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Molecules and Cells

Intracellular Degradation of *Fusobacterium nucleatum* in Human Gingival Epithelial Cells

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The role of *Fusobacterium nucleatum* in oral health and disease is controversial. We have previously shown that *F. nucleatum* invades gingival epithelial cells. However, the destiny of the internalized *F. nucleatum* is not clear. In the present study, the intracellular destiny of *F. nucleatum* and its cytopathic effect on gingival epithelial cells were studied. The ability of *F. nucleatum* and seven other oral bacterial species to invade immortalized human gingival epithelial (HOK-16B) cells were compared by confocal microscopy and flow cytometry. *F. nucleatum* had the highest invasive capacity, comparable to that of *Porphyromonas gingivalis*, a periodontal pathogen. Confocal microscopic examination revealed colocalization of internalized *F. nucleatum* with endosomes and lysosomes. Examination by transmission electron microscopy revealed that most intracellular *F. nucleatum* was located within vesicular structures with single enclosed membranes. Furthermore, *F. nucleatum* could not survive within gingival epithelial cells and had no cytopathic effects on host cells. Interestingly, endosomal maturation played a role in induction of the antimicrobial peptides human beta defensin (HBD)-2 and -3 by *F. nucleatum* from gingival epithelial cells. *F. nucleatum* is destined to enter an endocytic degradation pathway after invasion and has no cytopathic effect on gingival epithelial cells, which may cast new light on the role of *F. nucleatum* in the pathogenesis of periodontitis.

INTRODUCTION

Fusobacterium nucleatum is a Gram-negative, anaerobic, spindle-shaped or fusiform rod. Previous studies revealed that *F. nucleatum* not only induces antimicrobial peptides and IL-8, a chemokine for neutrophils, from gingival epithelial cells, but it is also highly susceptible to clearance by antimicrobial peptides and neutrophils (Ji et al., 2007a; 2007b; Krisanaprakornkit et al., 2000). These characteristics indicate that *F. nucleatum* could be a non-harmful or possibly beneficial member of the oral microflora. However, the prevalence and levels of *F. nucleatum* are significantly associated with increasing pocket depth (Socransky et al., 1998). *F. nucleatum* is also associated with preterm birth and has been isolated from amniotic fluid (Chaim and Mazor, 1992; Han et al., 2004).

Invasive capacity is one of pathogenic characteristics common to periodontal bacterial pathogens (Feng and Weinberg, 2006), and bacterial invasion has been suggested as a potential pathogenic factor of periodontitis for decades (Allenspach-Petrzilka and Guggenheim, 1983). We recently showed that the presence of *Porphyromonas gingivalis* in gingival tissues might be the cause of inflammatory infiltration (Kim et al., 2010). Bacteria internalized into non-phagocytic cells ultimately undergo degradation by endocytic or autophagic degradation pathways, whereas pathogenic bacteria have evolved various strategies to circumvent those degradation pathways and establish intracellular infections (Alonso and Garcia-del Protillo, 2004; Amano et al., 2006). It is noteworthy that *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, two well-defined periodontal pathogens, have the capacity to survive within the cytosol of epithelial cells and spread to adjacent cells (Lamont et al., 1995; Meyer et al., 1996; Yilmaz et al., 2006). Therefore, the ability of an invading bacterium to survive within its host cells is important for determining its pathogenicity. Furthermore, whether the surviving microbe is located in the cytosol or inside vesicles is an important issue for the ability of the immune system to cope with it. *F. nucleatum* can invade human gingival epithelial cells (Han et al., 2000), and Gursoy et al. recently reported that internalized *F. nucleatum* could multiply intracellularly in HaCa T human skin keratinocytes (Gursoy et al., 2008). However, the cellular response of skin keratinocytes to *F. nucleatum* is different from that of oral epithelial cells (Chung et al., 2004), and the fate of internalized *F. nucleatum* in oral epithelial cells could be different from that in skin keratinocytes.

We hypothesized that *F. nucleatum* may be a periodontal pathogen, provided it can survive inside gingival epithelial cells or has a cytopathic effect on its host cells. In the present study, we investigated the intracellular destiny of *F. nucleatum* and its cytopathic effect on gingival epithelial cells to clarify the role of *F. nucleatum* in the pathogenesis of periodontitis.

MATERIALS AND METHODS

Bacterial culture

Streptococcus sanguinis NCTC 10904, *Streptococcus gordonii* ATCC 10558, *Veillonella atypica* ATCC 17744, *F. nucleatum*

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ATCC 25586, *Prevotella intermedia* ATCC 25611, *P. gingivalis* ATCC 49417, *Tannerella forsythia* ATCC 43407, and *Treponema denticola* ATCC 33521 strains obtained from ATCC (USA) were cultured in the appropriate media, as described previously (Ji et al., 2007a), under an anaerobic atmosphere (5% H_2, 10% CO_2, and 85% N_2). For some experiments, bacteria were used after staining with 5 μM 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probe, USA).

Gingival epithelial cell culture

An immortalized human oral keratinocyte cell line (HOK-16B) was provided by Dr. N-H. Park (University of California Los Angeles, USA) and was maintained in keratinocyte growth medium (KGM) with a supplementary growth-factor bullet kit (Clonetics, USA). HOK-16B is immortalized by the transfection of human papillomavirus type 16 DNA into primary human oral keratinocytes cultured from excised gingival tissue (Park et al., 1991).

