Oral Microbiology
and Immunology

Bacterial internalization in periodontitis

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Background: Bacterial invasion of host epithelial cells plays an important role in the pathogenesis of periodontitis; however, the exact mechanism of the invasion has not been investigated.
Methods: Pocket epithelium biopsies in periodontitis were analysed via scanning and transmission electron microscopy using ultra-histochemical staining with ruthenium red for glycocalyx visualization.
Results: We demonstrated that oral bacteria adhered via fimbriae-mediated adhesion only. The bacterial internalization in periodontitis was marked by the hallmark of the fimbriae-induced zipper mechanism – the phagocytic cup formation – but we found no sign of the trigger mechanism of internalization. In addition, we frequently observed apoptosis in the phagocytizing epithelial cells.
Conclusion: Fimbriae-mediated adhesion is a prerequisite for bacterial invasion in periodontitis. This occurs by the fimbriae-induced zipper mechanism of internalization. As internalization induces apoptosis, the subsequent exfoliation might play a significant role in the clearance of periodontal pathogens.

Periodontitis is an inflammatory disease caused by the microorganisms of subgingival plaque (19) and those adhering to the sulcular gingiva (22). To establish and maintain a successful infection, microbial pathogens have evolved a variety of strategies to invade the host, avoid or resist the innate immune response, damage the cells, and multiply in specific and normally sterile regions (2). Internalization of putative periodontal pathogens, e.g. *Prevotella intermedia* (3), *Porphyromonas gingivalis* (4, 10, 16, 18), *Actinobacillus actinomycetemcomitans* (11, 12), and *Fusobacterium nucleatum* (8) has been observed *in vitro*. In addition, bacterial internalization by the junctional (7, 17) and pocket (1) epithelium in advanced periodontitis has been demonstrated by transmission electron microscopy. Two main entry mechanisms are known: zipper and trigger mechanism. The trigger mechanism is characterised by dramatic host membrane ruffling, whereas the zipper mechanism is typified by phagocytic cup formation and requires

fimbriae-mediated adhesion (2, 6). The only type of adhesion reported in periodontitis is the fimbriae-mediated one (22). Microbial pathogens using the zipper mechanism to enter nonprofessional phagocytes (e.g. epithelial cells) express fimbrial adhesins able to bind host surface

receptors involved in cell-to-cell adhesion, e.g. integrins. The expression of these adhesins leads to the formation of a vacuole that engulfs the bacterium through a 'zippering' process in which relatively modest cytoskeletal rearrangements and membrane extensions occur in response to
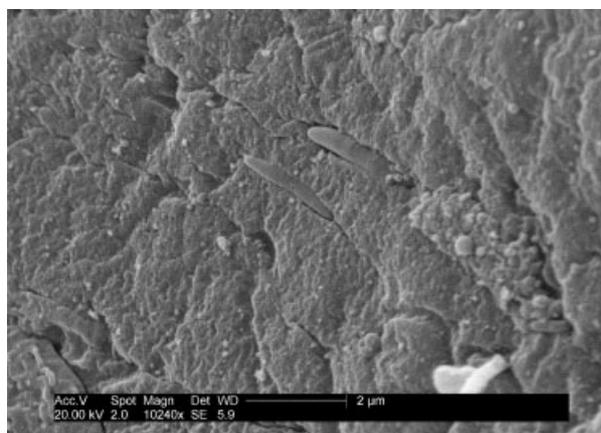

