Human Atherosclerotic Plaque Contains Viable Invasive Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis

To the Editor:

Because epidemiological evidence supports an association between cardiovascular and periodontal disease, we assessed whether periodontal pathogens were present in atherosclerotic lesions. To detect invasive bacteria, the natural tropism of the bacteria toward human tissues was exploited. Further, bacterial presence was demonstrated using quantitative polymerase chain reaction (Q-PCR). This confirms the presence of periodontal pathogens in atherosclerotic lesions, whereby the bacteria could contribute to the vascular pathology either directly through their cytotoxicity or indirectly by inducing or exacerbating inflammation.

Cardiovascular disease (CVD) is the leading cause of death in the United States. According to the American Heart Association’s statistics from 2003, there were no previous symptoms in 50% of men and 63% of women who died suddenly from CHD. In a 10-year follow-up study, ~25% of coronary deaths in males and 15% in females occurred in persons in the lowest two quintiles of the multivariate Framingham Heart Study risk scores. This and other data have led to an emerging paradigm shift from coronary heart disease having a purely hereditary/nutritional causation to possibly having an infectious component.

Many epidemiological studies strongly suggest that periodontitis may be a risk factor for coronary heart disease (CHD). Serologically, edentulousness and serum IgG-antibodies to Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in 1163 men were recently shown to be associated with CHD. In a larger prospective study of 6950 subjects, the same authors provide serological evidence that an infection caused by major periodontal pathogens is associated with future stroke. Previous studies have identified 16S rRNA of oral microbial pathogens, including P gingivalis and A actinomycetemcomitans, in atherosclerotic plaques using PCR. However, none of these studies provide evidence that the oral pathogens were viable at the vascular sites.

Methods

Detailed Methods are available online at http://atvb.ahajournals.org.

Results

Initially, we confirmed the presence of P gingivalis and A actinomycetemcomitans within the plaque at the first attempt by cell culture invasion assays and immunofluorescent microscopy (Figure). To confirm the findings of the invasion assays and microscopy, additional analyses were performed using Q-PCR. As expected, both organisms were detected using Q-PCR, P gingivalis (at log 4.273 in the DNA sample) and A actinomycetemcomitans (at log 4.779). As ~one-tenth of the plaque specimen was used for DNA isolation, this analysis indicated the presence of ~1.9×10^7 P gingivalis and 6.0×10^7 A actinomycetemcomitans in the resected tissue. As the specimen was washed immediately on resection, there is little doubt that the bacteria detected are not a bacteremic carryover from vascular channels or from hemorrhagic material. Control 10 ng of human DNA isolated from peripheral blood leukocytes turned negative in this test, thus demonstrating the primer specificity and the lack of cross-reactivity with the host DNA.

Attempts at plating the homogenate on blood agar plates to culture live colonies of P gingivalis were unsuccessful. Therefore, we set out to show that the pathogens from the plaque can invade host cells. As a result, both P gingivalis and A actinomycetemcomitans were detected within ECV-304 cells after incubation with a carotid atherosclerotic plaque homogenate. This indicates that the bacteria from the atherosclerotic plaque were viable because these two species need to be viable to invade nonphagocytic cells according to previous in vitro studies. In addition, the ECV-304 cells were counterstained with BiP, a lumenal endoplasmic reticulum protein.

Some, but not all, A actinomycetemcomitans detected within the ECV-304 cells colocalized with BiP. The detected P gingivalis did not colocalize with BiP. As a control, ECV-304 cells incubated with the plaque homogenate did not contain bacterial antigens from either species (data not shown). Further, plating lysed ECV-304 cells incubated with carotid atherosclerotic plaques as described above on blood agar plates was unsuccessful. Also unsuccessful were numerous attempts to obtain similar results with frozen plaques (stored in broth with 5% DMSO at −80°C). In terms of contamination, these organisms are fastidious (ie, sensitive to ambient environmental conditions) and thus do not survive under normal laboratory conditions, which makes the possibility of contamination unlikely. Also, the microscopy was performed in another, non-dental research building (University of Florida College of Medicine, Department of Anatomy and Cell Biology, Gainesville).

Several pathogens are currently being investigated for a potential role in the pathogenesis of CVD. A strong association exists between CHD and Chlamydia pneumoniae, a Gram-negative respiratory pathogen. Several groups have also isolated viable C pneumoniae from atherosclerotic plaques. Whereas C pneumoniae needs to be transported from the lung to the arteries through macrophages, oral organisms are introduced into the bloodstream multiple times daily in individuals with periodontitis through perturbations of the periodontal tissue such as toothbrushing. Therefore, the oral cavity represents a potentially large reservoir of Gram-negative pathogenic organisms that could readily interact with cardiovascular tissues. Indeed, P gingivalis was the predominant species among anaerobic bacteria in bacteremia after dental procedures. Further, P gingivalis heat shock protein–specific T-cell lines have been isolated from atheroma lesions, which independently supports our data.

