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Arteriosclerosis: The Novel Finding of Biofilms and Innate Immune System Activity within the Plaques

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Abstract

Atherosclerosis (AS) is a chronic disorder characterized by the formation and progression of plaques within arteries. Various microbes, most notably periodontal organisms, have been identified in plaques both epidemiologically and microbiologically, and have been deemed possible contributors to the disease. In this work, we have queried whether microbes acted similarly in AS, when compared to other chronic diseases such as atopic dermatitis, psoriasis, and Alzheimer's disease, by making biofilms to protect themselves and evade the immune system. In those diseases, the microbes created biofilms that activated the innate immune system reactant Toll-like receptor 2 (TLR2).

We examined 12 endarterectomy specimens using probes similar to those used in our previous examinations of the above diseases. Specifically, we stained the pathology specimens with hematoxylin and eosin (H+E), and periodic acid Schiff (PAS); the PAS stain would reveal the extracellular polysaccharides that forms the mass of the biofilm. Congo red, which stains the amyloid that forms the infrastructure of biofilms, was also performed. Immunostaining with CD 282 was performed on each specimen for evaluation of TLR 2.

Twelve of twelve atherosclerotic plaques showed the presence of biofilms and activation of TLR 2; this is entirely similar to our findings in atopic dermatitis, psoriasis and Alzheimer's disease. The TLR 2 seen in the specimens suggests that biofilms in atherosclerotic plaques may contribute to the progression of the disease as a result of their ability to contribute to chronic inflammation and continued immune system activation. Lipids have long been considered to be the major focus of atherosclerosis, but our recent work suggests that biofilms, due to their ability to induce a chronic inflammatory state, may be another determinant in the progression of atherosclerosis. In the future, we hope to characterize the microbes-with an initial focus on periodontal microbes because of the calcification in the plaques-that directly contribute to biofilm formation and propagation.

Keywords: Arteriosclerosis; Biofilms; Innate immune system; TLR2

Introduction

It has been well established that atherosclerosis is an inflammatory disorder characterized by the formation of plaques in arteries [1]. However, the etiology of chronic inflammation within atherosclerotic plaques has not been clearly defined. As regards pathogenesis of the disease, bacteria and viruses have been found in the plaques [1] and periodontal organisms have been linked epidemiologically [2]. However, none of these specific organisms has been fully implicated in causation. Moreover, the presence and impact of biofilms in the progression of atherosclerosis has not been addressed. Lipids have previously been considered to play the major role in the pathogenesis and progression of atherosclerosis, but it is likely that microbes and biofilms contribute significantly.

Our previous findings in atopic dermatitis have shown that *staphylococci* that are normal skin flora make biofilms, occlude sweat ducts and activate the innate immune system via TLR2. This contributes significantly to chronic inflammation [3]. Similar findings have been shown in psoriasis, Lyme disease, chronic arthritis, and Alzheimer's disease [4]. We have examined 12 endarterectomy specimens with similar probes as compared to atopic dermatitis, psoriasis, and Alzheimer's disease to evaluate whether biofilms were present in the arteriosclerotic plaques and whether TLR2 was activated. This gains significance inasmuch as the plaques have already been shown to contain microbes.

Methods

This was a preliminary observational study without blinding the observers, without utilizing controls, and without statistical analysis. The purpose was to determine whether biofilms and TLR2 could be found in arteriosclerotic plaques. Twelve endarterectomy specimens (4 of the right femoral artery, 1 of the left femoral artery, 3 of the right carotid artery and 4 of the left carotid artery) from patients (8M, 4F; ages 54-82) with severe atherosclerotic disease were assessed by 5 examiners. All specimens were stained with hematoxylin and eosin (H +E), periodic acid Schiff (PAS), and Congo red stains. Immunostaining with CD 282 (TLR2) antibody (BioLegend) at a titration level of 1:50 was utilized. Routine light microscopy was employed for evaluation.