Fig. 1. SEM. Bacteria partly engulfed by an epithelial cell. No membrane ruffles can be observed.
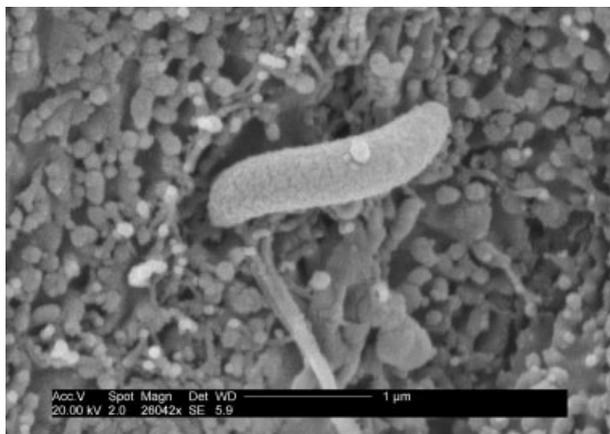
Fig. 2. SEM. Beginning of the internalization of a rod bacterium. Formation of a membrane invagination (phagocytic cup) that surrounds the bacterium is evident.
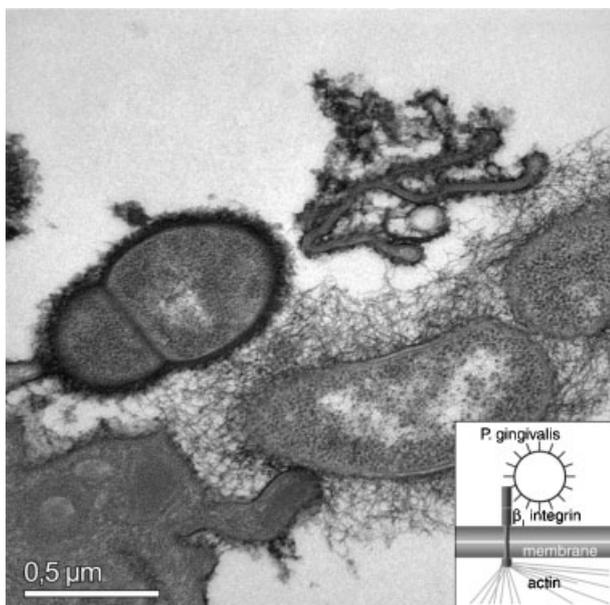

Fig. 3. TEM. Bacteria adhered to the epithelial surface by different types of fimbriae. The bacterial fimbriae are deeply blackened with the RR-OsO₄ technique. Fimbriae-mediated adhesion is the first stage of the zipper mechanism of internalization. Inset: a diagram illustrating the receptor binding by fimbrial adhesins. The signaling pathway is not activated yet.

engagement of the adhesin receptors (2). The fimbrial adhesion-induced zippering mechanism of internalization was observed in *P. gingivalis in vitro* (15); however, the internalization mechanism(s) of oral bacteria in periodontitis have not been investigated.

The aim of the present work was to examine, by scanning and transmission electron microscopy, the internalization mechanism in periodontitis.

Material and methods
Biopsies

Eight patients (mean age of 58 years) with progressive chronic periodontitis were selected for this study. In all patients, teeth extractions were indicated with the necessity to improve the contour of the alveolar bone by reducing bone prominences. Radiological examination revealed irregular bone resorption of more than 50% and depth-on-probing exceeding 6 mm. Exclusion criteria for patients were: systemic diseases related to subacute bacterial endocarditis prophylaxis, diabetes mellitus, antibiotic therapy in the last 6 months, steroid and radio-therapy. The study protocol was approved by the local ethics committee and informed written consent was obtained from all patients.

The gingival tissue situated between two of the extracted teeth was excised with a scalpel and immediately washed in 100 ml saline by gently swaying for 20 s to remove all the substances in contact with but not those attached to the epithelial surface. The biopsies were divided into two pieces along the root axis. One piece of each biopsy was processed for scanning and the other one for transmission electron microscopy.

Scanning electron microscopy (SEM)

The biopsies were fixed with 1.2% glutaraldehyde (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h at room temperature. Postfixation of the samples was performed with 1% osmium tetroxide (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h. The postfixed filters were dehydrated in ascending series of ethyl alcohol, critical-point-dried in a drying device CDP 030 (BAL-TEC, Balzers, Liechtenstein) and subsequently sputtered with gold (ca.. 10 nm). The specimens were examined in an environmental scanning electron microscope ESEM XL30 (FEI Company, Philips, Eindhoven, Netherlands) operating at 20 kV.

Transmission electron microscopy (TEM)

The biopsies were fixed with the ruthenium red-osmium tetroxide technique (RR-OsO₄ technique). This enables the visualization of fimbriae-mediated adhesion (22–24). The samples were fixed with 1.2% glutaraldehyde (buffered at pH 6.5 with 0.1 M sodium cacodylate) and 0.05% ruthenium red for 2 h at room temperature. Postfixation was performed with 1% osmium tetroxide (buffered at pH 6.5 with 0.1 M sodium cacodylate) and 0.05% ruthenium red for 2 h at room temperature. All specimens were routinely embedded in Epon 812. Ultrathin sections were cut on an ultramicrotome (Reichert Ultracut S, Optische Werke C. Reichert, Vienna, Austria), contrasted with LKB 2168 Ultrostainer (LKB Produkter AB, Bromma, Sweden) and examined in a transmission electron microscope LEO EM 910 (LEO Elektronenmikroskopie Ltd, Oberkochen, Germany) operating at 80 kV.

