

Autoimmunity in the Abdominal Aortic Aneurysm and its Association with Smoking

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Abstract

Smoking increases the risk of abdominal aortic aneurysm (AAA) in both humans and mice, although the underlying mechanisms are not completely understood. An adventitial aortic antigen, AAAP-40, has been partially sequenced. It has motifs with similarities to all three fibrinogen chains and appears to be connected in evolution to a large family of proteins called fibrinogen-related proteins. Fibrinogen may undergo non-enzymatic nitration, which may result from exposure to nitric oxide in cigarette smoke. Nitration of proteins renders them more immunogenic. It has recently been reported that anti-fibrinogen antibody promotes AAA development in mice. Also, anti-fibrinogen antibodies are present in patients with AAA. These matters are reviewed in the overall context of autoimmunity in AAA. The evidence suggests that smoking amplifies an auto-immune reaction that is critical to the pathogenesis of AAA.

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Key Words:

Abdominal aortic aneurysm • Smoking • Autoimmunity • Nitric oxide • Fibrinogen

Introduction

Compared with the relatively small effects of genetic mutations or polymorphisms on the risk for abdominal aortic aneurysm (AAA), smoking presents a much greater risk by as much as an order of magnitude. For example, the risk-odds ratio of a well-established polymorphism associated with AAA, DAB2IP

on chromosome 9q33.2, is 1.2 [1], and the risk-odds ratio for another polymorphism on 9p21 is 1.3 [2]. By contrast, the risk-odds ratio for heavy smoking is 12.0 [3], comparable only to the risks of age and male sex. Smoking also promotes aneurysm formation in laboratory mice [4, 5]. A molecular explanation for the effect of smoking has been proposed [6]. The purpose of this communication is to explain the hypothesis that nitric oxide (NO) in tobacco smoke nitrates a protein related to the fibrinogen superfamily in the aortic adventitia and thus enhances its antigenicity. In particular, I discuss four factors that are often associated with diseases of autoimmunity [7, 8]: genetic susceptibility, inflammation, matrix metalloproteinases (MMPs), and antibodies and autoantigens.

Human Genetics of AAA

Clifton reported three brothers affected with AAA in 1977 [9]. In 1978, the author operated on a friend and colleague to repair an AAA and bilateral popliteal artery aneurysms on separate occasions. The patient was otherwise healthy, fit, normotensive, and normocholesterolemic. His angiographic studies were unremarkable except for his aneurysms. There was no detectable atherosclerosis. His mother had died of a ruptured AAA, raising the question of whether a genetic predisposition was at work. In 1980, we reported on numerous differences between groups of patients undergoing aortic surgery for AAA versus occlusive (OCC) disease [10]. These differences (age, sex,



and other features) provided a basis for questioning the notion that atherosclerosis causes AAA. These anecdotes are just a few factors that drew me into a career-long interest in the human genetics of AAA and the pathobiology of the disease.

Our first effort at clinical genetics in 1984 was a small study of patterns of inheritance in 14 families with a total of 41 affected members [11]. The conclusion from this analysis was that there might be X-linked and autosomal dominant forms of the disease. Extending this study to include a total of 50 families, we concluded that if there was only a single locus explaining the inheritance of AAA disease, it was likely to be autosomal dominant [12].

Many confirmations of the hypothesis of a genetic association followed. The first study with a proper control group was performed by Johansen and Kopsell [13], who reported on the family histories of 250 AAA patients and 250 control patients. Whereas 19.2% of AAA patients reported an affected first-order relative, only 2.4% of control patients were aware of an affected relative. The possibility that being born into a smoking family might have a “paragenetic” effect was not considered in the early studies. I use the neologism “paragenetic” to suggest that a phenotype may run in families without a genetic basis. For example, if both parents are non-smokers and disapprove of smoking, it seems less likely that their children will take up the habit.

