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A Possible Role of Bacterial Biofilm in the Pathogenesis of Atherosclerosis

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Abstract

Multiple culture and molecular based studies have established the presence of bacteria in atherosclerotic plaques. Although bacteria are present within the plaque, there is no clear understanding or putative pathway as to what part bacteria might play, if any, in the pathogenesis of atherosclerosis. The current models for the pathogenesis of atherosclerotic plaque suggest that persistent inflammation is an important factor; however, the possible sources for this sustained inflammation are limited. The concept of biofilm infection, "a new paradigm of bacterial pathogenesis," is introduced to show that bacteria, organized into a biofilm phenotype mode of growth, produces a sustained hyper-inflammatory host niche. Biofilm produces an oxidative environment in a host infection. Samples of plaque from 10 patients were examined to compare 16S rDNA to 18S rDNA. Also 4 samples were evaluated in 2 separate locations to evaluate the homogeneity of bacteria within the sample. The 16S rDNA was also sequenced to identify the microorganisms present and their relative contribution to the sample. Several samples demonstrated large amounts of bacterial DNA. The spatial arrangement of bacterial DNA showed a very heterogeneous distribution of bacteria in the plaque. A heat map data analysis shows that for samples that were evaluated in 2 locations the bacteria identified closely correlated. For all the samples combined, the predominant microbial species identified have often been associated with the oral cavity. Several samples show bacterial DNA far exceeding what would be expected by contamination, suggesting the bacteria may be propagating in the plaque. If bacteria are propagating within the plaque, this would most likely be a biofilm phenotype mode of growth. Biofilm is known to produce a hyperinflammatory response in host environments, and therefore is a candidate for being the "engine" for the persistent inflammation necessary for the pathogenesis of atherosclerosis.

Keywords: Apolipoprotein B-containing lipoproteins; CXC chemokine receptor 1; Heme regulatory transport; Heme sensing system; Intracellular cell adhesion molecules; Low density lipoprotein; Oxidative low density lipoprotein; Type three secretion system; Vascular cell adhesion molecules

Abbrevations: ApoB-LP: Apolipoprotein B-containing Lipoproteins; CXCR1: CXC Chemokine Receptor 1; HRT: Heme Regulatory Transport; HSS: Heme Sensing System; ICAMs: Intracellular Cell Adhesion Molecules; LDL: Low Density Lipoprotein; OxLDL: Oxidative Low Density Lipoprotein; T3SS: Type Three Secretion System; VCAMs: Vascular Cell Adhesion Molecules

Introduction

Atherosclerosis, from an economic and individual patient standpoint, is the most important disease facing developed countries. Each year in the United States alone, over 500,000 people die from heart disease, suffer more than 50,000 strokes, and millions endure the complications of peripheral vascular disease. Because of this devastation, extensive research efforts have attempted to identify the cause of atherosclerosis. Yet, to date, there is no consensus on the molecular mechanisms which explain the persistent inflammation producing the downstream effects which characterize atherosclerosis [1-6].

It has been hypothesized that the hydrodynamic forces which affect the arterial system produce shear forces which damage the endothelial cells in specific regions [5]. This damage, in turn, upregulates intracellular adhesion molecules (ICAMs), along with vascular cell adhesion molecules (VCAMs), causes these injured areas of endothelium to sequester platelets and leukocytes. The attachment of platelets releases immunoregulatory agents such as microparticles, vasoactive substances, and cytokines [7,8] which recruit additional neutrophils and monocytes to the area.

Circulating monocytes quickly differentiate to activated macrophages under the effect of macrophage colony-stimulating factor (M-CSF) [9] producing macrophages that ingest apolipoprotein B-containing lipoproteins (apoB-LP). The sub endothelial accumulation of apoB-LP is felt to be an important initial step in plaque formation. Although macrophages can clear apoB-LP, studies have shown that activated macrophages can also release apoB-LP in the sub endothelial region in response to persistent inflammation which produces an unregulated feedback loop producing more apoB-LP and inflammation [10]. This may be one mechanism for persistent inflammation, yet in reviewing the macrophage's role in atherosclerosis, Moore writes "the situation in the atherosclerotic subepithelium is almost certainly more complex, and there is a significant gap between the in vitro and in vivo observations" [11].

