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A Brief Elevation of Serum Amyloid A is Sufficient to Increase Atherosclerosis

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A BRIEF ELEVATION OF SERUM AMYLOID A IS SUFFICIENT TO INCREASE ATHEROSCLEROSIS

Dissertation

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

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A BRIEF ELEVATION OF SERUM AMYLOID A IS SUFFICIENT TO INCREASE ATHEROSCLEROSIS

Cardiovascular disease is now the leading cause of death worldwide. Serum amyloid A (SAA), a positive acute phase reactant, along with C-reactive protein is used clinically as a marker of cardiovascular disease risk. However, recent data has shed light on a possible causal role of SAA in the development of atherosclerosis, the most pervasive form of cardiovascular disease. Several inflammatory diseases such as diabetes and obesity are known to confer increased risk of developing cardiovascular disease. Individuals with these diseases all have modest but persistent elevation of SAA. To determine if SAA caused the development of atherosclerosis, *apoe*$^{-/-}$ chow fed mice were injected with either an adenoviral vector expressing human SAA1 (ad-hSAA1), a null adenoviral vector (ad-Null) or saline. Human SAA levels rapidly increased, albeit briefly then returned to baseline within 14 days in mice that received ad-hSAA1. After 16 weeks, mice that received ad-hSAA1 had significantly increased atherosclerosis compared to controls on the aortic intimal surface ($p<0.0001$), aortic sinus ($p<0.05$) and the brachiocephalic artery ($p<0.05$). According to the "response to retention" hypothesis; lipoprotein retention by vascular wall proteoglycans is a key initiating event in the development of atherosclerosis. We previously reported that SAA-stimulated vascular smooth muscle cells expressed biglycan with increased glycosaminoglycan chain length and increased binding affinity for low density lipoprotein. To further test the role of biglycan on the development of atherosclerosis we generated biglycan transgenic mice. These mice were crossed to the *ldlr*$^{-/-}$ mouse on a C57BL/6 background and fed a pro-atherogenic western diet for 12 weeks. There was a significant increase in atherosclerotic lesion area on the aortic intimal surface ($p<0.05$) and the aortic

sinus ($p<0.006$), as well as a significant correlation between vascular biglycan content and aortic sinus atherosclerotic lesion area ($p<0.0001$). These data demonstrate that transiently increased SAA resulted in increased atherosclerosis compared to control mice, possibly via increased vascular biglycan content. In support of this we found that biglycan transgenic mice had significantly increased atherosclerosis compared to wildtype controls, likely through increased lipid retention in the vascular wall.

Keywords: Serum amyloid A, Biglycan, Transforming growth factor-β, Atherosclerosis

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1.1 Initiation of atherosclerosis: competing hypotheses

1.1.1 The Response to injury

The single initiating event in the development of atherosclerosis has yet to be defined. Over the years many theories have been tested providing a great deal of information regarding the origins of atherosclerotic plaques in the vasculature. One of the pioneers in the study of early atherosclerosis was Dr. Russell Ross. He championed the “response to injury” hypothesis of atherosclerosis which proposed that some insult to the endothelium represented the key initiating event in atherosclerosis. He argued that endothelial dysfunction caused by mechanical stress, homocysteine exposure, oxidized LDL, toxins as well as other triggers was sufficient to establish the beginnings of fatty streak formation, the first histologically visible manifestation of atherosclerosis. The body of his work focused on the activation of inflammatory signaling cascades in response to these endothelial insults (1). However, morphological analysis of lesion development revealed several concepts that contradict the theories of Ross et al. First, most lesions maintain an intact endothelial layer until they become very advanced, rupture-prone atheromas (2). Second, endothelial denudation, which is a known side effect of balloon angioplasty, does not result in plaque formation (3). Another major component of the “response to injury”

theory related to endothelial cells is the expression of vascular adhesion molecules. Vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) are both expressed on the luminal surface of endothelial cells in response to injury or inflammatory stimuli within the vessel wall. While this response is certainly necessary for the overall homeostasis of the vessel as it recruits leukocytes to points of injury, it can also be very problematic. If the stimuli driving adhesion molecule expression is itself dysregulated, the signaling of inflammatory cells will lead to excess leukocyte recruitment and inflammation; and the potential for disease development as seen in the progression of atherosclerosis. Inflammatory cytokines such as TNF-α and IL-1 are capable of stimulating the expression of adhesion molecules in endothelial cells; however, some signal must be present to drive the expression and secretion of these cytokines (4). The identification of the underlying driving force for inflammatory cytokine secretion is one area where the “response to injury” hypothesis remains incomplete. The “response to injury” also presents ox-LDL as a molecule capable of causing endothelial dysfunction (5). The limitation of this idea is that the plasma environment inhibits most oxidation of lipoproteins (6). Moreover, any amount of oxidized LDL found in circulation is cleared very rapidly by the liver (7), thus it’s not likely to interact with the vessel wall in appreciable amounts. It’s been demonstrated that LDL and other lipoproteins naturally flux in and out of the vessel wall (8), suggesting that lipoprotein flux is not pathogenic; however, the retention of those lipoproteins might be. All of the above mentioned processes, endothelial dysfunction,

adhesion molecule expression and the presence of ox-LDL are very important in the progression of atherosclerosis; however, the evidence simply does not support the idea that any of them are individually sufficient to initiate the development of atherosclerosis.

1.1.2 The Response to Retention

The gaps in explaining the development of atherosclerosis through vascular injury lead to the “response to retention” hypothesis of early atherosclerosis. It surmises that the retention of lipoproteins in the subendothelial space by vascular proteoglycans is an initiating event in the development of atherosclerosis (9). Much of the early evidence for retention as an initiating event in atherosclerosis was done in rabbit models. When made rapidly hypercholesterolemic via bolus injection of LDL, arterial lipid content increased within minutes to hours. Importantly, it was shown that the rate limiting step in lipid accumulation in the vessel wall was a lack of egress, meaning the LDL particles were being retained by some component of the subendothelial compartment (10). The molecules most implicated in the retention of LDL in the vessel wall are proteoglycans, which have negatively charged glycosaminoglycan side chains capable of interacting with positively charged motifs on apolipoprotein B (11). Evidence for this interaction has been demonstrated both histologically and biochemically.

The importance of this lipoprotein/proteoglycan interaction on the development of atherosclerosis was demonstrated by a series of experiments using mice transgenic for several mutations of apolipoprotein B. The mutations involved amino acid substitutions of several sites on the apolipoprotein B molecule identified as important in either LDL receptor binding or proteoglycan binding (11). In mice with apolipoprotein B lacking the ability to bind to proteoglycans, the degree of atherosclerosis was significantly less than those mice with normal proteoglycan binding, independent of the cholesterol levels. This implied that elevated total cholesterol was not sufficient to cause severe early atherosclerosis independent of proteoglycan binding (12). However, follow-up work related to proteoglycan binding defective apolipoprotein B demonstrated that, given enough time, the degree of atherosclerotic lesion development in all groups ultimately reached the same magnitude. Furthermore, lipoprotein lipase was identified to play a key role in bridging lipoproteins to proteoglycans in middle to late developing lesions; events that take place well after lipid retention has been established (13). Thus, lipoprotein/proteoglycan interaction is important for the initiation of atherosclerosis; however, it may not be required as other mechanisms such as molecules capable of bridging lipoproteins to proteoglycans have been identified (13).

1.1.3 Evidence that lipoprotein retention precedes inflammation

Franks et al demonstrated lipoprotein retention prior to histologically relevant lesion formation utilizing freeze-etch electron microscopy. Lipid droplets

in the subendothelial space of hyperlipidimic *apoe-/-* aortas, but not C57BL/6 control aortas, were clearly evident as early as three weeks of age (14).. Franks work was greatly extended by Nakashima et al, who investigated the progression of early atherosclerosis in human coronary arteries. They established a scoring system, with a scale of 0-3, based on the degree of lipid deposition in the subendothelial space of the arteries. Lesions scoring zero presented with only diffuse intimal thickening, a physical change in the vessel wall marked by increased matrix deposition. No lipid deposition was observed in lesions scored with zero. Conversely, lesions with a score of three had extensive lipid retention with accompanying macrophage infiltration. Their data demonstrated that lipid retention was only identified after diffuse intimal thickening, implying that matrix deposition was a key initiating event in the development of atherosclerosis. Once lipid retention progressed to some critical level, macrophages began to infiltrate the vessel wall, appearing to translocate exclusively from the luminal side of the vessel (15). Thus, their chronology of early atherosclerosis was: increased matrix deposition followed by lipid retention then followed by macrophage infiltration (16). The evidence clearly pointed to atherosclerosis as a disease of retention followed by inflammation.

1.2 Biglycan

1.2.1 Biglycan regulation

With regards to the “Response to Retention” hypothesis, the proteoglycan most associated with lipid retention in both human and murine atherosclerotic lesions is the small leucine rich proteoglycan biglycan (17). The gene for this small proteoglycan is located on the X chromosome, where it encodes a highly conserved core protein with a mass of about 42 kDa (18). Biglycan possesses two glycosaminoglycan side chains composed of chondroitin or dermatan sulfate disaccharide repeats (19), attached to the N-terminus of the core protein (20). The promoter region of the biglycan gene possesses AP2 sites, IL-6 response elements, an NF-κB site and a TGF-beta negative element. Actual regulation of the biglycan gene varies greatly depending on the cell type and developmental stage (21). For instance, in chondrocytes retinoic acid down regulates biglycan; however, in skeletal muscle retinoic acid had no effect on biglycan but did increase expression of the closely related proteoglycan decorin (22). It has also been clearly demonstrated that TGF-beta increases biglycan in most cell types (23) including glomerular mesangial cells (24). In osteoblastic cells, IGF-1 and IGF-2, both known to stimulate bone development have also been shown to increase biglycan expression (25).

1.2.2 **Biglycan and the extracellular matrix**

Within the vasculature, biglycan is expressed in many different cell types. However, the predominant site of synthesis is the vascular smooth muscle cells, where it is secreted as part of the extracellular matrix. When initially characterized some 30 years ago, biglycan’s normal physiological function within

the matrix, particularly bone, was thought to be interacting with and stabilizing matrix components such as collagen (26) and elastin fibers (27). Thus, biglycan was assumed to play an integral, albeit inert, role within the extracellular compartment. The importance of biglycan in stabilizing the extracellular matrix was demonstrated recently in biglycan deficient mice. These mice had an increased incidence of spontaneous aortic dissection and rupture due primarily to weakly organized collagen fibrils which resulted in aortas with low tensile strength (28). The rate of aortic dissection and rupture in biglycan deficient mice was further exacerbated by infusion of angiotensin II (29). Biglycan's residence in the extracellular matrix underscores many of its important, recently identified biological functions. Many studies now suggest that biglycan is a regulator of growth factors and inflammatory cytokines such that it is now regarded as a member of the innate immune system (30). Proteolytic enzymes such as bone morphogenic protein 1 (BMP1), matrix metalloproteinases (MMP) and granzyme B can cleave biglycan's core protein releasing it from the extracellular matrix where it then acts as a "danger signal" ligand for many different receptors. Mitogen activated protein kinase p38 (MAPK), extracellular signal-regulated kinase (ERK) and nuclear factor kappa-light chain enhance of activated B cells (NF-κB) are rapidly phosphorylated when soluble biglycan binds to Toll like receptor 2 and 4. Macrophages stimulated via biglycan/Toll like receptor interaction had a profoundly increased expression of macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1(MCP-1) resulting in the recruitment of more monocyte/macrophages to sites of tissue injury (31) (32).

Biglycan has also been shown to induce inflammation by clustering Toll like receptors with the receptor $P2X_7$ to activate the NLRP3 inflammasome ultimately leading to the secretion of mature IL-1β (33). Biglycan has certainly been established as a key mediator of tissue injury derived inflammation; however, recent reports also demonstrated that biglycan can activate the adaptive immune response in macrophages and dendritic cells. Once bound to Toll like receptors, biglycan was able to upregulate CXCL13, a major B cell attractant and biomarker of autoimmune inflammatory diseases (32).

1.2.3 Biglycan in atherosclerosis

Biglycan is the molecule most often associated with retention of lipids in early atherosclerotic lesions in both humans and mice (17). Biglycan is upregulated by several cytokines that have been implicated in the development of atherosclerosis including angiotensin II and TGF-β (34, 35). Studies have demonstrated that biglycan is present in both human and murine atherosclerotic lesions. Interestingly, in human coronary arteries, versican is the predominant proteoglycan. However, when human coronary atherosclerotic lesions were examined for lipoprotein and proteoglycan co-localization, biglycan was the proteoglycan most likely to co-localize with the lipoproteins apolipoprotein E and apolipoprotein B. Versican was associated with regions of low lipoprotein content (36). In both *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ mouse models, biglycan was present at all stages of aortic sinus lesion development co-localized with apolipoprotein A-I and apolipoprotein B lipoproteins (37). Interestingly, biglycan stimulated by cytokines

associated with atherosclerosis had longer, more negatively charged GAG chains (38). Such a modification would allow for a very strong ionic interaction between biglycan and the positive amino acid moieties found on lipoproteins such as apolipoprotein B (11). Thus, biglycan, possibly by way of retaining pro-atherogenic lipoproteins, is likely to play a role in the development of atherosclerosis. Interestingly, the evidence that biglycan is capable of activating the innate immune system; and the importance of inflammation in the development of atherosclerosis (39), likely provides a second possible means by which biglycan is atherogenic. As previously stated, TGF-β is a potent initiator of biglycan synthesis such that TGF-β warrants further discussion.

1.3 Transforming Growth Factor β

1.3.1 The TGF-β superfamily

TGF-β is a member of the eponymously named TGF-β superfamily of cytokines and growth hormones that play an immense role in all aspects of development and maintenance of most organisms. The family is comprised of several TGF-β isoforms as well as bone morphogenic proteins, activins, and nodals (40). These molecules play a role in processes such as cell proliferation and differentiation, which represent key elements of TGF-β's embryonic developmental programming. However, it is TGF-β's role in synthesis of extracellular matrix and regulation of immune cells that link it profoundly to the

development of atherosclerosis (41). There are three isoforms of TGF-β represented within this family, being TGF-β1, TGF-β2 and TGF-β3. They are all products of different genes and share approximately 80% sequence homology (42). To date, the vast majority of data collected on these isoforms pertains to TGF-β 1 function. Given its capacity to affect so many aspects of cellular homeostasis, TGF-β's expression and regulation are tightly controlled both within the cell and once secreted to the extracellular compartment. The TGF-β protein is synthesized as a pro-protein. The pro-protein segment of the molecule must be cleaved prior to TGF-β being exported; however, it remains ionically associated with TGF-β and the two molecules are secreted as a latent complex (43). Once in the extracellular compartment TGF-β is sequestered to the extracellular matrix by several molecules including biglycan. TGF-β remains inactive until it is proteolytically cleaved by one of several matrix proteases, including plasmin, integrins but most often thrombospondin 1 (44). Once liberated from the matrix, TGF-β becomes a ligand for the transforming growth factor β type two receptor (TβRII). This TGF-β complex then interacts with one of several transforming growth factor β type I receptors to initiate downstream signaling within the cell. Several members of the SMAD protein family are primarily responsible for canonical TGF-β signal transduction within the cell; however, other pathways have been identified. SMAD 2 & 3 are TGF-β receptor specific effector molecules that become phosphorylated upon TGF-β ligand binding and receptor complex formation. The phospho-SMAD2/3 then

complexes with SMAD4 ultimately acting as a nuclear transcription factor driving the expression of TGF-β target molecules (45).

The investigation of TGF-β function is made quite difficult by the fact that when knocked out, mice do not live more than a few weeks as a result of profound inflammation within the major organs, thus genetically derived loss of function studies are nearly impossible to perform (46). Furthermore, modulation of TGF-β in vivo involves the use of inhibitory antibodies and soluble receptors to inactivate the cytokine while in circulation. This fails to address the paracrine and autocrine capacities of TGF-β within the compartment for which it was synthesized and secreted (47). This is why TGF-β's physiological functions are loosely characterized as requiring the right cell type, the right cytokine concentration and the right timing (48).

1.3.2 TGF-β and atherosclerosis

TGF-β proteins are synthesized in many cells including most cells present in atherosclerotic lesions such as vascular smooth muscle cells, leukocytes and endothelial cells (48). A simplistic description of TGF-β action, particularly related to atherosclerosis, is to categorize it as anti-inflammatory and pro-fibrotic. However, again TGF-β signaling is very spatially and temporally dependent. Under homeostatic conditions, TGF-β inhibits cell proliferation and helps to maintain healthy extracellular matrix as its actions act in opposition to matrix degrading proteins such as matrix metalloproteinases(MMP) (45). TGF-β has

been shown to maintain vascular smooth muscle cells in a contractile state, thus preventing the cells from adopting a synthetic phenotype which would result in increased matrix deposition (49). Increased matrix accumulation including the deposition of the proteoglycan biglycan have been shown to associate with the early development of atherosclerosis by increasing the retention of lipoproteins in the vessel wall, thus setting off the cascade of events leading to fatty streak formation and ultimately the development of atheromas (50). Thus, maintaining homeostatic levels of TGF-β is key step in maintaining vascular wall integrity; however, many molecules with links to cardiovascular disease are capable of increasing TGF-β levels. Diseases such as diabetes are known to stimulate increased TGF-β expression (51). Interestingly, individuals with diabetes also have a greater risk of developing cardiovascular disease (52). Beyond disease states, several cytokines such as angiotensin II have been shown to increase plasma TGF-β levels. *Ldlr*$^{-/-}$ mice infused with angiotensin II for 28 days via alzet pump implantation had a rapid 3 fold increase in TGF-β levels by day three. These mice also had increased atherosclerosis compared to saline infused mice. When a subset of these mice was concurrently treated with angiotensin II and the TGF-β neutralizing antibody 1D11, the induction of TGF-β was prevented. Furthermore, the mice receiving TGF-β inhibition had less atherosclerosis quantified in the aortic sinus (53). Thus, TGF-β appeared to directly stimulate the development of atherosclerosis.

However a consensus on TGF-β's role in atherosclerosis is as yet undetermined. Work by Mallat et al, using a pan-TGF-β inhibitory antibody to

decrease circulating levels of TGF-β1, TGF-β2 and TGF-β3, resulted in increased plaque size in *apoe*$^{-/-}$ male mice. They also reported a much more inflamed plaque with increased macrophage content and decreased collagen fibers (54). Increased inflammation and a lack of fibrous continuity within a lesion represents the hallmark of an unstable, clinically significant atheroma (55). Conversely, Lutgens et al reported that TGF-β inhibition in *apoe*$^{-/-}$ male mice via injection of a soluble TGF-β receptor resulted in a dramatic change in lesion cellularity with an influx of inflammatory cells. However, when lesions were analyzed for atherosclerosis, the group that received TGF-β inhibition had a striking decrease in lesion area (56). In summary, three models of TGF-β inhibition and atherosclerosis were studied. One suggested that TGF-β was anti-atherogenic; two suggested that TGF-β was pro-atherogenic; however the two suggesting TGF-β is pro-atherogenic differed greatly in the resulting inflammatory effects of TGF-β inhibition. Obviously, there is still a great deal of work to be done to understand TGF-β's role in atherosclerosis.

1.4 Serum amyloid A

1.4.1 SAA background

Serum amyloid A (SAA) is a family of highly conserved apoproteins. They are expressed in almost every vertebrate animal studied to date, ranging from humans, goats, fox, to the arctic char, a salmon-like fish (57). This implies a significant evolutionary importance for this family of proteins. Humans have four

unique isoforms of SAA and mice have 5, each with some allelic variation. The genes for the human SAA isoforms are located on chromosome 11p15.1 within 150kb of each other (58). The genes for murine SAA are also located within very close proximity to one another, spanning approximately 45kb on chromosome 7. This chromosome locus is considered to be syntenic to the chromosome locus of human SAA (59). Murine and human SAA's are categorized based on their responsiveness to inflammatory or infectious stimuli. Mice and humans share two homologous acute phase isoforms of SAA; SAA1 and SAA 2 in humans and SAA1.1 and SAA 2.1 in mice, which are synthesized primarily in the liver (60). Though mice and humans have varying numbers of alleles of both of these genes, human SAA1 is considered homologous to murine SAA1.1 and human SAA2 is considered homologous to murine SAA2.1. During an acute phase response (APR) these protein variants can increase in concentration up to 1000 fold over baseline levels (61). Though the exact role for this substantial increase is not fully appreciated, it is thought to play a major role in activating the immune system to respond to acute stimuli. As an APR resolves there is an equally rapid resolution of the serum concentrations of the acute phase SAA's. Mice possess another less understood acute phase SAA, SAA3. This particular isoform is only modestly homologous to murine SAA1.1 and 2.1, sharing approximately 60% of the other acute forms' sequence (62). This is considered an extrahepatic acute SAA, expressed in macrophage and adipose tissue that does not contribute to circulating levels of SAA (63). Murine SAA3's homologue in the human genome was considered a pseudogene possessing a single

nucleotide mutation that inserts a premature stop codon into the sequence. This sequence disruption was thought to be sufficient to prevent the protein from being synthesized (64) ;however, studies have begun to identify human SAA3 by RT-PCR (65). Whether human SAA3 actually produces a functional gene product has yet to be determined. Both humans and mice have another isoform of SAA that is considered constitutively active as its serum levels do not change in the presence of acute inflammatory or infectious stimuli. These SAA's have an additional eight peptides generating a monomeric SAA about 2kDa larger than the acute isoforms (66). Though both humans and mice express these constitutive isoforms, their function remains unknown.

SAA has been most broadly investigated as the circulating precursor to complex amyloid plaques that form in tissue throughout the body during amyloidosis (67). Surprisingly though, little is known structurally about the SAA family of proteins. It had previously been reported that SAAs likely existed in an alternating helix- β–sheet conformation based on predictive structural analysis (68). However, recently, Jinghua et al have identified several structural components of human SAA that have helped to explain some the molecule's functions. First and foremost, they identified that the protein contains four alpha helices and no β-sheet motifs. Also, the structure has some homology to apolipoprotein E which is an interesting discovery given that SAA and apolipoprotein E have some similar binding capacities involving the bridging of lipoproteins to proteoglycan rich matrix (69). They also revealed that the two

regions of helix 1 and 3 in each monomer thought to be amyloidogenic are buried in the native conformation of human SAA1, thus implying that SAA does not naturally aggregate into amyloid plaques (70). Though the vast majority of work with SAA is focused on amyloidosis, a very robust body of literature is being cultivated investigating SAA's role in cardiovascular disease.