Infection of epithelial cells with oral bacteria

HOK-16B cells were plated at 6×10^4 cells/500 μl/well into 24-well plates one day before infection. At 80% confluence, cells were infected with various bacteria at m.o.i. 1000 (unless described otherwise) and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. In experiments using eight different oral bacterial species (Fig. 1), the GA1000 (50 μg gentamicin ml^{-1} and 50 ng amphotericin B ml^{-1}) in KGM was maintained to prevent facultative anaerobic bacterial growth. Under these conditions, 35-60% of bacteria survived without bacterial growth, as determined by Cytox Green staining (Ji et al., 2007b). In experiments using *F. nucleatum* alone, the GA1000 in KGM was removed to prolong bacterial survival.

Confocal microscopy

To examine bacterial invasion, cells were infected with CFSE-labeled bacteria for 24 h. The cells were stained with rhodamine-phalloidin (Sigma, USA) and Hoechst 33342 (Molecular Probes), as described previously (Ji et al., 2009). To examine the colocalization of *F. nucleatum* with endosomes, HOK-16B cells were infected with unlabeled bacteria by centrifuging at $900 \times g$ at 4°C for 10 min and further incubated for 90 min at 37°C. The cells were then fixed with 2% paraformaldehyde containing 0.1% Triton X-100 for 10 min, blocked with donkey serum (1:100) for 30 min, and stained with either FITC-conjugated mouse anti-human EEA1 (BD bioscience, USA) or an isotype control (eBioscience, USA) followed by Hoechst 33342. To examine the colocalization of *F. nucleatum* with lysosomes, HOK-16B cells were infected with CFSE-labeled bacteria for 4 h and stained with LysoTracker Red DND-99 (Molecular Probes), a freely permeant vital dye, 30 min before examination. All images were obtained at 3 μm from the bottom using an Olympus confocal microscope (FV300, Olympus, USA).

Flow cytometry

To measure bacterial invasion, HOK-16B cells were infected with CFSE-labeled bacteria for 4 h and detached with trypsin-EDTA. After quenching the fluorescence of bacterial bound to the surface with Trypan blue, cells were analyzed with FACSCalibur (BD bioscience). Complete quenching was confirmed using cells fixed with 3.7% formaldehyde. The percentage of HOK-16B cells containing invasive bacteria was determined based on live cells without bacterial infection, which presented higher autofluorescence than fixed cells.

To examine the effect of *F. nucleatum* infection on HOK-16B cell viability, HOK-16B cells were infected with *F. nucleatum* at m.o.i. 1000, 2500, 5000 or 10,000 for 24 h. Entire cells, including those in culture medium, were harvested, and their viability was analyzed by flow cytometry after staining with Trypan blue.

Transmission electron microscopy (TEM)

HOK-16B cells were infected with *F. nucleatum* for 2 h. The infected cells were harvested immediately or after killing extracellular bacteria by culturing in a medium containing antibiotics (100 μg amoxicillin ml^{-1} and 100 μg gentamicin ml^{-1}) for 2 h and further culturing for 22 h in fresh medium. The cells were prefixed in 2.5% buffered glutaraldehyde fixative and allowed to set in 2% low-melting agarose gel at 4°C. The gel was cut into blocks and post-fixed with 1% osmium tetroxide. After dehydration in graded ethanol (50-100%), the fixed cells were embedded in an Epon812 mixture. Thin sections (70 nm) were stained with uranyl acetate and lead citrate and examined with a TEM (JEM-1400, JEOL, Japan).

Antibiotics protection assay

HOK-16B cells (2.4×10^5) were infected with *F. nucleatum* at m.o.i. 1000 in an anaerobic chamber for 2 h. After washing with DPBS containing antibiotics (100 μg amoxicillin ml^{-1} and 100 μg gentamicin ml^{-1}), extracellular bacteria were killed by culturing the infected cells in fresh medium containing antibiotics in a humidified atmosphere of 95% air and 5% CO_2 for 2 h. No intracellular effect of amoxicillin has been reported previously (Hultén et al., 1996). The HOK-16B cells were washed with DPBS and further cultured in fresh medium without antibiotics for up to 22 h. At the indicated time points, cell lysates were prepared by lysing the cell monolayer with 400 μl sterile distilled water for 1 h. The lysates and accompanying culture medium were plated onto blood agar supplemented with hemin and menadione and cultured under anaerobic conditions for 4-5 days. Experiments were repeated four times.

Real-time reverse transcription polymerase chain reaction (RT-PCR) of HBD-2 and -3

HOK-16B cells were pretreated with vehicle or 300 nM bafilomycin A1 (Sigma) for 30 min and infected with *F. nucleatum* for 24 h. Total RNA from HOK-16B cells was extracted using TRIzol (Invitrogen, USA). HBD-2 and -3 expression levels were examined by real-time RT-PCR, as described previously (Ji et al., 2007b). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in parallel. The specificity of the PCR product was verified using a melting curve analysis and examination on a 3% agarose gel. Relative copy numbers compared to GAPDH were calculated using $2^{-\Delta Ct}$. Real-Time PCR was performed in triplicate for each RNA sample, and the experiment was repeated three times.

Statistics

Differences between the two groups were analyzed using the two-tailed, non-paired Student's *t*-test. Data were considered statistically significant at a *p*-value of < 0.05.