What is generally agreed upon is that increases in immune cellularity (macrophages and neutrophils) in the sub epithelium spreads proinflammatory cytokines and leads to an oxidative environment (i.e. reactive oxygen species, myeloperoxidase, lipases, etc). It is this oxidative milieu that is the putative "cause" for the downstream effects producing atherosclerotic plaques [12].

Likewise, the unregulated feedback loop between macrophages' release of apoB-LP and persistent inflammation does not seem sufficient

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to account for all the inflammation observed. As noted there are gaps in the pathway that causes macrophages to abandon their natural function of ingesting apoB-LP to primarily releasing apoB-LP and thus producing more inflammation. Widespread abnormalities in tightly controlled host regulatory pathways seem unlikely candidates to explain why the inflammation persists in the sub epithelium.

A similar explanation of the etiology of atherosclerosis is the Oxidative Modification Hypothesis. This hypothesis asserts that the oxidative milieu in the arterial vessel wall produces enzymatic and oxidative changes to low density lipoprotein micelles (LDL) producing sustainable levels of oxidized LDL (OxLDL) [13]. OxLDL is pointed to as one of the seminal molecular events producing the pathogenesis of atherosclerosis.

The Oxidative Modification Hypothesis suggests that LDL is transported across intact endothelial cells where it is oxidized through several different pathways including myeloperoxidase and other reactive oxygen species along with the participation of enzymes such as sphingomyelinase and secretory phospholipase 2 [12]. This important first step of conversion of LDL to OxLDL requires a persistent inflammatory environment characterized by the presence of interleukin 1 beta, tumor necrosis factor alpha, gamma interferon, interleukin 8, interleukin 6, reactive oxygen species, and myeloperoxidase. The largest and most readily available sources of reactive oxygen species and myeloperoxidase are neutrophils [14]. OxLDL is felt to be sufficient to produce all the downstream events for the production of an atherosclerotic plaque.

Adoption of the Oxidative Modification Hypothesis is limited because no sustainable "engine" producing an ongoing inflammatory milieu has been identified. One possibility based on in vitro findings of secretory phospholipase A2 (sPLA2) and secretory sphingomyelinase (S-SMase) causing release of apoB-LP producing an inflammatory feedback loop is more hypothetical than proven in an in vivo environment.

Bacteria have been associated with atherosclerosis for many years. Some epidemiologic literature suggests a strong link between high bacterial loads in the oral cavity with the development of atherosclerosis [1,15,16]. Also, numerous studies have identified bacteria in atherosclerotic plaque, [17-20] yet there is no clear understanding of whether these bacteria present in the plaque are non-propagating contaminants that have found shelter in an impaired host niche or if these bacteria are contributing to the pathogenesis of the atherosclerotic plaque.

Single organisms such as *Chlamydia pneumoniae* [21,22], *Porphyromonas gingivalis* [23,24], and several others have been implicated in the pathogenesis of atherosclerosis. Koren, utilizing the same pyrosequencing methodology used in this study, identified *Chryseomonas* in all atherosclerotic plaque samples, and *Veillonella* and *Streptococcus* in most of the 15 plaques studied [20]. However, when specific bacteria are addressed therapeutically, no improvement in clinical outcomes relative to atherosclerosis was noted [23,25]. There have been large studies to treat individuals with atherosclerosis with antibiotics which did not improve clinical outcomes [26]. This may have led to the misguided skepticism of bacteria playing any role in the pathogenesis of atherosclerosis.

In the 1990s, it became clear that bacteria forming a biofilm in a host environment produced an infection quite differently than bacteria that remained in a single cell (planktonic) mode of growth. Over the next decade, multiple experiments demonstrated that host antibodies [27], white blood cells [28] and compliment were ineffective against the bacteria encased in a biofilm matrix. That is, host immunity seems to be ineffective against biofilm infection.

Bacteria in biofilm phenotype are also much more resistant to antibiotics and biocides than the same bacteria grown under planktonic conditions [29,30]. When a biofilm community is fully established, it exhibits powerful tolerance to antibiotics up to 1,000 times MIC [31]. In vivo studies confirm that biofilm phenotype bacteria are also much more resistant to antibiotics and biocides in a host setting [32].

