Role of Polymorphonuclear Leukocyte-Derived Serine Proteinases in Defense against *Actinobacillus actinomycetemcomitans*

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Periodontitis is a chronic destructive infection of the tooth-supportive tissues, which is caused by pathogenic bacteria such as *Actinobacillus actinomycetemcomitans*. A severe form of periodontitis is found in Papillon-Lefèvre syndrome (PLS), an inheritable disease caused by loss-of-function mutations in the cathepsin C gene. Recently, we demonstrated that these patients lack the activity of the polymorphonuclear leukocyte (PMN)-derived serine proteinases elastase, cathepsin G, and proteinase 3. In the present study we identified possible pathways along which serine proteinases may be involved in the defense against *A. actinomycetemcomitans*. Serine proteinases are capable to convert the PMN-derived hCAP-18 into LL-37, an antimicrobial peptide with activity against *A. actinomycetemcomitans*. We found that the PMNs of PLS patients released lower levels of LL-37. Furthermore, because of their deficiency in serine proteases, the PMNs of PLS patients were incapable of neutralizing the leukotoxin produced by this pathogen, which resulted in increased cell damage. Finally, the capacity of PMNs from PLS patients to kill *A. actinomycetemcomitans* in an anaerobic environment, such as that found in the periodontal pocket, seemed to be reduced. Our report demonstrates a mechanism that suggests a direct link between an inheritable defect in PMN functioning and difficulty in coping with a periodontitis-associated pathogen.

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severe reduction in serine protease activities in PLS patients. The importance of elastase, cathepsin G, and protease 3 in the immune system has been studied extensively. Studies of knockout mice reveal a crucial role in the defense against pathogens such as Staphylococcus aureus (57) and Escherichia coli (4, 48), Candida albicans (57), or Klebsiella pneumoniae (4). In addition, in vitro experiments showed that cathepsin G and elastase are able to kill A. actinomycetemcomitans and Capno-cytophaga spp. (3). As mentioned above, elastase and cathepsin G are able to neutralize the leukotoxin of A. actinomycetemcomitans (34). In vitro, all three serine proteases are able to convert the PMN-derived hCAP-18 (which is normally stored in the specific granules (44) into the antimicrobial peptide LL-37 (65). After exocytosis, however, only protease 3 seems to be capable of processing hCAP-18 (65). LL-37 has activity against a broad range of pathogens such as S. aureus, E. coli, Pseudomonas aeruginosa, and K. pneumoniae (25, 52, 64, 78) and also to A. actinomycetemcomitans (56, 69). In humans, the absence of LL-37 is associated with the Morbus Kostmann syndrome (56). These patients suffer from a congenital neutropenia and face severe periodontal disease during adulthood (56). No other deficiencies in LL-37 in human PMNs have been reported thus far.

Serine proteases (e.g., elastase, cathepsin G, and protease 3), together with antimicrobial peptides (e.g., LL-37), form the basis of the oxygen-independent machinery PMNs can use to kill bacteria (12, 49). Since the periodontal pocket is characterized by a reduced oxygen tension (50), the defense against pathogens in this environment may depend predominantly on oxygen-independent means. Based on our previous finding that PLS patients lack elastase, cathepsin G, and protease 3 activity (16), we hypothesize that the etiology of periodontitis in these patients is due to a PMN defect, resulting in a compromised innate immune response to A. actinomycetemcomitans. It was the aim of the present study to identify the importance of elastase, cathepsin G, and protease 3 in the defense against A. actinomycetemcomitans.

MATERIALS AND METHODS

Reagents. All chemicals were obtained from Sigma (Sigma Chemicals Co, St. Louis, MO) unless stated otherwise.

Patients and controls. Three families with PLS patients participated in the present study. Samples of family A included two female PLS patients (AP1, 19 years old) and her parents (AC1 and AC2); samples of family B included one female PLS patient (BP1, 19 years old) and her parents (AP1, 27 years old; AP2, 29 years old) and their parents (AC1 and AC2); samples of family C included one female PLS patient (BP1, 19 years old) and her parents (AP1, 27 years old; AP2, 29 years old) and their parents (AC1 and AC2). Blood samples were available from family B, due to too-frequent sample collection, decided to stop participating. The Institutional Review Board on Human Studies approved this study, and all individuals involved gave their written consent, in accordance with the World Medical Association Declaration of Helsinki.