1.4.2 SAA and cardiovascular disease

Modestly elevated SAA is a characteristic of many inflammatory diseases such as diabetes and obesity (71) (72). Individuals who suffer from such diseases have an increased risk of developing heart disease, particularly atherosclerosis, though the exact etiology remains unknown (73). SAA and high sensitivity C-reactive protein (hs-CRP) have long been regarded as very useful markers of inflammation, and both are quite robustly associated with cardiovascular disease outcomes (74). hs-CRP is more widely accepted as a better marker for overall cardiovascular risk (75); however, SAA may be a better indicator of the extent of disease present as shown by data analyzed as part of the Women's Ischemia Syndrome Evaluation (WISE) trial (74).

Not until recently though has SAA's potential causal role in atherosclerosis been investigated. Though SAA's exact native biological role in both humans and mice is not fully understood, several potential pathologic features of SAA as they relate to the development of atherosclerosis have been identified. Lee at al demonstrated that human monocytes in culture responded to SAA stimulation

with a dose dependent increase in CCL2, a potent monocyte chemoattractant found in atherosclerotic lesions. They extended this work to reveal that SAA acted through the receptor FPRL1 to activate NF-κB to drive the observed CCL2 synthesis (76). We too have demonstrated that SAA's effects on biglycan synthesis in vascular smooth muscle cells was dependent upon interaction between SAA and the FPRL1 receptor as competitive inhibition of the receptor rendered the cells insensitive to exogenous SAA (38). It is well known that recruitment of inflammatory cells, particularly macrophages, into developing lesions is a key step in the progression of atherosclerosis from a disease of lipid retention to a disease of inflammation. Once in the lesion, macrophage uptake of modified lipid particles results in the formation of lipid laden foam cells (9). Lee et al continued their work on SAA to demonstrate that not only could SAA increase the recruitment of macrophages to lesions via increased CCL2 expression, it could also directly stimulate the transition from macrophage to foam cell via LOX1 receptor activation (77). Work by others has revealed that not only can SAA recruit macrophages, but other leukocytes as well (78). SAA is an apoprotein most commonly associated with HDL. Furthermore during inflammation, SAA becomes the predominant protein associated with HDL particles (79). One of the main athero-protective functions of HDL is to promote reverse cholesterol transport (RCT), which involves the trafficking of peripheral lipids to the liver for excretion either as bile or feces (80). SAA was previously thought to reduce RCT though it may simply be SAA associated inflammation driving that effect (81), as a recent study utilizing a murine model in which both

SAA1.1 and SAA2.1 were knocked out revealed that the effect of inflammation on RCT was independent of SAA (82). The previously mentioned events surrounding atherosclerosis are not regarded as initiating events, but reveal a role for SAA in progression of the disease.

Beyond its physiological effects on cytokines and inflammatory cells, SAA has also been visualized via immunohistochemistry in atherosclerotic lesions co-localized with lipoproteins such as apoA-I and apoB in two murine models of atherosclerosis (83). Interestingly SAA was also shown to interact with a class of molecules of particular interest in our lab, proteoglycans, and in particular biglycan. Studies have revealed that HDL's rate of retention within atherosclerotic lesions via interaction with vascular biglycan is directly related to the amount of SAA found associated with the HDL particle (84).

The question of SAA's direct role in increasing atherosclerosis was first addressed by the work of Dong et al when they overexpressed SAA in *apoe*$^{-/-}$ mice via injection of a lentiviral vector encoding murine SAA1.1. Their data revealed that increased SAA directly accelerated the progression of atherosclerosis independent of all other risk factors. Thus SAA for the first time was shown to be directly causal in the development of atherosclerosis. Their mechanistic assessment of SAA mediated atherosclerosis found that SAA drove the expression of vascular cell adhesion molecules thus increasing inflammatory cell recruitment to the lesion (85). This phenomenon is thought to contribute to

progression, but not initiation, of atherosclerosis as lipid deposition has been shown to precede leukocyte infiltration (15). Thus, their model did not address SAA's capacity to initiate the disease.

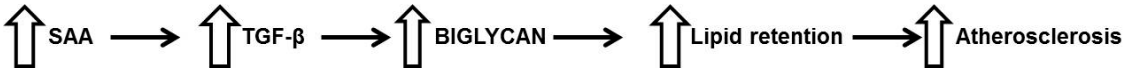
Previous work from our lab also investigated the role of SAA in the development of atherosclerosis. Vascular smooth muscle cells treated with recombinant SAA demonstrated a dose dependent increase in proteoglycan synthesis, primarily due to an increase in biglycan synthesis. The SAA stimulated biglycan molecule had longer, more negatively charged glycosaminoglycan side chains (38). This modification of biglycan is thought to increase LDL retention (17). This effect on biglycan synthesis was shown to be TGF-β dependent as TGF-β inhibition nullified the observed increase in biglycan synthesis. We repeated the experiment in a murine model of atherosclerosis by injecting *apoe*$^{-/-}$ mice with an adenoviral vector containing cDNA for human SAA1, a control adenoviral vector (ad-Null) or saline. Mice injected with ad-hSAA1 had dramatically increased plasma SAA after only three days. Interestingly, these mice also had dramatically increased TGF-β over the same timeframe. After 28 days, western blot analysis of whole aortas demonstrated increased biglycan content in ad-hSAA1 injected mice, possibly predisposing the aorta to increased lipid retention and atherosclerosis (38). This work represented the foundation of the experiments presented in this thesis.

1.5 Rationale for the studies presented

Inflammation associated diseases such as diabetes and obesity are present in a very large portion of westernized societies. Individuals suffering with such maladies have an increased risk of developing cardiovascular disease compared to age matched healthy peers though the exact etiology remains elusive. A useful diagnostic tool to evaluate the extent of inflammation in these patients are assays for the plasma proteins hs-CRP and SAA. Though much attention was given to hs-CRP and its potential causal role in atherosclerosis, no definitive causal association was determined. Conversely, early working into SAA's biology within the vasculature revealed several features that could potentiate cardiovascular disease. SAA is found in lesions associated with apolipoprotein B and apolipoprotein A1 containing lipoproteins, it is chemotactic for molecules found in atherosclerotic lesions and it's elevated in diseases known to confer greater risk of developing atherosclerosis. One molecule for which SAA exudes potent synthetic upregulation is the small leucine rich, ubiquitously expressed proteoglycan biglycan (38). Cells treated with recombinant SAA produced more biglycan, possessing long GAG chains and having a greater affinity for LDL particles. SAA's effects on biglycan synthesis were dependent on the action of TGF-β as inhibition of the cytokine with an inhibitory antibody resulted in no increase in biglycan synthesis compared with cells treated with SAA alone. The previous experiments were conducted using a recombinant SAA molecule from Peprotech Inc. (NJ, USA). This variant of SAA is a combination

of human SAA 1 and 2 with two amino acid substitutions. Many of the pro-inflammatory effects attributed to SAA have been deduced using this molecule for in vitro culture system analysis. Recently, the Peprotech recombinant human SAA was directly compared with another Peprotech SAA variant, recombinant human SAA 1. This molecule was based entirely on the human SAA 1 protein; however, it too has an amino acid substitution, thus a direct comparison to endogenous human SAA was not made. The authors claim that the commonly used recombinant human SAA molecule is distinctly more inflammatory than the SAA variant based on human SAA 1 alone. Their data suggested that recombinant human SAA 1 did not activate IL-8 or NF-κB to the same magnitude as the commonly used recombinant human SAA. They conclude by stating that experiments using the recombinant forms of SAA should also be performed using endogenous forms for verification of the results (86). We feel confident that the in vitro work using recombinant human SAA reflects SAA's true biological functions as the data was corroborated in vivo using *apoe*$^{-/-}$ male mice, injected with an adenoviral vector containing the cDNA for human SAA1. These mice exhibited a rapid induction of SAA with a concordant increase in TGF-β. Western blot analysis 28 days after treatment revealed that there was an increase in vascular biglycan content. Based on the response to retention hypothesis, an increase in modified biglycan within the subendothelial space of atherosclerosis prone arteries would increase the potential for increased lipid retention and atherosclerosis. However, definitive evidence that an SAA mediated increase in

biglycan content would result in more plaque burden remained to be determined. Thus we set out to investigate the following model by testing five hypotheses:



- SAA, expressed chronically would result in increased atherosclerosis
- A brief increase in plasma SAA would also increase the development of atherosclerosis via upregulation of vascular biglycan content
- Increased vascular biglycan is individually sufficient to increase atherosclerosis independent of inflammation
- Vascular biglycan is required for the development of atherosclerosis
- TGF-β is a necessary intermediate between SAA and biglycan in the development of SAA mediated atherosclerosis

Chapter 2: Materials and Methods

2.1 Murine Models

The work presented in this thesis utilized several murine models including the apolipoprotein E deficient (*apoe*$^{-/-}$) mouse, the recombination activating gene 1 x apolipoprotein E (*rag1*$^{-/-}$ *x apoe* $^{-/-}$*)* double knockout, the biglycan transgenic and the biglycan x apolipoprotein E (*bgn*$^{-/-}$ x *apoe* $^{-/-}$) double knockout. The *apoe* $^{-/-}$ and the *rag1* $^{-/-}$ mice were purchased from The Jackson Laboratory (stock # 002052 and stock # 003729, respectfully), and crossed in house to generate the *rag1*$^{-/-}$ *x apoe* $^{-/-}$ mouse. The *apoe* $^{-/-}$ deficient mouse was utilized for all the SAA mediated atherosclerosis studies for two reasons. First, apolipoprotein E can bridge lipoproteins to proteoglycans in a manner similar to SAA, thus interpretation of SAA's role in lipoprotein retention would be difficult to isolate in the presence of apolipoprotein E. Second, the *apoe* $^{-/-}$ mouse spontaneously develops atherosclerosis without the need for a high fat/high cholesterol diet, as these diets are capable of elevating SAA levels. The deletion of the *rag1* gene established a mouse unable to generate mature B and T lymphocytes, meaning the mouse had very little immune function present. When the *apoe* $^{-/-}$ mouse was crossed to the *rag1*$^{-/-}$ mouse, it created an *apoe* $^{-/-}$ mouse tolerant to repeated adenoviral injections.

Vascular biglycan content was elevated in mice with increased plasma levels of SAA. However, SAA has multiple mechanisms by which it is suspected

to increase atherosclerosis. Therefore, in the presence of SAA it was not possible to determine the exact individual role that elevated biglycan had on the development of atherosclerosis. Therefore, the biglycan transgenic mouse was generated. This mouse allowed elevated biglycan to be investigated independent of the other possible confounding factors associated with elevated SAA. The biglycan transgene construct was created by inserting human biglycan cDNA downstream of the smooth muscle alpha actin promoter in the pBS-HSMA construct. This construct was generously provided to us by Sasamura et al (87). It was immediately sent to Xenogen Biosciences (NJ, USA), were they performed microinjections of the construct into fertilized C57BL/6 ova. Three founders, all male were returned to the University of Kentucky for further breeding. No offspring resulted from in-house breeding with the male founders. As a result, one male was shipped to Charles River Laboratory (MI, USA) for in vitro fertilization of C57BL/6 female mice. Viable pups were produced, returned to the University of Kentucky and crossed with *ldlr*$^{-/-}$ mice. Transgenic mice were identified by PCR using the following primer sequences: forward primer, 5′-TCC TGG GAC TTC ACC CTG GA-3′ and reverse primer, 5′-TTG AAG TCA TCC TTG CGG AG-3′. Mice positive for the transgene were bred together to establish the transgenic line, mice negative for the transgene were bred to establish wildtype control mice for the studies. Interestingly, the difficulty we experienced establishing the biglycan transgenic mouse was not experienced by the original investigator. Sasamura reported no fertility issues in generating this mouse. We postulate that the increased biglycan content led to increased matrix deposition

and blockage of the vas deferens resulting in infertility of the three male founders.

Preliminary studies revealed that elevated SAA resulted in increased vascular biglycan content which was hypothesized to increase atherosclerosis (38). However, the absolute necessity of biglycan in SAA mediated atherosclerosis was not known, therefore the *bgn*$^{-/-}$ *x apoe* $^{-/-}$ double knockout was generated. The *bgn*$^{-/-}$ mouse was kindly provided by Dr. Marian Young (National Institutes of Health).). *bgn*$^{-/o}$ males carry only a single copy of the gene for biglycan, located on the X chromosome. In order to generate the *bgn*$^{-/-}$ *x apoe* $^{-/-}$ double knockout mouse, males deficient in *bgn*$^{-/-}$ were crossed to female *apoe* $^{-/-}$ deficient mice establishing biglycan wildtype males(*bgn*$^{+/o}$*)* and biglycan heterozygous *(bgn*$^{+/-}$*)* females, that were now all heterozygous for apolipoprotein E (*apoe* $^{+/-}$). Male biglycan wildtype by apolipoprotein E heterozygous (*bgn*$^{+/o}$ *x apoe* $^{+/-}$) were crossed to female biglycan x apolipoprotein E heterozygous mice (*bgn*$^{+/-}$ *x apoe* $^{+/-}$). The offspring from this cross provided the first opportunity to establish a male biglycan knockout mouse that was either deficient or heterozygous for apolipoprotein E (*bgn*$^{-/o}$ *x apoe* $^{+/-}$ or *bgn*$^{-/o}$ *x apoe* $^{-/-}$). The previous cross also established a female mouse that was heterozygous for biglycan but deficient in apolipoprotein E (*bgn*$^{+/-}$ *x apoe* $^{-/-}$). The double knockout mouse was established by crossing the male biglycan deficient x apolipoprotein deficient (*bgn*$^{-/o}$ *x apoe* $^{-/-}$) to the female biglycan heterozygous x apolipoprotein E knockout (*bgn*$^{+/-}$ *x apoe* $^{-/-}$).

All studies were initiated in eight week old mice housed in specific pathogen free caging systems with 12 hour light/dark cycles in accordance with the University of Kentucky Animal Care and Use Committee.

2.2 Human SAA1 adenoviral vector

The human SAA1 and null adenoviral vectors were generated and kindly provided by Nancy Webb, University of Kentucky. Briefly, cDNA for human SAA1 was cloned into the plasmid vector pAdCMVlink. Plasmids which contained properly inserted cDNA were co-transfected into HEK293 cells with adenoviral DNA from H5.100CMVlacZ (88). After 15 days, plaques were selected and screened by PCR. Those plaques possessing SAA DNA were then further purified and used to produce a large scale preparation of the adenoviral vector stocks (89). To commence the sustained SAA studies, eight week old male *rag1*$^{-/-}$ x *apoe*$^{-/-}$ mice were injected via the lateral tail vein every 21 days with 1.5 x 10^{11} plaque forming units of an adenoviral vector encoding human SAA1(ad-hSAA1), a control empty adenoviral vector (ad-Null) or saline as previously reported (38). The *rag1*$^{-/-}$ x *apoe*$^{-/-}$ mice were utilized as the deletion of the *rag1* gene made the mice tolerant to repeated viral injections necessary to facilitate chronic expression of SAA (*model of sustained elevation in SAA*). For the brief elevation of SAA, eight week old *apoe*$^{-/-}$ male mice, also from Jackson Laboratory, received a single lateral tail vein injection of 1.5 x 10^{11} plaque forming units of ad-hSAA1, ad-Null or saline (*model of brief elevation in SAA*). The *bgn*$^{-/-}$ *x apoe*$^{-/-}$ mice as well as the *apoe*$^{-/-}$ mice which received virus concurrently with

TGF-β inhibition also received a single injection of ad-hSAA1, ad-Null or saline as described above. All experiments involving adenoviral vectors were performed in biological safety level II (BSL2) isolation cabinets following the Division of Laboratory Animal Research's standard operating procedures. To perform the injections, adenoviral stocks, either ad-hSAA1 or ad-Null, were transported on dry ice to the appropriate procedure room within the vivarium, equipped with a BSL2 safety cabinet. The mice were assessed for overall health as part of a pre-enrollment screen prior to initiating the study. Once all mice were deemed healthy, the supplies (syringes, alcohol swabs, etc.) were arranged in the hood in such a way as to minimize the time required to perform the injections as the virus was only stable for a few minutes once resuspended in sterile PBS. For the injection, 100μl of sterile saline diluted viral stock was drawn into a 0.3cc insulin syringe with a 28 ½ gauge needle. The mouse was then restrained in a rotating tail injector (Braintree Scientific; MA, USA) and the tail was warmed with a chemical hand warming pouch for 30 seconds. The lateral tail vein was visualized and the resuspended virus was injected into the mouse. In the event that the vein became compromised do to mouse movement or needle infiltration, the contralateral vein was quickly accessed and injected. The restraint was then loosened and hemodynamic control at the injection site was gained if necessary. The mouse was then returned to its home cage and monitored for 20 minutes post injection.

2.3 TGF-β Neutralizing Antibodies

Inhibition of TGF-β was utilized to determine the necessity of TGF-β in SAA mediated atherosclerosis. We had previously shown in vitro that SAA increased biglycan in a TGF-β dependent manner (38). However, it has yet to be determined if TGF-β is required for SAA mediated upregulation of biglycan in vivo and/or atherosclerosis; thus, we designed an experiment where SAA levels would be elevated in the presence or absence of TGF-β. Inhibition of TGF-β was achieved using R and D Systems TGF-β 1, 2, 3 antibody (clone 1D11, MAB1835). This antibody was selected as it was very specific to TGF-β, and did not affect other members of the TGF-β superfamily (90). *apoe*$^{-/-}$ mice received antibody injections once concurrently with the administration of the adenoviral vectors; ad-hSAA1 or ad-Null. The procedure for administration of SAA is described in Section 2.2, Human SAA1 adenoviral vector. That procedure concluded with the mouse being removed from the tail vein injector and returned to the home cage. For this experiment, the mouse would receive the tail vein injection, then immediately be removed from the restraint and be physically restrained by scruffing the excess skin between the shoulder blades while concurrently grasping the tail. This position provided easy access to the midline of the ventral surface of the mouse. Once sufficient control of the animal was achieved it was injected intraperotineally with 1D11 antibody (2mg/kg) in sterile PBS via a 0.3cc syringe with a 28 ½ gauge needle. As a control for the TGF-β inhibition, each study included a group of mice similarly injected with the 13C4

irrelevant control antibody (2mg/kg; GenScript, Piscataway, NJ, USA) also via the intraperotineal route. The 13C4 antibody was directed against Shigella toxin and shown not to interfere with TGF-β signaling (91). Mice were then returned to their home cage and monitored for 20 minutes.

2.4 Diets

For the studies investigating the role of SAA overexpression, all mice were maintained on normal rodent chow (product #8604) from Harlan Teklad. Throughout the study, mice had ad libitum access to food and water. The Biglycan transgenic mice, crossed to the *ldlr*$^{-/-}$ mouse on a C57BL/6 background, were fed “western diet” from Harlan Teklad (TD.88137). The notable changes in this diet vs. standard chow are the high percent of calories from fat (42% vs. 4.7% in normal chow) and 0.2% cholesterol. The western diet was utilized for the transgenic studies as the biglycan transgenic x *ldlr*$^{-/-}$ mouse required dietary modification to develop atherosclerosis (92). Because of the high fat content of the “western” diet, it would spoil quite rapidly. As such, fresh food was provided every three to four days. Mice ate approximately 4 grams of food per day.

2.5 Blood collection

For all studies, blood was collected via submandibular vein route for all samples except the terminal blood collection. The submandibular approach is preferred when small volumes of blood are required with short intervals between

collections. In the SAA studies, blood was collected on days 0, 1, 3, 7, 10, 14, 21, 28 and then weeks 8, 12 and 16, depending on the duration of the study. The increased frequency of collection of blood at the beginning of the studies allowed for the accurate determination of human SAA, murine SAA and TGF-β levels, as these molecules tend to have rapid induction with an equally rapid reduction in plasma concentration. However, the increased frequency of blood collection required that we staggered the mice such that not all mice were bled at each timepoint. For example, if a study group had 9 mice; then the first 3 would be bled on day 0, the next three on day 1 and the final three on day 3. The staggered approach to blood collection prevented the use of repeated measures analysis as the data did not represent each mouse at each timepoint. For the biglycan transgenic study, blood was collected at day 0 and then at weeks 4, 8 and 12. To collect the blood, unanesthatized mice were scruffed by the excess skin between the shoulder blades to immobilize the mouse's head. The submandibular vein typically resides just caudal and slightly superior to the sebaceous gland located just caudal to the mandible. The skin was lanced superficially with a 5mm stainless steel lancet (Medipoint; NY, USA). As blood began to flow it was collected in EDTA treated tubes. Once an appropriate volume of blood had been collected, pressure was applied to the wound until hemostasis was achieved. The animal was then returned to its home cage and monitored for 20 minutes. Terminal blood was collected by cardiac puncture for all studies. At the conclusion of all studies, mice were adequately anesthetized and a thoracotomy performed to gain access to the heart. A 1.0cc syringe with a

28 gauge needle was inserted into the right ventricle and approximately 1ml of blood was collected. All blood samples were stored on ice until they could be centrifuged to separate the plasma from the red blood cells. All analyte measurements were performed on plasma.

2.6 Plasma analyte measurements

Blood samples were collected throughout all studies described in this thesis. For studies involving the sustained overexpression of SAA, the concentration of the following analytes were measured: human and mouse SAA, TGF-β, total cholesterol and triglycerides. For studies involving the brief overexpression of SAA, the concentration of the following analytes were measured: human and mouse SAA, TGF-β, total cholesterol, triglycerides and liver alanine transaminase. For the biglycan transgenic study, TGF-β, total cholesterol and triglycerides were measured. Human and mouse SAA were measured by species specific ELISA from Anogen Corp. (EL10015) and Invitrogen (KMA0021) respectfully. TGF-β was measured by the Promega TGF-β1 Emax ImmunoAssay System (G7590). Cholesterol (Cholesterol E) and triglycerides (L-Type TG M) were measured via colorimetric assay using kits from Wako Diagnostics (Richmond, VA). Alanine transaminase was measured using Therma Scientific Infinity ALT/GPT assay reagent. All assays were performed per manufacture's instruction. A BioRad X-mark spectrophotometric plate reader was utilized to determine analyte concentration.

2.7 Western Blot

Vascular tissue was homogenized in Trizol reagent (Invitrogen; NY, USA) and proteins purified per the manufacturer's protocol. Proteins were treated with Chondroitinase ABC to digest the glycosaminoglycan side chains of the proteoglycans overnight at 37°. Total protein was measured using the Lowry method and equal amounts of protein from each sample was then separated on 4-20% precast gradient gels (Bio-Rad; CA, USA) at 150 volts for 45 minutes. The samples were then transferred to PVDF membranes at 100 volts for 90 minutes. The membranes were then probed for biglycan (AF2667, R & D systems, 1:1000) or the loading control β-actin (A2066, Sigma-Aldrich; MO, USA, 1:1000). The membranes were then incubated with horseradish peroxidase conjugated secondary antibody at 1:25,000 dilution and proteins detected by film exposure using West Pico Super Signal chemiluminescent detector (34080, Pierce; IL, USA). Densitometry was performed on individual bands using imageJ (NIH, USA) software's western blot analysis plug-in.