These findings were subsequently woven into a comprehensive vision for biofilm infection [33]. It was suggested that biofilm infections in a host environment may start with either biofilm detachment fragments or planktonic bacteria attaching to exposed epitopes in an impaired host environment. The radical transformation from planktonic molecular machinery to biofilm phenotype is dependent on the up regulation of over 800 different genes and is usually measured in minutes [34]. During the metamorphosis from planktonic phenotype to biofilm phenotype bacteria, early extracellular polymeric substances are secreted around the biofilm phenotype bacteria firmly attaching the bacteria to the host surface, but also protecting the individual constituents from host immunity. Once a sufficient number of bacteria, a quorum, are established, quorum-sensing pathways are upregulated. Quorum-sensing directs the phenotypic expressions in discreet regions which lead to mature biofilm.

Once bacteria are attached, there is an upregulation of operons responsible not only for production of biofilm matrix but also for the establishment of a hyperinflammatory milieu. Biofilm infections such as chronic rhinosinusitis, cystic fibrosis, periodontal disease and wounds can all be defined by a similar local host response. These infections produced by biofilm have been shown to exhibit elevated proinflammatory cytokines (tumor necrosis factor alpha, gamma interferon, interleukin 1 beta, interleukin 6 and interleukin 8), along with excessive neutrophils and dysfunctional macrophages.

Bacterial type three secretion system, type four secretion system, and type six secretion system utilized a myriad of effectors (small peptides) to inhibit phagocytosis [35], cause actin disorganization (preventing migration and shedding of host cells) [36], suppression of gene transcription, manipulate adaptive immunity [37] and block apopotosis [38]. These biofilm strategies target host cellular functions in order to produce senescent cells to which the biofilm can remain attached. This allows the biofilm to produce long-term persistent inflammation which prevents healing and provide sustainable nutrition through inflammatory exudate.

The molecular foundations are now well-established for biofilm infections. These molecular mechanisms define how bacteria (even commensals) recognize a vulnerable host environment and quickly form an early biofilm. At the same time, molecular mechanisms are upregulated to kindle host inflammatory pathways which stimulate pro-inflammatory cytokines and recruit inflammatory cells. This persistent oxidative milieu is to provide the biofilm sustainable nutrition through plasma exudate, as well as preventing repair of the local host environment [39]. In other biofilm infections, the bacteria growing in biofilm phenotype produces host lesions characterized by increased proinflammatory cytokines, matrix metalloproteases, reactive oxygen species, elastase, myeloperoxidase, and excessive neutrophils and macrophages. Simply put, biofilm phenotype bacteria producing a host infection in other locations produce the same inflammatory biochemistry and cellular findings consistent with that found in atherosclerosis. Therefore biofilm may be an excellent candidate for the "engine" driving the inflammatory process critical to the pathogenesis of atherosclerosis.

With the understanding of biofilm's ability to produce persistent

human infection, this study was undertaken as a first step to reveal the diversity as well as the amount of bacteria present in atherosclerotic plaque. Further inferences may be able to be made as to the source of certain bacterial species.

Materials and Methods

Ten patients were identified and underwent the consenting process as per institutional review board (Texas Tech University Health Science Center IRB # L08-085) approved protocol.

Patients who were scheduled to undergo atherectomy utilizing a silverhawk device and its well-defined procedure [40] were approached to participate in this study. During the procedure, the segments of atheromatous plaque that were removed were placed directly into a sterile container utilizing strict aseptic procedures. The samples were then immediately placed in a -4°C freezer in the surgery department until a courier picked up the sample later that day. The maximum time in the -4°C freezer was no longer than 8 hours and each sample was hand carried, on ice, to the evaluating laboratory where the sample was immediately stored at -80°C until being processed for the study.

Sample preparation

Plaques were washed twice in molecular grade water and sectioned aseptically into 3 equal proportions. Two of these sections were subjected to molecular analysis.

DNA extraction

Plaque sections were homogenized and 200mg of these sections were asceptically suspended in 500µl RLT buffer (Qiagen, Valencia, CA) with β-mercaptoethanol. A sterile 5mm steel bead (Qiagen, Valencia, CA) and 500µl volume of sterile 0.1mm glass beads (Scientific Industries, Inc., NY, USA) was added to ensure complete bacterial lyses performed using a Qiagen TissueLyser (Qiagen, Valencia, CA) run at 30Hz for 5 minutes. Samples were centrifuged and 100µl of 100% ethanol added to a 100µl aliquot of the sample supernatant. This mixture was then added to a DNA spin column, and DNA recovery protocols followed as noted in Qiagen DNA Tissue Kit (Qiagen, Valencia, CA) starting at step 5 of the Protocol. DNA was eluted from a DNA spin column with 50µl water and samples were diluted accordingly to a final nominal concentration of 20 ng/µl. DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France).