RESULTS

***F. nucleatum* has the highest invasive ability among eight oral bacterial species studied**

We previously showed that *F. nucleatum* efficiently invades gingival epithelial cells (Ji et al., 2007b). The ability of *F. nucleatum* to invade gingival epithelial cells was compared with three non-periodontopathic and four periodontopathic bacterial spe-

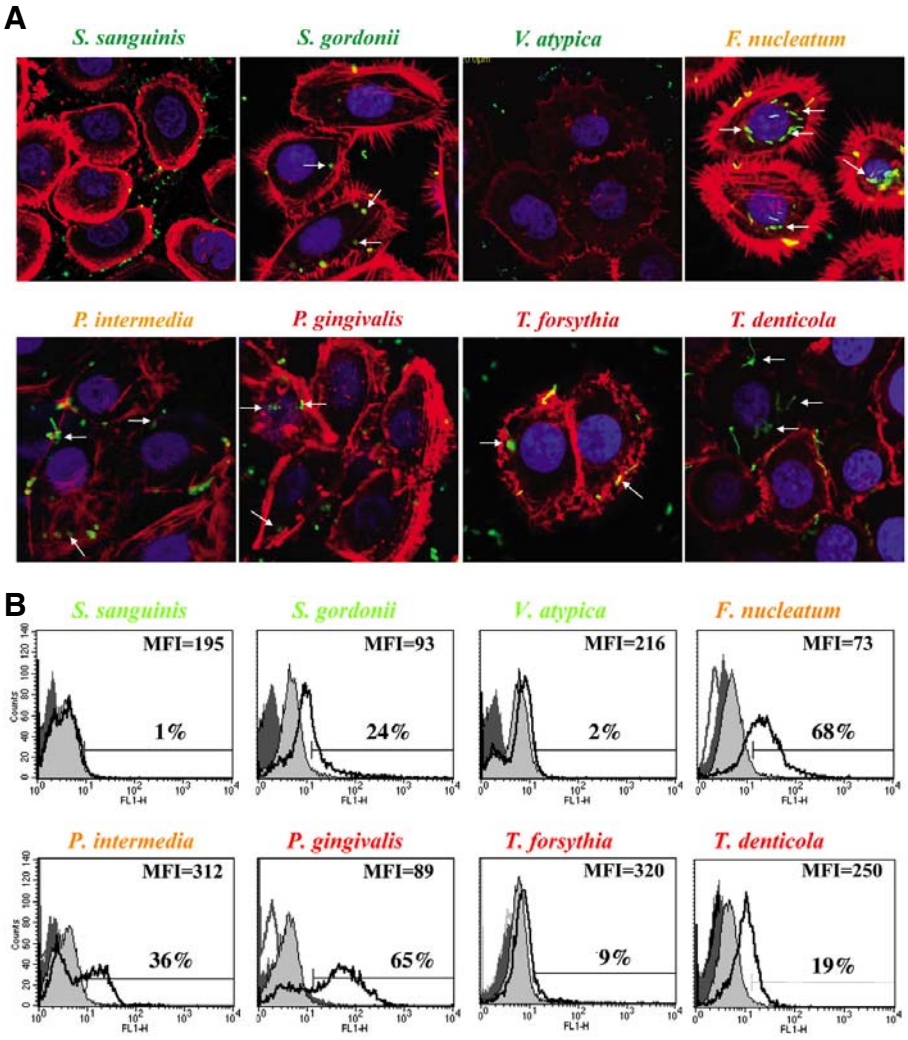

Fig. 1. Invasive capacity of several oral bacterial species on immortalized human gingival epithelial HOK-16B cells. (A) HOK-16B cells were infected with various CFSE-labeled oral bacteria at m.o.i. 1000 for 24 h. After fixation and permeabilization, cells were stained with rhodamine-phalloidin and Hoechst 33342 and examined under a confocal microscope. Arrows indicate intracellular bacteria. (B) HOK-16B cells were infected with CFSE-labeled bacteria at m.o.i. 1000 for 4 h. After quenching the fluorescence of the bacteria bound on the surface with 500 μl Trypan blue, cells were analyzed by flow cytometry (solid empty line). Fixed HOK-16B cells co-cultured with the bacteria (filled with dark gray) confirmed the quenching efficiency. The percentage of cells containing bacteria was determined based on live HOK-16B cells alone (filled with light gray). MFI at the right upper corner indicates the mean fluorescence intensity of each labeled bacterium. The names of non-periodontopathic early colonizers, periodontopathic bridging colonizers, and periodontopathic late colonizers are written in green, orange, and red colors, respectively. Data shown are representative of three similar results.

cies by confocal microscopy and flow cytometry. HOK-16B cells infected with CFSE-labeled bacteria for 24 h were stained with rhodamine-phalloidin and Hoechst 33342. Examination by confocal microscopy revealed the presence of internalized bacteria within the cell boundary surrounded by actin filaments. Among the non-periodontopathic bacteria, only *S. gordonii* displayed invasive ability; by contrast, all periodontopathic bacteria, including *F. nucleatum,* invaded gingival epithelial cells. HOK-16B cells infected with *F. nucleatum* presented numerous bacteria in the perinuclear region (Fig. 1A).