2.8 Atherosclerosis analysis

The aortic sinus, brachiocephalic artery and whole aorta from the ascending arch to the iliac bifurcation were collected at the conclusion of all studies. Aortic roots and brachiocephalic arteries were frozen in a cryogenic cutting compound and sectioned at 10um thickness using a Leica CM1850

cryostat. Collection of aortic root sections began once all three valve leaflets of the aortic valve were in view and proceeded distally away from the valves towards the ascending aorta. Sections were collected in series such that each aortic root was collected on eight slides containing nine sections each. Each section on an individual slide was 80μm from the previous section. Given that the brachiocephalic artery is longer than the aortic root, a total of 12 sections per slide were collected across eight slides. The start point of brachiocephalic artery collection was the convergence of the right subclavian artery and the right common carotid. Once this point was reached sections were collected proximally towards the heart. Again, the sections were 80μm apart on each slide.

Prior to staining, aortic root and brachiocephalic sections were allowed to dry at room temperature and then fixed in 10% neutral buffered formalin for 5 minutes. Lipids were visualized by staining the sections with Oil Red O' (O0625, Sigma-Aldrich; MO, USA) for 20 minutes as previously described (34). Sections were then counterstained with Mayer's Hematoxyin (26043, Electron Microscopy Sciences; PA, USA) and coverslipped. Aortic root (4x magnification) and brachiocephalic artery (10x magnification) sections were visualized using a Nikon Eclipse 80i bright field microscope and Nikon Digital Sight Ri1 camera. One slide from the aortic root and brachiocephalic artery from each mouse was quantified, thus nine aortic root sections and twelve brachiocephalic sections were quantified per mouse. Using Nikon NIS elements software suite, all lesions per tissue section were traced and summed to generate the total lesion area per

section. After all nine (aortic root) or twelve (brachiocephalic) sections were measured, they were averaged to give an average lesion area per mouse. Data were then presented as the average lesion area for each mouse as well as the group average.

Whole aortas were cleaned in vivo and removed from the mice and fixed in 10% neutral buffered formalin overnight, then transferred to saline prior to en face analysis to soften the tissue. Using very fine tipped spring scissors, the vessels were first cut along the greater curvature of the aortic arch distal to just past the left subclavian bifurcation. The aorta was then cut along the lesser curvature from the ascending arch to the iliac bifurcation. The tissue was then pinned en face in premade black wax dishes and stained with 1% Sudan IV (S4261, Sigma-Aldrich; MO, USA) dissolved in 70% ethanol/acetone for 20 minutes. The aortas were washed several times with PBS, and then visualized using a Nikon SMZ800 stereo microscope with a Nikon Digital Sight DSFi1 camera.

For en face analysis, the aortic arch is analyzed. To establish the total arch area, a line extended 3mm from the left subclavian artery bifurcation distally along the aorta. A line is then created perpendicular to the aorta along the end of the 3mm line. Any tissue superior to this line was considered part of the total aortic arch area and used in quantification. The first en face measurement is the total area achieved by simply tracing the entire perimeter of the tissue previously

identified as arch. Then each lesion is traced and surface area summed to establish total lesion area. Data were presented as the percent lesion area for each mouse as well as the mean for each group. All data was analyzed by 1-way ANOVA.

2.9 Fluorescent Immunohistochemistry

Aortic root and brachiocephalic sections were immunostained with antibodies against murine biglycan (AF2667,R & D systems R & D systems, 1:50), apolipoprotein B (K23300R, Biodesign, 1:50), Perlecan (BMDV10187, Accurate, 1:100), CD68 (ab53444, Abcam, 1:100) and smooth muscle alpha actin (NB300-978, Novus, 1:50) as previously described (53). Sections were then probed with host specific secondary antibodies conjugated to either 488nm or 594nm fluorophore (Jackson ImmunoResearch) and counterstained with Vectashield mounting media containing DAPI (H-1200, Vector Labs). Sections were visualized fluorescently via a Nikon Eclipse 80i fluorescent microscope or a Leica AOBS TCS SP5 inverted laser scanning confocal microscope.

2.10 Bright field Immunohistochemistry

Aortic root sections were incubated with primary antibodies for murine biglycan (AF2667, R & D systems), then incubated with a biotinylated secondary antibody (Jackson ImmunoResearch). The presence of biglycan antigen was

visualized using an AEC chromogen kit (SK-4205, Vector Labs) and counterstained with Meyers hematoxilyn (26043, Electron Microscopy Sciences; PA, USA). Images were captured using a Nikon Eclipse 80i light microscope at 4x and 20x magnification.

2.11 LDL binding assay with *apoe*$^{-/-}$ VSMC's

Primary vascular smooth muscle cells were isolated from ~3 week old apolipoprotein E (*apoe*$^{-/-}$, stock # 002052) mice and grown to confluence without passaging. Cells were incubated with SAA (25μg/ml) isolated from acute phase murine plasma (kindly provided by Frederick and Maria de Beer, University of Kentucky) ± TGF-β neutralizing antibody 1D11 (10μg/ml, MAB1835, R & D Systems; Minneapolis, MN) or control antibody 13C4 (10μg/ml, GenScript, Piscataway, NJ) for 24 hours. The cells were washed and then labeled with Alexa-fluor 594 (A10237; Life Technology; Grand Island, NY) labeled LDL (0.5mg/ml) for 2 hours at 4°C. Cells were washed twice in cold PBS, fixed in 4% formaldehyde and then coverslipped with Vectasheild mounting media containing DAPI (H-1200, Vector Labs). The cells were then imaged using a Nikon Eclipse 80i fluorescent microscope. For each experimental condition, 5 20x fields were captured in both the 594nm and the 405nm channels and quantified using ImageJ software (93). LDL binding is expressed as Alexa-fluor 594 surface area normalized to DAPI surface area from 5-10 20x microscope fields. Negative controls included wells with no cells and/or no Alexa-fluor 594 labeled LDL.

2.12 Fast performance liquid chromatography

Terminal plasma samples (50ul) were separated for analysis of lipoprotein cholesterol content chromatographically using a BioRad Biologic Duoflow FPLC machine equipped with a Superose size exclusion column. Fractions, each containing about 200μl of eluate were collected on a BioRad Biofrac fraction collector and assayed for total cholesterol as previously described (93) using a Wako Diagnostic Cholesterol E colorimetric assay kit.

2.13 Statistical analysis

Statistical differences were assessed by one-way ANOVA to assess effects of treatment with ad-hSAA1, ad-Null or saline; or two-way ANOVA to assess effects of ad-hSAA1 or ad-Null ± 1D11 or 13C4. LDL binding was assessed using a student's t-test. Linear regression was used to assess the relationship between vascular biglycan and aortic sinus atherosclerosis. $P<0.05$ was considered statistically significant.

Chapter 3: Sustained elevation of SAA results in increased atherosclerosis

3.1 Results

We previously investigated the effect of SAA on vascular smooth muscle cells. Cells treated with increasing doses of SAA had a dose-dependent increase in biglycan synthesis as well as increased binding affinity of biglycan for LDL. SAA's effect on biglycan synthesis was recapitulated in vivo in *apoe*$^{-/-}$ mice injected with an adenoviral vector containing the gene for human SAA (38). Interestingly, the effect of SAA was inhibited by concurrently treating the cells (38) or mice (Thompson et al, in submission) with a TGF-β inhibitory antibody. We hypothesized, based on the response to retention hypothesis, that the increased vascular biglycan would lead to increased lipoprotein retention in the vessel wall stimulating the development of atherosclerosis. To test this hypothesis, *rag1*$^{-/-}$ *x apoe* $^{-/-}$mice were injected with ad-hSAA1, ad-Null or saline once every 21 days for 12 weeks. *rag1* deficiency was necessary for the animals to tolerate repeated viral injections. These mice had persistently elevated human SAA throughout the study (Figure 3.1A) with no difference in murine SAA between groups at any timepoint (Figure 3.1B), indicating that our experimental manipulations did not induce an endogenous inflammatory response. We previously determined in vitro that SAA's increase in vascular biglycan content was TGF-β dependent (38). Thus we measured TGF-β in all groups to determine

in vivo whether elevated SAA had any effect on TGF-β levels. Mice that received ad-SAA also had increased TGF-β compared to ad-Null and saline injected mice throughout the study (Figure 3.2).Consistent with Dong et al, ad-hSAA1 treated mice had increased atherosclerosis on the aortic intimal surface (Figure 3.3A, $p<0.001$) and within the brachiocephalic artery (Figure 3.3B, $p<0.05$) compared with ad-Null or saline groups. There was a strong trend towards increased atherosclerosis in the aortic root in ad-hSAA1 treated mice compared to ad-Null or saline groups; however it did not reach statistical significance (Figure 3.3C, $p=0.09$). Biglycan and apoB co-localized in aortic sinus lesions, further supporting the response to retention hypothesis (Figure 3.4). The induction of human SAA had no effect on plasma cholesterol or triglycerides (Figure 3.5AB). Lipoprotein distribution was measured using fast performance liquid chromatography and did not differ between any of the groups (Figure 3.5C). Body weight did not differ at any point throughout the study (data not shown).

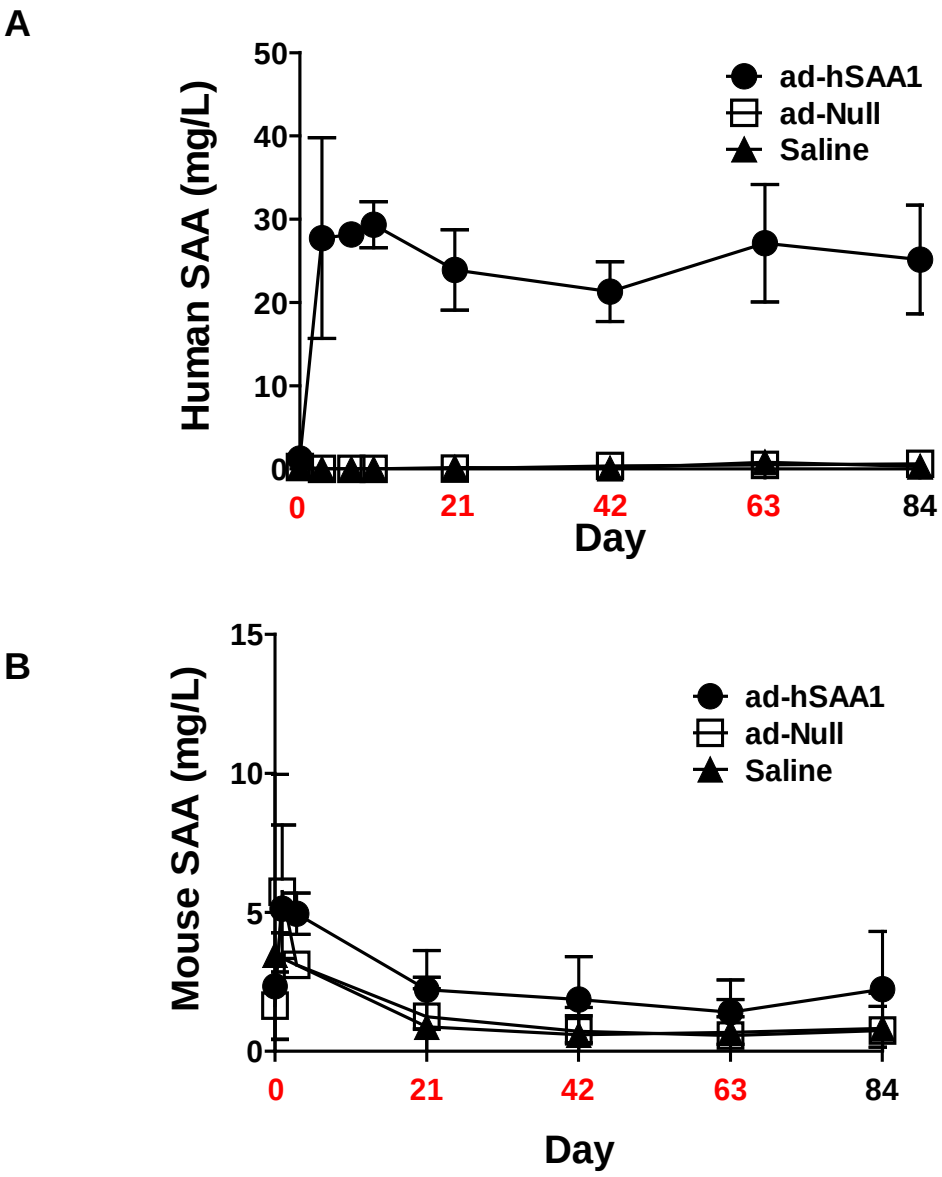


Figure 3-1: **Human, but not murine SAA increased when mice received multiple injections of ad-hSAA1 compared to control mice**

rag1$^{-/-}$ *x apoe*$^{-/-}$ male mice were injected with ad-hSAA1 (black circles), ad-Null (open squares) or saline (black triangles) every 21 days (as indicated by the red numerals on the x axis) and fed normal rodent chow for 12 weeks. A. Mice receiving ad-hSAA1 had a dramatic, persistent elevation of human SAA1. Shown are mean ± SEM for n=2-5 per group. B. Murine SAA did not increase nor did it differ between groups throughout the study. Shown are mean ± SEM for n=2-7 per group.

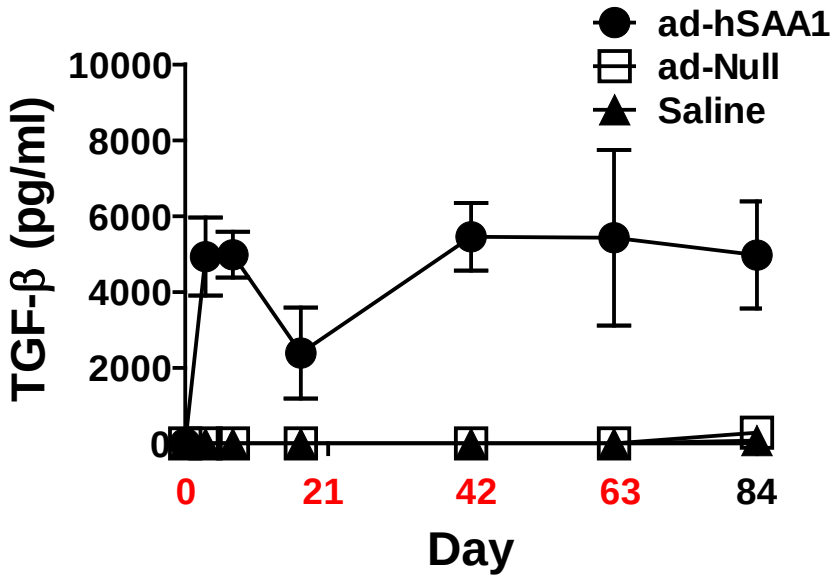


Figure 3-2: Murine TGF-β increased in mice injected with ad-hSAA1
rag1$^{-/-}$ *x apoe*$^{-/-}$ male mice were injected with ad-hSAA1 (black circles), ad-Null (open squares) or saline (black triangles) every 21 days (indicated by red numerals on the x axis) and fed normal rodent chow for 12 weeks. Ad-hSAA1 injected mice had persistently increased TGF-β throughout the study compared to mice injected with ad-Null or saline. Shown are means ± SEM from n= 2-6 mice/group per timepoint.

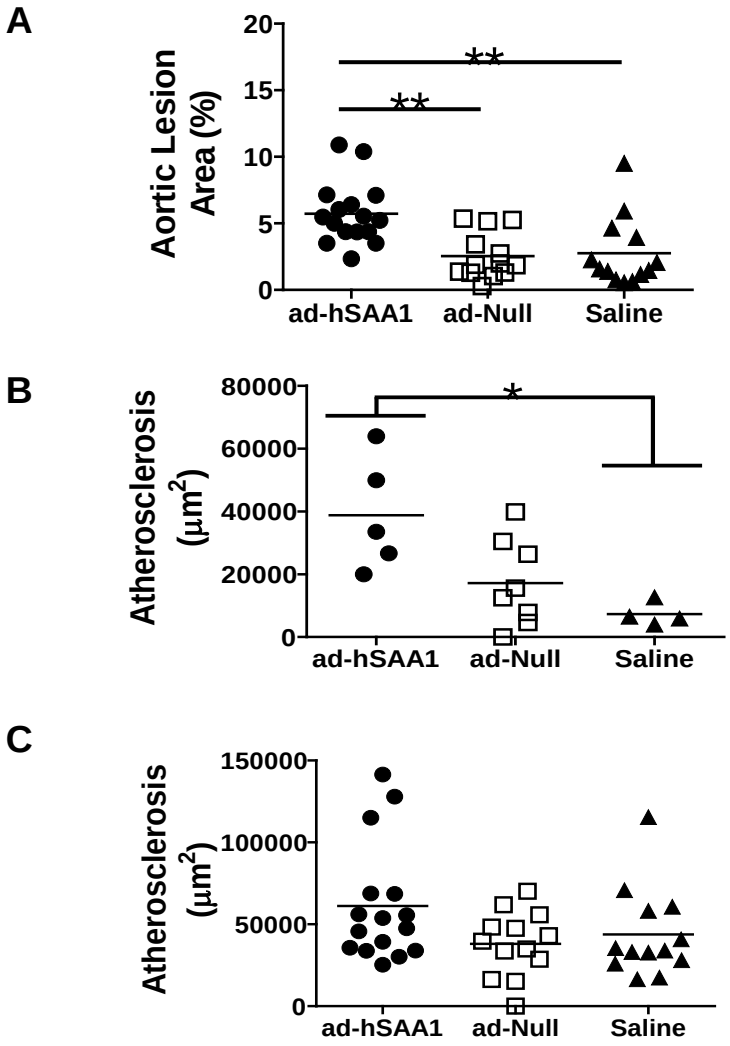


Figure 3-3: Atherosclerosis increased in mice chronically overexpressing human SAA

rag1$^{-/-}$ *x apoe*$^{-/-}$ male mice were injected with ad-hSAA1 (black circles), ad-Null (open squares) or saline (black triangles) every 21 days and fed normal rodent chow for 12 weeks. Ad-hSAA1 injected mice had increased atherosclerosis on the aortic intimal surface (A) and in the brachiocephalic artery (B). There was a trend towards increased atherosclerosis in the aortic root that did not reach significance (C). Data points represent individual mice with the horizontal bar representing means of n =4-16 mice per group analyzed by 1-way ANOVA and Tukey's multiple comparisons test. *p<0.05, **p<0.01.

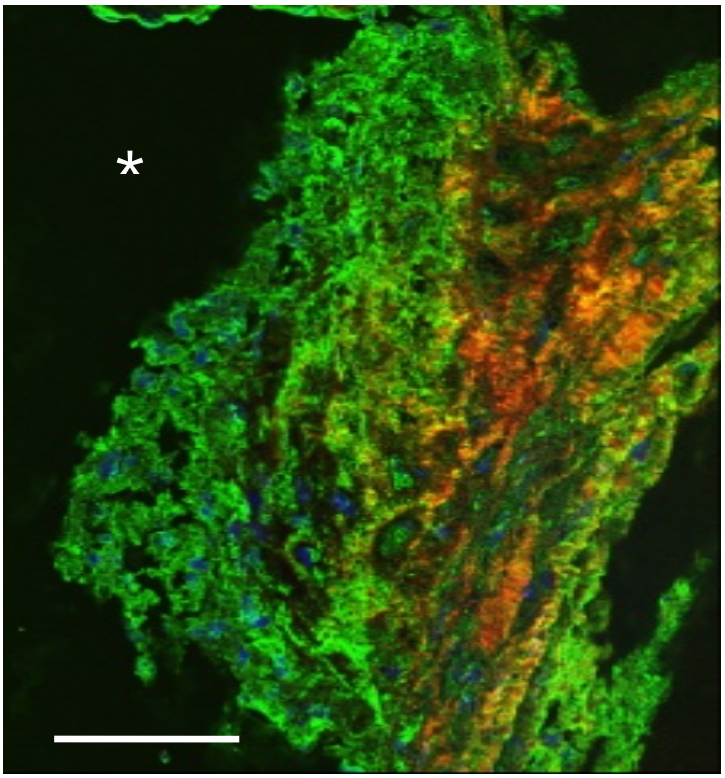


Figure 3-4: Biglycan and apolipoprotein B colocalized in *rag1-/- x apoe -/-* murine aortic sinus lesions

rag1$^{-/-}$ *x apoe*$^{-/-}$ male mice were injected with ad-hSAA1, ad-Null or saline every 21 days and fed normal rodent chow for 12 weeks. Representative confocal image of a shoulder lesion in an aortic root from an ad-hSAA1 injected *rag1*$^{-/-}$ *x apoe*$^{-/-}$ mouse (magnified 40x). ApoB and biglycan were visualized with green and red fluorescent antibodies respectfully. Co-localization is indicated by yellow. Image is representative of 4-5 mice per group. Asterisk is within the lumen of the vessel, scale bar 50μm.