Quantitative PCR

Using a commercial broad range bacterial quantitative PCR assay and a human specific quantitative PCR approach (Research and Testing Laboratory, Lubbock, TX) the relative ratios of bacterial and human host genetic content were measured in the plaques. With efficiencies of 0.92 and 0.94 respectively the quantitative PCRs were performed in triplicate on each of the plaque samples and the relative ct numbers utilized to determine the ratio of human and bacterial genetic content.

Massively parallel bTEFAP

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) and analyses were performed as described previously using Gray28F 5'TTTGATCNTGGCTCAG and Gray519r 5' GTNTTACNGCGGCKGCTG [41-50] Tag-encoded FLX amplicon pyrosequencing analyses utilized Roche 454 FLX instrument with Titanium reagents, titanium procedures performed at the Research and Testing Laboratory (Lubbock, TX) based upon RTL protocols (www.researchandtesting.com).

Bacterial diversity data analysis

Following sequencing, all failed sequence reads, low quality sequence ends, tags, short reads, and primers were removed and sequence collections were depleted of any non-bacterial ribosome sequences and chimeras [45] as has been described previously [41,42,44,46-51]. To determine the identity of bacteria in the remaining sequences, sequences were clustered, and then queried using a distributed BLASTn algorithm (www.krakenblast.com) against a database of high quality 16s bacterial sequences derived from NCBI (version 02-01-2011). Using a .NET and C# analysis pipeline, the resulting BLASTn outputs were compiled and data reduction analysis performed. Only sequences which were common between each atherosclerotic section from each individual plaque were utilized to provide further stringency.

Bacterial identification

Sequences with identity scores, to known or well characterized 16S sequences, 97% or greater were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family, between 85% and 90% at the order level, 80 and 85% at the class level, and 77% to 80% at phyla level. After resolving based upon these parameters, the percentage of each bacterial taxonomic designation was individually analyzed for each sample providing relative percentage information within and among the individual samples based upon total numbers of reads within each sample. Evaluations presented at each taxonomic level, including percentage compilations represent all sequences resolved to their primary identification or their closest relative [50].

Results

Molecular findings showed that bacterial genetic content was present in all atherosclerotic plaques. The proportion of bacterial genetic content in relation to host genetic content ranged from 4% to 98% within the individual sections of the plaques based upon qPCR evaluations (Table 1). There was surprising diversity of microbes with a total of 564 unique genetic signatures identified. Upon increasing the strin-

qPCR 16S (bacterial) and 18S (human) rDNA results																
	11	**12**	**13**	**14**	**15**	**15a**	**15b**	**16a**	**16b**	**17a**	**17b**	**18**	**18a**	**18b**	**19**	**20**
16S	53%	98%	7%	3%	4%	10%	11%	29%	62%	84%	4%	21%	90%	6%	10%	6%
18S	47%	2%	93%	97%	96%	90%	89%	71%	38%	16%	96%	79%	10%	94%	90%	94%

Table 1: qPCR 16S (bacterial) and 18S (human) rDNA results.

Table 1 demonstrates the percent 16S (bacterial) versus 18S (human) ribosomal DNA from each plaque sample. The samples obtained were long ribbons of atherosclerotic plaque obtained from the Silverhawk procedure. For several samples, the entire plaque was homogenized and evaluated. For other samples, the analysis was done at discreet locations (A and B) within the plaque. The findings were quite interesting. Bacterial DNA is not uniformly distributed throughout the plaque as would be expected from random contamination. For example, 16B, 17A, and 18A show overwhelming bacterial (16S) ribosomal DNA as compared to the human DNA found in that area while samples from a different location 16 A, 17 B, and 18 B showed just the opposite. The cycle threshold numbers for the 16S contribution to samples 16B, 17A, and 18A was quite low suggesting very high numbers of bacteria, far more than would be expected from contamination. In fact, sample 12 showed 98% bacterial DNA for the entire homogenized sample. Studies with scanning electron microscopy along with molecular studies suggest that biofilm infections are heterogeneous with the same bacterial species located throughout the infection, yet very condensed in certain portions of the sample and very rarified in other locations of the sample. This is the exact pattern identified for these 10 plaques.

