Invasion of vascular cells \textit{in vitro} by \textit{Porphyromonas endodontalis}

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Abstract


Aim The objective of this study was to determine whether laboratory strains and clinical isolates of microorganisms associated with root canal infections can invade primary cultures of cardiovascular cells.

Methodology Quantitative levels of bacterial invasion of human coronary artery endothelial cells (HCAEC) and coronary artery smooth muscle cells (CASMC) were measured using a standard antibiotic protection assay. Transmission electron microscopy was used to confirm and visualize internalization within the vascular cells.

Results Of the laboratory and clinical strains tested, only \textit{P. endodontalis} ATCC 35406 was invasive in an antibiotic protection assay using HCAEC and CASMC. Invasion of \textit{P. endodontalis} ATCC 35406 was confirmed by transmission electron microscopy.

Discussion Certain microorganisms associated with endodontic infections are invasive. If bacterial invasion of the vasculature contributes to the pathogenesis of cardiovascular disease, then microorganisms in the pulp chamber represent potential pathogens.

Keywords: coronary cells, endodontic, invasion, \textit{Porphyromonas endodontalis}, \textit{Prevotella}.

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Introduction

There is an emerging paradigm shift from coronary heart disease (CHD) having a purely hereditary/nutritional causation to having an infectious component (Libby et al. 1997, Coles et al. 1998, Nieto 1998). Some potential pathogens include \textit{Chlamydia pneumoniae}, \textit{Helicobacter pylori}, Cytomegalovirus, and oral bacteria (Mattila et al. 1998, Epstein et al. 1999). Some epidemiological studies have demonstrated an association between periodontal disease and coronary heart disease (DeStefano et al. 1993, Mattila et al. 1993, Mattila et al. 1995, Beck et al. 1996, Arbes et al. 1999, Morrison et al. 1999). Conversely, several studies have reported no significant associations between oral disease and CHD (Joshipura et al. 1996, Hujoele et al. 2000, Mattila et al. 2000). Two of the latter studies that reported no significant association did find associations between the two diseases in younger age groups. Thus, there may be no overall association, but there may be associations within subsets of the population, for example, younger age groups and specific human genotypes (Beck et al. 2000). Invasion of the cells of the arterial wall by oral bacteria could represent the injury that either initiates and/or more probably exacerbates atherogenesis.

Transient bacteremias occur in patients after toothbrushing, mastication, extraction of teeth, periodontal therapy, and endodontic treatment (Sconyers et al. 1973, Silver et al. 1977, Carroll & Sebor 1980, Debelian et al. 1995), and this dissemination of oral bacteria may be a possible route of infection to those arterial tissues. Oral bacteria have been found in atherosclerotic plaques (Chiu 1999, Haraszthy et al. 2000). A few periodontal pathogens have been reported to invade oral epithelial tissues. The periodontal pathogens \textit{Porphyromonas gingivalis} and \textit{Prevotella intermedia} also invade primary
cultures of human coronary artery endothelial cells (HCAEC) and coronary artery smooth muscle cells (CASMC) (Dorn et al., 1999).

Anaerobic bacteria are found within root canal infections and may have a role in the pathogenesis of pulpal disease (Assed et al., 1996). Endodontic infections are frequently associated with black-pigmented bacteria (BPB) as a group. Porphyromonas and Prevotella spp., both BPBs, occur within 79% of endodontic lesions (Haapasalo 1989), and P. nigrescens is the most common of the BPBs in the pulp canal (van Winkelhoff et al., 1988, Bae et al., 1997). Several periodontal pathogens, including P. gingivalis and P. intermedia, are frequently found in root canal lesions. Additionally, some microorganisms associated with endodontic lesions (Porphyromonas endodontalis and Prevotella nigrescens) are closely related to invasive periodontal pathogens. Therefore, the objective of this study was to determine whether microorganisms associated with endodontic lesions have the ability to invade primary cultures of human coronary artery endothelial cells and coronary artery smooth muscle cells. In addition to the laboratory strains, isolated bacterial strains from root canal lesions from patients at the University of Florida, USA were tested for invasive ability. The bacteria from root canal infections have the potential to be involved in the development of atherosclerosis and CHD since they have access to the systemic circulation.

Materials and methods

Bacterial and cell culture

In addition to laboratory strains, black-pigmented bacteria (BPB) were isolated from patients undergoing root canal treatment as described previously (Debelian et al., 1995). Strains of BPBs (Table 1) were grown on tryptic soy agar (Difco Laboratories, Detroit, MI, USA) supplemented with 5.0% sheep blood (Lampire Biological Laboratories, Pipersville, PA, USA). 0.5% yeast extract (Difco), haemin (5 µg mL⁻¹), and vitamin K (5 µg mL⁻¹). Overnight cultures of BPB were grown in brain heart infusion (BHI) broth (Difco) supplemented with 0.5% yeast extract, 0.1% cysteine (Sigma Chemical Co., St. Louis, MO, USA), haemin (5 µg mL⁻¹), and vitamin K (5 µg mL⁻¹) under anaerobic conditions. E. coli MC1061 (a gift of A. S. Bleiweis) was grown in Luria-Bertani (LB) medium consisting of Bacto Tryptone (10 g L⁻¹; Difco), Bacto yeast extract (5 g L⁻¹), and NaCl (10 g L⁻¹).