To quantify the invasive ability of the bacteria, a modified flow cytometric phagocytosis assay was applied. In the preliminary time course experiment, the percentage of cells containing *F. nucleatum* decreased slightly after 5 h of infection. Therefore, HOK-16B cells were infected with CFSE-labeled bacteria for 4 h. At this time point, *F. nucleatum* showed the highest invasive ability among the eight species, comparable to that of *P. gingivalis*. *P. intermedia, T. denticola,* and *S. gordonii* had substantial invasive capacity. *S. sanguinis* and *V. atypica* were hardly invasive, confirming the results of the confocal microscopy study (Fig. 1B). In summary, these results confirmed the highly invasive ability of *F. nucleatum*.

Intracellular *F. nucleatum* colocalizes with endosomes and lysosomes in gingival epithelial cells

To determine the destiny of internalized *F. nucleatum*, colocalization of intracellular *F. nucleatum* with endosomes or lysosomes was examined by confocal microscopy. HOK-16B cells infected with *F. nucleatum* were stained with FITC-conjugated antibody to EEA1 (a marker of early endosomes) and Hoechst 33342 (to visualize *F. nucleatum*). In HOK-16B cells without bacterial infection, round endosomes were scattered in the perinuclear region (Fig. 2A). However, colocalization of EEA1 with intracellular rod-shaped *F. nucleatum* was observed after 90 min of infection (Fig. 2B).

Colocalization of CFSE-labeled *F. nucleatum* with lysosomes was determined using LysoTracker Red. Lysosomes were also examined at the perinuclear region. Colocalization of *F. nucleatum* with lysosomes was best examined at 3 or 4 h of infection; at that time, 77% of intracellular bacteria that were located around the perinuclear region colocalized with lysosomes (Fig. 3). Taken together, these results demonstrate that internalized *F. nucleatum* is trafficked to early endosomes and late endosomes/lysosomes within 4 h of infection.

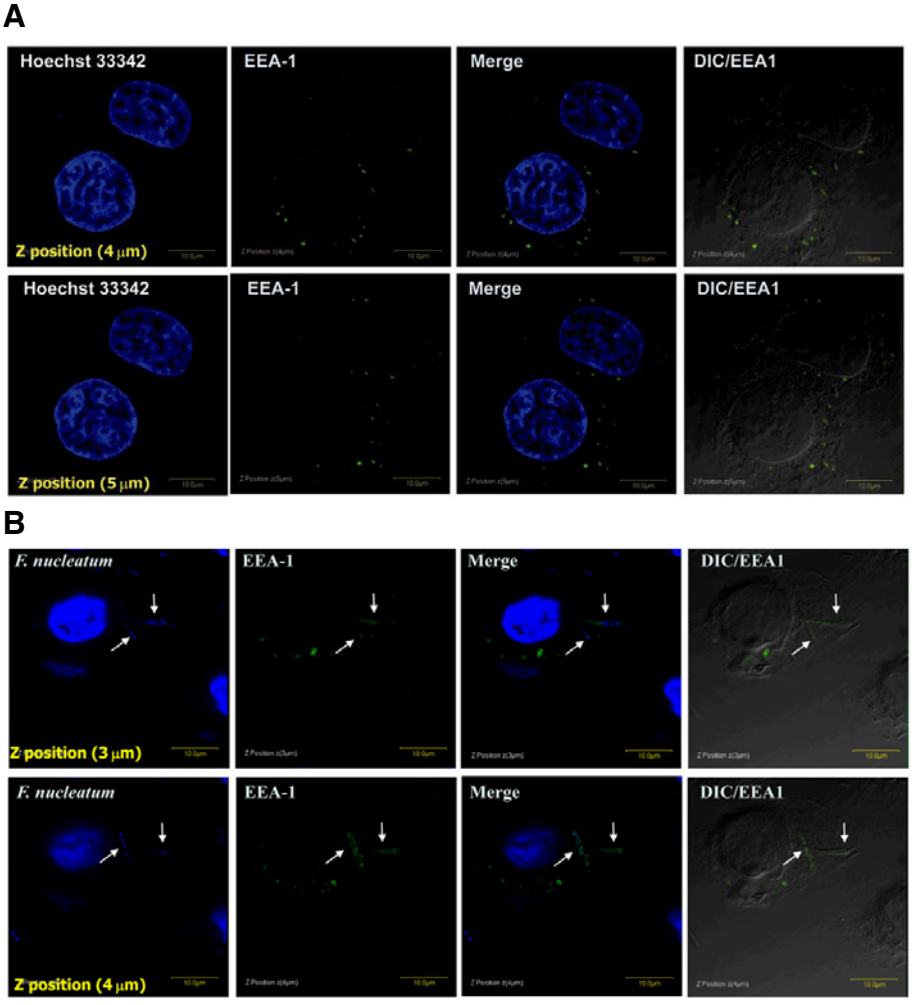

Fig. 2. Colocalization of intracellular *F. nucleatum* and EEA1. Uninfected HOK-16B cells (A) or cells infected with unlabeled *F. nucleatum* at m.o.i. 1000 for 90 min (B) were stained with FITC-conjugated mouse anti-human EEA1 (green) and Hoechst 33342 (blue) to visualize *F. nucleatum* and then examined under a confocal microscope with serial z-sections. Arrows indicate colocalized *F. nucleatum* and endosomes. Data shown are representative of two similar results.