Results
SEM analysis

Bacterial internalization was evident in the pocket epithelium (Fig. 1 and 2). Under high magnification the formation of a phagocytic cup around the internalising bacteria was revealed, which is the hallmark of the zipper mechanism of

internalization (Fig. 2). No membrane ruffling was observed.

TEM analysis

The samples from the pocket epithelium were characterized by extensive fimbrial adhesion (Fig. 3), bacterial biofilm formation, and lymphocyte transmigration. Bacterial engulfment by both nonprofessional (epithelial cells) (Fig. 4) and professional (leukocytes) phagocytes was evident in the pocket epithelium. For the zipper mechanism of bacterial internalization the fimbriae are characteristically the only bacterial part touching the epithelial cell membrane. The latter was deeply invaginated to engulf the bacterium, i.e. forming a phagocytic cup. The epithelial membrane forming a vacuole was evident in the last stage of internalization (Fig. 5). Neither membrane ruffling nor adhesion mediated by afimbrial adhesins was observed. Massive internalization was frequently accompanied by apoptosis of the phagocytizing pocket epithelium cells. The apoptosis was marked by the characteristic alterations: cellular detachment (Fig. 6), plasma membrane blebbing (Fig. 7), and nuclear condensation as well as fragmentation culminating in the formation of apoptotic bodies (Fig. 8). As nuclei were infrequently observed in the outer cell layers, the latter two features were conclusive. The bacterial glycocalyx in the vital epithelial cells was not blackened because of the impermeability of the intact epithelial membrane for ruthenium red. By contrast, the glycocalyx of some bacteria within apoptotic cells was strongly blackened.

Discussion

The present study provides, for the first time, ultrastructural evidence of fimbriae-induced zippering internalization in periodontitis. In addition, our findings confirm earlier reports (22) that only fimbriae-mediated adhesion has been observed in periodontitis. The adhesion in the human oral cavity is crucial for the microbial survival, as microbes have to attach themselves to a host surface before being able to withstand the saliva flow, mastication, and stress resulting from swallowing, breathing, speaking, etc. The adhesion is a result of noncovalent binding between bacterial adhesins and receptors expressed on the membranes of the eukaryotic cells. Depending on the expression of the adhesins, which are either directly on the bacterial outer surface or on the append-
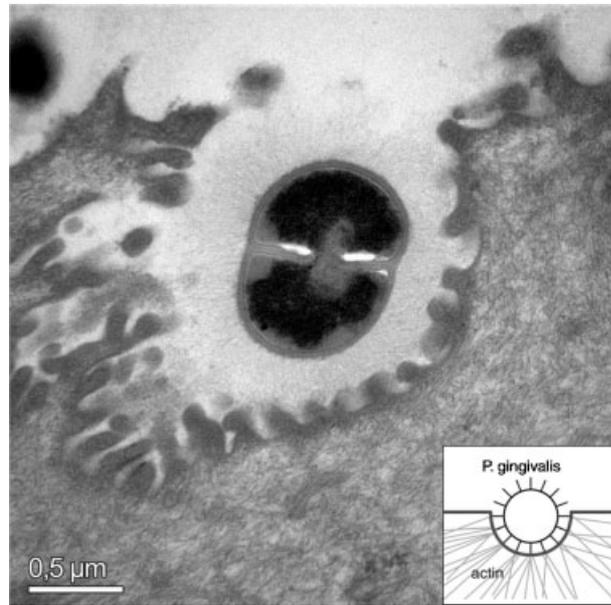

Fig. 4. TEM. Engulfing of a bacterium via a phagocytic cup formation by an epithelial cell. Phagocytic cup formation is the second stage of the internalization. The fimbriae are distinctly visualized with the RR-OsO$_4$ technique. Inset: a diagram illustrating the phagocytic cup formation as a consequence of actin polymerization and membrane extension due to the transient signals by the formation of adhesin–receptor complexes.
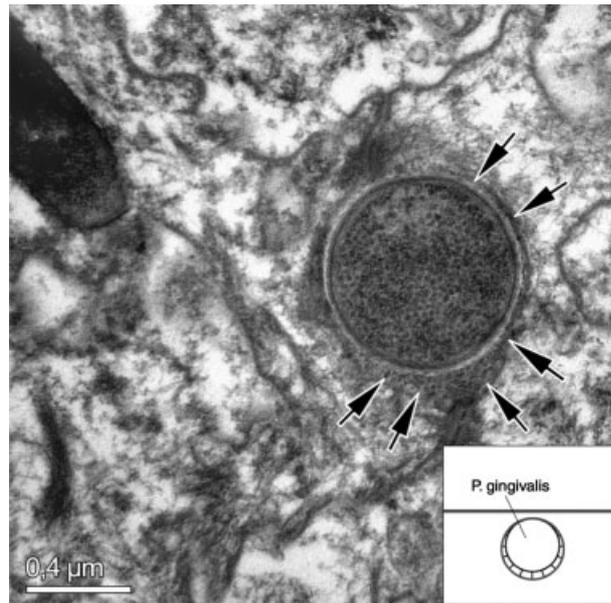