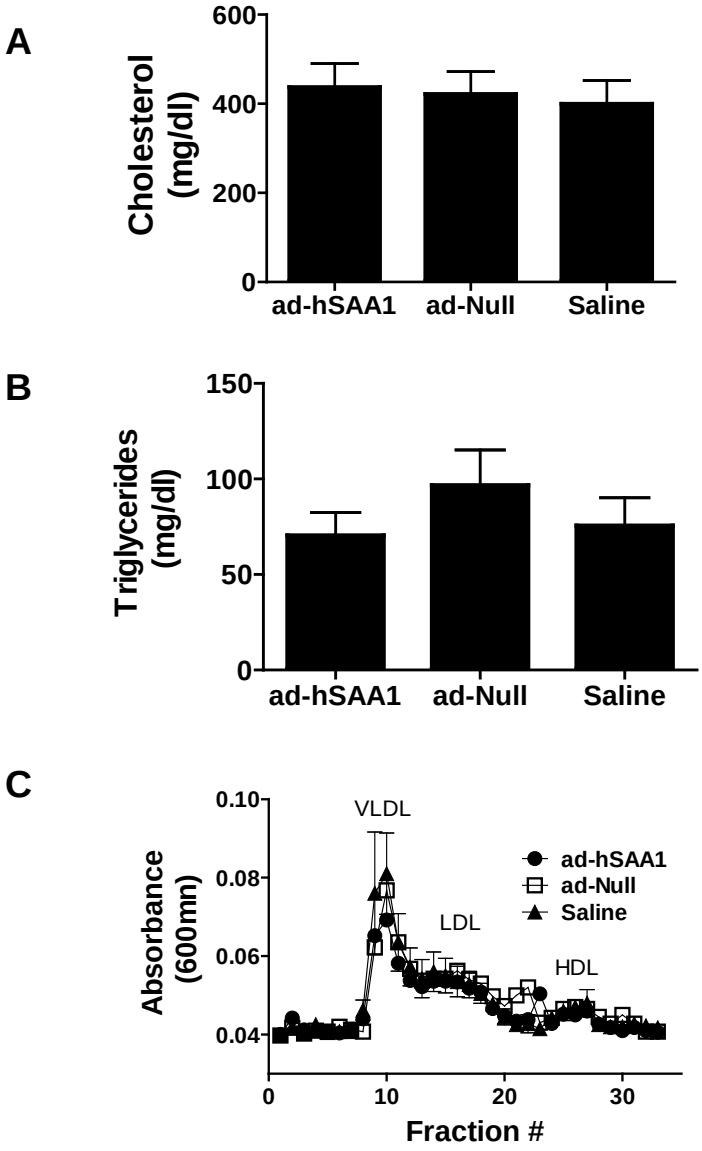


Figure 3-5: Chronically elevated hSAA1 did not affect plasma lipids
rag1$^{-/-}$ *x apoe*$^{-/-}$ male mice were injected with ad-hSAA1, ad-Null or saline every 21 days and fed normal rodent chow for 12 weeks. After 12 weeks, there was no difference in plasma cholesterol (A, n=9-11 mice per group) or triglycerides (B, n=8-10 mice per group). A & B data presented as mean ± SEM analyzed by 1-way ANOVA and Tukey's multiple comparisons test. Lipoprotein distribution was measured in plasma from n=4-5 mice per group collected after 12 weeks of study and separated by FPLC. No differences were observed between groups [C, ad-hSAA1 (black circles), ad-Null (open squares) or saline (black triangles), data presented as mean ± SEM].

3.2 Discussion:

Persistently elevated human SAA in *rag1*$^{-/-}$ *x apoe*$^{-/-}$ mice resulted in increased atherosclerotic lesion area. There were no differences between groups in cholesterol or triglyceride levels, and the adenovirus did not induce endogenous inflammation demonstrated by the lack of elevation of murine SAA. Mice that received ad-hSAA1 had dramatically elevated TGF-β compared to mice receiving ad-Null or saline.

Biglycan is thought to be the proteoglycan most responsible for the retention of pro-atherogenic lipoproteins in the vessel wall leading to the development of atherosclerosis (9). Thus we propose that at least one mechanism by which SAA is pro-atherogenic is by induction of vascular biglycan in a TGF-β dependent manner leading to increased retention of atherogenic lipoproteins. The co-localization of apolipoprotein B with biglycan in a shoulder lesion of the aortic sinus supports this concept.

However, the exact role of TGF-β in atherosclerosis remains quite controversial. Mallat et al used an inhibitory antibody to decrease TGF-β content and observed increased plaque size in *apoe*$^{-/-}$ male mice. They also reported a much more inflamed plaque with increased macrophage content and a decrease in collagen fibers (54). Similarly, Lutgens et al reported that TGF-β inhibition in *apoe*$^{-/-}$ male mice via injection with a soluble TGF-β receptor resulted in a dramatic change in lesion cellularity with an influx of inflammatory cells.

However, when lesions were analyzed for atherosclerosis, the group receiving TGF-β inhibition had a striking decrease in lesion area (56). To add to the confusion surrounding TGF-β in atherosclerosis, we have previously shown that *ldlr*$^{-/-}$ mice infused with angiotensin II (angII) for 28 days had increased vascular biglycan content. We also demonstrated that the increased biglycan content was TGF-β dependent, (similar to SAA induction of biglycan) as the inhibition of TGF-β in the setting of angII infusion resulted in less vascular biglycan and less atherosclerotic lesion area. However, there was no change in inflammatory cell content within lesions (53). Thus further study into the role of elevated TGF-β in our SAA overexpression model is necessary to elicit its exact role in SAA mediated atherosclerosis.

Our results support the previous findings from Dong et al (85) that SAA is proatherogenic. In their study, murine SAA was overexpressed by lentiviral vector leading to significantly increased atherosclerosis on the aortic intimal surface and the aortic sinus in chow fed *apoe-/-* mice. The increased atherosclerosis coincided with upregulation of vascular adhesion molecules and chemotactic factors capable of increasing leukocyte migration into atherosclerosis susceptible regions of arteries. The study presented here addresses events prior to vascular wall leukocyte infiltration, including vascular wall remodeling and lipoprotein retention (9). Our model indicated that SAA is acting on the vasculature prior to the cascade of inflammatory events observed in

Dong's work. We have shown that treatment with SAA, both in vitro and in vivo, increased vascular biglycan content leading to increased lipid retention.

In further support of the hypothesis that lipid retention precedes inflammatory infiltration of atherosclerosis prone regions of arteries, Nakashima et al investigated human coronary vessels to establish a chronology of events thought to provoke atherosclerosis development. In a very effective use of histology and immunohistochemistry, their study demonstrated very convincingly that lipid retention preceded leukocyte infiltration in the development of lesions in human coronary arteries. They investigated vessels from individuals whose average age of death was 36 years. These individuals died from trauma or other non-cardiovascular causes. In all the lesions investigated, diffuse intimal thickening (DIT), the non-pathologic remodeling of the vessel wall preceded both lipid and macrophage infiltration. They identified several locations in the coronary artery were there was DIT but no lesion development; however, they did not find regions with lesions and no DIT. The deposition of matrix was clearly an initial step in the development of atherosclerosis. They also demonstrated that lipid accumulation became quite substantial in regions with DIT prior to macrophage infiltration suggesting that lipid retention preceded the inflammatory response in the development of atherosclerosis, strongly supporting our model of atherosclerosis development (15). Thus, our work taken together with Dong's data suggest that SAA's actions leading to the development of atherosclerosis

may be multifactoral in nature, playing a role in both early (lipid retention) and more advanced lesion development (inflammatory infiltration).

Changes in the vessel wall, such as diffuse intimal thickening prior to fatty streak formation are thought to be paramount in the development of atherosclerosis in regions of the vessel prone to lipid deposition (94). In diffuse intimal thickening there is a dramatic increase in subendothelial matrix deposition including proteoglycans such as biglycan which we observed in our model and is thought to play a major role in lipid retention in atherosclerotic lesions. The vasculature is divided into two groups based on the presence or absence of diffuse intimal thickening. First are the atherosclerosis prone vessels such as the aorta, carotids and coronaries. In humans, these vessels all exhibit diffuse intimal thickening as early as late fetal development (16). Conversely atherosclerosis resistant arteries are located much farther down the vascular tree and tend to deliver blood to specific organs such as the splenic and hepatic arteries. Observations of these vessels reveal no diffuse intimal thickening at any timepoint observed (16). Though there is not always atherosclerosis at sites of diffuse intimal thickening, in the human vasculature there are is always subendothelial matrix expansion including increased biglycan at sites of atherosclerosis (15).

Beyond the observation that SAA increases biglycan deposition in a proatherogenic manner, it has been reported that SAA co-localized with both biglycan and apolipoprotein B containing lipoproteins (83). SAA has amino acid

clusters that allow it to simultaneously bind proteoglycans and lipoproteins which could make SAA directly atherogenic via directing the binding of lipoproteins to biglycan in the subendothelial space of atherosclerosis prone arteries(95) . Certainly more research is required to demonstrate that SAA is directly mediating such an interaction. Thus, SAA could contribute to atherosclerosis through multiple mechanisms, including vascular wall remodeling, by directing chemotaxis of circulating leukocytes to the region of lipoprotein retention (85), and by potentially bridging lipoproteins to vascular wall proteoglycans (83) (95).

Chapter 4: Briefly elevated SAA resulted in increased atherosclerosis

4.1 Results

We have clearly shown that sustained elevation of SAA is proatherogenic (Chapter 3), providing a possible link between chronic inflammatory diseases and the increased risk of cardiovascular disease. However, many people have dramatic, albeit brief increases in SAA via an acute phase response to infection or injury (96-98). Survivors of such an event have increased long term all cause mortality, including cardiovascular disease. Thus we sought to determine if a brief increase in SAA also increased atherosclerosis, as we have previously demonstrated that even a brief increase in SAA resulted in increased vascular biglycan content (38). To test the hypothesis that even brief exposure to increased SAA was proatherogenic, *apoe*$^{-/-}$ mice received a single injection of Ad-hSAA1, Ad-Null or saline at the initiation of the study. The mice that received ad-hSAA1 had a dramatic increase in human SAA, albeit not to the level seen in an acute phase response. The increased human SAA returned to baseline levels in less than two weeks (Figure 4.1A). Murine SAA did not differ between groups at any timepoint (Figure 4.1B). In mice that received ad-hSAA1 there was a transient increase in TGF-β that resolved in a similar timeframe to human SAA (Figure 4.2). Interestingly, despite only observing a transient increase in human SAA levels, these mice developed significantly more atherosclerosis on the aortic

intimal surface (Figure 4.3A, $p<0.001$), the aortic root (Figure 4.3B, $p<0.05$) and the brachiocephalic artery (Figure 4.3C, $p<0.05$) compared with ad-Null or saline treated mice. This result could be explained by the fact that mice receiving ad-hSAA1 had increased vascular biglycan content at the conclusion of the study even though SAA was only increased for a brief period of time (Figure 4.4). The induction of human SAA had no effect on terminal plasma lipids (Figure 4.5AB). Lipoprotein distribution was measured using fast performance liquid chromatography, and no differences were observed between groups (Figure 4.5C). Body weight measured at the completion of the study did not differ between groups (Figure 4.6A). Alanine transaminase (ALT) was measured for the first two weeks of the study to determine if injecting the mice with an adenoviral vector that traffics to the liver led to hepatitis. There was a brief increase in ALT in the mice receiving ad-hSAA1 that did not exceed the normal range of 5-60 U/L typically observed in mice (Figure 4.6B). The ad-Null and saline groups did not deviate from baseline ALT values. Aortic root sections were double stained for BGN and apoB and immunofluorescent analysis demonstrated colocalization of apolipoprotein B with biglycan within the vascular wall (Figure 4.7). To determine if the retention of ApoB containing lipoproteins by vascular matrix is a direct result of increased plasma SAA, primary *apoe*$^{-/-}$ aortic vascular smooth muscle cells were incubated with mouse SAA for 24 hours, followed by incubation with Alexa-594 labeled LDL for two hours. Cells treated with SAA had a 4 fold increase in LDL binding compared to cells not treated with SAA (Figure 4.8A). To determine if the role of TGF-β in SAA mediated

lipoprotein retention, the experiment was repeated with the additional conditions; SAA + TGF-β inhibitory antibody 1D11 or SAA + 13C4 irrelevant control antibody. In the absence of TGF-β signaling, the amount of bound LDL was significantly reduced compared to SAA treated cells (Figure 4.8B).

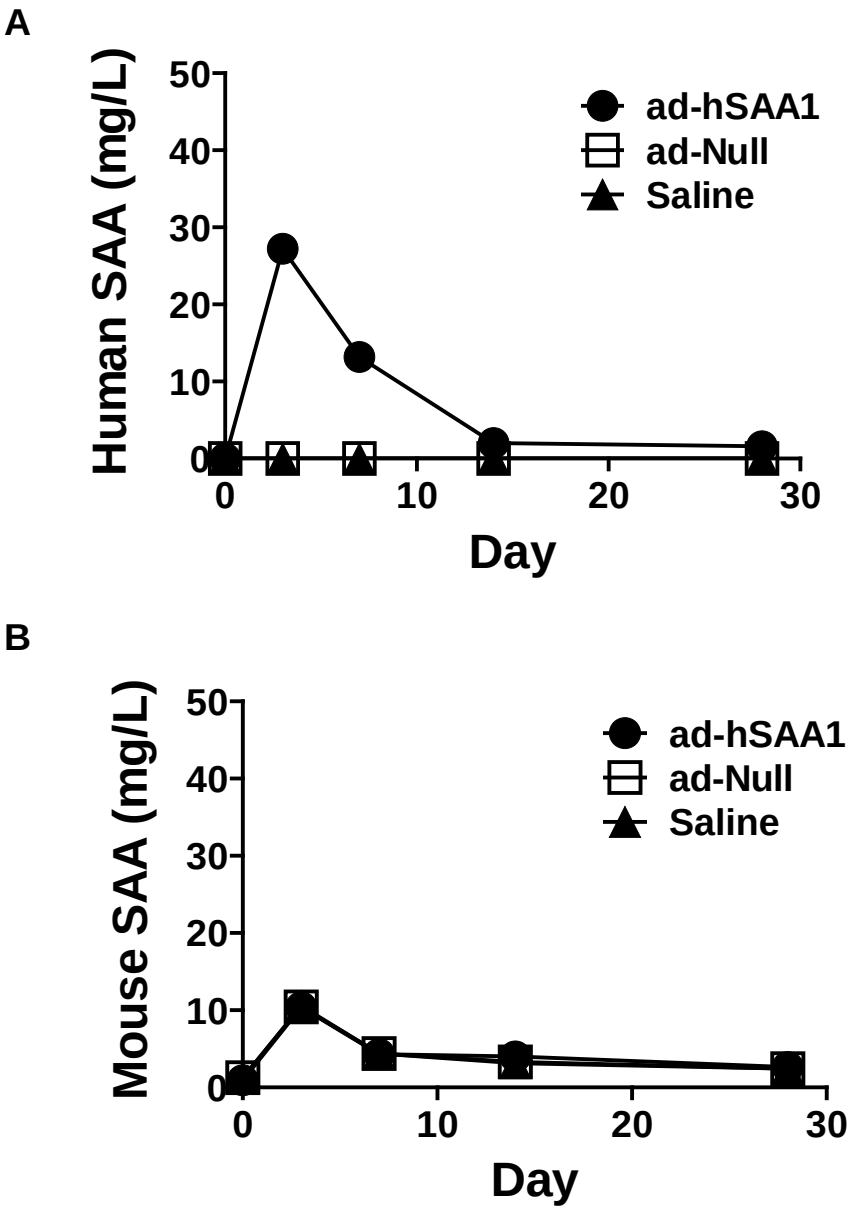


Figure 4-1: Human, but not murine SAA increased in mice that received a single injection of ad-hSAA1 compared to control mice

apoe$^{-/-}$ male mice were injected at day 0 with ad-hSAA1 (black circles), ad-Null (open squares) or saline (black triangles) and fed normal rodent chow for 16 weeks. Mice receiving a single injection of ad-hSAA1 had a dramatic albeit transient increase in human SAA1. (A, data presented as mean ± SEM for n=2-3 mice per group). All groups had a small, transient increase in murine SAA. (B, data presented as mean ± SEM for n=2-6 mice per group).

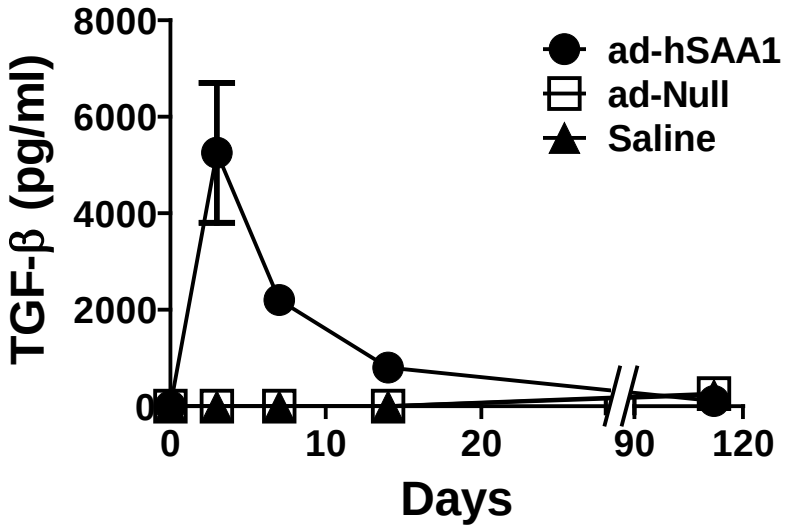


Figure 4-2: **TGF-β increased dramatically in mice overexpressing SAA**

apoe$^{-/-}$ male mice were injected with ad-hSAA1 (black circles), ad-Null (open squares) or saline (black triangles) and fed normal rodent chow for 16 weeks. TGF-β was transiently but dramatically increased in ad-hSAA1 mice compared to ad-Null or saline mice. Data presented as means ± SEM from n= 4-6 mice per group.

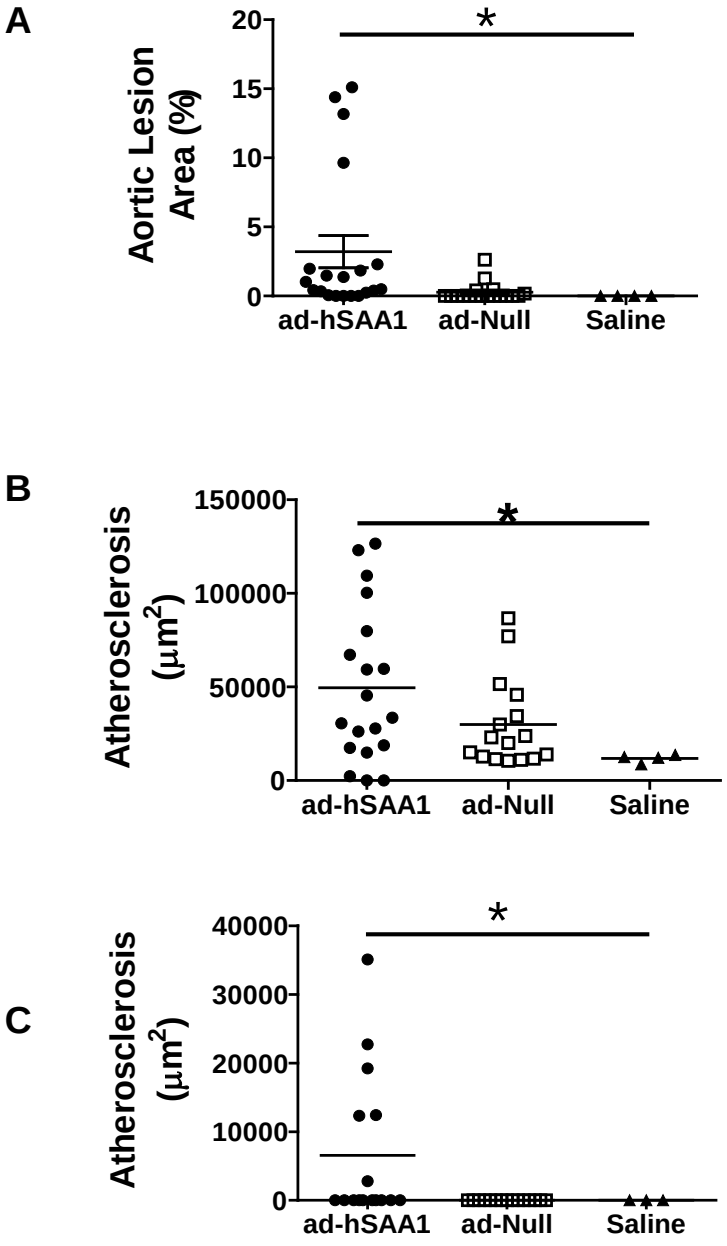


Figure 4-3: A brief elevation of human SAA1 was associated with increased atherosclerosis after 16 weeks

apoe$^{-/-}$ male mice were injected with ad-hSAA1, ad-Null or saline and fed normal rodent chow for 16 weeks. Atherosclerosis was measured at three sites: the aortic intimal surface (A), the brachiocephalic artery (B) and the aortic sinus (C). Data points represent individual mice with the horizontal bar representing means of n =4-16 mice per group analyzed by 1-way ANOVA with Tukey's multiple comparisons. *p<0.05.

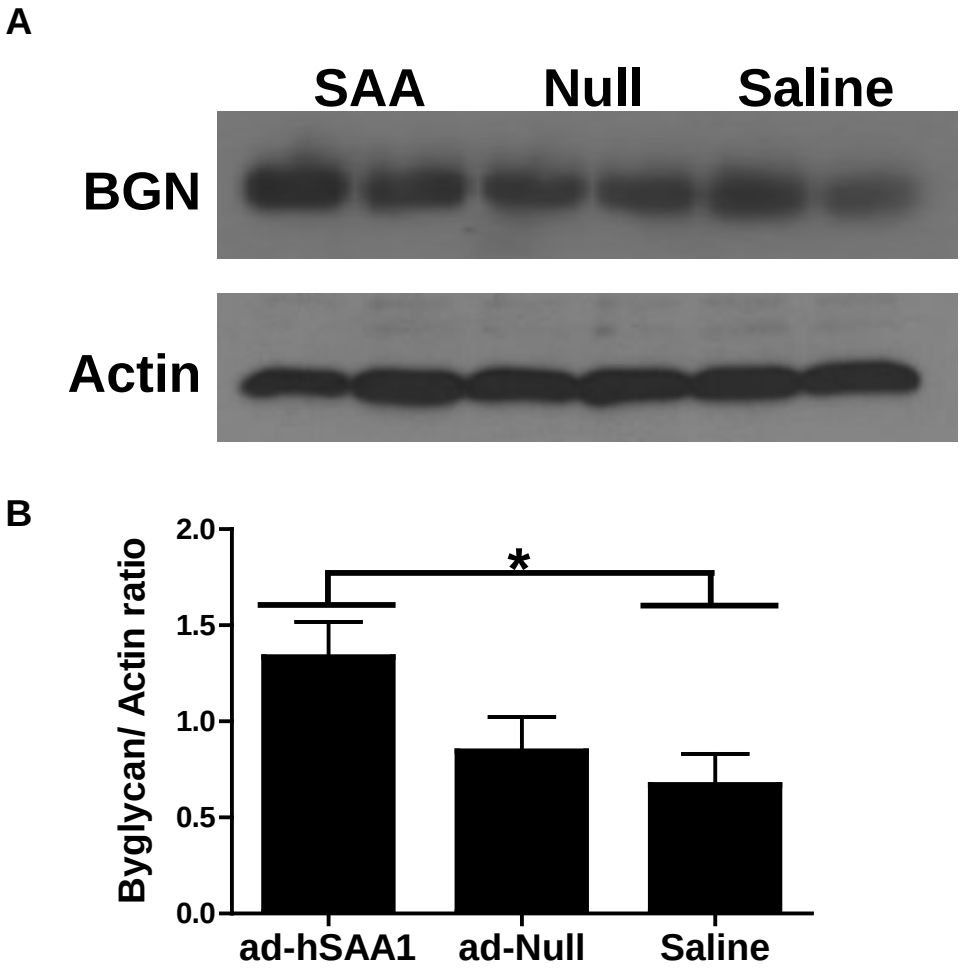


Figure 4-4: A brief increase in human SAA1 resulted in increased vascular biglycan content after 16 weeks.

apoe$^{-/-}$ male mice were injected with ad-hSAA1, ad-Null or saline and fed normal rodent chow for 16 weeks. Carotid arteries were collected and immunoblotted for biglycan (BGN) or actin (A) then analyzed by densitometry using ImageJ software (B). Each lane shows protein from an individual mouse; densitometry shows means ± SEM for 5-7 mice per group analyzed by one-way ANOVA and Tukey's multiple comparisons. *$p<0.05$.