gency of the analysis by requiring that any bacterial identification be present in both halves of a given plaque, this number fell to 121 unique taxonomic signatures (Top 50 listed in Figure 1). The most predominant genera identified were *Flavobacterium, Pseudomonas*, *Clostridium, Streptococcus*, and *Acinetobacter*. This occasional high bacterial load, along with diversity, is very consistent with biofilm infection. Many of the species identified were consistent with oral source of bacteria.

Discussion

These findings confirm that bacteria are present in atherosclerotic plaques. This is important because current theories (abnormal hydrodynamic forces, deposition of apoB-LP, genetically abnormal macrophages, etc.) explaining the initiation of pathogenesis of atherosclerosis suffer from their low prevalence in the general population with atherosclerotic lesions; yet bacteria is always present in atherosclerotic plaque. This point cannot be overstated since the proximate cause producing the pathogenic process leading to atherosclerosis must be present in each individual suffering from atherosclerosis.

Also, many of the individual species that were identified by this study have previously been reported as organisms contributing to oral plaque and/or periodontitis. This again raises the question of oral bacteria's role in atherosclerosis. The literature has indisputably linked oral bacteria to frequent transient bacteremia [52,53]. Also, there is epide-

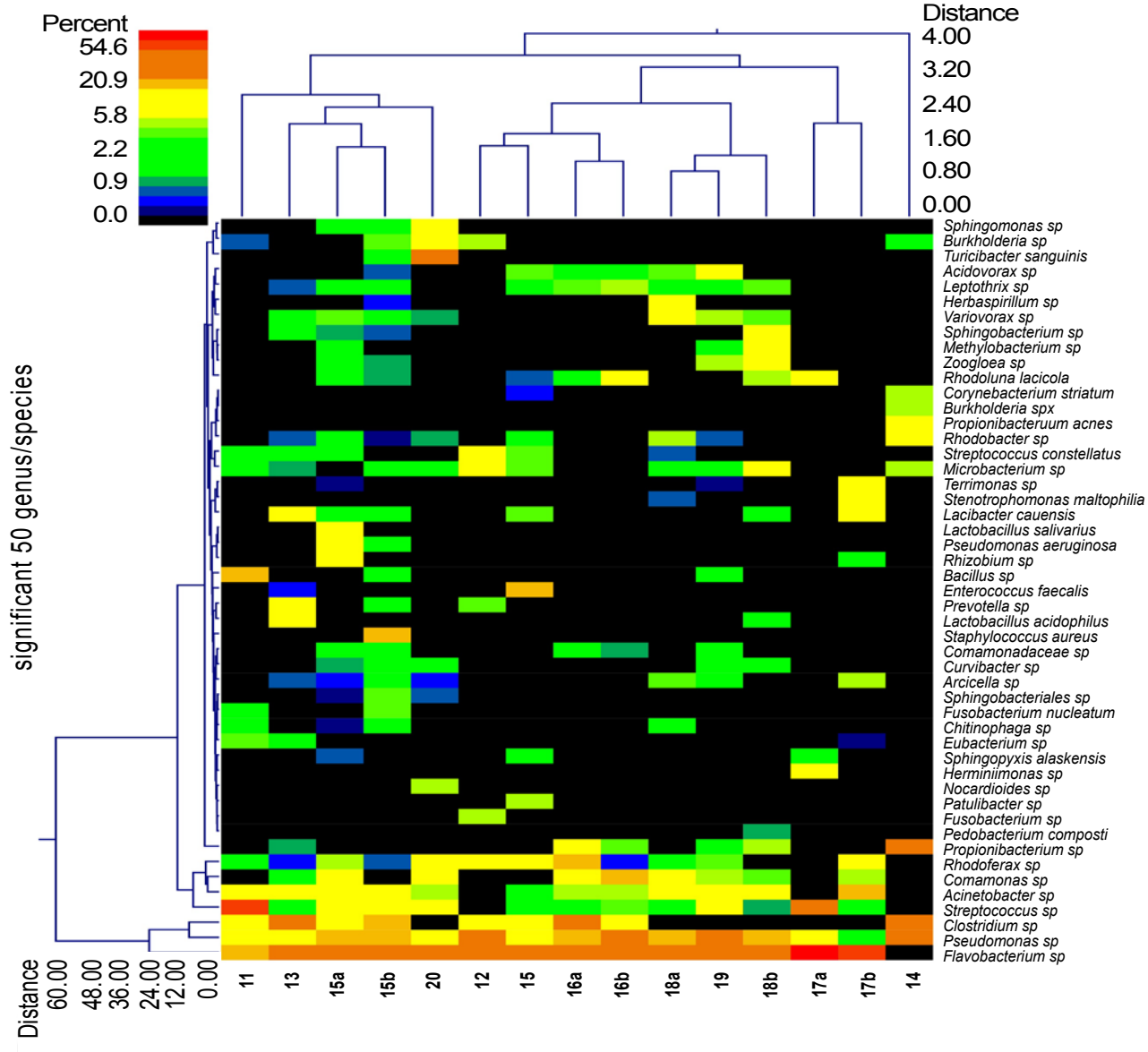

Figure 1: Cluster analysis (dendrogram) of sequencing results. This is a heat map of the species identified by pyrosequencing present in each atherosclerotic plaque sample analyzed and the number of copies for each species identified (relative quantification). This information is then mapped to identify how close the samples correlate to one another. It is interesting that for samples 15A and B, 16A and B, and 17A and B that the same species were found in each different location within the same plaque, and their relative prevalence remain the same from the A location to the B location. The only thing that differed was the absolute number of bacteria between the locations. For contamination it would be expected that the species would be random and their relative quantification within the different locations of the same sample would definitely be quite different. The heat map clearly shows that the dominant species in one location remains the dominant species in the second location, raising the possibility that the presence of the bacteria is not random contamination but rather propagation of bacteria in situ.