Fig. 5. TEM. An epithelial cell with an engulfed bacterium within a vacuole. Arrows: vacuole wall. This is the third and last stage of the internalization. On the left a bacterium, probably escaped from the vacuole, is evident. The vacuole lysis is a common pathogen mechanism to evade phagocytosis (2, 6). Inset: a diagram illustrating phagocytic cup closure and retraction as well as actin depolymerization.

ages (fimbriae or pili) attached to it, two morphologic types of adhesion can be differentiated: fimbriae-mediated adhesion and adhesion mediated by afimbrial adhesins. The fimbriae (pili) are long, thin, filamentous, multimeric macromolecules attached to the bacterial surface. The entirety of the fimbriae forms an additional polysaccharide-rich surface layer of the cell wall denoted as the glycocalyx (5). It is peripherally located on the outer membrane in gram-negative bacteria and to the
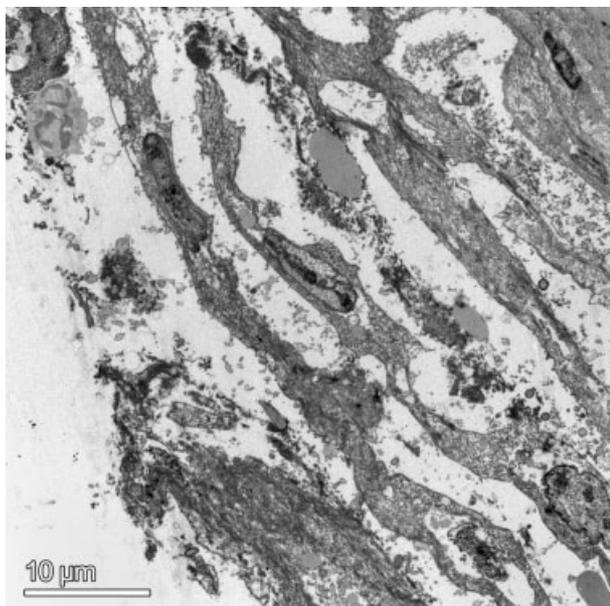
Fig. 6. TEM. Detachment of apoptotic epithelial cells. Clearance by exfoliation (detachment and subsequent shedding) might be an epithelial cell response to the bacterial invasion. A multitude of blebs between the cells are evident.
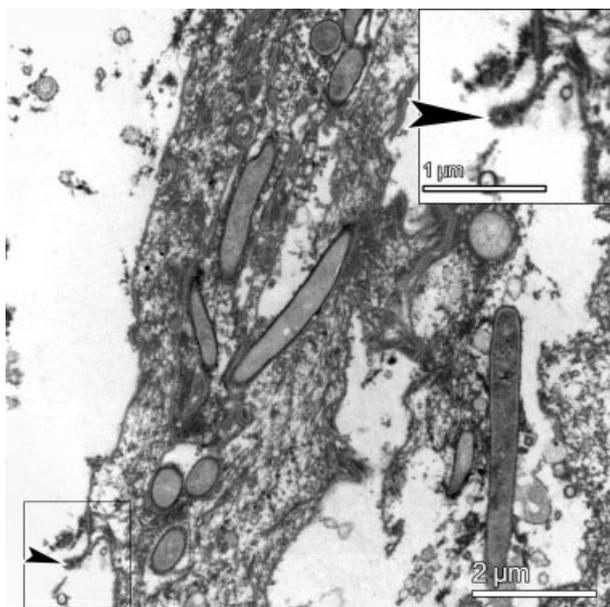

Fig. 7. TEM. A detail from Fig. 6. Many plasma membrane blebs are evident. Inset: a detail. Arrowhead: a bleb formation. The glycocalyx of the internalized bacteria is strongly blackened by ruthenium red. Some of the bacteria are lying in vacuole-like formations.