Initial efforts to root the genetic hypothesis in a molecular basis involved the candidate gene approach [14]. Our first contribution implicated the TIMP-1 gene [15], which was subsequently confirmed by a larger study [16]. Another interesting candidate was the major histocompatibility complex molecular locus DR-15 (originally protein DR-2) [17, 18]. Other investigators also showed interest in the major histocompatibility complex [19]. In addition, we found an identical amino acid substitution in the ferritin light chain gene in 2 of 19 AAA patients [14].

Collagen XI-alpha 1 was of special interest to our laboratory for multiple reasons. It was overexpressed 38-fold in a AAA-derived fibroblast cell line (a clear outlier) relative to a cell line from normal aortic fibroblasts. It was subject to alternative slicing, with cartilage- and aorta-specific variants in exon 6. Furthermore, it was selectively expressed in the adventitia

of the normal aorta. Sequencing of exon 6 revealed a heterozygous G>T signal 70 bp upstream from exon 6 in 14 of 19 consecutive AAA samples [14].

A plethora of additional genes were proposed by others, and the situation became more complicated. In 2007, Sandford et al. summarized a decade of effort [20], concluding that “while the candidate gene approach has led to significant advances in understanding the pathogenesis of AAA, it is unlikely that a single gene polymorphism will hold the key to aneurysm formation. Whole gene studies are likely to be required...”. These studies are now referred to as genome wide association studies (GWAS).

An advantage of GWAS is that no knowledge of AAA pathobiology is required, as it is a brute force approach. A disadvantage is that it is inherently large-scale and resource-intensive. A recent meta-analysis of six GWAS datasets with a total of 10,204 AAA patients and 107,766 control patients had 116 multinational co-authors [21]. Four novel single nucleotide polymorphisms (SNPs) were identified and added to a list of five others identified from previous studies. The pathobiological roles of all nine SNPs in AAA disease remain unknown. The summary situation is mostly unchanged since the review by Saratzis et al. in 2015 [22], which concludes that AAA represents a “multifactorial disease, with the likelihood that there are multiple variants of very low effect contributing to the overall genetic disease risk.” Thus, the genetic approaches that seemed so promising 25 years ago have failed to uncover a “smoking gun.”

This discussion would be incomplete without mentioning that we are now entering a “post-GWAS” era [23]. Another problem with GWAS is that the variations detected so far occur in non-coding DNA sequences, so their significance is not obvious. An alternative has been adoption of the epigenetic approach. Epigenetics refers to stable differences in gene expression that are “not attributable to DNA sequence variation” [24]. Joehanes et al. [25] report that the “epigenetic signatures of cigarette smoking” reflect “a broad impact on genome-wide methylation.” In addition to methylation, the study of microRNAs is another example of an epigenetic modification. No doubt there will be interesting developments in these areas in the future.

The Role of Inflammation

In 1986, Beckman proposed that autoimmunity plays a role in the pathogenesis of AAA [26]. Beckman observed an infiltration of plasma cells in 31 of 156 AAA specimens from patients who were not thought on clinical grounds to be examples of the “inflammatory variant” of aneurysms. He speculated that there was an immune reaction against atherosclerotic elements in the AAA wall, consistent with the view at that time that atherosclerosis causes non-specific aneurysms (the term “non-specific” is used because the Joint Committee on Reporting Standards, sponsored by the Society of Vascular Surgery and International Society for Cardiovascular Surgery, recommended in 1991 that this terminology replace the conventional usage “atherosclerotic aneurysm” due to insufficient evidence that atherosclerosis actually caused the disease [27]).

Another example of a Th2 (allergic or autoimmune) response appeared in 1990 [28], when I was invited to write a book chapter on the pathology of AAA. The assistance of a pathologist at Yale University School of Medicine was enlisted (G. J. W. Smith), and a “séance” was arranged to compare a tray of AAA slides with a tray of atherosclerotic aortic slides. One of the most conspicuous findings was the virtual absence of iron-hematoxylin-reactive elastin in the media of AAA specimens. Another finding was that there was inflammatory infiltrate in AAA at the junction of the outer media with the adventitia. In some cases, this infiltrate was so impressive that our pathologist remarked that it was reminiscent of that seen when syphilis was common. The infiltrate was primarily plasmacytic, and there were Russell bodies within areas of inflammation.