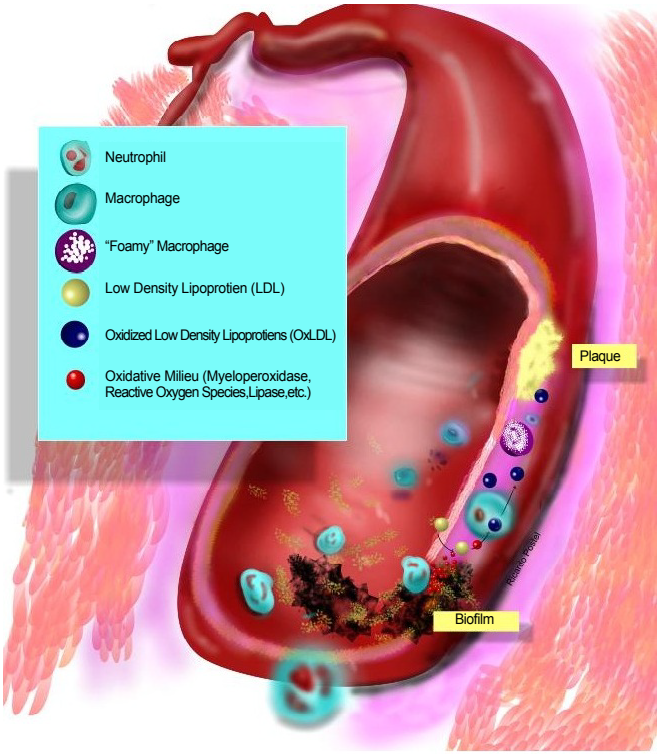

Figure 2: Model of biofilm's possible role in the pathogenesis of atherosclerosis.
This illustration outlines one possible pathway by which bacteria may participate in the pathogenesis of the formation of atherosclerotic plaques. It is possible that not only white blood cells but also bacteria can attach to the areas of endothelial cells within the arteries that have upregulated ICAMs and VCAMs due to hydrodynamic injuries. Once bacteria have attached to these sticky areas, they are committed to biofilm phenotype mode of growth. Biofilm phenotype bacteria quickly produce senescence of the host cells to which they are attached, produce a protective extracellular matrix, and downregulate virulence factors. This leads to a tightly bound polymicrobial community of bacteria producing intense inflammation through the inducement of proinflammatory cytokines such as interleukin 1, interleukin 8, interleukin 6, gamma interferon and tumor necrosing factor alpha. The proinflammatory cytokines produce an oxidative milieu through the production of nonphysiologic levels of myeloperoxidase, reactive oxygen species, lipases, and many other oxidative products. It has been generally agreed that this type of oxidative milieu is sufficient to produce oxidative low density lipoproteins (OxLDL) which, in turn, produce macrophages capable of ingesting these degradated LDLs. Foamy macrophages then deposit in the area incasing the biofilm thus sequestering the biofilm in such a way as to allow some repair. Because of the persistent, intense inflammation produced by the biofilm, the "healing" over the plaque is often fibrotic. Although there is much speculation in this scenario, a biofilm role in the pathogenesis of atherosclerosis sheds light on many of the unanswered questions of atherosclerosis.

miologic evidence that increased oral bacterial load is related to individuals with atherosclerosis [1,16,17,24,54].

The variability in the densities is notable (absolute bacterial DNA amounts per 200 mg of sample), but no significant difference in the species identified in the analysis of different locations within the same sample. The heat map (Figure 1) shows that the species from two different locations of the same sample closely correlate with one another lining up side by side in all but one sample. This demonstrates that the same species of bacteria are represented throughout an individual plaque, but are heterogenous in their distribution. This spatial heterogenicity is also seen in other biofilm infections such as wounds [55]. It seems unlikely that the pockets of high numbers of bacteria could be present through contamination but rather suggests that these bacteria are actually propagating within the atheromatous plaque.