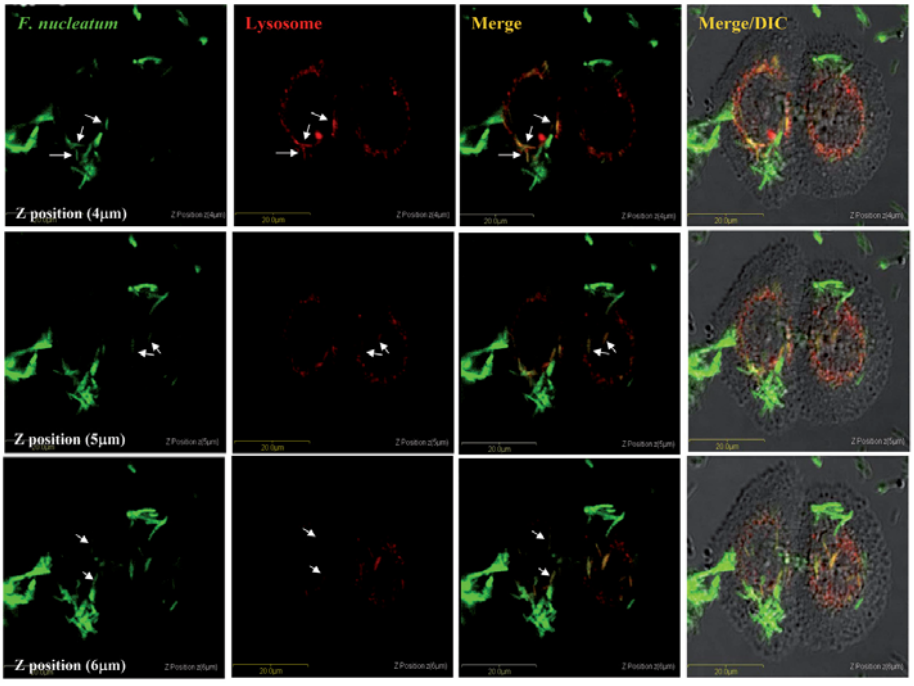

Fig. 3. Colocalization of intracellular *F. nucleatum* and lysosomes. HOK-16B cells were infected with CFSE-labeled bacteria (green) at m.o.i. 1000 for 4 h in the presence of LysoTracker Red DND-99 (red) and examined under a confocal microscope with serial z-sections. Arrows indicate colocalized *F. nucleatum* and lysosomes. Data shown are representative of two similar results.

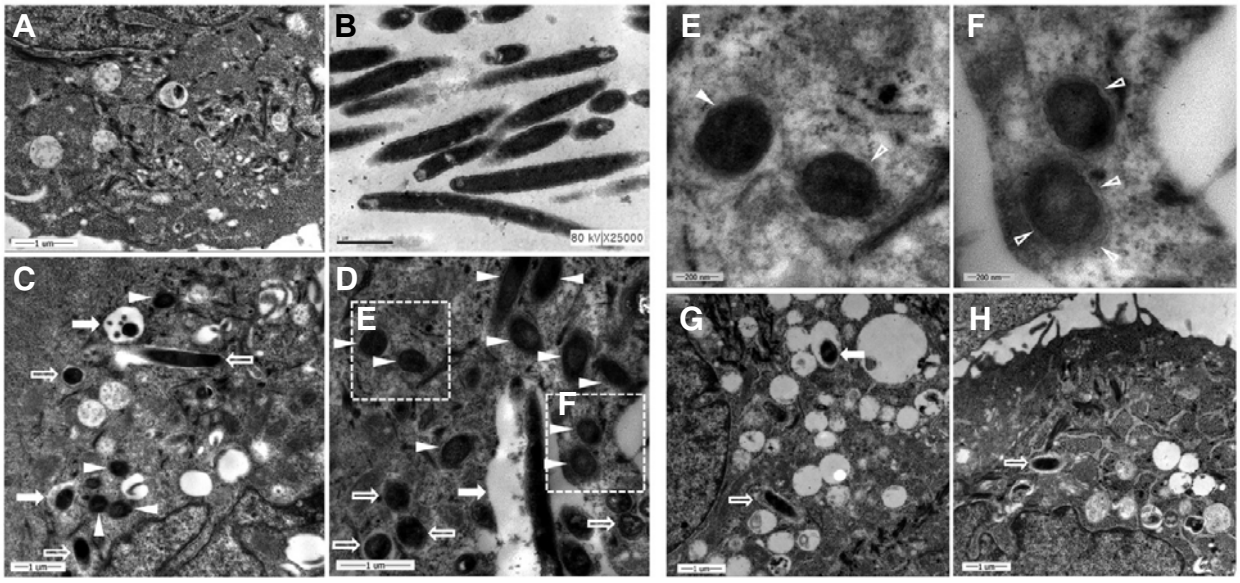

Fig. 4. TEM examination of internalized *F. nucleatum* within HOK-16B cells. Non-infected HOK-16B cells (A), *F. nucleatum* alone (B), and HOK-16B cells infected with *F. nucleatum* (C-H) were examined under TEM. Infected cells were harvested immediately (C-F) or after further culturing in the presence of antibiotics for 2 h and then in the absence of antibiotics for 22 h (G, H). Filled arrows: bacteria in vacuole; unfilled arrows: bacteria in vesicular structure with an enclosed membrane; filled arrowhead: bacteria in the cytosol; unfilled arrowhead: membranous structure adjacent to bacteria under higher magnification. Scale bars: 1 μm (A-D, G, and H) and 200 nm (E and F).