Sample isolation, sequencing, and mutation analysis. Peripheral heparin anticogulated blood was collected by standard venipuncture from family C for mutation analysis of the cathepsin C gene. Genomic DNA was isolated from the blood samples by using the Puregene kit (Gentra Systems, Inc., Michigan) according to the manufacturer’s protocol. Sequencing was performed as described previously (16). In family C, a previously reported mutation, c.815G>C/p.R272P, was found (72). Patients CP1 and CP2 were homozygous for this mutation, whereas the mother (CC1) was a heterozygous carrier. The patients showed no cathepsin C activity, and the mother had reduced (50%) levels. We confirmed that the patients were deficient for elastase, cathepsin G, and protease 3 activity (16).

Isolation of PMNs from peripheral blood. Peripheral blood (heparin anticogulated) was obtained from patients, parents, and healthy unrelated controls by standard venipuncture. The blood was diluted with phosphate-buffered saline (PBS) and layered on Percoll (density, 1.076 g/ml) or Ficoll (both from Pharmacia, Uppsala, Sweden). After centrifugation for 30 min at 1,000 × g, the supernatant was removed, and the red cell pellet was treated with lysis buffer (155 mM ammonium chloride, 0.1 mM EDTA, 10 mM KHCO3) to remove the erythrocytes. The PMNs were washed twice with PBS, collected by centrifugation for 5 min at 300 × g at 4°C, and resuspended in assay buffer (20 mM HEPES, 152 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.2 mM KH2PO4, 1 mM CaCl2, 5.5 mM glucose [pH 7.4]) (28). Cell viability was assessed by trypan blue exclusion and was generally more than 98%.

Enzyme activity. Proteinase 3 activity was measured by the hydrolysis of N-t-but-Ala-Ala-Nva-thiobenzylester (Elastin Products Co., Missouri) (39). The reaction buffer consisted of 100 mM HEPES (Gibco Laboratories, Grand Island, NY), 500 mM NaCl, 5 mM DTTN [5′-dithiothreitol-2-nitro- benzoylamidomethyl], and 250 μM N-t-but-Ala-Ala-Nva-thiobenzylester (pH 7.0) (Al-Aia-Nva-thiobenzylester). The PMNs were incubated with 10 ng/ml elastase, 4.0 ng/ml cathepsin G, and 1.3 μg/ml proteinase 3, together with antimicrobial peptides (e.g., LL-37), for 5 min at 300 × g at 4°C, and resuspended in assay buffer (20 mM HEPES, 152 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.2 mM KH2PO4, 1 mM CaCl2, 5.5 mM glucose [pH 7.4]) (28). Cell viability was assessed by trypan blue exclusion and was generally more than 98%.

Leukotxin degradation by PMN lysates. Leukotxin degradation by PMN lysates was studied as follows. Leukotxin, purified from the highly leukotoxic A. actinomycetemcomitans HK 1519 strain, was kindly provided by A. Johansson (University of Umeå, Sweden) (36). PMN lysates of PLS patients and their parents from family A and family B and a healthy male (family A and family B and a healthy male) were used as PMN lysates. Samples from PLS patients and parents were diluted with PBS to protein content, which was determined by using a BCA assay kit (Pierce, Rockford, Illinois). An aliquot of 2 μg of protein was incubated with 150 ng of leukotxin and incubated at 37°C for 3 h. Proteins in the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. PMNs from five healthy individuals (22 to 45 years old) were treated similarly, with comparable results (data not shown).