The presence of significant numbers of bacteria with high diversity in the small area of a single sample from an atherosclerotic plaque indicates biofilm phenotype infection [56]. Routine broad-spectrum antibiotics would be effective against many of the species identified in this study if they were in a planktonic mode of growth. Given biofilm tolerance for antibiotics, it is not surprising that studies utilizing empiric antibiotics had little effect on atherosclerosis. The failure of antibiotics in these studies, far from suggesting that atherosclerosis is not related to bacteria, more likely indicates the bacteria associated with atherosclerotic plaques is in a highly antibiotic resistant biofilm mode of growth.

Bacteria have been shown to gain access to the host vascular system through multiple mechanisms including eating, oral hygiene, bowel movement and many other routine daily events [57,58]. Bacteria from oral sources would most reasonably be fragments of biofilm which continue to possess colony defenses against host immunity and antibiotics [59]. Once these bacteria are in the circulatory system they should be quickly sequestered. However, because of the frequency that bacteria enter the circulatory system, it seems reasonable that a few of these fragments could quickly attach to damaged endothelial cells with expressed intracellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs).

By taking control of host immunity, biofilms are able to produce a hyperinflammatory host milieu which provides the biofilm nutrition as well as preventing host repair, thus maintaining the host niche. This strategy of infection employed by biofilm phenotype bacteria can allow the bacteria to survive and thrive for the life of the host. The biochemical and cellular milieu associated with biofilm infections should be sufficient to produce the oxidative milieu needed to produce OxLDL. Biofilm producing a local infection on vascular endothelium is a reasonable candidate for the sustained oxidative milieu that is necessary to drive all the well known pathogenic mechanisms necessary to produce atherosclerotic plaque.

Conclusions

These findings, utilizing PCR and sequencing methods to analyze atherosclerotic plaque, raise the question of bacteria's role in the pathogenesis of atherosclerosis. The demonstration of large numbers of multiple species of bacteria coexisting in a small host area may suggest a local biofilm infection in the arterial wall. Since biofilm infections are known to produce sustained inflammation which produces an oxidative milieu then this may be sufficient to drive the molecular pathways generally accepted for the Oxidative Modification Hypothesis. Also, the species identified are most consistent with periodontal disease and well known to produce frequent transient bacteremia, as well as being epidemiologically linked to atherosclerotic disease.

Biofilm infection on the arterial surface possesses all the requisite properties necessary to be an "engine" driving the pathogenic process of atherosclerosis. Applying generally accepted principles from other host biofilm infections integrated with the findings of this present study raises the possibility of bacteria, in a biofilm mode of growth, contributing to the pathogenesis of atherosclerosis.

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