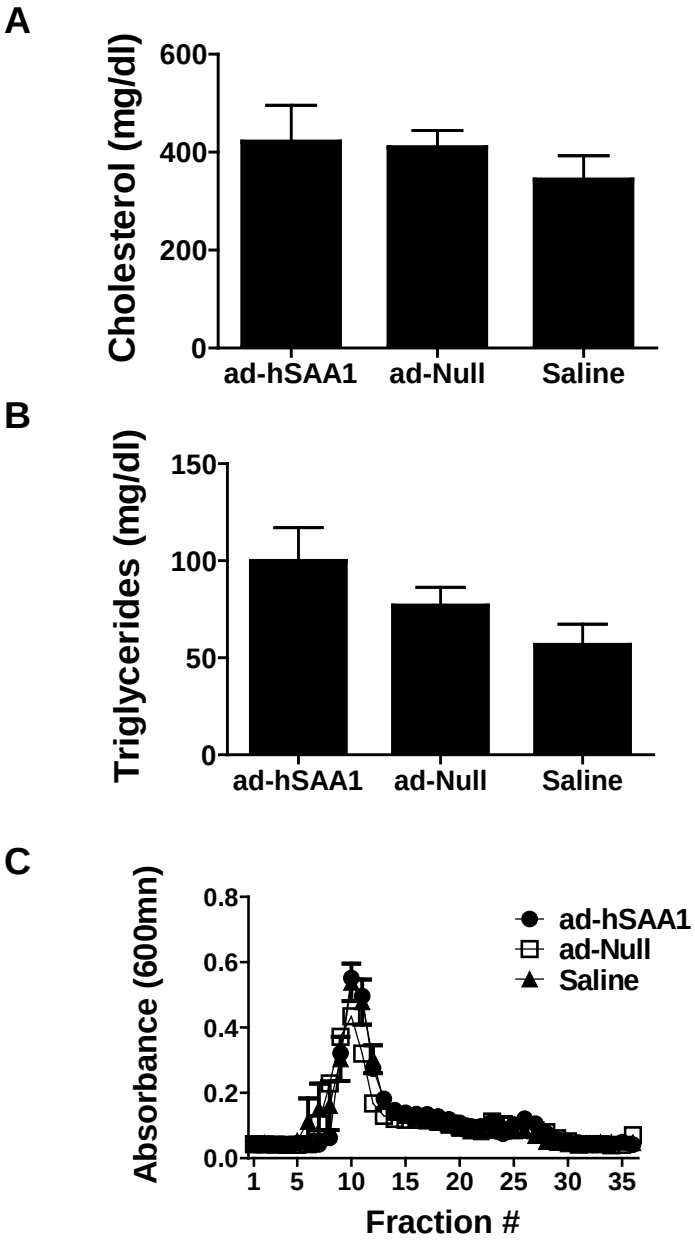


Figure 4-5: **Briefly elevated human SAA1 did not alter plasma lipids or lipoprotein distribution**

apoe$^{-/-}$ male mice were injected once with ad-hSAA1 , ad-Null or saline. After 16 weeks, total cholesterol (A) and triglycerides (B) did not differ between groups. Data presented as mean ± SEM for 4-18 mice per group analyzed by one-way ANOVA and Tukey's multiple comparisons. The distribution of lipoproteins did not differ between groups (C), data represent mean ± SEM for n=2-9 mice per group, ad-hSAA1 (black circles), ad-Null (open squares), saline (black triangles).

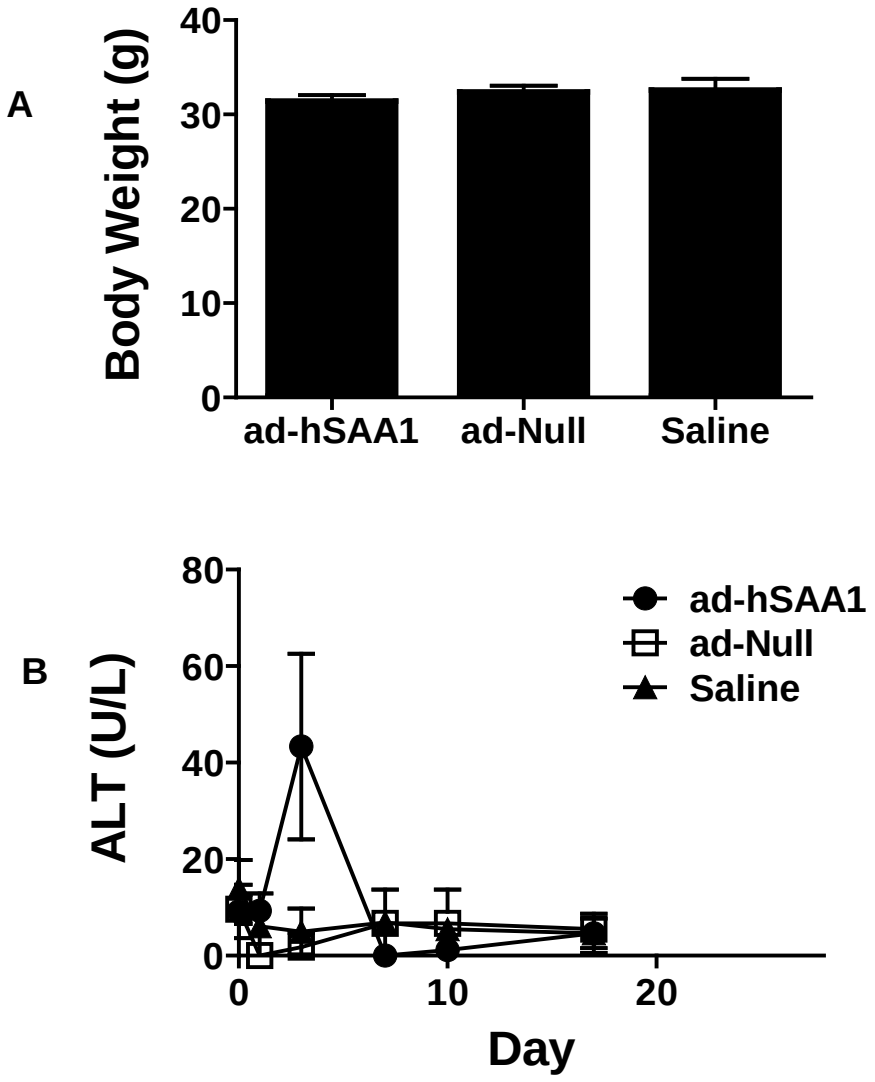


Figure 4-6: Body weights did not differ; however, ALT increased slightly as a result of increased human SAA1 compared to control mice.

apoe$^{-/-}$ male mice were injected once with ad-hSAA1, ad-Null or saline. Body weight did not differ between groups at the conclusion of the study (A). Data represent mean ± SEM for n=4-11 mice analyzed by one-way ANOVA and Tukey's multiple comparisons. Mice injected with ad-SAA had a slight increase in ALT liver enzymes compared to mice injected with either ad-Null or saline (B). Data represent mean ± SEM for n=2-3 mice per group.

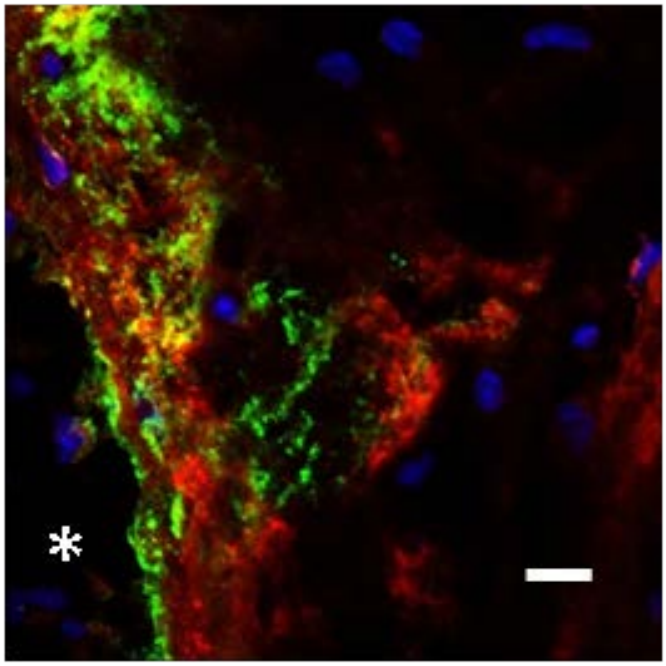


Figure 4-7: **Biglycan and apolipoprotein B co-localized in aortic sinus lesions of mice overexpressing human SAA1.**
An atherosclerotic lesion in an aortic root from an ad-hSAA1 injected *apoe*$^{-/-}$ mouse was double stained for apoB (green) and biglycan (red). Co-localization is indicated by yellow. Shown is a confocal image magnified 63X, representative of 4 mice. The asterisk indicates the lumen of the aortic sinus. Scale bar 10μm.

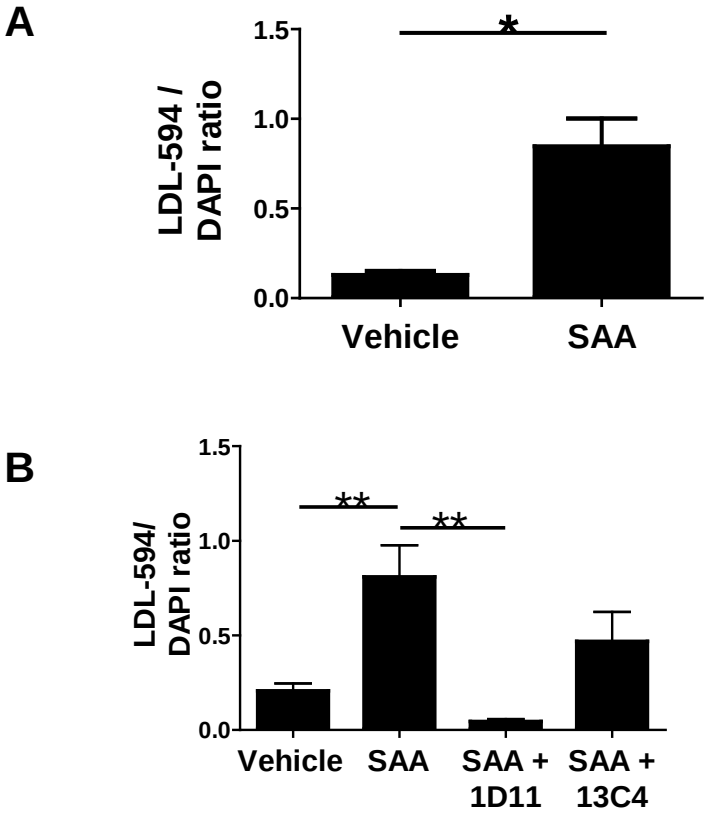


Figure 4-8 VSMC cultures treated with murine SAA had increased TGF-β dependent LDL binding

apoe$^{-/-}$ aortic VSMC cultures were treated with vehicle or SAA for 24 hours, then washed and incubated with Alexa-594 labeled LDL (0.5mg/ml) for 2 hours. LDL binding is expressed as Alexa-fluor 594 surface area normalized to DAPI surface area quantified by fluorescent microscopy using ImageJ software (NIH, USA). A. SAA treated cells had significantly increased LDL binding compared to controls. Data analyzed by student's t-test. B. To determine the role of TGF-β in LDL retention, *apoe*$^{-/-}$ aortic VSMCs were treated with vehicle, SAA or SAA with the TGF-β neutralizing antibody 1D11 or control antibody 13C4. 1D11 prevented the SAA increase in LDL binding; there was no effect of 13C4. Data are presented as mean±SEM from 5-10 20x regions/condition analyzed by one-way ANOVA and Tukey's multiple comparisons. *$p<0.05$ **$p<0.01$. Controls included cells treated with 1D11 inhibitory antibody or 13C4 antibody without concurrent SAA treatment.

4.2 Discussion

In this model, we investigated the potential atherogenic effect of a single transient increase (<14 days) of ad-hSAA1, such as seen in an intensive care hospitalization. Similar to mice with sustained overexpression of SAA, mice in this study had increased atherosclerosis at all three vascular sites investigated. Furthermore biglycan and apolipoprotein B co-localized in aortic sinus lesions of mice exposed to a brief increase in SAA. Also similar to the sustained elevation group, these mice had no metabolic variability in such lipid parameters as cholesterol, triglycerides or lipoprotein distribution to explain the difference in atherosclerosis. Thus, this increase in atherosclerosis is presumably driven directly by increased SAA. It is noteworthy to point out that the atherosclerosis data for the aortic intimal surface appears to be biphasic. The data for the mice receiving ad-hSAA1 appears to form two unique groups, one with a much higher average lesion area than the other, suggesting that the mice in the lower group did not respond to the administration of ad-hSAA1 to the same degree as those in the high atherosclerosis group. This could be considered a confounding factor in concluding that SAA is universally causing the development of atherosclerosis. However, the aortic sinus and brachiocephalic atherosclerosis data looks normally distributed such that the aortic intimal surface is likely an anomaly.

This study investigated the role of acutely increased SAA and its effect on atherosclerosis. This induction of SAA, though lower in total plasma SAA concentration, is similar in duration to what a human could experience during an acute phase response to infection or injury. However, in our study, we utilized an

adenoviral vector to mediate increased SAA gene expression rather than LPS or sodium nitrate, which would increase plasma SAA, but also broadly stimulate the innate immune system. The adenoviral method avoided introducing the milieu of inflammatory cytokines seen in LPS or sodium nitrate stimulated immune response that would make it nearly impossible to elicit SAA's individual effect on atherosclerosis. A better understanding of the impact of brief but significant inflammation is necessary as clinical data suggests that individuals suffering acute severe illness resulting in intensive hospitalization have increased mortality post discharge (96-98) . These patients have increased SAA as part of the APR experienced in the hospital, thus it is plausible that the increased mortality, particularly related to cardiovascular disease could be driven by the downstream effects of elevated SAA. This idea is supported by a study investigating myocardial infarct (MI) and progression of atherosclerosis. Dutta et al investigated the potential for MI, with the associated APR, to accelerate the development of atherosclerosis. The common dogma of atherosclerosis following MI is that the disease progression continued at the pre- MI rate. This assumption failed to consider the risk of acute inflammation, thus they investigated the possibility that MI related inflammation could alter the progression of atherosclerosis. Using an *apoe*$^{-/-}$ mouse model of left coronary artery ligation, they compared atherosclerosis development between infarcted and sham operated animals. The data demonstrate that mice in the MI group had a subsequent increase in atherosclerosis, likely the result of the acute phase response to the infarct leading to increased monocyte recruitment to the

developing lesions (99). Our work here investigated SAA, a single component of the acute phase response and its role in the development of atherosclerosis. It is certainly possible that the increased atherosclerosis observed in Dutta's study was in part the result of increased SAA during the post MI acute phase response. SAA is known to be chemotactic for macrophages, thus SAA in the lesion could have explained the observed increase in monocyte recruitment. Thus SAA's elevation in the acute inflammatory response following MI could certainly contribute to accelerated atherosclerosis (78). It is also well established beyond just myocardial infarction that brief but significant episodes of in-hospital inflammation increases mortality after discharge. Brinkman et al analyzed data from intensive care units (ICU) which treated patients admitted for many different illnesses. They determined that post-discharge mortality was much higher for ICU discharged patients than age and gender matched non hospitalized individuals (100). Thus, we propose that beyond indicating risk, elevated plasma SAA plays a causal role in atherosclerosis development and prevention of increased SAA may be a therapeutic target. A number of currently available agents can lower SAA including statins. Individuals with a history of myocardial infarction and elevated plasma SAA are at greater risk for a second cardiovascular event than similar patients with lower SAA. However, treatment with pravastatin reduced the increased risk, perhaps in part by lowering SAA, (though SAA levels were not a specific endpoint of the study and thus were not individually analyzed) (101). Given the pleiotropic effects of statins it is difficult to dissect the mechanisms by which they lower CVD risk. However, it is possible

that beyond lowering LDL cholesterol, statins may have beneficial effects by lowering SAA. Beyond statins, other approaches have been found to lower SAA, such as weight loss (72) and aspirin based therapies (102). However, further clinical research is necessary to validate SAA as a therapeutic target in humans, and to determine if therapies targeted specifically to elevated SAA in humans can be protective.

To summarize, we have shown that both sustained and brief elevations of SAA result in increased atherosclerosis. We propose that SAA is increasing vascular biglycan content, resulting in increased lipid retention in the vasculature leading to increased atherosclerosis. To examine the specific role of increased vascular biglycan independent of manipulation to plasma SAA, we developed a mouse model overexpressing biglycan under the control of the smooth muscle α actin promoter.

Chapter 5: Biglycan overexpression and atherosclerosis

5.1 Results

In our models of sustained and briefly increased SAA, mice developed increased atherosclerosis. Biglycan, whose vascular content is increased by SAA, is thought to play a central role in the development of atherosclerosis by retaining apolipoprotein B containing lipoproteins in the subendothelial space of atherosclerosis prone arteries. The purpose of the following experiment was to determine if increased biglycan, such as seen following an increase in SAA, was individually sufficient to increase atherosclerosis, independent of any potential confounding factors introduced by elevated SAA. This study utilized the biglycan transgenic mouse. For a detailed description of the mouse model, please review section 2.1, Murine models. At eight weeks of age, male and female biglycan transgenic x *ldlr-/-* and control mice were fed western diet for 12 weeks. The presence or absence of the transgene had no effect on body weight; however, male mice significantly out gained females in both genotypes (Figure 5.1). Similar to body weights, the presence of the transgene did not affect total cholesterol; however, there was a significant difference between male and female mice (Figure 5.2A). Interestingly, the transgene did affect plasma triglycerides. Gender as well as genotype resulted in significant changes; again males had higher triglycerides as did mice not carrying the transgene (Figure 5.2B). FPLC fractionation revealed that lipoprotein profiles did not differ between genotypes (Figure 5.3). TGF-β was elevated with western diet, but did not differ

between transgenic and non-transgenic mice (Figure 5.4). Atherosclerosis was analyzed at three sites; the aortic intimal surface, the aortic sinus and the brachiocephalic artery. There were no differences in atherosclerotic lesion area between genders, so male and female mice within genotypes were combined for analysis. Biglycan transgenic mice had increased atherosclerosis on the aortic intimal surface (Figure 5.5A, $p<0.05$) and in the aortic sinus (Figure 5.5B, $p<0.006$). There was no difference in atherosclerosis measured in the brachiocephalic artery (Figure 5.5C) after 12 weeks; however, there was a trend towards increased atherosclerosis in the brachiocephalic artery in mice analyzed after four weeks of diet (data not shown). Such regional and chronological differences in the development of atherosclerosis are well described in the literature and could certainly explain the lack of atherosclerosis in the brachiocephalic artery (103).

Immunohistochemical analysis of aortic root sections demonstrated increased biglycan content in transgenic mice (figure 5.6A). Vascular biglycan content was measured by western blot (Figure 5.6B) and was increased in transgenic mice, which correlated with the aortic sinus lesion area (Figure 5.6C, $p<0.0001$). Biglycan is thought to be a key mediator of lipid retention in the vessel wall, so aortic root sections were double labeled with biglycan and apoB antibodies. Mice transgenic for biglycan had increased co-localization with apoB containing lipoproteins (Figure 5.7). To further test the effect of increased vascular biglycan, vascular smooth muscle cells were cultured from transgenic

and wildtype aortas and incubated with fluorescently labeled LDL. There was a strong trend towards increased binding in cells from mice possessing the biglycan transgene; however, it did not reach significance (Figure 5.8).