P gingivalis did not colocalize with BiP, contrary to what would be expected of bacteria that traffic to the autophagic pathway. At the time of the experiments, the ECV-304 cells were classified as a human umbilical vein endothelial cell line. Since the time of the experiments, the ECV-304 cells have been identified as a derivative of T24 human bladder epithelial cells. P gingivalis traffics differen-

Deconvolution micrograph of ECV-304 cells infected with P gingivalis (left) and with A actinomycetemcomitans (right) from a carotid atherosclerotic plaque. P gingivalis is fluorescein isothiocyanate (FITC)-labeled (primary Mab is 61B31.3) whereas the host cell is TRITC-labeled (primary rabbit anti-BiP antibody). P gingivalis–specific staining is indicated in green, and BiP–, a lumenal endoplasmic reticulum protein, specific staining is indicated in red. Negative control was ECV-304 cells without plaque homogenate inoculation (not shown). The A actinomycetemcomitans is TxR-labeled (primary antibody is purified rabbit IgG fraction against strain SUNY463) whereas the host cell is FITC-labeled with FITC-conjugated goat anti-rabbit BiP. A actinomycetemcomitans–specific staining is indicated in red, and BiP–specific staining is indicated in green. Colocalization of A actinomycetemcomitans and BiP is indicated in yellow because of the overlap of the red- and green-specific staining. Negative control was ECV-304 cells without plaque homogenate inoculation (not shown). Bar, 15 μm.
ently in epithelial cells than their intracellular trafficking to the autophagic pathway in endothelial cells.

*Actinobacillus actinomycetemcomitans* escapes its initial vacuoles and is free within the cytoplasm. However, cells may use the autophagic machinery to defend against bacteria that escape their vacuoles. Thus, the colocalization between *Actinobacillus actinomycetemcomitans* and BiP may be a result of host cell defense. Therefore, these data are consistent with other studies regarding the in vitro intracellular life cycle of *P. gingivalis* and *Actinobacillus actinomycetemcomitans*.

Detection of periodontal pathogens in atherosclerotic plaques by PCR does not provide evidence as to the bacteria’s viability within the plaque. This is the first report to provide evidence for the presence of invasive periodontal pathogens at the sites of atherosclerotic disease. In addition, their presence was demonstrated at the DNA levels. The intracellular bacteria must have been viable because only viable *P. gingivalis* and *Actinobacillus actinomycetemcomitans* can invade host cells. Notably, the images presented here are all from the same patient. The patient apparently harbors periodontal organisms, judging from his oral health (partial dentition only). Further investigatory work needs to be performed to determine whether periodontal pathogens truly have a role in the pathogenesis of atherosclerotic disease and, if so, how the bacteria contribute to the progression of this disease. Nevertheless, establishing such an unequivocal physical link between these two prevalent conditions will certainly support the notion of periodontitis as an exacerbating factor in cardiovascular pathologies. Identifying the inflammatory bacteria associated with vascular pathogenesis will be beneficial to understanding the epidemiological link between periodontal disease and CVD as well as in developing novel therapies for CVD.

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Methods

Patients and specimens. A diseased tissue specimen was obtained at the Division of Vascular Surgery of Shands Hospital, Gainesville, Florida from a male endarterectomy patient, 74-year old with dentures and 80% carotid occlusion upon approval of the protocol by the Institutional Review Board. After surgical removal, the specimen was immediately washed in prereduced TSB broth to remove blood from vascular channels or hemorrhagic material, placed on ice and processed in a lab free of *P. gingivalis* and *A. actinomycetemcomitans*, as follows.

Cell invasion assay. A longitudinal portion of a carotid atherosclerotic plaque was homogenized in cold PBST (phosphate buffered saline, 10mM phosphate, 2.7mM KCl and 0.14M NaCl, pH 7.4, with 0.05% Tween 20) followed by sonication for 30 sec at level 3 (Model 100 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA). The homogenate was added to a T-25 flask of ECV-304 cells (ATCC; a gift of M.I. Phillips) in antibiotic-free M199 medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). The addition of the carotid atherosclerotic plaque homogenate to the M199 medium occurred within 4-6 hours of the carotid plaque’s removal from the patient. The homogenate and the ECV-304 cells were incubated for 24 h when the cells were washed, trypsinized, seeded onto coverslips and incubated overnight. The cells were then washed three times with phosphate buffered saline (PBS) and then fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. This was followed by washing twice in PBS and quenching in NH₄Cl (50 mM)/0.3% Tween 20/ PBS for 10 min at room temperature. After quenching, the ECV-304 cells were washed two times in PBS. The primary antibodies, 1/50 dilution in
PBS/5% normal goat serum/0.3% Tween 20, were applied for 2 h at room temperature. The antibodies used were the mouse monoclonal anti-\textit{P. gingivalis} antibody 61BG1.3 (a kind gift of R. Gmür)\(^1\), the mouse monoclonal anti-\textit{A. actinomycetemcomitans} antibody 13(10B\(_2\)) conjugated to Texas Red (a gift of W. McArthur and M. Handfield), and rabbit polyclonal anti-BiP, an endoplasmic reticulum protein (a gift of S. Frost).