The HCAEC were maintained in Microvascular Endothelial Growth Medium-2 (BGM-2; Clonetics, Inc., San Diego, CA, USA) consisting of endothelial cell basal medium-2 supplemented with fetal bovine serum, hydrocortisone, human recombinant fibroblast growth factor, vascular endothelial growth factor, recombinant insulin growth factor-1, ascorbic acid, human recombinant epidermal growth factor, gentamicin, and amphotericin (Clonetics). The CASMC (Clonetics) were maintained in smooth muscle basal medium-2 (SmmG-2) consisting of smooth muscle basal medium-2 supplemented with insulin, human recombinant fibroblast growth factor, fetal bovine serum, human recombinant epidermal growth factor, gentamicin, and amphotericin (Clonetics). Cells were cultured in 7.5-cm² flasks at 37 °C in a humidified atmosphere of 5% CO₂. Both the HCAEC and CASMC were obtained cryopreserved third passage and were passaged an additional two or three times before use.

Sampling from the root canal

The protocol for collection of bacteria from infected root canals was that described by Debelian et al. (1995). Clinical isolates from infected root canals were identified as described by Moore et al. (1994) using the MIDI identification system (Microbial ID, Inc., Newark, DE, USA).

Invasion assay

For the invasion assays, the bacteria were grown in BHI broth, centrifuged at 3500 g, and resuspended in antibiotic-free media to a concentration of 10⁷ cells mL⁻¹ as determined spectrophotometrically (Shimadzu UV-1201, VWR, Marietta, GA, USA). Approximately 10³ human cells per well in a 24-well tissue culture plate

Table 1 Bacterial strains used

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Source</th>
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<tbody>
<tr>
<td>E. coli MC1061</td>
<td>A. S. Bleiweis⁴</td>
</tr>
<tr>
<td>P. endodontalis ATCC 35406</td>
<td>D. Grenier⁵</td>
</tr>
<tr>
<td>P. endodontalis H11a−e</td>
<td>D. Grenier</td>
</tr>
<tr>
<td>P. endodontalis R-41</td>
<td>D. Grenier</td>
</tr>
<tr>
<td>P. gingivalis 381</td>
<td>SUNY-Buffalo Collection⁶</td>
</tr>
<tr>
<td>P. intermedia 27</td>
<td>PDRC Collection</td>
</tr>
<tr>
<td>P. loeschei FAB1</td>
<td>This study</td>
</tr>
<tr>
<td>P. nigrosens ATCC 9336</td>
<td>This study</td>
</tr>
<tr>
<td>P. tannerae FAB1</td>
<td>This study</td>
</tr>
</tbody>
</table>

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were washed three times with phosphate-buffered saline (PBS) prior to incubation with 1.0 mL of the bacterial suspension at 37 °C aerobically for 90 min. In order to more closely approximate in vivo conditions, the bacteria were not centrifuged onto the cells to promote intimate contact. The media were aspirated off infected cells after 90 min, and the cells were washed three times with PBS. Medium containing gentamicin (300 µg mL⁻¹) and metronidazole (200 µg mL⁻¹) was then added to each well to kill any extracellular bacteria, and the plates were incubated for 60 min aerobically at 37 °C. Finally, the media were removed, the cells washed three times with PBS, and lysed by a 20-min incubation with 1.0 mL sterile distilled water at 37 °C under aerobic conditions. Dilutions of the lysates of cells infected with BPBs were plated in triplicate on Tryptic Soy Agar (Difco) plates supplemented with 5.0% sheep blood, 0.5% yeast extract, haemin (5 µg mL⁻¹), and vitamin K (5 µg mL⁻¹) and cultured anaerobically. The CFU of strain 35406 recovered following the antibiotic incubation was 1.8 ± 0.6 × 10⁴ in HCAEC. Although the invasive ability of P. endodontalis ATCC 35406 was invasiveness, it is considered moderately invasive according to the classification previously described (Dorn et al. 2000). Invasion was confirmed by transmission electron microscopy (Fig. 2).

Transmission electron microscopy

Following the 90 min incubation of eukaryotic cells with bacteria, the cells were washed two times with PBS, fixed in 2% PBS buffered glutaraldehyde at room temperature for 1 h, centrifuged for 5 min at 200 × g, and washed with PBS (pH 7.3). Three drops of 3% low-gelling agarose were then added to the pellet and allowed to solidify at 4 °C for 10 min. The agarose-embedded pellet was then washed twice for 10 min in PBS, incubated in 1% osmium tetroxide for 1 h, and washed three times in distilled H₂O. The washed cell pellets were dehydrated in a graded series of ethanol and stained overnight en bloc with 2% uranyl acetate. Finally, the pellets were infiltrated and embedded in EM Bed-812 (Electron Microscopy Sciences, Ft. Washington, PA, USA). Thin sections were cut, poststained with uranyl acetate and lead citrate, and examined in a Hitachi 7000 transmission electron microscope.