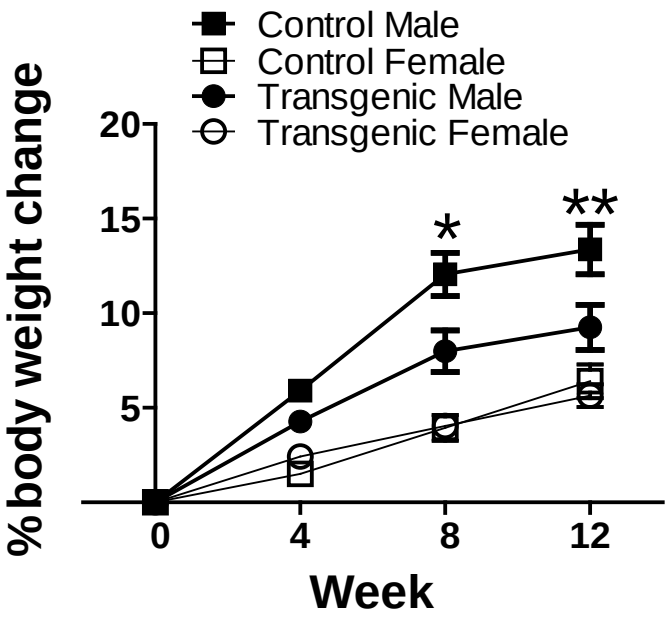


Figure 5-1: Biglycan transgenic male mice gained less weight than control males; however, genotype did not affect female weight gain

Biglycan transgenic x *ldlr-/-* and control mice were fed a western diet for 12 weeks (Control male, closed square; Control female, open square; transgenic male, closed circle; transgenic female, open circle). Body weight was measured at the indicated timepoints throughout the study. Control male mice gained significantly more weight by week 8 compared to biglycan transgenic mice. This effect persisted for the duration of the study. Body weight in female control mice did not differ from biglycan transgenic females at any time throughout the study. Data shown is mean ± SEM for n=11-16 per group analyzed by two-way repeated measures ANOVA with Bonferroni multiple comparisons. * $p<0.05$, ** $p<0.001$

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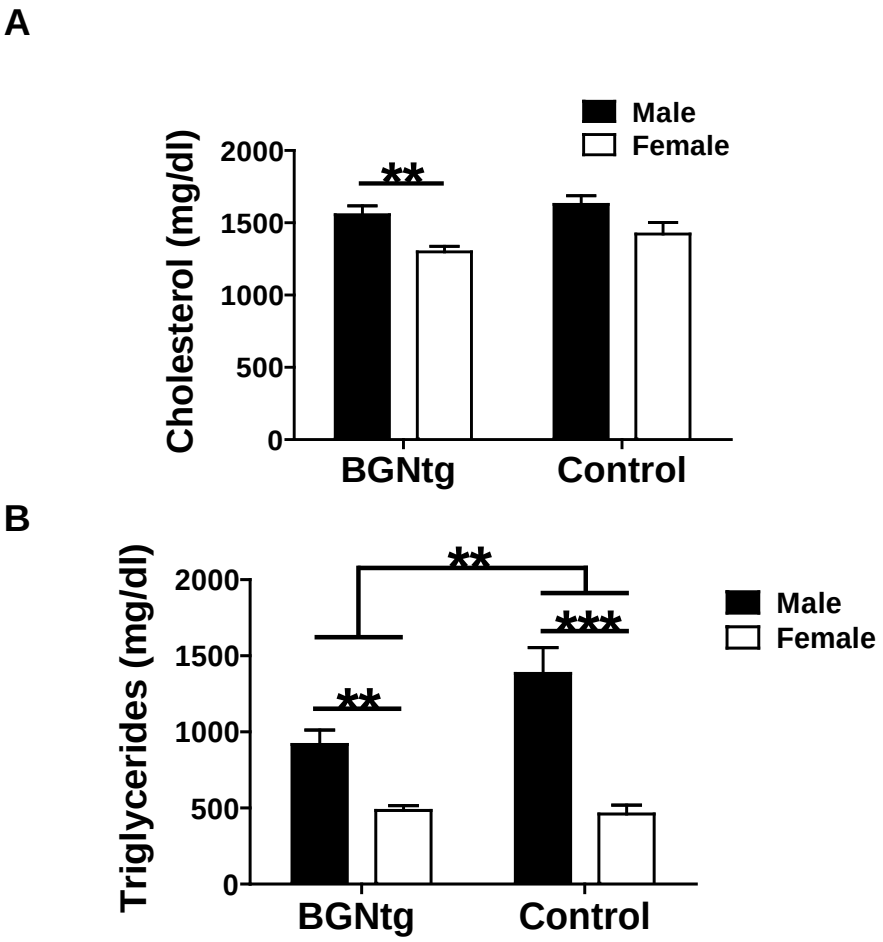

Figure 5-2: Biglycan transgenic mice had lower triglycerides compared to control mice; however, there was no difference in cholesterol

Biglycan transgenic (BGNtg) and control mice were fed a western diet for 12 weeks. Plasma collected at the end of 12 weeks was analyzed for cholesterol and triglycerides. Cholesterol did not differ between transgenic and control mice; however, male transgenic mice did have higher total cholesterol than female transgenic mice (A). Data shown is mean ± SEM for n=12-16 mice per group, ** $p<0.01$. Triglycerides differed between transgenic and control mice, between male and female transgenic mice, and between male and female control mice (B). Data shown is mean ± SEM for n=12-16 mice per group. Data analyzed by two-way ANOVA with Bonferroni multiple comparisons. **$p<0.01$, ***$p<0.001$.

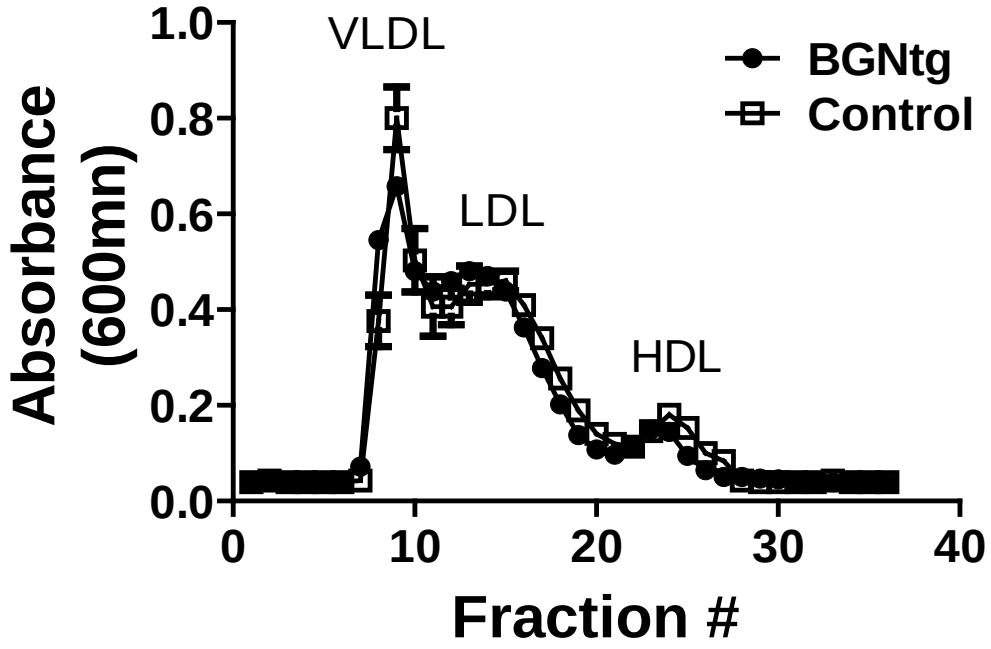

Figure 5-3: **Lipoprotein cholesterol distribution did not differ between biglycan transgenic and control mice.**

Biglycan transgenic (BGNtg, closed circles) and control (open squares) mice were fed a western diet for 12 weeks. Terminal plasma samples were separated by fast performance liquid chromatography to analyze lipoprotein distribution. No difference was observed between transgenic animals (black circle) and controls (open squares). Data shown is mean ± SEM for n=3-4 mice per group.

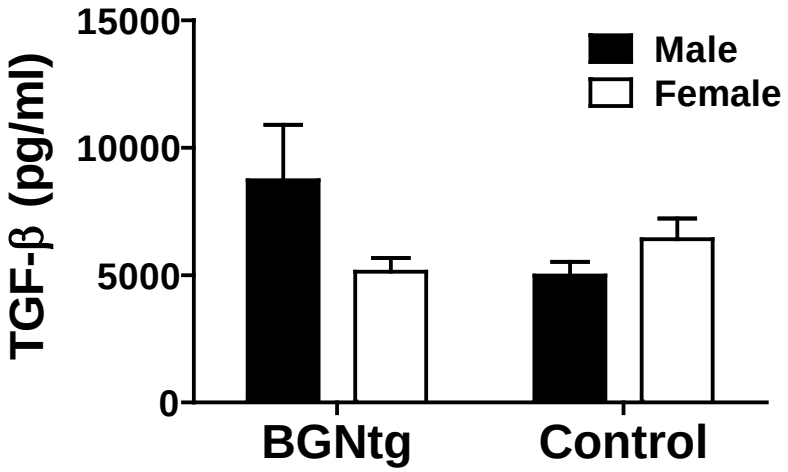

Figure 5-4: **TGF-β measured after 12 weeks on study did not differ between biglycan transgenic and control mice**

Biglycan transgenic (BGNtg) and control mice were fed a western diet for 12 weeks. Terminal plasma was collected and assayed for TGF-β. No difference was observed between transgenic and control mice. Data shown is mean ± SEM for n=9-13 mice per group analyzed by two-way ANOVA with Bonferroni multiple comparisons.

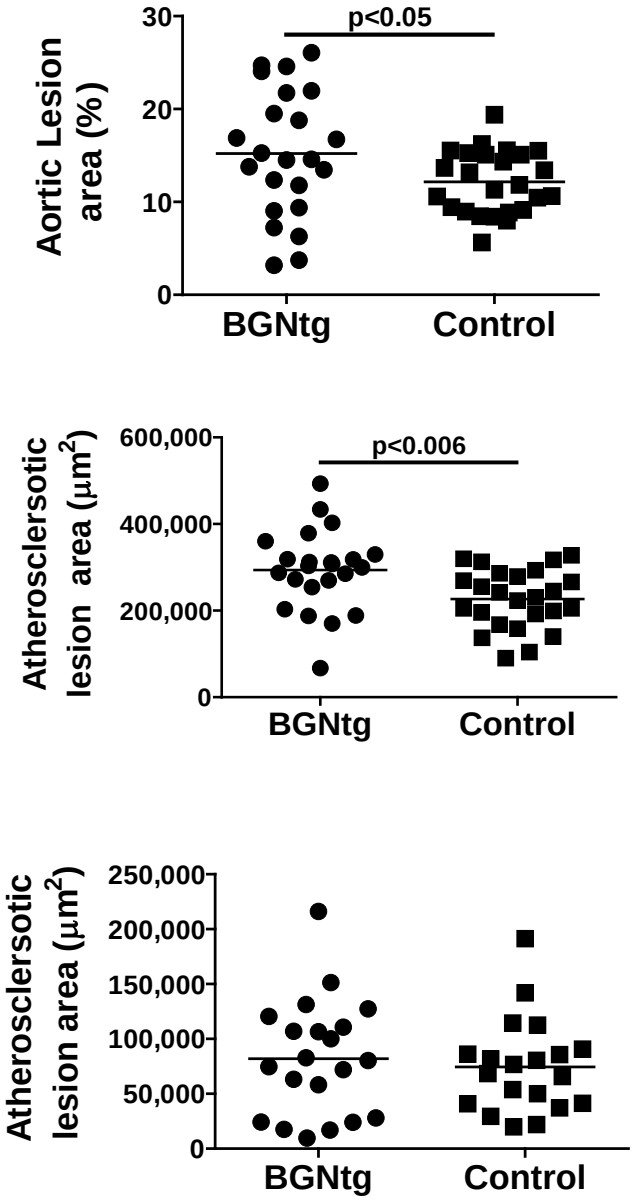

Figure 5-5: **Biglycan transgenic mice had increased atherosclerosis in the aortic sinus and on the aortic intimal surface**

Biglycan transgenic (BGNtg, closed circles) and control mice (closed squares) were fed a western diet for 12 weeks. Transgenic mice had increased atherosclerosis on the aortic intimal surface (A) and in the aortic sinus (B). There was no difference atherosclerosis between transgenic and control mice in the brachiocephalic artery (C). Lesions were analyzed using Nikon NISelements software. Data points represent individual mice with the horizontal bar representing means of n =20-25 mice per group analyzed by students t-test. *p<0.05.

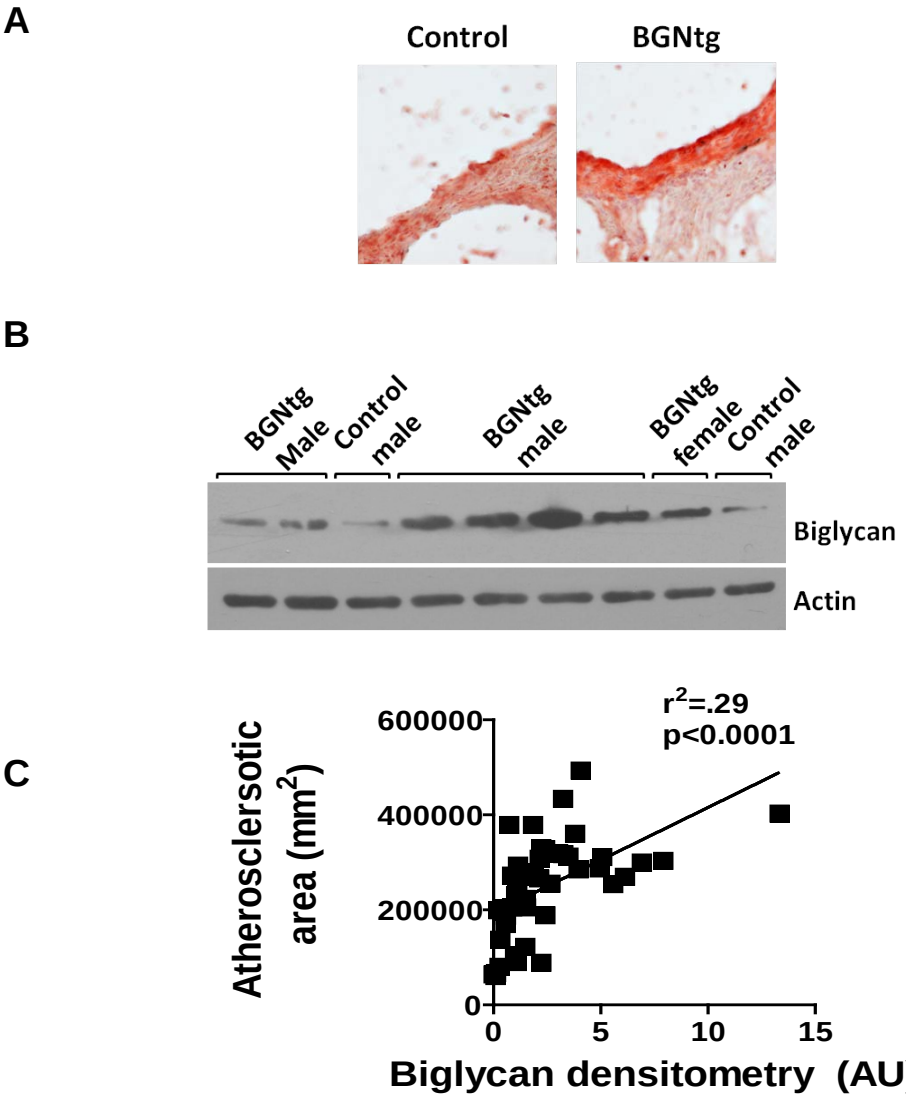

Figure 5-6: **Biglycan transgenic mice had increased vascular biglycan content which correlated with aortic root lesion area**

Biglycan transgenic (BGNtg) and control mice were fed a western diet for 12 weeks. Aortic root sections from biglycan transgenic and control mice were immunostained for biglycan, revealing a striking increase in biglycan content in transgenic mice (A). Shown are representative images from n=3-4 mice per group. Total protein from carotid arteries was immunoblotted for biglycan to determine the extent of transgene expression. The data revealed an increased in vascular biglycan content in transgenic mice (B). The magnitude of increased vascular biglycan correlated strongly with aortic sinus atherosclerosis in both transgenic and control mice (C, r^2=0.29, p<0.0001).

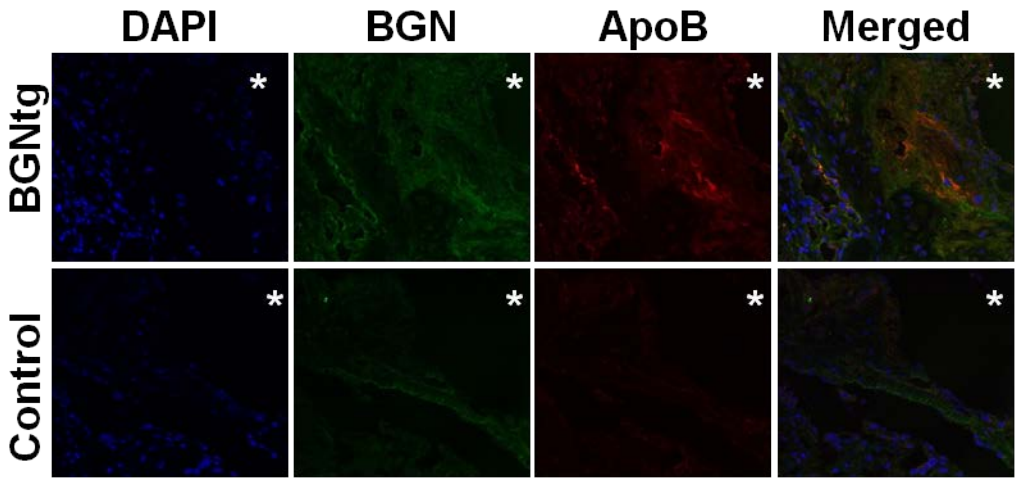

Figure 5-7: **Biglycan and apolipoprotein B co-localized to a greater extent in biglycan transgenic mice compared to control mice**

Biglycan transgenic and control mice were fed a western diet for 12 weeks. Representative confocal images of a shoulder lesion from biglycan transgenic (BGNtg) and control mouse aortic root section (magnified 40x). Apolipoprotein B (apoB) and biglycan (BGN) were visualized with red and green fluorescent antibodies respectfully. Nuclei were stained blue with DAPI. Co-localization is indicated by the presence of yellow in the merged image. Images are representative of n=4-10 per group. Asterisks indicate the lumen of the vessel.

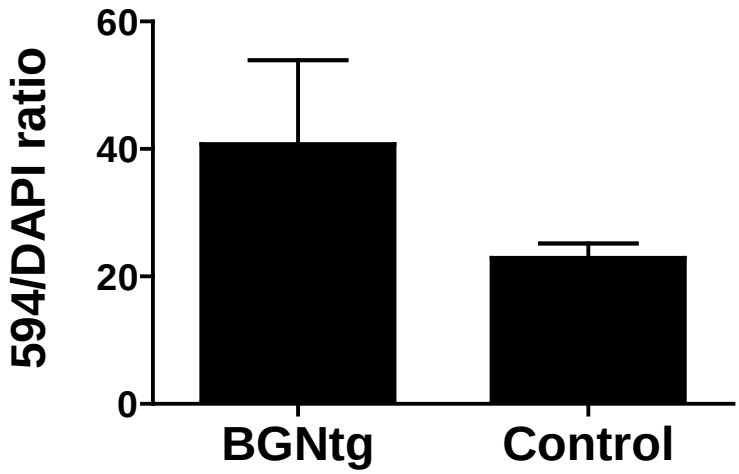

Figure 5-8: **Vascular smooth muscle cell cultures from biglycan transgenic mice did not differ in LDL binding compared to control mice**

Vascular smooth muscle cells from biglycan transgenic and control aortas were cultured in chamber slides to near confluence. Cells were then incubated with Alexafluor-594 labeled LDL for two hours at 4^{o}C. Bound LDL was quantified by quantitative fluorescent microscopy and normalized to total DAPI area. Biglycan transgenic animals had a trend towards increased LDL binding; however, it did not reach significance. Data are presented as mean±SEM for n=5-10 regions/condition analyzed by students t-test.

5.2 Discussion

Ldlr$^{-/-}$ mice expressing the transgene for human biglycan and wildtype controls were fed a western diet for 12 weeks. Mice possessing the biglycan transgene had increased atherosclerosis in the aortic sinus and on the aortic intimal surface compared to wildtype mice. Atherosclerotic lesion area did not reach significance in the brachiocephalic artery after 12 weeks of western diet.

Biglycan has been proposed to be a key mediator of lipid retention in both murine and human atherosclerosis. It is found in atherosclerotic lesions and we and others have demonstrated its colocalization with apolipoprotein B containing lipoproteins. Many mechanisms have been shown to increase vascular biglycan content. Individuals with diabetes for instance have increased biglycan expression (104), and peptides and cytokines such as angiotensin II (105), and serum amyloid A (38) also lead to increased vascular biglycan content. Biglycan synthesized under stimulation of such cytokines had longer, more negatively charged glycosaminoglycan side chains that interacted more strongly with the positively charged amino acid repeats on the surface of apolipoprotein B (17). However, diabetes and these cytokines have other potential atherogenic effects. For instance, hyperglycemia has been shown to down regulate eNOS derived NO in vascular endothelial cells (106), which in turn led to activation of the NF-kB inflammatory pathways. AngII is a small peptide best studied in the renin-

angiotensin system of blood pressure regulation. However, angII has a potent proinflammatory effect on vascular cells found within lesions leading to induction of many cytokines linked to the development of atherosclerosis (107). SAA has been described extensively in this thesis and it too can stimulate the expression of many proinflammatory cytokines known to activate cells found in lesions. Thus to truly identify the potential role of biglycan in the development of atherosclerosis, the biglycan transgenic mouse model was created. It allowed for the study of lipid retention mediated by vascular biglycan independent of other confounding factors that could also stimulate lesion development.

It has been demonstrated that biglycan synthesis under the influence of inflammatory cytokines has increased glycosaminoglycan (GAG) chain length with increased sulfate incorporation (38). Here biglycan synthesis was under the control of the smooth muscle alpha actin promoter and not elevated cytokines. Thus it could be argued that the study did not accurately reflect the environment previously described in biglycan mediated lipid retention. We did not measure GAG chain length or sulfate incorporation in the transgenic animal. However, this study was performed in *ldlr*$^{-/-}$ mice fed a proinflammatory western diet meaning that the mice may have been in an inflamed state, and biglycan synthesized under these conditions may have had changes in the GAG chains; however, since control mice were under the same conditions they would have the same response.

One potentially confounding factor in the analysis of atherosclerosis in this study was the elevated triglycerides in the littermate control animals. Triglycerides are well documented as a marker of cardiovascular disease and their increase is seen as a robust indication of disease risk (108). Individuals that receive intensive statin therapy, in which their LDL cholesterol levels decreased to clinically optimal levels still had continued risk of acute coronary events if dyslipidemia in the form of hypertriglyceridemia remained (109), suggesting the elevated triglycerides are an independent risk factor for CVD. This risk may be due to the association of triglycerides with atherogenic lipoproteins and lipoprotein remnants, especially those carrying apo C-III (110). Typically these remnant particles are quickly cleared by the liver; however, in hypertriglyceridemia, the clearance is greatly reduced and the accumulation of triglyceride rich particles on endothelial cells has been documented (111). These particles then translocate to the subendothelial space, binding proteoglycans in the matrix and enhance the retention of proatherogenic lipoproteins. Thus the mechanism by which triglycerides may enhance atherosclerosis is slightly different from traditional lipid retention; however, the end product is subendothelial lipoprotein particle retention. Taken together the increased triglycerides supports our model as the increase in both vascular biglycan and translocation of triglyceride rich particles ultimately ends with increased lipoprotein retention and increased transit time in the subendothelial space leading to the milieu that drives the

inflammatory component of atherosclerosis. These findings lend credence to triglyceride's causal role in atherosclerosis; however, clinical data has not fully supported triglycerides as an independent risk factor (109). Statin monotherapy has been shown to decrease triglycerides and LDL-C; however, statins have many functions beyond lipid lowering and a clear reduction in CVD based explicitly on the reduction in triglycerides has yet to be proven (112) (113). Other classes of drugs are also effective at lowering triglycerides. Both niacin and fibrates have been shown to reduce triglycerides in dyslipidemca individuals. Both drugs in large trials as monotherapies for the reduction of CVD have been shown to reduce both triglyceride levels and acute cardiovascular events; however, the effect of lower triglycerides has not been clearly shown to drive the decrease in CVD (114) (115) . Given that significantly increased atherosclerosis was observed in the transgenic animals compared to the wildtype controls whose triglycerides were elevated, the role of triglycerides will not alter the conclusion that proteoglycan mediated retention of lipoproteins is responsible for the increase in atherosclerosis.

The purpose was to determine if increased biglycan content was individually sufficient to increase atherosclerosis over control mice. The data indicated that biglycan was increased in transgenic mice as was the extent of atherosclerotic lesion development. Thus increased biglycan is pro-atherogenic likely by increasing the retention of proatherogenic lipoproteins.