After two hours, the ECV-304 cells were washed four times in PBS for 5 min each time. The secondary antibodies (1/200 dilution in PBS/5% normal goat serum/0.3% Tween 20) were applied for 1 h at room temperature. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (Sigma Chemical Co., St. Louis, MO) secondary antibody was used to detect \textit{P. gingivalis}. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated and FITC-conjugated goat anti-rabbit BiP (Sigma) was the secondary antibody used to visualize the host cells in \textit{P. gingivalis} and \textit{A. actinomycetemcomitans} imaging, respectively. The ECV-304 cells were then washed twice with PBS before mounting with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) onto glass microscope slides and sealing with nail polish. Images were viewed using an Olympus IX70 deconvolution microscope and Deltavision software (Applied Precision, Inc., Wepahah, WA). Raw images were captured by a CCD camera in both color channels in a series of ten 0.2 \(\mu\)m increments. The Deltavision software converted the stack of images into a computational three-dimensional view using a constrained iterative deconvolution algorithm.

\textit{DNA isolation.} Another portion of the specimen was used to purify total DNA. DNeasy\textsuperscript{®} Tissue kit (Qiagen Sciences, Valencia, CA) was used for the isolation of DNA according to the supplier’s protocol for further PCR analysis.
Primer Design. The primers used were, as follows. Pg forward: 5’-catagatatcacgaggaactcgcgatt-3’; Pg reverse: 5’-aaactgttagcaactacgatgtgg-3’; Aa forward: 5’-ggcacgtaggcggacctt-3’; Aa reverse: 5’-accaggctaaagcccaatc-3’. The amplification primers were designed using PrimerExpress™ software (Applied Biosystems, Foster City, CA). Briefly, sequences for the 16S rRNA genes of the organisms of interest were aligned and inspected for regions of conserved and variable sequences. Regions specific for target bacteria were selected and evaluated for melting temperatures and several other characteristics using the software. Sequences which satisfied that criteria were evaluated using BLAST² and/or Gapped BLAST³ searches. Sequences with appropriate specificity were used to design amplification primers, again using the PrimerExpress™ software. All primers were tested for specificity using 40-50 laboratory strains of bacteria and human genomic DNA.

Q-PCR. A Master Mix of all components for the PCR amplification reaction except the target DNA was prepared (PCR SYBR Master Mix, Qiagen Inc., Valencia, CA). The components were combined and 23 µl of Master Mix dispensed into the wells of a 96 well assay plate (MicroAmp™ Optical 96 Well Reaction Plate, PE/ABI). Triplicate samples of target DNA (2µl, 7.5 ng/µl) from plaque samples or dilutions of target organism standards were added to the wells. The samples were cycled in a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) for 15 minutes at 95°C followed by 40 cycles of 95°C for 30 seconds and 60 seconds at 60°C). During the thermal cycling run, data were collected by the iCycler in each well of the 96-well plate. The reduced data were obtained from the computer immediately after each run. The software calculates a C_t value for each well, which is the number of amplification cycles a particular well
requires to accumulate more reporter dye signal than the mean of the negative control plus 10 standard deviations \(^4\). The \(C_t\) is proportional to the number of bacterial genes in each sample. The exact relationship is estimated using a standard curve of purified bacterial genomic DNA.

Q-PCR standard curve was obtained, as follows. Genomic DNA from \(P.\) \textit{gingivalis}, \(A.\) \textit{actinomycetemcomitans} and \(E.\) \textit{coli} were purified from mid-log phase cultures using a QIAmp Tissue Kit (Qiagen), and the concentration determined by a DNA dye-binding assay (Pico-Green, Molecular Probes, Eugene, OR). DNA was demonstrated to be free of RNA by comparison of OD\(_{260}\) readings before and after treatment with DNase-free RNase. The concentration of DNA was adjusted to 2 \(\mu\)g/ml in water and then 6 additional serial dilutions (1:10) were made. Ten replicate samples of each dilution (5\(\mu\)l/sample) were then assayed as described above for the subject DNA above. The sensitivity of each assay was determined to be the lowest dilution of DNA that produced statistically significant (\(p<0.001\)) average signal higher than the no template control using a t-test. In order to attach some notion of the number of bacteria these data might represent we assumed that the average cell contained about 10 fg of genomic DNA and about 7 copies of each 16S rRNA gene \(^5\).
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