MR signal in this study as described below.

In comparing MR images acquired prior to sacrifice with average histological values, we found that information extracted from T1-weighted, high-resolution MR imaging with gadofosveset is strongly associated with average vessel wall microvessel count, intimal area, and macrophage area. Correlation coefficients for the comparisons had $p$ values below 0.05 in some cases, but the small sample size ($n=4$) of this study precludes any claims of statistical significance.

Owing to the largely intravascular nature of the agent, T1-shortening effects by the albumin-bound complex would normally be assumed to associate with vascularity or blood volume. Therefore, regions of signal enhancement in the vessel wall may indicate regions containing microvessels, whether they are vasa vasorum in the adventitia or neovessels that have extended into the plaque itself. The technique employed in this study does not weigh the pixels belonging to the vessel wall enhancement area according to signal intensity and likely reflects the total area of neovascularization in the vessel wall as opposed to differences in microvessel density.

In addition to T1-shortening within the blood pool, signal increase from gadofosveset may occur when bound or free gadofosveset molecules pass through the endothelium and enter
the vessel wall. The bound form of gadofosveset may cross the endothelium in a manner similar
to native albumin and infiltrate diseased vessel walls more easily due to loss of endothelial
integrity. On the other hand, small, unbound (15% of circulating gadofosveset at 5 minutes post-
injection\textsuperscript{16}, gadolinium-containing molecules seem to penetrate both healthy and diseased vessel
walls easily\textsuperscript{12}. These free gadofosveset molecules in the vessel wall may bind again to albumin,
a protein that has been shown to increase with intimal area\textsuperscript{17}. Supporting the retention of
gadofosveset by albumin in the plaque is the persistence of MRI vessel wall enhancement 24
hours after gadofosveset injection\textsuperscript{18}. Upon binding, the T1 relaxivity of the complex increases
nearly two-fold at 3.0 T\textsuperscript{19}, with a similar increase in signal at gadofosveset concentrations on the
order of 1µM and at sequence parameters used in this study. Plaque neovessels could serve as
another route of contrast agent leakage deep in the plaque itself, while macrophages could
increase permeability of the endothelium of both plaque neovessels and the macrovessel
endothelium. Based on these possible contributors to signal enhancement, our measure of vessel
wall enhancement area would be expected to reflect endothelial permeability and albumin
content in addition to plaque neovascularization.

Regarding the second hypothesis of this study, the results suggest that gadofosveset-
enhanced MR images can track plaque progression over time. Images taken before balloon
denudation (week 9), 5 weeks after balloon injury (week 15), and 10 week after balloon injury
(week 20) show an increasing trend between each time point, with most vessels exhibiting
greater plaque progression between weeks 15 and 20.

Measures of plaque enhancement from blood pool MR contrast agents\textsuperscript{10,12,13}, as well as
parameters extracted from dynamic contrast-enhanced MRI with extravascular agents\textsuperscript{6,14}, have
previously been correlated with presence of atherosclerosis, neovessel density, and macrophage
density. Novel to this study was the method in which vessel wall boundaries were determined.
While previous studies have used ROIs that include the adluminal enhancing ring, we excluded this region as its signal intensity is dependent on flow velocity adjacent to the endothelium and contrast agent concentration within and adjacent to the endothelial surface. Furthermore, qualitative observations that the additional enhancing regions appear in conjunction with plaque neovascularization suggested that focusing on these regions would yield a more sensitive surrogate marker of plaque neovascularization.

Another novel component to this study was the temporal comparison involving serial, non-invasive measures in the same animals. While comparison with histology is needed to properly characterize imaging techniques, a non-invasive and reproducible imaging measure that correlates with the feature of interest can reduce the number of animals needed in a study by eliminating the need for multiple time points for sacrifice. The vessel wall enhancement area measured with MRI in this study may have the potential to monitor plaque response to therapy in preclinical studies. Serial measures taken in the same subject both before and after the initiation of therapy would allow for the severity of disease to be taken into account when assessing response to therapy. Non-invasive measures may also assist in animal model development to deduce which subjects possess the desired phenotype and which subjects require additional intervention.

A significant limitation of the measurement techniques used is that they give global estimates despite local variations in these measures along the lengths of each vessel. When individual MRI slices and histological sections were compared, their correlations demonstrated a low strength of association. This may be due to misregistration between the locations of the image slices and histological sections along the length of each aorta. The histological sections were taken from within a 5 mm long block, leaving uncertainty as to which MRI slice (1.6 to 2.0 mm thick) was the best match.
One major drawback of the vessel wall enhancement area parameter used in this study is the high degree of subjectivity, leading to moderate interobserver agreement. Signal inhomogeneity caused by the surface coil sensitivity pattern and receiver overflow artifact precluded the use of a reference tissue intensity and pre-contrast images were not available for all of the animals. Ideally, high-resolution T1 mapping would provide an absolute measure of contrast agent concentration.