This data supports a role for biglycan in the development of atherosclerosis; however, it remains to be seen whether biglycan is absolutely necessary for the development of atherosclerosis. To determine this, further studies were conducted using biglycan/apolipoprotein E double knockout mice injected with ad-hSAA1 or controls.

Chapter 6: Biglycan deficiency and atherosclerosis

6.1 Results

Many studies have suggested a proatherogenic role for biglycan; however, its absolute requirement for the development of atherosclerosis has not been investigated. Here we investigate the necessity of biglycan in atherosclerosis. Biglycan deficient mice, five times backcrossed to the low density lipoprotein receptor knockout mouse on a C57BL/6 background, were obtained from Williams et al (Thomas Jefferson University, Philadelphia, PA, USA) and crossed with *apoe*$^{-/-}$ mice from Jackson Laboratory. Generating this mouse was challenging as the *bgn*$^{-/o}$ males carry only a single copy of the gene for biglycan, located on the X chromosome. In order to generate the *bgn*$^{-/-}$ *x apoe* $^{-/-}$ double knockout mouse, males deficient in *bgn*$^{-/o}$ were crossed to female *apoe* $^{-/-}$ deficient mice establishing biglycan wildtype males(*bgn*$^{+/o}$) and biglycan heterozygous (*bgn*$^{+/-}$) females, that were now all heterozygous for apolipoprotein E (*apoe* $^{+/-}$). Male biglycan wildtype by apolipoprotein E heterozygous (*bgn*$^{+/o}$ *x apoe* $^{+/-}$) were crossed to female biglycan x apolipoprotein E heterozygous mice (*bgn*$^{+/-}$ *x apoe* $^{+/-}$). The offspring from this cross provided the first opportunity to establish a male biglycan knockout mouse that was either deficient or heterozygous for apolipoprotein E (*bgn*$^{-/o}$ *x apoe* $^{+/-}$ or *bgn*$^{-/o}$ *x apoe* $^{+/-}$). The previous cross also established a female mouse that was heterozygous for biglycan but

deficient in apolipoprotein E (*bgn*$^{+/-}$ *x apoe* $^{-/-}$). The double knockout mouse was established by crossing the male biglycan deficient x apolipoprotein deficient (*bgn*$^{-/o}$ *x apoe* $^{-/-}$) to the female biglycan heterozygous x apolipoprotein E knockout (*bgn*$^{+/-}$ *x apoe* $^{-/-}$). Littermate control mice were established by crossing a male mouse possessing a single copy of biglycan but deficient in apolipoprotein E *(bgn*$^{+/o}$ *x apoe* $^{-/-}$) to a female homozygous for expression of biglycan while deficient in apolipoprotein E *(bgn*$^{+/+}$ *x apoe* $^{-/-}$)

There was an immediate and persistent phenotype observed with the *bgn*$^{-/-}$ *x apoe*$^{-/-}$ mice. Approximately 80% of offspring born to *bgn*$^{-/-}$ *x apoe*$^{-/-}$ females exhibited hydrocephaly and required euthanasia by the third week of life. However, the 20% of offspring that reached weaning age while remaining asymptomatic for hydrocephaly appeared to be healthy for the duration of their life. We have successfully crossed the *bgn*$^{-/-}$ mouse to the *ldlr*$^{-/-}$ mouse for studies investigating the role of biglycan in a model of angiotensin II mediated atherosclerosis (29). These mice exhibited no obvious health issues, thus the combination of biglycan deficiency and hyperlipidemia cannot explain the health issues observed in the *bgn*$^{-/-}$ *x apoe*$^{-/-}$. This study utilized the *bgn*$^{-/-}$ *x apoe*$^{-/-}$and *apoe*$^{-/-}$ male mice that survived to eight weeks of age. Both the *bgn*$^{-/-}$ *x apoe*$^{-/-}$and *apoe*$^{-/-}$ mice were injected once with ad-hSAA1 or ad-Null and fed normal rodent chow for 16 weeks. Human SAA increased dramatically in the ad-hSAA1 injected mice (Figure 6.1 A). Strikingly, *bgn*$^{-/-}$ *x apoe*$^{-/-}$mice receiving ad-hSAA1 had significantly more atherosclerosis in the aortic sinus compared to *bgn*$^{-/-}$ *x*

apoe$^{-/-}$ ad-Null injected mice (Figure 6.1B). The *bgn*$^{-/-}$ *x apoe*$^{-/-}$ mice injected with ad-hSAA1 had a trend toward increased atherosclerosis on the aortic intimal surface compared to *bgn*$^{-/-}$ *x apoe*$^{-/-}$ ad-Null injected mice (Figure 6.1C).

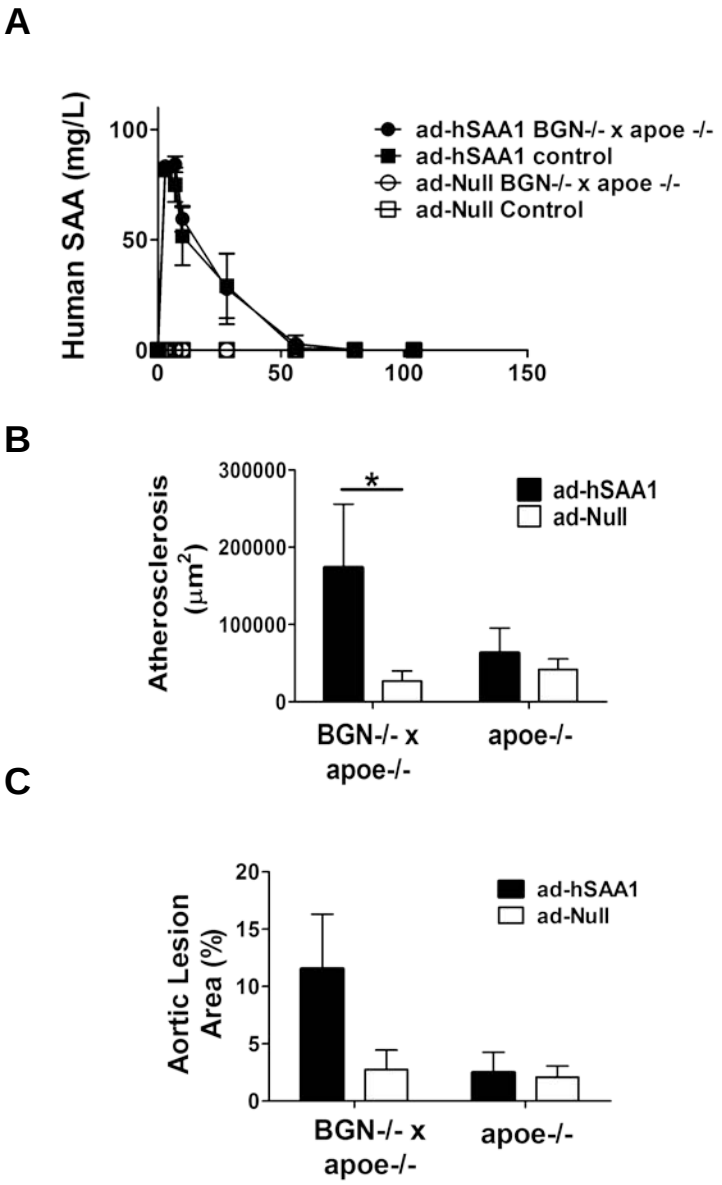

Figure 6-1: **Biglycan deficiency, combined with elevated hSAA1 resulted in increased atherosclerosis**

bgn$^{-/o}$ *x apoe*$^{-/-}$ and *bgn*$^{+/o}$ *x apoe*$^{-/-}$ were injeced once with ad-hSAA1 or ad-Null (ad-hSAA1 DKO, closed circle; ad-hSAA1/control, closed square; ad-Null/DKO, open cirlce; ad-Null/control, open square) and bled at the timepoints indicated. Human SAA1 was measured by species specific ELISA. Mice receiving ad-hSAA1 had dramatically elevated human SAA1 (A). Data shown is mean ± SEM for n=3-11 mice per group. Atherosclerosis, after 16 weeks, was significantly increased in *bgn*$^{-/-}$ *x apoe*$^{-/-}$ ad-hSAA1 injected mice in the aortic sinus (B) Data shown are mean ± SEM for n=7-9 mice per group, $p<0.05$. There was a trend towards increased atherosclerosis in *bgn*$^{-/-}$ *x apoe*$^{-/-}$ ad-SAA injected mice on the aortic intimal surface (C) Data shown is mean ± SEM for n= 6-7 analyzed by two-way ANOVA with Bonferroni multiple comparisons. *$p<0.05$

6.2 Discussion

The analysis of this study was stopped as concerns about the mice enrolled in the study became more apparent. The *bgn*$^{-/-}$ *x apoe*$^{-/-}$ double knockout mouse was produced in our colony by initially crossing biglycan deficient males to apolipoprotein E deficient females. As biglycan is an X-linked gene, generation 1 offspring were all heterozygous or wildtype; the lines were then split such that knockout and littermate controls were produced from the same founders. This was the same strategy our lab used to produce the *bgn*$^{-/-}$ *x ldlr*$^{-/-}$ double knockout (93). However, the combination of biglycan deficiency and apolipoprotein E deficiency resulted in hydrocephaly rates for pre-weaned double knockout offspring greater than 80%. It took much longer than initially anticipated to enroll the study because of the extensive amount of breeding required.

Initial analysis also revealed some atherosclerosis patterns that were not observed in other studies that made us believe we were observing a possible “healthy survivor” effect, also known as survivorship bias (116). This statistical concept can be applied to many fields from finance to meta-analysis of medical studies. Basically we are biasing our data by not considering the mice that did not survive and thus the probability that they may have somehow influenced the data differently than what we actually observed. Given the excessive mortality we may have been sampling a sub-

population of double knockout mice that was very different from the double knockout population as a whole, making it impossible to draw conclusions about the entire population. Both *bgn*$^{-/-}$ *x apoe*$^{-/-}$ and *apoe*$^{-/-}$ mice injected once with ad-hSAA1 had a profound increase in human SAA levels compared to mice injected with ad-Null, and the human SAA did not return to baseline levels for more than 8 weeks. Previous experiments using *apoe* $^{-/-}$ mice and a single injection of ad-hSAA1 have consistently shown resolution in human SAA levels by 10-14 days post injection. Interestingly, in this study, *apoe* $^{-/-}$ mice with a prolonged increase in human SAA did not have increased atherosclerosis in the aortic sinus or on the aortic intimal surface compared to ad-Null injected *apoe* $^{-/-}$ mice. This too is quite different than in previous studies as ad-hSAA1 injected mice consistently had more atherosclerosis than ad-Null mice. This study also failed to recapitulate the atherosclerosis data within the littermate controls. In previous work from our lab, *apoe* $^{-/-}$ mice that received ad-hSAA1 consistently had more atherosclerosis at all three site of atherosclerosis analysis compared to mice receiving ad-Null. However, in this study the two groups have almost identical levels of atherosclerosis in their aortic sinus and on their aortic intimal surface.

The hypothesis that SAA is proatherogenic is supported by not only this thesis but also by Dong et al that revealed increased SAA alone was sufficient to increase atherosclerosis (85). Furthermore, we hypothesized that biglycan deficiency would be atheroprotective. Biglycan is found in both

murine and human lesions and co-localizes with apolipoprotein B containing lipoproteins (36). It is the basis for the well supported “response to retention” hypothesis of atherosclerosis that states that lipoprotein retention by proteoglycans, particularly biglycan, is an initiating event in the development of atherosclerosis. Recently we published that increased biglycan resulted in increased atherosclerosis using a biglycan transgenic model {Chapter 5 & (93)}. Thus, we hypothesized that the absence of biglycan should attenuate SAA mediated lesion development. However, data in our *bgn*$^{-/-}$ *x apoe*$^{-/-}$ mice demonstrated that biglycan deficiency in the presence of elevated SAA is proatherogenic. These mice had significantly more atherosclerosis in the aortic sinus than controls ($p<0.05$). There was a trend towards increased atherosclerosis on the aortic intimal surface that did not reach significance. This data, though contradictory to our overall hypothesis in the lab that biglycan is pro-atherogenic, is becoming more supported. In unpublished data from another study examining biglycan deficiency in diabetic *ldlr*$^{-/-}$ mice we observed an increase in atherosclerosis in mice deficient in biglycan compared to mice expressing bgn regardless of glycemic status (data not shown). The likely confounding factor in biglycan deficiency seems to be proteoglycan compensation. Biglycan is a very relevant molecule responsible for biological processes from initial development in utero through maintenance of collagen fibers, teeth and bones throughout adult life (30). Previous work in our lab examined the role of angiotensin II on the development of atherosclerosis in biglycan deficient and biglycan wildtype

mice crossed to *ldlr*$^{-/-}$ mouse on a C57BL/6 background. We demonstrated that mice lacking biglycan actually developed more atherosclerosis compared to biglycan wildtype mice. Further analysis revealed that perlecan; another vascular proteoglycan, is upregulated and co-localizes with apolipoprotein B containing lipoproteins in the biglycan deficient mice. This perlecan upregulation is not observed in biglycan wildtype mice nor is it TGF-β dependent as the treatment of these mice with the TGF-β inhibitory antibody 1D11 did not alter perlecan's expression (29). These data suggest that perlecan can compensate for biglycan's absence by achieving the same if not greater degree of lipoprotein retention in the vessel wall. However, interpretation of this data is complicated by the fact that perlecan is thought to not play a role in the development of human atherosclerosis and is in fact down regulated in human disease (117).

Another possible contributor to increased atherosclerosis in biglycan deficiency is TGF-β. Hyperglycemia, increased AngII and increased SAA have all been shown to increase TGF-β. Interestingly TGF-β is maintained in its latent state in the matrix by interactions with biglycan (44) and decorin (118). Thus, the absence of biglycan could allow for increased available active TGF-β, as we have previously shown (53). However, as discussed in the introduction section 1.4.1, TGF-β has such complex and interconnected signaling pathways that deducing its exact role in a particular disease may be beyond the scope of our current technologies (48).

These data, though not conclusive, do point to the concept that biglycan may not be necessary for the development of atherosclerosis. To further investigate our mechanism for the development of atherosclerosis, the role of TGF-β was investigated using an *apoe*$^{-/-}$ mouse model of atherosclerosis coupled with TGF-β inhibition.

Chapter 7: The necessity of TGF-β signaling in SAA mediated atherosclerosis

7.1 Results

We have previously demonstrated that both brief and sustained elevations of SAA directly increased atherosclerosis. Our data suggests that increased atherosclerosis resulted from SAA increasing TGF-β which in turn increased vascular biglycan content followed by an increase in lipid retention. We have previously shown in vitro that inhibition of TGF-β prevented SAA induction of vascular biglycan content (38); however, the downstream effect of TGF-β inhibition on the development of atherosclerosis remains unknown. Thus, we now aimed to determine the necessity of TGF-β in SAA-induced atherosclerosis.

For this study, eight week old male *apoe* $^{-/-}$ mice were injected once with ad-hSAA1 or ad-Null via tail vein method, and simultaneously injected via intraperotineal route with the TGF-β inhibitory antibody 1D11 or 13C4 control antibody. Human and murine SAA were measured throughout the study using species specific ELISA's. Human SAA increased only in the ad-hSAA1 group receiving 13C4 control antibody (Figure 7.1 A). Murine SAA did not differ between groups at any timepoint (Figure 7.1 B). Subsequent data analysis was stopped given the lack of SAA expression in the ad-hSAA1/1D11 group.

Certainly the question could be asked as to whether the concurrent administration of 1D11 with ad-hSAA1 specifically could have resulted in the inability of the virus to infect the mice. However, we had previously carried out a very small pilot study investigating SAA's effect on vascular biglycan content in the absence of TGF-β signaling. In that study *apoe*$^{-/-}$ male mice were injected with ad-hSAA1 or ad-Null and then concurrently injected with either 1D11 or 13C4; and vascular tissues collected 28 days later. This pilot SAA/1D11 study utilized the same lot of ad-hSAA1 that all other studies presented here utilized. Plasma was collected for measurement of human SAA and TGF-β at several early timepoints. Ad-hSAA1 administration increased human SAA to levels well within what we typically see when administering ad-hSAA1 without 1D11 (Figure 7.2A). The data clearly demonstrates that 1D11 antibody did not affect the expression of human SAA in these mice. Levels of TGF-β were undetectable in the mice receiving 1D11 indicating that antibody was fully functional as well (Figure 7.2A). Furthermore, western blot analysis of aortas taken 28 days after administration of ad-Null, ad-hSAA1, ad-hSAA1 + 1D11 or ad-hSAA1 + 13C4 revealed a striking decrease in biglycan synthesis in mice receiving 1D11, compared to mice receiving ad-hSAA1 + 13C4 (Figure 7.2B). So based on this small study the administration of ad-hSAA1 and 1D11 do not interfere with one another.

To attempt to resolve this issue, another small study was performed using eight week old *apoe*$^{-/-}$ male mice from The Jackson Laboratory (Bar Harbor, Maine, USA). *apoe*$^{-/-}$ mice (n=12) were divided into three groups. The first group

received a single injection of ad-hSAA1 and acted as a control for the expression of SAA. The second group was injected with ad-hSAA1 via tail vein and then concurrently injected with 1D11 antibody via IP route. For 1D11 to truly be an issue, the antibody would likely have to alter a pathway involved in SAA secretion from hepatocytes as the virus is being delivered intravenously and would likely bind to and infect the hepatocytes prior to the antibody being absorbed into the lymphatics and then systemic circulation where it could interfere with the virus directly. There are no documented TGF-β dependent SAA secretory pathways described in the literature so this is either not the issue or would represent a novel interaction between human SAA and antibodies against murine TGF-β. The third group was injected with ad-hSAA1 and then 24 hours later received an IP injection of 1D11. This group investigated the possibility of 1D11 inhibitory antibody to destabilize SAA in circulation such that it is rapidly cleared. If such an effect is taking place the SAA levels in this delayed 1D11 group should be much lower than the ad-hSAA1 only group on day three indicating the increased SAA clearance. Plasma was analyzed from samples collected on days 0, 1 and 3 to determine the time course expression of human SAA. All three groups had dramatically, equivalently elevated expression of SAA over the first three days (Figure 7.3). This experiment demonstrates that 1D11 does not interfere with SAA expression and thus we have not resolved why the 16 week SAA 1D11 experiment failed. This research is ongoing.

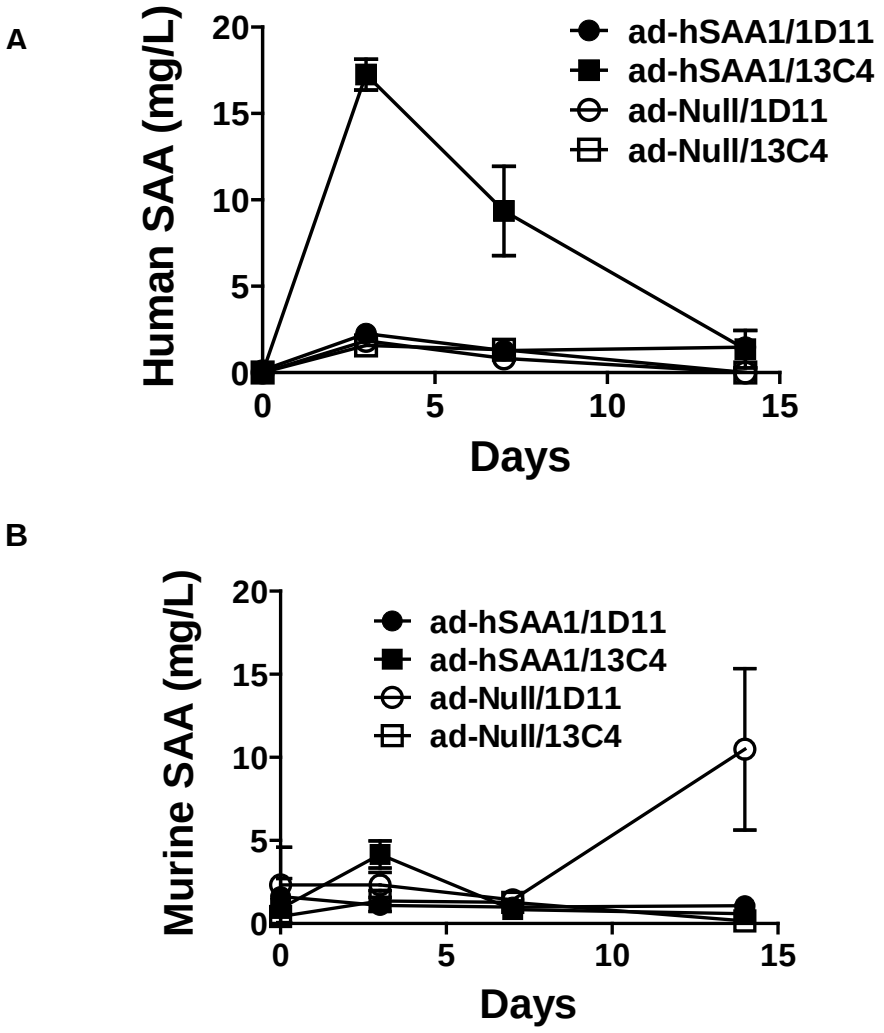

Figure 7-1: **Human SAA1 did not increase in mice concurrently injected with ad-SAA and TGF-β inhibitory antibody 1D11**

apoe$^{-/-}$ mice were injected once with ad-hSAA1 or ad-Null and concurrenlty injected with either TGF-β inhibitory antibody 1D11 or 13C4 control antibody (ad-hSAA1/1D11, closed circle; ad-hSAA1/13C4, closed square; ad-Null/1D11, open cirlce; ad-Null/13C4, open square) and bled at the timepoints indicated. Human and murine SAA were measured by species specific ELISA. Only mice receiving ad-hSAA1 + 13C4 had dramatically elevated human SAA1 (A). Data shown is mean ± SEM for n=3-9 mice per group. Murine SAA did not differ between groups at any timepoint (B). Data shown is mean ± SEM for n=2-9 mice per group.