Russell bodies are eosinophilic aggregates of immunoglobulin G (IgG) seen in plasma cells undergoing excessive synthesis of antibodies in autoimmune conditions. They reflect an overstuffed endoplasmic reticulum. The role of inflammation in the etiology of AAA was further elaborated by Brophy et al. in 1991 [29]. A conspicuous mononuclear infiltrate was present in 8 of 10 specimens, primarily located at the adventitial/medial junction. The presence of Russell bodies was confirmed. In addition, large quantities of immunoglobulins were extractable from AAA tissue by affinity to protein A.

Evidence for a Th-1 (cytotoxic) response appeared in 1990, when Koch et al. formally proposed that “immunophenotypic analysis suggested an immune-mediated response” in non-specific AAA [30]. The analysis was based on 32 specimens: 4 normal aortas (NLs), 6 OCC aortas, 17 AAAs, and 5 inflammatory AAAs (IAA). Monoclonal antibodies were used to identify the various cell types. The extent of inflammatory infiltrate was IAA > AAA > OCC > NL. The authors interpreted these findings as a spectrum, suggesting that a single disease progressed from OCC to AAA. I advanced the notion that AAA and OCC are two separate disease processes with common risk factors like smoking and hypertension. The aneurysm might actually initiate atherosclerotic degeneration of the subendothelium [31, 32]. A study in mice suggests that aortic dissections precede the onset of atherosclerosis [33]. Other investigators have suggested that the two processes may be “running in parallel” [34].

More recent studies have significantly expanded the evidence for a Th-1 response in AAA [35, 36]. Amplification of beta-chain T-cell receptor transcripts from AAA lesions suggests that 9 of 10 patients have substantial proportions of identical polymerase chain reaction products. The authors conclude that “AAA is a specific antigen-driven T-cell disease.”

Matrix Metalloproteinases

MMP activation or deregulation is a typical feature of autoimmune diseases. Loss of elastin is a sentinel histochemical feature of AAA, and Reilly et al. were the first to report that the “killer elastase” is a metalloproteinase with a molecular weight of ~80 kDa [37]. We subsequently identified it as gelatinase B or MMP-9 [38]. Mammalian collagenase (MMP-1) [39] and stromelysin (MMP-3) [40] are also present. Immunohistochemically, MMP-9 is co-distributed with macrophages, whereas MMP-1 is associated with endothelial cells of neovascularizing vessels and mesenchymal-like cells in the infiltrate [41]. MMP-2 is expressed by a fibroblast cell line cultured from a human AAA explant but not by a normal aortic cell line. The MMP-2 cell line is stable in its expression of MMP-2 over multiple passages (unpublished observations). Cytokines that regulate these MMPs are also elevated in AAA [42].

These observations are interesting, but they are phenomenological and do not establish causal relationships. The development of gene knockout (KO) technology in inbred mice has led to a new era in AAA experimentation. For example, Pyo et al. [43] report that AAA does not develop in MMP-9 null/null mice in the elastase-perfusion model. The AAA-susceptible phenotype is "rescued" by bone marrow transplantation (macrophages) from wild-type mice. These studies show that MMP-9 is a necessary condition for AAA. A similar strategy was employed by Longo et al. [44] in the abluminal (peri-adventitial) calcium chloride model. No aneurysms occurred in MMP-9 or MMP-2 KO mice, suggesting that both macrophage- and mesenchymal-derived MMPs are required and may work in concert to produce AAA.

It is possible that the nicotine in burning tobacco (and not some other component of the smoke) initiates or promotes the smoking/AAA association. Nicotine exposure equivalent to plasma levels in smokers augments MMP-9 and MMP-2 expression by macrophage and vascular smooth muscle cell lines *in vitro* [45]. Of course, *in vitro* findings do not always translate smoothly to the situation *in vivo*. Bergoeding et al. [4] found that cigarette smoking increases aortic dilatation *in vivo* without affecting the expression of MMP-9 or MMP-12. Wang et al. [46] report that acute infusions of nicotine produce aneurysms in Apo-E-deficient mice, but the aneurysms that occur in this model resemble those induced by angiotensin (Ang) II. In our experience, these AAAs begin as aortic dissections and not as a typical fusiform AAAs. Guo et al. [47] report that the JNK inhibitor, SP600125, attenuates the nicotine plus Ang II model of AAA formation, but again, these AAAs are false aneurysms.