Most intracellular *F. nucleatum* is found within vesicular structures with single enclosed membranes

The subcellular localization of internalized *F. nucleatum* was further verified by TEM. Han et al. (2000) previously reported that *F. nucleatum* is confined to a membrane-bound vacuole after internalization. However, in their report, epithelial cells were incubated with bacteria for 3 h; some bacteria may escape the vacuole after longer incubation times. Therefore, HOK-16B cells were examined immediately after infection with *F. nucleatum* for 2 h and after further culturing the infected cells for 24 h. Under TEM, *F. nucleatum* appeared as electron-dense objects of 0.3 to 0.5-μm in diameter with an outer membrane; such structures were not found in non-infected HOK-16B cells (Figs. 4A and 4B). Immediately after infection, numerous bacteria were found within a single host cell (Figs. 4C and 4D) and appeared to be localized to large vacuoles (filled arrow), to membranous structures tightly enclosing bacteria (unfilled arrow) or to the cytosol (filled arrowhead). Higher magnification revealed a membrane surrounding the bacteria in most (but not all) cases (Figs. 4E and 4F). After 24 h of further culture, it was difficult to find cells containing bacteria, and all intracellular bacteria were found in large vacuoles or membranous structures tightly enclosing bacteria (Figs. 4G and 4H).

Internalized *F. nucleatum* does not survive more than 12 h

Because some bacteria appeared to be localized in the cytosol, *F. nucleatum* survival within its host cells was studied by an antibiotics protection assay. At an antibiotics concentration that does not affect HOK-16B cell viability, extracellular *F. nucleatum* could not be killed completely. The extracellular bacteria died slowly, and no viable counts were detected after 24 h culture under aerobic conditions. The number of intracellular *F. nucleatum* decreased most rapidly between 4 h and 6 h, and no viable counts were detected 24 h after infection (Fig. 5A). In this experiment, HOK-16B cells and culture medium were taken from different wells for each time point. Furthermore, due to

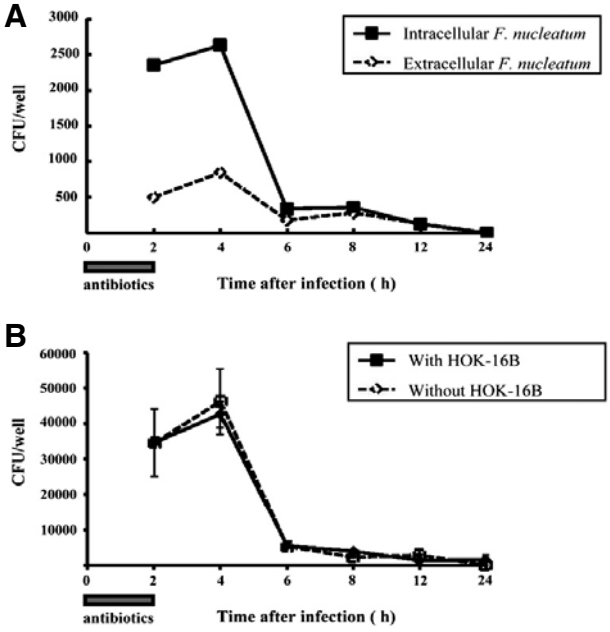

Fig. 5. Antibiotics protection assay of internalized *F. nucleatum*. HOK-16B cells were infected with *F. nucleatum* at m.o.i. of 1000. (A) After killing extracellular bacteria by culturing in medium containing antibiotics for 2 h, the HOK-16B cells were washed with DPBS and further cultured in fresh medium without antibiotics for up to 22 h. At the indicated time points, the cell lysates and accompanying culture medium were plated onto blood agar and cultured under anaerobic condition for 4-5 days. (B) After killing extracellular bacteria with antibiotics, the infected cells were fed with fresh medium. Half of the medium was immediately transferred to a new well, and survival of the remaining extracellular bacteria was examined over time in the presence or absence of host cells.

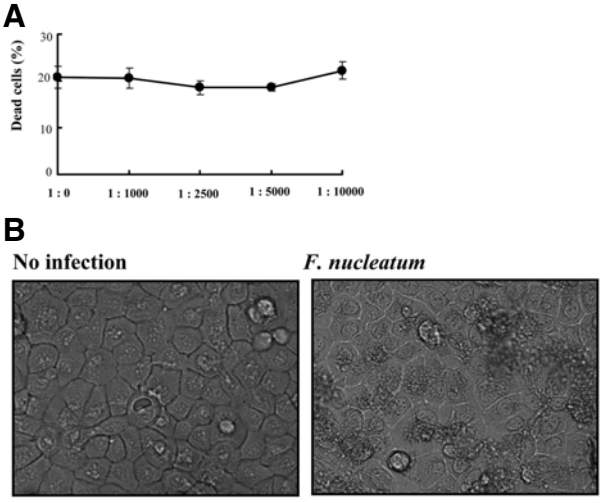

Fig. 6. Effect of *F. nucleatum* infection on the viability and morphology of HOK-16B cells. (A) HOK-16B cells were infected with *F. nucleatum* at m.o.i. 0, 1000, 2500, 5000 or 10000 for 24 h, and host cell viability was analyzed by flow cytometry after staining with Trypan blue. Data shown are the mean ± SEM of three independent experiments. (B) HOK-16B cells infected with *F. nucleatum* at m.o.i. 0 and 10,000 were observed under light microscopy.