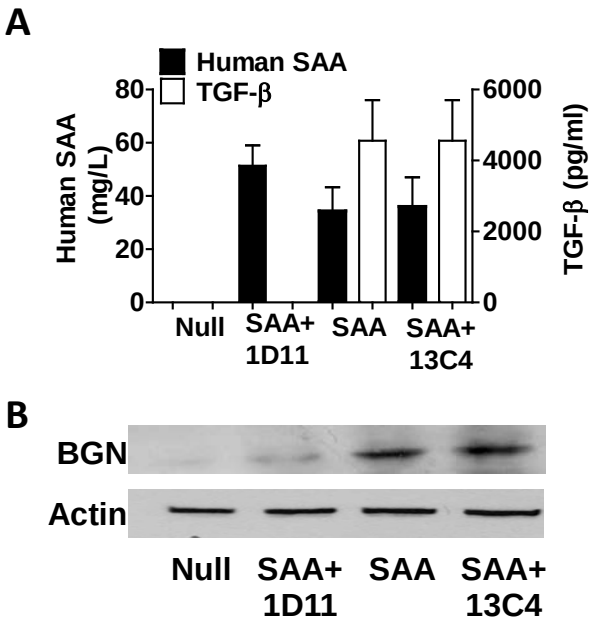

Figure 7-2: TGF-β inhibitory antibody 1D11 suppressed TGF-β expression, resulting in decreased vascular biglycan content in mice concurrently injected with ad-hSAA1 and 1D11 antibody.

apoe$^{-/-}$ male mice, eight weeks of age, were injected with ad-hSAA1 or ad-Null and simultaneously injected with either the TGF-β inhibitory antibody 1D11 or control antibody 13C4 via IP route. Human SAA (black columns) was measured by species specific ELISA. No difference in SAA was detected in mice 1 day after receiving ad-hSAA1 regardless of the presence or absence of TGF-β antibody (A) Data shown is mean ± SEM for n=4-5 mice per group analyzed by one-way ANOVA with Tukey's multiple comparisons. TGF-β (open columns) was elevated in mice receiving ad-hSAA1 with or without 13C4 control antibody. However, mice receiving 1D11 antibody had no detectable TGF-β, similar to ad-Null injected mice (A). Data shown is mean ± SEM for n=4-5 mice per group analyzed by one-way ANOVA with Tukey's multiple comparisons. Aortas were collected 28 days after injections and immunoblotted for biglycan (BGN) or actin. 1D11 prevented the ad-hSAA1 induction of vascular biglycan but 13C4 had no effect (B). Each lane shows protein from an individual mouse representative of n=4-5 mice per group.

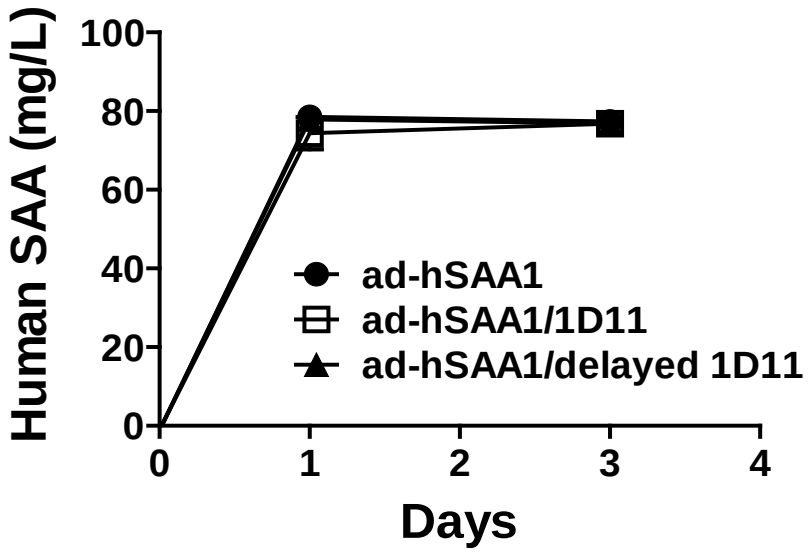

Figure 7-3: **TGF-β inhibitory antibody 1D11 did not affect human SAA expression in ad-SAA injected mice.**

Eight week old male *apoe*$^{-/-}$ mice were injected with ad-SAA (closed circle), ad-SAA + 1D11 (open square) or ad-SAA + 1D11 24hrs after ad-SAA (delayed group, closed triangle). Plasma was drawn at the indicated timepoints and assayed for human SAA. All mice had rapid induction of human SAA regardless of the presence or timing of administration of the TGF-β inhibitory 1D11. Data shown is mean ± SEM for n=4 mice per group.

7.2 Discussion:

apoe$^{-/-}$ deficient male mice were injected with ad-hSAA1 or ad-Null concurrently with either TGF-β inhibitory antibody 1D11 or an irrelevant control antibody 13C4. However, the group receiving ad-hSAA1 concurrently with 1D11 did not have the anticipated increase in human SAA as seen in the ad-hSAA1 mice concurrently injected with 13C4. There are several possible explanations for this. First could be a problem with the adenovirus' ability to infect the mice. The virus is very sensitive to freeze thaw cycles and must be stored correctly, transported to the animal room correctly and resuspended as close to administration of the injections as possible. The virus is stored in a -80^{o} freezer. When aliquots are removed from the freezer, they are immediately placed on dry ice for transport to the animal facility. In my preparation to do adenoviral injections, this is always the last step so as to minimize the time the virus is out of the freezer. Once everything in the animal facility is set-up to perform the injections the virus is resuspended in sterile saline, then immediately injected into the animals. This has been my ritual for every set of injections I have done. I have utilized the tail vein injection procedure for hundreds of injections. I have a great deal of experience with tail vein injections and make notes as part of the animal record as to the quality of the injection such that a mouse can be removed based on poor delivery of the virus on my part. So it does not appear to be a procedural deficiency that led to the lack of SAA expression. Thus, it is highly unlikely that one of these steps resulted in the absence of SAA expression. The adenoviral construct was produced in the Webb lab as previously described (89).

They have extensive knowledge of large scale adenoviral production and storage. All viral aliquots are immediately stored in a designated -80 freezer once prepared. Throughout our lab's investigation of SAA's effects on atherosclerosis we have used aliquots of the same large scale viral prep produced in the Webb lab. This implies that all steps from production to initial storage were taken appropriately as we have used dozens of aliquots and had only one issue potentially linked to their production. Several years ago, we injected a group of mice with ad-hSAA1 and had most of the mice collapse, appearing very ill in a short period of time (<10 minutes) post injection. Mice were given supportive therapy such as warmed 0.9% saline subcutaneously and monitored. All of the mice recovered after about 2 hours and remained healthy for the duration of the study they were participating in. One possible explanation for such a dramatic acute collapse would be LPS contamination in the viral aliquot likely from a contaminated tube that the viral aliquot was stored in, although a definitive cause of the animals' distress was never pinpointed.

It is also worth noting that the mice from both the 1D11 group (which failed to express human SAA) and the 13C4 groups received ad-hSAA1 from the same pooled aliquots of virus. The 13C4 group did express SAA so the virus was obviously sufficiently virulent to infect the mice. This implies that every step prior to injection was done accordingly and the problem with ad-hSAA1 expression in the 1D11 group lies elsewhere.

In my thesis work and the pilot SAA/1D11 study mentioned above, the 1D11 antibody was from the same manufacturer, though the lots were different. Of note, we have noticed in the past that manufacturers will change storage conditions (such as the inclusion of azide in buffer) and not communicate them well to the end user. It is highly unlikely that this would result in a problem but certainly worth mentioning. So at this point we have three studies using both ad-hSAA1 and 1D11 concurrently. Two of the three studies had elevated SAA expression as would be expected. The third study was designed to investigate the requirement of TGF-β in SAA mediated atherosclerosis. In that study ad-hSAA1 and 1D11 antibody were concurrently injected; however, elevation of human SAA was not achieved, thus no conclusion regarding TGF-β's role in SAA mediated atherosclerosis can be made. These results are certainly unsettling and will constitute a major portion of the future directions of this thesis.

Chapter 8: Conclusions and future directions

8.1 Conclusions

The work presented in the thesis is summarized in the model below:

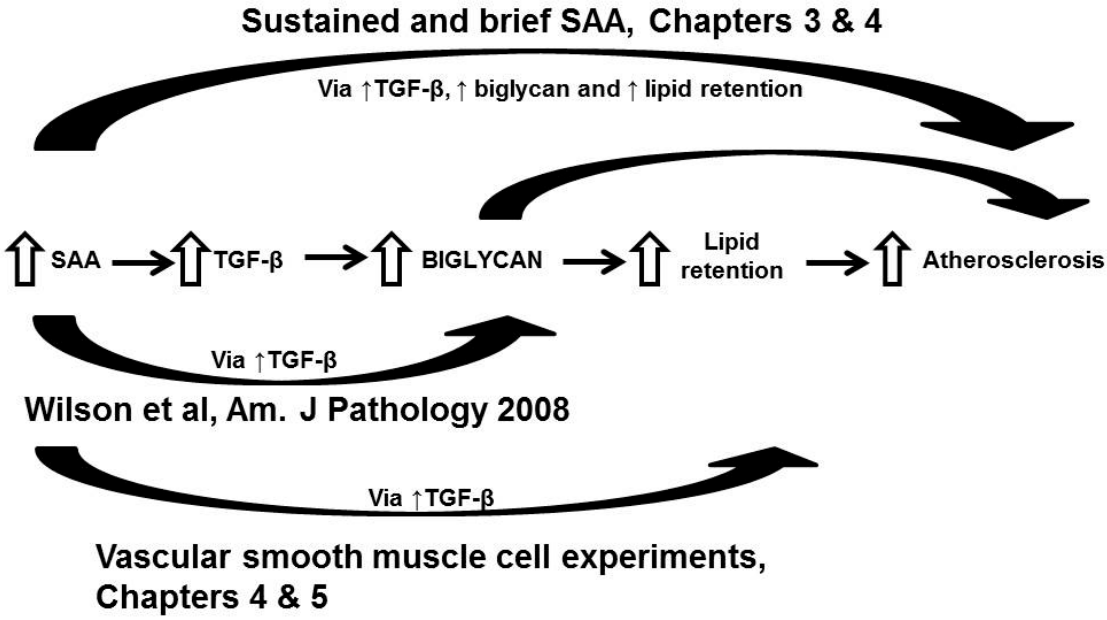

Figure 8-1: Working model

The data in this thesis definitively characterizes serum amyloid A as a proatherogenic cytokine capable of orchestrating vascular wall remodeling. In both *apoe*$^{-/-}$ and *rag1*$^{-/-}$ x *apoe*$^{-/-}$ male mice, increased SAA led to an increase in atherosclerosis at three common sites of analysis. This data is supported by previous work by Dong et al who demonstrated for the first time that chronic overexpression of SAA by lentiviral transfection accelerated the progression of atherosclerosis (85). Their conclusions were based on changes to the vascular

endothelium known to contribute to atherosclerosis such as increased VCAM and ICAM, leading to increased monocyte adhesion and translocation into the developing lesion. Here we expanded upon their findings by demonstrating that chronically elevated SAA, via adenoviral infection, is atherogenic via the upregulation of vascular biglycan leading to increased lipid retention. Both our study and Dong et al clearly implicate SAA as a proatherogenic molecule; however, timing of our observed effects differed greatly from that presented in Dong's work. We demonstrated that SAA was acting early to remodel the vessel wall such that proatherogenic lipoproteins would be retained leading to the inflammatory infiltration observed by Dong. Taken together these data indicate that SAA may be mediating the development of atherosclerosis through multiple mechanisms at multiple distinct developmental timepoints in the disease progression. Right now there are literally hundreds of millions of people currently living with diseases that include chronically elevated SAA as a hallmark feature. These diseases, such as diabetes and obesity confer greater risk of developing cardiovascular disease compared to healthy age matched individuals, though that risk remains largely unexplained. Our data demonstrated that chronically elevated SAA is pro-atherogenic could certainly provide an explanation for their increased CVD risk and warrants further investigation.

In our preliminary studies we observed increased vascular biglycan content 28 days after a single transient increase in SAA. Biglycan, stimulated by SAA has increased GAG chain length and affinity for apolipoprotein B containing

lipoproteins. As Nakashima demonstrated, lipid retention following increased matrix deposition precedes inflammatory infiltration into the developing lesion (15). Interesting was the revelation that even a single transient elevation of SAA could lead to sustained, stable remodeling of the vasculature such that biglycan levels were still elevated 16 weeks after the SAA exposure. Furthermore, mice with only a brief exposure to SAA and subsequent sustained increase in biglycan displayed increased atherosclerosis compared to mice with no change in SAA.

In both sustained and briefly elevated SAA studies, increased SAA did not alter lipids or lipoprotein distribution, nor did body weight or other anthropometric parameters differ.

To further characterize the importance of biglycan in early lesion development, we developed the biglycan transgenic mouse and crossed it onto the *ldlr*$^{-/-}$ background. This model provided a platform to investigate increased biglycan independent of the potential confounding effects of elevated SAA. Vascular biglycan content was increased in both male and female transgenic mice. These mice exhibited a strong correlation between vascular biglycan content and aortic sinus atherosclerosis and also a striking co-localization of aortic sinus biglycan and apolipoprotein B containing lipoproteins.

Furthermore, in support of our hypothesis that biglycan is proatherogenic, biglycan transgenic mice had increased atherosclerosis within the aortic root and

on the aortic intimal surface compared to their wildtype controls. The increased atherosclerosis was independent of other risk factors such as altered cholesterol or lipoprotein distribution.

Interestingly our data also revealed that biglycan, clearly pro-atherogenic when present, is not absolutely required for the development of atherosclerosis. *bgn*$^{-/-}$ mice injected with ad-hSAA1 actually had increased atherosclerosis compared to *bgn*$^{-/-}$ mice injected with ad-Null or wildtype mice injected with either ad-hSAA1 or ad-Null. Superficially this data appears to contradict our working hypothesis that biglycan is an important molecule in the development of atherosclerosis; however, the absence of biglycan does not negate the important pathological role it is playing in atherosclerosis when present. As such, we have recapitulated this finding in two other models of biglycan deficient atherosclerosis. In both a biglycan deficient model of AngII-stimulated atherosclerosis (53) and a biglycan deficient model of diabetes (in preparation), the lack of biglycan resulted in either no difference or increased atherosclerosis compared to biglycan wildtype controls, respectfully. The data presented in this thesis regarding the absence of biglycan appears to support those findings; however, there are caveats attached to the *bgn*$^{-/-}$ data (as discussed in chapter 6)

Our studies also demonstrated that *apoe*$^{-/-}$ VSMC's treated with murine SAA and subsequently incubated with alexa-fluor labeled LDL had increased LDL binding compared to control cells. The increased LDL binding was ameliorated with cells were concurrently treated with murine SAA and TGF-β

inhibitory antibody 1D11; implicating TGF-β as an intermediate in SAA stimulated lipoprotein retention by vascular smooth muscle cell secreted matrix.

8.2 Future directions

8.2.1 **Repeat the TGF-β inhibition study, Chapter 7**

Our model of SAA mediated increased biglycan leading to increased vascular lipid retention and increased atherosclerosis remains incomplete. Previous data from our lab established that SAA acted through TGF-β to increase biglycan expression as TGF-β inhibition resulted in decreased vascular biglycan. A thoughtful experiment to clearly establish the role of TGF-β in SAA mediated biglycan expression was performed. However, post experimental analysis of human SAA revealed that the group concurrently receiving ad-hSAA1 and the TGF-β inhibitory antibody 1D11 did not express human SAA. A thorough discussion of this experiment can be found in Chapter 7 (Discussion, page 95). This data represents the biggest gap in knowledge within this thesis and also represents the first step in moving forward. To further assess our proposed mechanism we need to understand TGF-β's role in SAA mediated atherosclerosis. Therefore the experiment presented in chapter 7 needs to be repeated. This data is necessary prior to addressing any additional future directions with this project.

8.2.2 Cross the biglycan transgenic mouse to the *apoe*$^{-/-}$ mouse

Beyond addressing the TGF-β inhibition experiments, the fact that the biglycan transgenic mice presented in Chapter 5 were crossed to the *ldlr*$^{-/-}$ is the next issue to resolve. The biglycan transgenic mouse needs to be crossed to the *apoe*$^{-/-}$ mouse, thus a direct comparison of non-stimulated, increased vascular biglycan can be more thoroughly related to the other experiments presented here, were the mice were all *apoe* deficient. The current biglycan transgenic model, crossed to an *ldlr* $^{-/-}$ mouse, was developed for investigating biglycan overexpression as a means of furthering research already begun into the role of angiotensin II increased biglycan expression in the vasculature. It proved to be a very useful tool for eliminating the confounding effects of angiotensin II which are many when discussing atherosclerosis development (53). Although *apoe*$^{-/-}$ and *ldlr* $^{-/-}$ mice are considered the gold standards for modeling atherosclerosis and are quite similar, there are differences worth noting. *apoe*$^{-/-}$ mice will spontaneously develop lesions independent of dietary modification, but another pro-atherogenic insult such as diet is required for atherosclerosis development in *ldlr* $^{-/-}$ mice. *apoe*$^{-/-}$ mice tend to carry the bulk of their cholesterol on remnant particles whereas *ldlr* $^{-/-}$ mice utilize LDL for cholesterol transport. However, the most crucial attribute to *apoe*$^{-/-}$ lesion development is that it closely mimics the progression and severity of human lesions; thus, is more representative of the disease we are ultimately investigating (119). Given that *apoe*$^{-/-}$ mice carry the

bulk of their lipid on chylomicrons and VLDL remnants which are predominantly apoB48 particles, it is plausible that biglycan mediated lipid retention is not the only mechanism by which lipoproteins are retained in an *apoe*$^{-/-}$ mouse. Heparin and lipoprotein lipase have both been implicated in lipid retention as well (120). As it stands now, biglycan transgenic mice on an *ldlr* $^{-/-}$ background have more atherosclerosis independent of other confounding factors, we will need to conduct experiments in the biglycan transgenic *apoe*$^{-/-}$ mouse to see if those data are replicated. The previously proposed experiments would resolve the issues directly related to the thesis work presented. However, several observations made within studies related to this thesis and other work being conducted in our lab introduced questions that should also be investigated as part of a bigger more comprehensive evaluation of SAA mediated atherosclerosis that was beyond the scope of this thesis.

8.2.3 Determine the role of perlecan in SAA mediated atherosclerotic lesions

Our hypothesis that biglycan was the predominant lipid retaining molecule in the vasculature was brought into question by findings in this thesis and other work in our lab. Within our SAA model, we demonstrated a significant increase in atherosclerosis in mice lacking biglycan. In a model of diabetic nephropathy and one investigating angiotensin II induced atherosclerosis, both using a biglycan deficient mouse and wildtype controls, the amount of atherosclerosis was either not different (angiotensin II) or strikingly higher (diabetic model) when

biglycan was absent. In the model of diabetic nephropathy, male *bgn*$^{-/-}$ *x ldlr*$^{-/-}$ and bgn wildtype mice were injected with streptozotocin, to induce a type 1 diabetic phenotype, or citrate buffer and maintained on a 0.12% cholesterol diet for 26 weeks. Atherosclerosis was analyzed in two sites, the aortic intimal surface and the aortic sinus. In both sites, the extent of atherosclerosis was significantly greater in the diabetic mice lacking biglycan (manuscript in preparation). In the model of angiotensin II induced atherosclerosis, *bgn*$^{-/-}$ *x ldlr*$^{-/-}$ mice were given angII or saline infusion followed by western diet for 6 weeks. Interestingly the lack of biglycan was not atheroprotective as was hypothesized. The mice lacking biglycan had the same extent of atherosclerosis in the aortic sinus and on the aortic intimal surface as the wildtype angII infused mice (29). We had previously demonstrated that infusion of angII via alzet minipump increased both biglycan and perlecan expression in mice (34). The angII mediated increase in perlecan was not TGF-β dependent as mice co-treated with angII and the TGF-β inhibitory antibody 1D11 only demonstrated reduced vascular biglycan content, but no effect on perlecan content (29). It is therefore plausible that biglycan exhibits some as yet described regulatory role over perlecan expression. Thus, within our SAA model of atherosclerosis, findings that mice lacking biglycan had increased atherosclerosis could be explained by increased perlecan content. To address this question, vascular tissues should be probed for perlecan using multiple approaches including western blot and immunohistochemistry to determine what is happening to perlecan levels in the presence or absence of biglycan. However, there is a significant caveat that

must be considered when addressing perlecan in atherosclerosis. It has also been shown that, at least in murine lesions, apolipoprotein B containing lipoproteins colocalize with perlecan (37). However, even though perlecan is the primary heparan sulfate proteoglycan in human arteries its expression is markedly decreased within human atherosclerotic lesions (117, 121) .

8.2.4 **Determine what role locally synthesized vs. systemic SAA is playing in the development of atherosclerosis.**

Our study focused on SAA1, one of two isoforms of SAA closely associated with the acute phase response. SAA1 is mainly produced in the liver then secreted into circulation after hepatic stimulation via inflammatory molecules (60). SAA's association with inflammation and its utility as an inflammatory marker for diseases such as atherosclerosis are based on measuring alterations in SAA levels in plasma (122). We and others have demonstrated that, when elevated even briefly, the acute isoforms of SAA are proatherogenic (Chapters 3 & 4, (85). However, the necessity of acute phase isoforms of SAA in the development of atherosclerosis was recently questioned. Male and female *apoe*$^{-/-}$ mice were compared to *apoe*$^{-/-}$ mice also deficient in both SAA 1.1 and SAA 2.2. All mice were fed a chow diet for 50 weeks. Atherosclerosis was analyzed on the arch, thoracic and abdominal intimal surface and no differences were observed between mice regardless of the presence or absence of SAA. The experiment was repeated, replacing the normal rodent chow previously used with western diet for 12 weeks. The results were the same; there were no

observed differences in atherosclerosis between groups (123). These data indicate that acute SAA may be important for the progression of early atherosclerosis; however, it may not be required.

SAA has been found in atherosclerotic lesions of both *apoe*$^{-/-}$ *and ldlr*$^{-/-}$ mice co-localized with apolipoprotein-AI and apolipoprotein B containing lipoproteins (83). We have also demonstrated SAA's presence in lesions of *apoe*$^{-/-}$ mice (data not shown); however, the specific isoform of SAA within the lesion has never been determined. Interestingly, many cell types found in atherosclerotic lesions have been shown to express SAA including macrophages, endothelial cells, vascular smooth muscle cells and adipocytes (124). Thus, further investigation of the lesional content and specific isoforms of locally synthesized SAA within atherosclerotic lesions would be beneficial. I propose utilizing laser capture microscopy to investigate lesions of *apoe*$^{-/-}$ mice compared to lesions of *apoe*$^{-/-}$ mice also deficient in SAA 1.1 and SAA 2.1 to determine if the expression of SAA3, the extrahepatic, acute isoform of SAA in mice is altered between the different groups. The SAA3 isoform does not contribute to circulating SAA so its expression levels cannot simply be accessed by plasma analysis using ELISAs. If SAA3's expression levels were higher in the SAA 1.1 and SAA 2.1 deficient mice it would imply that the acute phase SAAs of hepatic origin may exhibit some down regulation of SAA3 that is not observed when SAA 1.1 and 2.1 are absent. This could also imply that SAA3 is conditionally proatherogenic in the absence of SAA 1.1 and 2.1

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Publications:

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