peptidoglycan in gram-positive ones. From the cytochemical view of point, the bacterial fimbriae are glycoproteins. As their carbohydrate part is not stainable by routine electron microscopic staining, glycocalyx cannot be easily monitored in TEM unless special staining techniques are used. In the present study, the RR-OsO$_4$ technique for visualization of bacterial fimbriae, as applied for human oral biopsies (22–24), was used. This technique relies on the ability of the carbohydrate part of the fimbriae to be semiselectively stained by the cationic dye ruthenium red (5, 20). The high differentiation ability of this method is due to the combination of cytochemical and morphologic characteristics resulting from the application of the RR-OsO$_4$ technique to tissue samples (22–24) .

Unlike fungi (24), oral bacteria are unable to penetrate the membrane of the host cells by themselves. Thus, bacterial invasion is correctly referred to as internalization. In the case of the zipper mechanism, the internalization is induced by the bacterial fimbriae, but is fulfilled by the host cells. Thus, *P. gingivalis* fimbriae attached to latex microspheres elicit the internalization of the latter (15). The zipper mechanism of entry can be divided into three successive steps (2):

- contact and adhesion: this step is independent of the actin cytoskeleton and involves only the bacterial adhesin and its receptor and leads to receptor clustering;
- phagocytic cup formation: this step is triggered by the transient signals occurring after formation of the first adhesin-receptor complexes and propagating around the invading microbe. These signals induce actin polymerization and membrane extension;
- phagocytic cup closure and retraction as well as actin depolymerization.

β1 integrins are identified as human epithelial receptors for *P. gingivalis* fimbriae (25). Interestingly, the affinity of the integrin for its ligand can be altered by signals from within the cell (inside-out signaling) in a process referred to as integrin activation (9). Thus, inside-out signaling of the gingival epithelial cells might be the crucial factor in periodontal pathology; however, future investigations in molecular biology will have to determine this. The internalization shields the bacteria from the host immunity. Using the altered permeability of the epithelial cell membrane for ruthenium red (10, 13) and characteristic cellular alterations, we demonstrated on the ultrastructural level that the internalization induced apoptosis of the phagocytizing epithelial cells in periodontitis. The apoptosis observed in the superficial epithelial layers in periodontitis has been suggested as a relevant process for the maintenance of local immune homeostasis at sites of chronic bacterial challenge (21). Bacterial clearance in bladder epithelium by exfoliation as a consequence of internalization has been demonstrated in an animal model (14). As *P. gingivalis* and other periodontal pathogens are also found in periodontal healthy persons, the question arises whether clearance by apoptosis occurs in the crevices of these persons. Probably, apoptosis induced by internalization might be of significance for the pathogenesis of periodontitis.
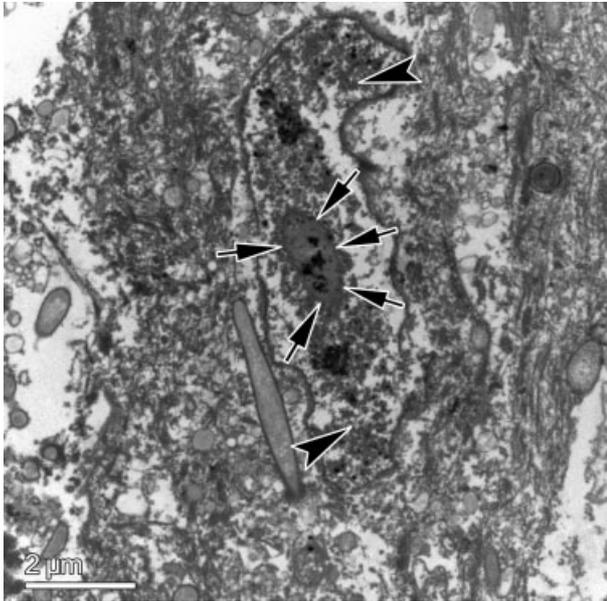
Fig. 8. TEM. Central: an apoptotic nucleus showing advanced nuclear fragmentation and condensation. Arrows: an apoptotic body. Arrowheads: ruthenium red-positive substances, which cannot be visualized by routine staining.

The ultrastructural evidence presented for bacterial internalization in periodontitis demonstrated that bacterial adhesion to human gingival epithelium in periodontitis is a prerequisite for invasion. Consequently, antiadhesive therapy might be of clinical interest. The detection of internalization-induced apoptosis provides new perspectives on the pathogenesis of periodontitis.

Acknowledgments

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