incomplete killing of extracellular bacteria, the source of bacteria was unclear. The possibility that intracellular *F. nucleatum* was released into the medium was further investigated. After killing extracellular bacteria with antibiotics, infected cells were fed with fresh medium. Half of the medium was immediately transferred to a new well, and survival of the remaining extracellular bacteria was examined over time in the presence or absence of host cells. The presence of infected host cells did not affect the survival curve of extracellular bacteria (Fig. 5B).

F. nucleatum has no cytopathic effects on gingival epithelial cells

Although *F. nucleatum* could not survive for a long period of time within gingival epithelial cells, bacterial infection may induce morphological changes or even death in host cells. HOK-16B cells were infected with *F. nucleatum* at various m.o.i. for 24 h, and host cell viability was analyzed by flow cytometry after staining with Trypan blue. Infection with *F. nucleatum* had no significant effect on HOK-16B cell viability (Fig. 6A). In addition, no morphological changes were observed (Fig. 6B). These results indicate that *F. nucleatum* has no cytopathic effects on its host cells.

Endosomal maturation plays a role in the induction of HBDs by *F. nucleatum* from gingival epithelial cells

We recently reported the role of bacterial invasion in induction of human beta defensin (HBD)-2 and -3 by *F. nucleatum* from gingival epithelial cells (Ji et al., 2009). In the present study, the role of endosomal maturation in HBD induction by *F. nucleatum* was examined. Interestingly, pretreatment of HOK-16B cells with bafilomycin A1, an inhibitor of the vacuolar H^+ pump and endosomal acidification (Hanada et al., 1999), abolished the induction of HBD-2 and -3 by *F. nucleatum* (Fig. 7A), while *F. nucleatum* invasion was not affected (Fig. 7B).

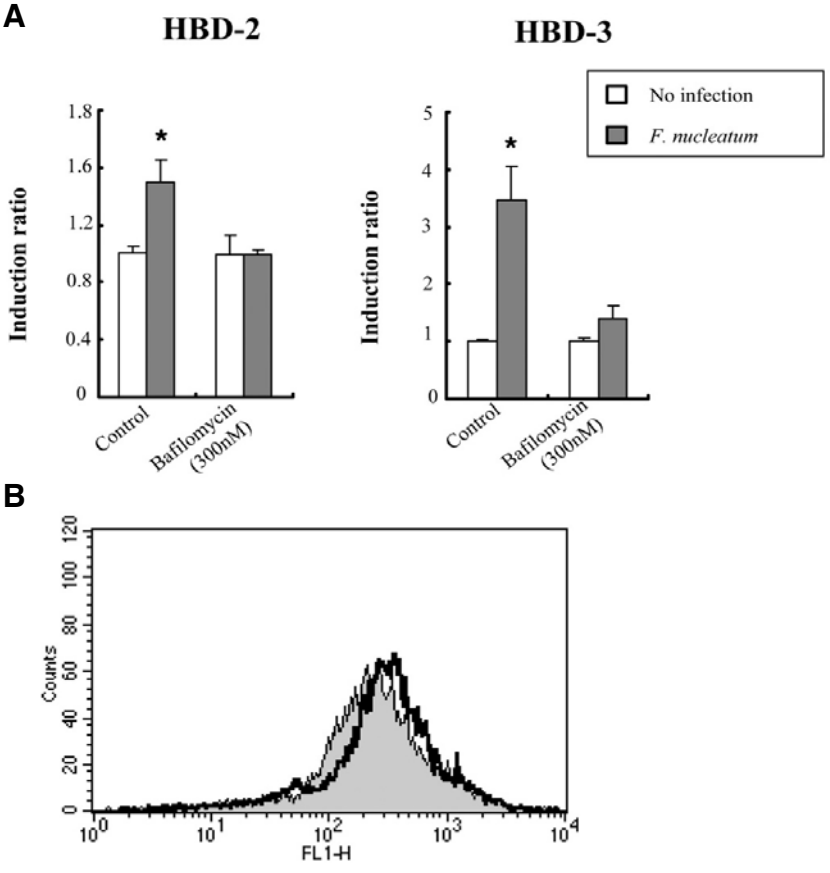

Fig. 7. Role of endosomal maturation in HBD-2 and HBD-3 induction by *F. nucleatum*. (A) HOK-16B cells were pretreated with 300 nM bafilomycin A1, an inhibitor of endosomal maturation, for 30 min and then infected with *F. nucleatum* at m.o.i. 1000 for 24 h. The effect of 300 nM bafilomycin A1 on HBD-2 and -3 induction by *F. nucleatum* was evaluated by real-time RT-PCR. The mean ± SEM values from nine real-time RT-PCR assays were expressed as an induction ratio compared to control culture without bacteria. *$p < 0.05$ versus control. (B) HOK-16B cells were pretreated with vehicle (filled with light gray) or 300 nM bafilomycin A1 (solid empty line) for 30 min and infected with CFSE-labeled *F. nucleatum* at m.o.i. 1000 for 2 h. The effect of bafilomycin A1 on *F. nucleatum* invasion was assessed by flow cytometry as in Fig. 1B.