Results

The atherosclerotic plaques showed fragments of smooth muscle wall, intracellular and extracellular lipid, along with an extracellular matrix, including collagen, some elastic fibers with various degrees of degeneration, calcification and biofilms. These components occurred in varying proportions and configurations in different lesions. Usually, there was a superficial fibrous cap composed of smooth muscle cells and dense collagen. Some specimens displayed more cellular areas containing macrophages, lymphocytic infiltrate and plump fibroblasts. Underneath the cap there were necrotic cores, containing lipid (cholesterol clefts), debris from dead cells, and lipid-laden macrophages. Variably organized thrombus formation and biofilms were present. Some specimens demonstrated neovascularization, while others were composed almost exclusively of smooth muscle and sclerotic tissue. The PAS and Congo red stains highlighted the presence of biofilms, sometimes intimately associated with the calcified areas (Figures 1-4). (Congo red stains the amyloid fibers that form an infrastructure for biofilms) [2] CD 282, expressive of TLR2, showed areas of reactivity within the extracellular matrix containing the biofilms (Figures 5 and 6).

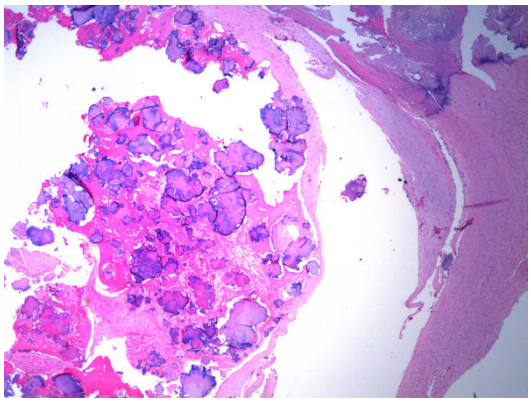

Figure 1: In the lumen of this artery, there is a plaque which shows cholesterol clefts and calcium deposits. PAS positive material (biofilm), surrounding the calcium deposits, is present (PAS 2.5X).

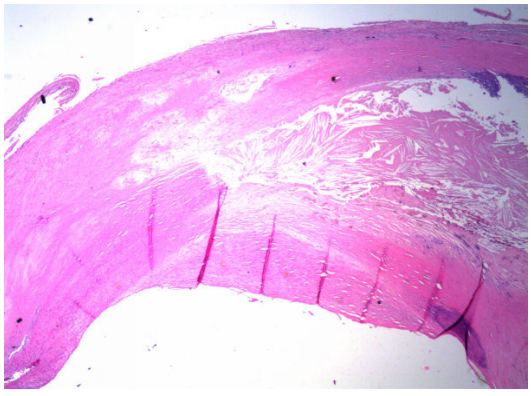

Figure 2: In the arterial wall, the cholesterol clefts are engulfed with PAS (biofilm) positive material (PAS 4X).

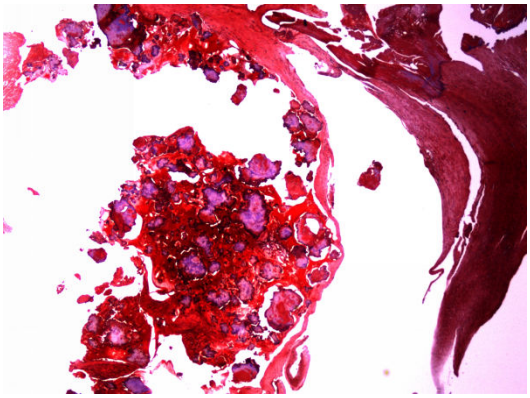

Figure 3: Same vessel as Figure 1; Congo red positive material (amyloid within biofilm), surrounding the calcium deposits, is present (Congo re 2.5X).

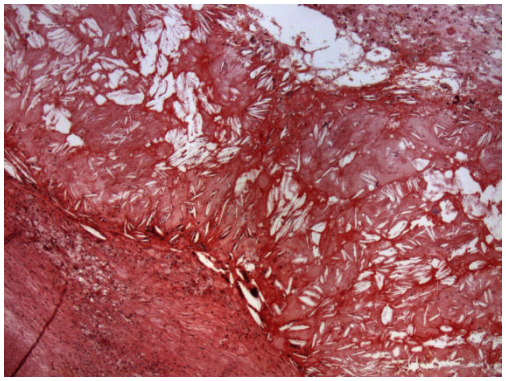

Figure 4: Same vessel as Figure 2; Congo red staining material (amyloid within biofilm), surrounding the cholesterol clefts, is present (4X).