Results

Five laboratory strains of microorganisms (P. endodontalis strain 35406, P. endodontalis strain R-41, P. endodontalis strain H11 a–e, P. intermedia strain 27, and P. nigrescens strain 9336) associated with endodontic infections were tested for the ability to invade primary cultures of endothelial and smooth muscle cells. Of these, only P. endodontalis ATCC 35406 was invasive (the CFU were lower than non-invasive E. coli MC1061) in the antibiotic protection assay (Fig. 1). The CFU of strain 35406 recovered following the antibiotic incubation was 1.8 ± 0.6 × 10⁴ in HCAEC. Although the invasive ability of P. endodontalis ATCC 35406 was higher than non-invasive E. coli MC1061) in the antibiotic protection assay (Fig. 1). The CFU of strain 35406 recovered following the antibiotic incubation was 1.8 ± 0.6 × 10⁴ in HCAEC. Although the invasive ability of P. endodontalis ATCC 35406 was approximately 10-fold less than P. gingivalis 381. It is considered moderately invasive according to the classification previously described (Dorn et al. 2000). Invasion was confirmed by transmission electron microscopy (Fig. 2). Although P. endodontalis is closely related to P. gingivalis, its intracellular location following invasion of HCAEC is different.
from the \text{P. gingivalis} vacuole. \text{P. gingivalis} is localized within autophagosomes, whereas \text{P. endodontalis} is located in large endosomes contained by a thin membrane. \text{P. endodontalis} ATCC 35406 also invaded CASMC (1.1 ± 0.04 × 10^4) in the antibiotic protection assay. All of the other strains tested had invasion levels below the non-invasive control \text{E. coli} MC1061. \text{P. endodontalis} strain R-41 and strain H11a–e were not invasive. Thus, the invasion ability of \text{P. endodontalis} varies between the strains. The type strain of \text{P. nigrescens}, the most frequently isolated BPB from endodontic lesions and a close relative of \text{P. intermedia}, was also not invasive.

Figure 2 Transmission electron micrograph of internalized \text{P. endodontalis}. Internalized \text{P. endodontalis} (arrow) in HCAEC following a 90-minute infection.

Microorganisms from infected root canals were isolated, identified, and tested for their invasive ability. Four strains were positively identified as \text{P. intermedia}, \text{Prevotella loeschii}, and \text{Prevotella tannerae} (from two different patients). None of these four strains were invasive (Fig. 3). A fifth strain was isolated and was also non-invasive; however, tests could not definitively determine whether it was \text{Prevotella melaninogenica} or \text{P. nigrescens}. None of the bacteria isolated from infected root canals were invasive in the antibiotic protection assay.

Discussion

Microorganisms from oral focal infections may disseminate throughout the body and subsequently colonize extraoral tissues. During sepsis, microorganisms from the oral cavity may attach to and invade sites along the arterial tree. This theory was originally formulated to address the putative association between periodontal disease and cardiovascular disease. The theory could also apply to microorganisms associated with endodontic infections due to bacteraemia after endodontic therapy (Debelian et al. 1995, Debelian et al. 1998). The interactions between the bacteria and the vascular cells could initiate, or more likely, exacerbate the inflammatory lesion of atherosclerosis (Ross 1999).

In contrast to the focal infection theory, microorganisms from endodontic infections may not be seeding distant sites in the body, but rather the dental pulp may be infected by a general bacteraemia (Drancourt et al. 1998, Aboudharam et al. 2000). Therefore, bacteria found in the dental pulp could be a reflection of microorganisms found systemically. These bacteria may be indicators of pathogens causing morbidity at extraoral sites. More specifically, invasive bacteria in the dental...
pulp may be indicative of bacteria causing disease at the endothelium.

The invasion ability of strains of *P. endodontalis* in this study was heterogeneous. Of the three strains tested, only *P. endodontalis* ATCC 35406 was invasive. This finding was not unexpected, since previous studies have demonstrated that the invasion abilities of both *P. gingivalis* and *P. intermedia* vary amongst the different strains (Dorn et al. 1998). Conversely, certain strains of *P. loescheii*, *P. nigrescens*, and *P. tannerae* that were not tested in this study may be invasive. The different strains of BPPs from the oral cavity vary in their ability to invade mononuclear cells. The intracellular location of *P. endodontalis* was different from that of *P. gingivalis*. Although both are invasive, the two microorganisms have evolved different mechanisms of controlling their intracellular environment in order to survive (Hackstadt 2000).

In summary, we have demonstrated that some, but not all, microorganisms associated with endodontic infections can invade human coronary artery endothelial cells (HCAEC) and coronary artery smooth muscle cells (CASM C). A next step would involve animal models to determine whether the bacteraemic conditions would occur. The different strains of BPPs may be indicative of bacteria causing disease at the endothelium.

**References**


K otherwise indicated, items were retrieved from the University of Florida College of Medicine Electron Microscopy Core Laboratory of the Interdisciplinary Center for Biotechnology Research for the transmission electron micrographs.

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