DISCUSSION

In this study, we investigated the intracellular destiny of *F. nucleatum* in gingival epithelial cells and its cytopathic effect on host cells. *F. nucleatum* efficiently invades gingival epithelial cells, but the internalized bacteria are quickly trafficked to an endocytic degradation pathway without harming host cells.

The highly invasive characteristic of *F. nucleatum* was shown by a modified flow cytometric phagocytosis assay (Fig. 1B). Compared with the conventional method, which enumerates the number of viable bacteria inside host cells, this method is fast and adequate for assessing the invasive ability of bacteria in a manner that is independent of their intracellular survival (Pils et al., 2006). One limitation is that the fluorescence intensity of labeled bacteria can affect the results. Despite differences in methodology and bacterial strains, our results agree with those of another group (Han et al., 2000) who showed that *F. nucleatum* and *P. gingivalis* demonstrated the highest invasive capacity, followed by *P. intermedia* and *T. forsythia*. *T. denticola* has been previously reported to invade periodontal tissue through the intercellular space (Lux et al., 2001); in this study, we have shown its ability to directly invade cells.

Although *F. nucleatum* demonstrated a highly invasive capacity, comparable to that of *P. gingivalis*, the destinies of these two bacterial species within their host cells were quite different. In contrast to *P. gingivalis,* which escapes endosomes immediately after invasion and survives in the cytoplasm (Lamont et al., 1995), *F. nucleatum* was shuttled to endolysosomes. By examination with TEM, some internalized *F. nucleatum* appeared to be located in the cytosol immediately following infection. This observation can be interpreted in two possible ways: 1) the bacteria had escaped the endosomes and 2) the resolution was not sufficient to discriminate a membrane surrounding the bacteria. Since all intracellular bacteria were found within membrane-enclosed vesicles after 24 h culture, the latter possibility is more likely. The fact that *F. nucleatum* invades gingival epithelial cells via a zipping mechanism in close contact with the host cell membrane (Han et al., 2000) also supports the latter possibility. Although the antibiotics protection assay has limitations due to incomplete killing of extracellular bacteria, it is clear that *F. nucleatum* cannot survive more than 12 h within host cells. The number of intracellular bacteria rapidly decreased between 4 h and 6 h after infection, which coincides with the fusion with lysosomes at 4 h and the dramatic decrease in intracellular bacteria at 24 h, as observed by TEM. Gursoy et al. (2008) reported intracellular replication and release of *F. nucleatum* into medium in skin keratinocytes. We did not examine intracellular replication or release into the medium. Whether or not such a discrepancy is attributed to host cell differences requires further investigation. Gursoy et al. (2008) cultured infected cells only up to 11 h; longer culture may lead to different results. The ability of bacteria to survive inside host cells is an important pathogenic factor, and mutants that have lost this ability demonstrate a reduction in pathogenicity (Fuller et al., 2008; Grabenstein et al., 2004). Furthermore, *F. nucleatum* induced neither cell death nor changes in morphology of gingival epithelial cells, whereas *P. gingivalis* changes the morphology of host cells from spread to rounded by interfering with cell-cell adhesion (Hintermann et al., 2002). Thus, *F. nucleatum* is expected to have reduced pathogenicity compared with *P. gingivalis*, a well-defined periodontal pathogen.

Although internalized *F. nucleatum* was eventually killed, its invasion and subsequent degradation in endolysosomes was important for the induction of HBD-2 and -3 from gingival epithelial cells. We recently reported that TLR2 and NALP2 mediate HBD-2 and -3 induction by *F. nucleatum* (Ji et al., 2009). TLR2, normally expressed on the cell surface, is often enriched in endosomes after activation, which leads to increased recognition of pathogen-associated molecular patterns within endosomes (Tricker and Cheng, 2008). Breakdown of *F. nucleatum* in the endolysosomes may also provide access to NALP2 located in the cytoplasm.

Collectively, *F. nucleatum* is expected to play a minor role in periodontal tissue destruction in the presence of an intact epithelial barrier. *F. nucleatum* degradation within gingival epithelial cells demonstrates the active role of an epithelial barrier in protection against bacterial invasion to the underlying tissue. Furthermore, invasion of *F. nucleatum* into gingival epithelial cells may actually enforce the epithelial barrier by enhancing the expression of HBDs. Thus, *F. nucleatum* might only play an indirect role in the development of periodontitis by facilitating colonization and invasion of other periodontal pathogens (Saito et al., 2009). In periodontal lesions where the barrier is no longer intact, however, *F. nucleatum* may gain access to the connective tissue and aggravate tissue destruction directly through proteases or indirectly through the induction of potent inflammatory responses (Bachrach et al., 2004; Rossano et al., 1993). In addition, an organism can be 'a friend' in one place but 'a foe' in another (Feng and Weinberg, 2006), and *F. nucleatum* may act as a pathogen in the placenta, liver, or lung (Bauer et al., 2000; Hsu and Luh, 1995).

In conclusion, *F. nucleatum* is destined to the endocytic degradation pathway after invasion and has no cytopathic effect on gingival epithelial cells, which may cast new light on the role of *F. nucleatum* in the pathogenesis of periodontitis.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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