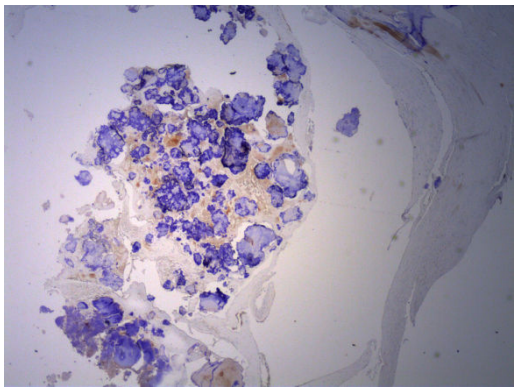

Figure 5: Same vessel as Figure 1; TLR 2 staining positively amongst the calcium deposits. CD 282 2.5X.

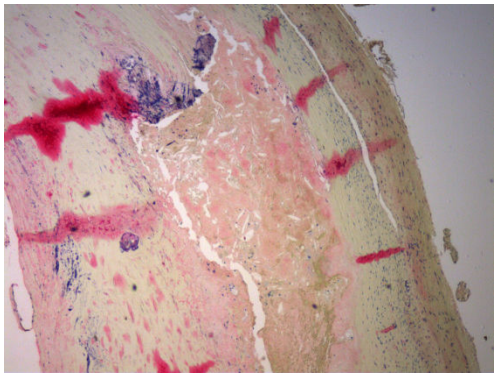

Figure 6: Same vessel as Figure 2; TLR 2 staining positively (red chromophore) amongst the cholesterol clefts (CD 282 4X).

Discussion

Our findings indicate biofilms are present in the arteriosclerotic plaques in the pathology specimens from patients undergoing endarterectomies. These biofilms show the same pathological staining characteristics as those that we previously have noted in atopic dermatitis, psoriasis and Alzheimer's disease. In the skin diseases, the biofilms were created by *staphylococci* and *streptococci*; in Alzheimer's disease, spirochetes created them. We have also shown the activation of TLR2 in each previously studied disease and postulated how that activation fit into the pathogenesis of each individual disorder [4]. In the current study, it is of interest that the biofilms are present in the same location as is the calcification. It is intriguing to postulate that they are produced by dental organisms because these organisms are capable of producing both the calcification and the biofilms.

Despite once being considered a disease of progressive plaque formation, atherosclerosis is now also considered to be a chronic inflammatory condition. After decades of sub-endothelial accumulation of LDL particles, the LDL particles become susceptible to oxidative modifications that lead to activation of endothelial cells and induction of the expression of adhesion molecules and secretion of chemokines [5-7]. Leukocytes are recruited and migrate to the location of the damaged endothelium, and inflammation progresses with up-regulation of pro-inflammatory mediators. This is one potential mechanism for plaque formation. Notably, TLRs can be activated by oxidized LDL, an endogenous ligand, and contribute to the inflammatory cascade [6,8].

TLRs respond to a wide range of pathogen associated molecular proteins (PAMPs). Classically, TLR2 has been thought to respond to gram-positive organisms, while TLR4 responds to the lipopolysaccharide component of the gram-negative cell wall [3]. In our exploration of atopic dermatitis, we focused on the role of TLR2 as the primary responder to gram-positive organisms [3]. In our work investigating the role of biofilms in Alzheimer's disease, we described the activation of TLR2 in response to the extracellular polysaccharide slime [9]. Recently, TLR2 has also been shown to be attracted to Curli fibers-a functional amyloid component of the biofilm matrix that allows bacteria to bind to surfaces. Specifically, TLR2/1 complexes recognize Curli fibers and are capable of initiating the immune response [10,11]. TLR2 generates TNFα and NFκB by means of the myeloid differentiation 88 (MyD88) pathway. Because TNFα cannot penetrate biofilms, it has been theorized that TNFα instead damages the surrounding tissue [9]. Thus, it is likely the immune system damages the tissue and not the microbes or the biofilm [9].

Our endarterectomy specimens demonstrated the presence of TLR2, which supports our previous findings that biofilms initiate the activation of TLR2 [9]. Our finding is also consistent with previous studies that have shown up-regulation TLR1, TLR2, and TLR4 in human atherosclerotic plaques [12]. TLR2 has been shown to cause macrophage lipid accumulation, induce proliferation in vascular smooth muscle cells, and initiate an inflammatory reaction in endothelial cells [13-16].