Maegdefessel et al. [48] report that nicotine pellets (versus placebo) augment aneurysmal expansion in the elastase-perfusion model. This model avoids some of the difficulties of the Ang II model. It also avoids the problem of "smoker's hypertension" that develops in chronically smoke-exposed mice [49], which may independently promote AAA formation. Blood pressure measurements at 14 days showed no difference between nicotine and placebo groups, but more extensive observations might be useful to rule out this potentially misleading possibility. Of course, a direct effect of nicotine in pellets and enhancement

of autoimmunity by smoke are not mutually exclusive as valid explanations of the smoking/AAA association.

Antibodies and Autoantigens

Capella et al. [50] carried out experiments to determine whether increases in IgG in AAA tissue are subclass-specific (by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies) and whether the IgG complex is associated with increases in complement C3. Seven AAA, four OCC, and two NL aortic specimens were evaluated. Comparing by subclass, IgGs were elevated in AAA over NL by 193× for IgG1, 160× for IgG2, 389× for IgG3, and 627× for IgG4. Increases in IgGs in AAA specimens over NL and OCC specimens by subtype were statistically significant ($p < 0.01$). ELISA showed a 125× increase in immunoreactive C3 in soluble extracts of AAA, and Western immunoblots revealed multiple C3 immunoreactive isoforms or degradation products.

With evidence that immunoglobulins are recoverable from AAA tissue, interest intensified to find the aortic autoantigen(s). Gregory et al. [51] purified Ig from serial AAA specimens and found that it was immunoreactive with a protein of ~80 kDa in Western blots of 11 of 14 (79%) AAA patients versus 1 of 9 (11%) control patients ($p = 0.002$, Fisher's exact test). By immunohistochemistry, antibodies extracted from AAA specimens were immunoreactive with a microfibrillar protein in the matrix of the adventitia of the normal aorta.

This experiment was repeated by Chew et al. [52], who confirmed that AAA immunoglobulins are immunoreactive with a matrix protein of ~80 kDa, extractable from aneurysmal aortas by high concentrations of guanidinium hydrochloride. We inferred that this was a dimeric form of an AAA antigenic protein with a molecular weight of ~40 kDa, which had been affinity-purified with antibodies from AAA specimens [53]. This protein was named AAAP-40 for aneurysm-associated antigenic protein 40 kDa.

The partial amino acid sequence of AAAP-40 was determined. It had homology to a protein in pig with an aorta-specific tissue distribution. The porcine protein had been discovered and named microfibril-associated glycoprotein 36 kDa by Kobayashi et al. [54]. AAAP-40 has seven tyrosine residues, including one

tyrosine doublet, which are prime sites for nitration [55]. AAAP-40 also has short homologies with all three chains of fibrinogen, suggesting that its evolutionary history placed it close to the common ancestor of the three modern chains. We calculated the evolutionary distance of AAAP-40 from fibrinogen-beta (measured in point-accepted mutation rates [PAMs]; among fibrinogen-related proteins [FRePs], a PAM unit is about a million years). Only 30 PAMs separated AAAP-40 from fibrinogen-beta [56]. These results agreed with a neighbor-joining tree computing the relatedness of MAGP-36, AAAP-40, MFAP-4, and a “fibrinogen-like protein of the sea cucumber” [57].