While the vessel wall enhancement area correlates with plaque neovascularization, this relationship may be confounded other factors as outlined above. Using an animal model with less closely connected plaque angiogenesis and inflammation may determine whether steady-state imaging with gadofosveset correlates more strongly with one over the other. Alternatively, techniques such as dynamic contrast-enhanced MRI or the use of strictly intravascular contrast agents may assist in separating the effects of angiogenesis and inflammation.

In conclusion, MR vessel wall imaging with gadofosveset generates a measure that correlates with histologically measured vessel wall neovascularization, intimal area, and macrophage area in an animal model of atherosclerosis. This MR vessel wall measure also increases over multiple time points in the same animals, likely in concert with plaque growth, inflammation, and neovascularization.
References


CHAPTER 4:

Summary and Future Directions

4.1 Thesis Summary

The work in this thesis was geared towards producing an animal model with plaque angiogenesis leading to neovessel hemorrhage that is amenable to observation by noninvasive imaging techniques. Intraplaque hemorrhage can destabilize an atherosclerotic plaque in several ways and render it more susceptible to rupture and subsequent thrombus formation, which is the basis of a large portion of cardiovascular clinical events. While hemorrhage products have been imaged with MRI in patients, there is currently no animal model available for the longitudinal study of IPH and its natural history.

Chapter 2 of the thesis detailed the steps taken to test and optimize combinations of strategies for accelerating plaque formation and encouraging plaque angiogenesis. A rabbit model was developed in the end that forms atherosclerotic plaques whose size and degree of neovascularization are predictable to a certain extent. Rabbits on the high cholesterol diet for 10 weeks prior to endothelial denudation of the abdominal aorta by balloon catheter tended to develop large plaque with extensive neovascularization in all layers of the vessel wall when their plasma cholesterol level was above a certain level at the time of balloon injury. Previous rabbit injury models have not explicitly shown the consistent development of neovessel-rich plaques with a single injury, likely due to a shorter cholesterol feeding period prior to injury.

The limitations of the model were also outlined in Chapter 2. One of the major drawbacks is the long time needed for cholesterol feeding, which may limit the amount of time available for the study of plaque progression following the production of plaque angiogenesis
before the rabbits succumb to diet-related illness. Further investigation may determine whether neovessel-rich plaques can be generated and maintained with lower concentrations of cholesterol. As well, the use of endothelial denudation may permanently damage the endothelium to an extent that is not seen in natural atherosclerotic plaque formation in humans. Therefore, the testing of any drugs on the model whose delivery is significantly affected by endothelial permeability may not translate to clinical research.

Chapter 3 of the thesis demonstrates that the animal model developed in the previous chapter can be monitored noninvasively using contrast-enhanced MRI. Using the intravascular injection of a contrast agent that stays mostly within the circulation, MRI was used to visualize regions in the vessel wall where the contrast agent accumulated. These regions on MRI, measured using a novel method, correlated with intimal area, macrophage area, and microvessel count in corresponding histological sections. The MRI-derived measure also demonstrated an increasing trend over time with plaque development. Drawbacks pertaining to this study include the subjective nature of the MRI quantification method, the lack of signal specificity for plaque neovascularization, and the use of only 4 animal subjects. The subjective nature was partly due to signal inhomogeneity in most images (from receiver overflow artifact) and the lack of pre-contrast images for one of the subjects. These issues can be corrected to provide a reference tissue or signal enhancement ratio, either of which may eliminate the need for manual threshold setting and decrease interobserver variability. Weighting the vessel wall enhancement area by normalized post-contrast intensity may provide a measure that more closely associates with intimal area, macrophage area, and microvessel count.

Taken together, chapters 2 and 3 outline the development of an animal model that demonstrates consistent plaque angiogenesis that can be monitored noninvasively using contrast-enhanced MRI. Not only can the model be used to refine imaging techniques for the detection of
plaque angiogenesis in patients, but the proposed MRI technique can be used to guide animal model development and monitor the natural history of and the effects of interventions targeted towards plaque angiogenesis. With a standardized MRI technique for measuring plaque angiogenesis, the hypothesis that plaque neovessels lead to IPH can be tested in patients via long term study. One of the advantages of using gadofosveset is that it is already clinically approved for patients with suspected peripheral vascular disease, unlike other MR blood pool contrast agents.

