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Human Atherosclerotic Plaque Contains Viable Invasive *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*

Emil V. Kozarov, Brian R. Dorn, Charles E. Shelburne, William A. Dunn, Jr and Ann Progulske-Fox

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Letter to the Editor

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 in the United States.[1] 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.[2] 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.[3]

Many epidemiological studies strongly suggest that periodontitis may be a risk factor for coronary heart disease (CHD).[4] Serologically, edentulousness and serum IgG-antibodies to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in 1163 men were recently shown to be associated with CHD.[5] 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.[6] Previous studies have identified 16S rRNA of oral microbial pathogens, including *P gingivalis* and *A actinomycetemcomitans*, in atherosclerotic plaques using PCR.[7] 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.

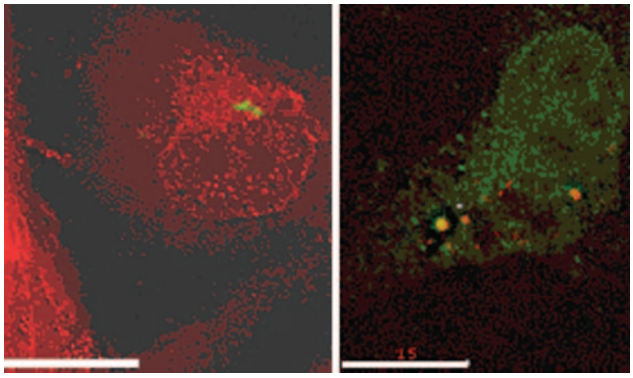
Results

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 $\approx 1.9\times10^5$ *P gingivalis* and 6.0×10^5 *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.[8,9] 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 not 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.[10] Several groups have also isolated viable *C pneumoniae* from atherosclerotic plaques.[11–13] 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.[14] 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.[15] Further, *P gingivalis* heat shock protein–specific T-cell lines have been isolated from atheroma lesions,[16] 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.[17] 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 differ-



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 61BG1.3) 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 SUNY465) 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.

A actinomycetemcomitans escapes its initial vacuoles and is free within the cytoplasm.[18] However, cells may use the autophagic machinery to defend against bacteria that escape their vacuoles.[19] Thus, the colocalization between *A 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 *A 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 *A actinomycetemcomitans* can invade host cells.[8,9] 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 investigative 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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Emil V. Kozarov
Brian R. Dorn
Charles E. Shelburne
William A. Dunn, Jr.
Ann Progulske-Fox
College of Dental Medicine (E.V.K.), Nova Southeastern University, Fort Lauderdale, Fla; Center for Molecular Microbiology (E.V.K., B.R.D., W.A.D., A.P.-F.), Department of Oral Biology and the Periodontal Disease Research Center, College of Dentistry (E.V.K., B.R.D., A.P.-F.), and Department of Anatomy and Cell Biology, College of Medicine (W.A.D.), University of Florida, Gainesville; and University of Michigan School of Dentistry (C.E.S.), Ann Arbor.
B.R.D. is currently affiliated with Merchant & Gould, P.C., Minneapolis, Minn.

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