Antibody against a unique amino acid sequence of AAAP-40 (not found in any other mammalian protein) was immunoreactive with adventitial microfibril of the human aorta and selected other vessels [58, 59]. This antibody was also immunoreactive with a microfibrillar protein in mouse aortic adventitia [60]. Figure 1 illustrates that a commercial antibody against fibrinogen-beta is immunoreactive with a microfibrillar protein in the human aortic adventitia. Figure 2 shows

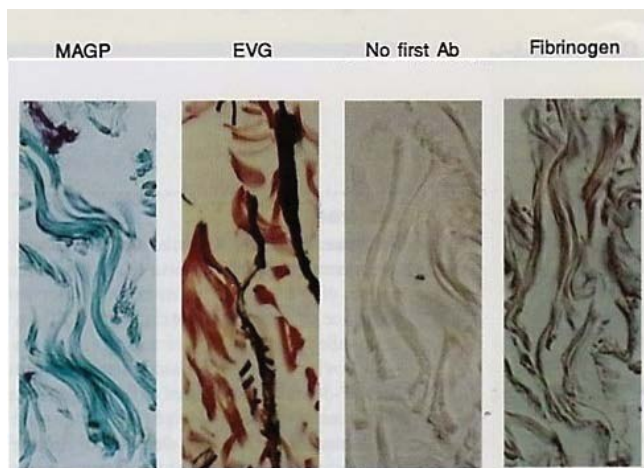


Figure 1. Tissue sections from the adventitia of a normal human (cadaver donor) aorta photographed at 400x. An immunohistochemical stain (Gomori's aldehyde fuchsin) was used for microfibril-associated glycoprotein (MAGP). Verhoeff's elastic stain (EVG) was used for collagen (pink) and elastin (black). Immunohistochemistry employed rabbit anti-human fibrinogen as a primary antibody and goat anti-rabbit Ig as a secondary antibody. No primary antibody was used as a control. MAGP and fibrinogen co-distributed with collagen (pink) in EVG staining. The elastin of the adventitia did not have the lamellar organization characteristic of the arterial media.

that AAAP-40 is site-specifically expressed in the human arterial tree. It is abundant in the aorta, common iliac, internal iliac, and popliteal arteries but is barely detectable in the external iliac artery. This distribution corresponds to the susceptibility of the different vessels to aneurysm formation [10, 61]. Taken together, these observations suggest that the AAA autoantigen is a normal constituent of the aortic adventitia and not a product of atheromatous degeneration.

Smoking Promotes AAA in Mouse Models

Stolle et al. [5] report that cigarette smoke promotes AAA in an Ang II/Apo-E-deficient mouse model. Although it is notable that smoke exerts effects in this model, we found that APO-E deficiency is not required for AAA formation. We had success in inducing AAA in normal C57/BL6 mice that were selected only for advanced age (i.e., retired breeders) [62, 63].

Bergoeing et al. [4] adapted the elastase perfusion mouse model to determine whether tobacco smoke lowers the threshold of aortic injury required for AAA development. The adaptation involved changing the concentration of elastase in the perfusate, resulting in high-dose, standard-dose, and low-dose regimens. Exposure to tobacco smoke began 2 weeks before perfusion and continued until sacrifice 2 weeks after

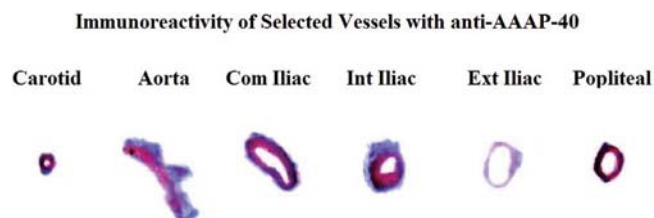


Figure 2. Immunohistochemical studies of human arterial segments from the autopsy of a patient with no abdominal aortic aneurysm (AAA) and minimal occlusive disease. The antibody was rabbit anti-human AAAP-40, raised against a synthetic peptide, based on a unique sequence not found in any other mammalian protein (-GMAKEYDGFQYT). Staining was performed in one batch for all sections. Immunoreactive protein stained blue in the adventitia of the aorta and the aneurysm-prone popliteal, internal, and common iliac arteries. The aneurysm-resistant external iliac artery exhibited minimal staining. We were surprised to note staining of the carotid artery, but this finding may explain the elongation often seen in AAA patients.

perfusion. Smoking resulted in larger aneurysms in low-dose mice (aortic diameter increased by 134%) compared with non-smoking low-dose mice. There was no difference in MMP-9 expression between smoking and non-smoking mice.