TLR2, widely known to recognize components of the gram-negative cell wall, is required for the innate response to *Porphyromonas gingivalis* [17]. In animal studies, *P. gingivalis*-mediated TLR2 activation has been shown to contribute to atherosclerosis progression. In human studies, TLR4 activation was also implicated in the signaling cascade resulting in the activation of NFκB transcription factor and pro-inflammatory proteins, which propels the inflammatory reaction and causes destabilization of atherosclerotic plaques [18]. Systemic TLR4 expression, mRNA and protein, on circulating monocytes were increased in patients with acute myocardial infarction and stable angina as compared to non-ischemic patients [19]. These findings suggest the TLR4 is another possible area for investigation in our future work.

Recent studies have hypothesized that bacteria within plaques are likely a result of colonization by biofilms resistant to antimicrobial treatment which allows them to be contributors to a chronic pro-inflammatory state [20,21]. Many organisms, including *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, *Helicobacter pylori*, influenza A virus, hepatitis C virus, and HIV, have been associated with a higher risk of cardiovascular disease, though the strength of the supporting data varies. Additionally, researchers have been unable to isolate viable organisms from atherosclerotic plaques for all of the previously listed pathogens, with the exception of *Chlamydia pneumoniae*, an obligate, intracellular, anerobic pathogen [1]. Moreover, the identification of *Chlamydia pneumoniae*, *Helicobacter pylori*, and *Porphyromonas gingivalis* DNA within atherosclerotic plaques suggests that these agents are able to gain access to the vasculature and contribute to the state of chronic inflammation [7].

Periodontitis is a biofilm-induced chronic inflammatory disease that destroys the periodontium and has long been associated with cardiovascular disease [22]. There are many factors that contribute to the severity of periodontal disease, including genetic heritability and immune system dysregulation [23]. There are also many bacteria in the oral cavity, and the progression of periodontal disease has been linked to multiple organisms, including *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* [7]. More recently, a model of polymicrobial synergy and dysbiosis has been proposed as the mechanism for disruption of host-microbe homeostasis that leads to deregulation of the inflammatory response development of disease [24]. Though the presence of DNA from periodontal pathogens has been demonstrated in atherosclerotic lesions, the mechanism by which the bacterium reaches systemic sites remains unclear.

We have described the role of biofilms and the innate immune system in the pathogenesis of atopic dermatitis, psoriasis, chronic arthritis, Lyme disease, and Alzheimer's disease [4,25]. Almost all organisms are capable of producing biofilms, which consist mostly of

extracellular polysaccharides. TLR2 is activated by biofilm-forming microbes, and generates an inflammatory response.

The conclusion from our findings is that we have found biofilms in atherosclerotic plaques in all the arteriosclerotic plaques from endarterectomy specimens. The innate immune system in the presence of TLR2 was also present. These findings likely contribute to the progression of the disease as a result of their ability to contribute to chronic inflammation and continued immune system activation. Lipids have long been considered to be the major focus of atherosclerosis, but our recent work suggests that biofilms, because of their ability to help induce a chronic inflammatory state, may be another determinant of the progression of atherosclerosis. In our future work, we hope to characterize the microbes within atherosclerotic plaques that directly contribute to biofilm formation and propagation.

Limitations of this study include small sample size and lack of control specimens. This also limits statistical evaluation. If we were to include negative controls (as one would see with normal arteries) the "p" value would be 0.001 where all the specimens were positive and all the controls were negative.

As an aside, there are many commonly prescribed medications that have additional abilities to act as systemic biofilm dispersers. These include piperidines (donepezil, haloperidol, risperidone), thiophenes (olanzapine), furans (citalopram), and pyrroles (leflunomide, itraconazole, celecoxib, and atorvastatin) [26-30]. The capability of atorvastatin to act as a biofilm disperser (in addition to being a cholesterol lowering agent) is of particular interest; biofilm dispersion that occurs near the proximal end of the fibrous cap in an atherosclerotic plaque may affect plaque stability, which may increase the risk of plaque rupture and adverse downstream cardiovascular events [31].

Funding

All studies were done with the approval of the Drexel University College of Medicine Institutional Review Board and the Drexel Pathology Tissue Bank.

Conflicts of Interest

No author has conflicts

Authors Contribution

D Morales and S Ballal and H Allen are pathologists; S. Joshi is a microbiologist, J Boles is a medical student working with Dr Allen, Drs Allen and Joshi were involved with study design; the pathologists interpreted the specimens; all had input into writing and editing the document.

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