4.2 Future Directions

4.2.1 Animal Model of Intraplaque Hemorrhage

Now that an animal model that demonstrates plaque angiogenesis in a predictable manner has been defined, the next logical step in creating an animal model of IPH would be to somehow trigger hemorrhage from these neovessels. Frank hemorrhage has been performed in a rabbit model previously, but the mechanism of hemorrhage was the disruption of the overlying fibrous cap with a combination of viper venom, which is toxic to the endothelium, and histamine, a vasoconstrictor in rabbits. To date, there are no established techniques for triggering leakage or rupture of plaque neovessels.

One possible way to accomplish local microvascular hemorrhage may be high intensity focused ultrasound (HIFU) with microbubbles, a technique that is attractive in its targeted and noninvasive nature. As previously mentioned, the disruption of microbubble contrast with low frequency ultrasound can result in extravasation of albumin, microspheres, and mostly importantly, red blood cells. An ultrasound- or MRI-guided HIFU with a low-frequency spherical transducer can be used to optimize ultrasound intensity and localization to the vessel. While convenient for simultaneous imaging and bubble disruption, a clinical system such as the one used in this thesis is limited to higher frequencies (mostly 3.5 MHz and higher) and
intensities that are deemed safe. A HIFU system, if calibrated properly, should be able to trigger hemorrhage in plaque neovessels while sparing surrounding healthy tissue and vasa vasorum because of the restriction of microbubbles, which lower the intensity threshold for damage, to the circulation and the greater susceptibility of plaque neovessels to hemorrhage. Plaque neovessels are documented to be more fragile due to their lack of pericytes and tight junctions and spatial association with lipid-rich cores.

4.2.2 Therapeutics for Intraplaque Hemorrhage

With an animal model of triggered IPH, both the natural history of IPH and the effects of therapeutics could be studied using histological methods. IPH would be expected to increase plaque inflammation and lipid core size, both of which are easily measured with histology. These characteristics could also be examined in response to iron-chelating and anti-oxidant therapeutic agents that would be expected to attenuate lipid oxidation and other effects of oxidative stress, leading to a reduction in inflammation and lipid core size. Candidates for therapeutic agents include desferrioxamine, an iron chelator that would be expected to decrease the oxidant activity of ferric iron in the setting of IPH and alpha lipoic acid, whose non-specific anti-oxidant and chelating properties would likely exhibit anti-atherogenic effects in general.

4.2.3 Imaging Plaque Neovascularization and Intraplaque Hemorrhage

4.2.3.1 Contrast-enhanced Magnetic Resonance Imaging

While the contrast-enhanced MRI technique described in chapter 3 did correspond with plaque neovascularization, the use of a strictly intravascular contrast agent could determine whether the additional relationships with plaque size and inflammation were consequences of the animal model itself or the use of a contrast agent that extravasates to a limited extent. An intravascular agent could determine the specificity of our technique with the clinically approved gadofosveset for plaque neovascularization.
4.2.3.2 Magnetic Resonance Imaging of Intraplaque Hemorrhage

Once IPH is triggered in the animal, the previously mentioned clinical MRIPH sequence could potentially be correlated with histological specimens to verify the origin of the bright signal. Longitudinal studies could also be performed with this sequence as a tool to study the evolution of a plaque with IPH without perturbing the plaque. However, the MRIPH sequence relies on the presence of methemoglobin, which may decreased in rabbits because of superior hemoglobin scavenging capabilities. Rabbits do not possess the 2-2 phenotype of scavenging protein haptoglobin, which is bulky and cannot access and bind hemoglobin effectively. If this issue interferes with our ability to identify MRIPH positive plaques, then an alternate animal model may be needed to investigate the MRIPH signal.

4.2.3.3 Contrast Ultrasound Imaging

While ultrasound contrast agent has been mentioned mainly in the context of bubble disruption and its bioeffects in this thesis, its primary use is for visualizing blood vessels. The bubbles stay in the circulation and can be detected with an ultrasound system at the level of a single bubble. Using the nonlinear echoes that the bubbles uniquely emit when insonated, it is possible to isolate the signal coming from the bubbles only. Contrast ultrasound has been used to image plaque neovascularization in patient carotid arteries and may be applied to this model to noninvasively monitor plaque neovascularization. However, the carotid artery is easier to image with ultrasound because of its superficial placement, unlike the rabbit aorta. An alternative approach may be to employ an intravascular ultrasound (IVUS) transducer, which is a more invasive procedure. However, the transducer operates at a much higher frequency (20 MHz), allowing for higher resolution imaging that is more suited for detecting the small neovessels.

4.3 Conclusion

With the use of a high cholesterol diet and endothelial denudation in the abdominal aorta of
rabbits, plaque angiogenesis can be induced in a rabbit model. The presence of plaque angiogenesis in this model can be identified and monitored over time using noninvasive, contrast-enhanced MRI.
References