In parallel with human AAA disease, after a mouse model has been initiated by smoke exposure, enhanced expression of the aneurysm phenotype persists even after a smoke-free interval. Jin et al. [64] found that after smoke exposure for 6 weeks followed by no smoke exposure for another 6 weeks, the promoting effect was still in force. They proposed three possible explanations for the smoking effect: 1) a persistent increase in MMP activity; 2) a persistent direct injury to the aorta; or 3) promotion of an immune response. They found that smoking overrode MMP effects, as the smoking effect persisted even in doxycycline-treated mice or those with null/null elastolytic deficiencies. Injury to the aorta was ruled out by direct electron microscopic studies. Thus, the promotion of an immune response was favored because leukocytes from smoke-exposed mice localized to the aneurysms of smoke-free mice and increased their aortic diameters. Apparently, after leukocytes are initiated, possibly by exposure to nitro-tyrosine in aortic FRePs, they acquire memory for promoting an autoimmune response.

Zhou et al. [65] identified a natural IgG antibody in mice that binds to fibrinogen and initiates the inflammatory response that culminates in AAA development. They found that a mouse anti-fibrinogen antibody enhances AAA formation. Also, they reported that AAA patients have circulating antibodies against "fibrinogen or fibrinogen-associated epitopes in human aneurysmal tissue". This finding confirms the rationale for the use of ELISA to detect antibodies as a screening test for the disease as proposed by Knoetgen et al. in 1997 [66].

Smoking-Induced Nitration of Plasma and Tissue Proteins

Tyrosine nitration is a modification that may change the rate of proteolytic degradation of nitrated proteins. Protein nitration in cardiovascular diseases has been extensively reviewed by Turko and Murad [67]. The au-

thors note that the process is selective (e.g., residue-, protein-, and tissue-specific). Not all tyrosine residues of a protein are nitrated, and not all proteins are targets for nitration. Typically, one or two tyrosine residues are site-specifically nitrated. The product of nitration is 3-nitro-tyrosine, which has been established as a biomarker of "nitro-oxidative stress" [68].

Nitrotyrosine in plasma proteins is increased in smokers with chronic obstructive pulmonary disease, consistent with "increased nitration associated with inflammatory processes" [69]. Nitrated plasma proteins are also increased in lung cancer patients [70]. Fibrinogen is among the plasma proteins that are nitrated.

Our interest in this subject matter arose in the context that cigarette smoke contains substantial amounts of NO [71]. We found that the nitrite content of AAA tissue is comparable to levels that were once considered possibly carcinogenic in smoked sausage [72]. This concentration of nitrite (but not nitrate) damages the aortic matrix *in vitro* [73] and leads to tyrosine depletion in a solution of collagen [74]. Immunogenicity of tyrosine-nitrated self-proteins has been reviewed in the context of understanding the mechanisms leading to autoantibody production [55]. Structurally modified self-proteins give rise to new epitopes to which T and B lymphocytes are not tolerant. These nitrated proteins elicit cellular and humoral responses in mice.

The Smoking/Autoimmune Hypothesis

AAA has features of an autoimmune disease. Inflammation is rampant, and autoantigens are identifiable with purified auto-antibodies. The principal auto-antigen appears to be a microfibrillar protein with similarities to fibrinogen localizing to adventitia of the aorta. It has seven loci for potential tyrosine nitration. NO in tobacco smoke may nitrate this protein, which would be expected to enhance its immunogenicity. Anti-fibrinogen antibodies promote AAA in mouse models. Finally, antibodies against fibrinogen are detectable in humans with AAA. These findings suggest that smoking heightens autoimmunity in AAA in humans and mice. The autoimmune hypothesis does not exclude other possible explanations for the smoking/AAA association.

Conflict of Interest

The author has no conflict of interest relevant to this publication.

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