Leukotoxic activity. PMNs were resuspended in assay buffer at a concentration of 3.6 × 106 cells/ml. A 450-μl aliquot of cell suspension was incubated with 50 μl (150 ng) of leukotxin. The samples were incubated at 37°C in air with 5% CO2 for 1 h. After the incubation, the samples were placed on ice to stop the reaction. PMNs were collected after centrifugation (300 × g, 5 min, 4°C). PMNs expressing leukotxin were fixed in 1% glutaraldehyde and 4% formaldehyde for 1 h, washed three times with 0.1 M sodium cacodylate buffer (pH 7.4). The cells were washed and postfixed in 1% OsO4 in 0.1 M sodium cacodylate buffer. Next, the cells were dehydrated through a graded series of ethanol, pelleted, and embedded in epoxy resin (LX-112). Semithin sections were made with a diamond knife and stained according to the method of Richardson et al. (58). Sections were randomized and examined by using a ×60 objective lens on a Leica DM LB microscope (Leica, Wetzlar, Germany). According to their morphology, cells were classified as either viable or nonviable. Viable cells had intensely stained cytoplasm, normal-appearing nuclei, and discernible organelles; nonviable cells had weakly stained cytoplasm and were without clearly defined organelles (including the nucleus). At least 100 cells were counted per patient or control. The number of nonviable cells was expressed as a percentage of the total number of counted cells. Ultra-thin sections were stained with uranyl and lead and then examined in a Philips EM 420 electron microscope (Philips Medical Systems, Eindhoven, the Netherlands). Images were digitized, and figures were prepared by using Adobe Photoshop (Adobe Systems, San Jose, CA).

Degranulation of PMNs. For degranulation experiments, PMNs were resuspended in assay buffer to a concentration of 106 cells/ml. Cells were preincubated at 37°C for 5 min with 10 μg of cytochalasin B/ml and subsequently stimulated with 2.5 μM formylmethionine/leucylphosphatidylalanine (MLP) or 4 μg of phorbol 12-myristate 13-acetate (PMA)/ml for 15 min. Similarly, cells were preincubated at 37°C in assay buffer and then stimulated with 1 μM ionomycin for 15 min. Cells were placed on ice water to stop the stimulation and pelleted by centrifugation (300 × g, 10 min, 4°C). The supernatants were collected and stored at −20°C.
RESULTS

Serine proteinases are essential in the neutralization of leukotoxin. Serine proteinases have been demonstrated to be able to neutralize the leukotoxin produced by \textit{A. actinomycetemcomitans} (34). We studied the lack of serine proteinase activity in the PMNs of PLS patients on the degradation of leukotoxin. PMN lysates were obtained from two unrelated families, family A (parents AC1 and AC2, patients AP1 and AP2) and family B (parents BC1 and BC2, patient BP1), and a healthy control (H1) and incubated with 150 ng of leukotoxin for 3 h (Fig. 1A). In both families the PMN lysates were equally capable of cleaving the leukotoxin compared to the control. After 3 h of incubation the intact leukotoxin was no longer detectable, and fragments of the leukotoxin were detectable. In contrast, the samples of the PMN lysates of the PLS patients of both families showed fragments of the leukotoxin, but the greater part of the intact leukotoxin was still detected.

Next, we studied the importance of serine proteinases in the protection of PMNs to exposure to the leukotoxin (Fig. 1B). For this purpose, PMNs were isolated from PLS patients from two unrelated families (AP1, AP2, CP1, and CP2) and their parents (AC1, AC2, and CC1) and incubated with leukotoxin at 150 ng. Since there was no sample available from the father of family C, a sample of a female healthy (H2, aged 45) donor was used instead. The supernatants were analyzed for the presence of leukotoxin (Fig. 1B). In the samples of the parents (AC1, AC2, and CC1) and the healthy donor (H2) there was almost no intact leukotoxin at 116 kDa or fragments of the leukotoxin present, indicating that the leukotoxin had been degraded. The samples of the patients (AP1, AP2, CP1, and CP2) clearly showed the presence of the intact leukotoxin. Based on the intensity of the staining, the amount of leukotoxin was comparable to the initial quantity of leukotoxin (Fig. 1B, right lane). By using microscopy we studied in detail the condition of the PMNs after exposure to the leukotoxin (Fig. 1C). Electron microscopy revealed that the PMNs of the parent (AC1) had not been heavily affected by the leukotoxin compared to cells that were not exposed to the leukotoxin (Fig. 1C, left graphs). The majority of the PMNs had a “viable” appearance, as signified by an intense staining of cytoplasm and nuclei. The nuclei had a normal multilobed appearance. Furthermore, granules and other intracellular compartments were clearly visible. A similar result was found for the other parents and the healthy donor. In contrast, the PMNs of the patient (AP1) were severely damaged (Fig. 1C, right graphs). The majority of the PMNs of the patients stained vaguely both in the cytoplasm and in the nucleus. No organelles or granules were observed in the cytoplasm. PMNs of other patients (AP2, CP1, and CP2) showed similar phenomena. We quantified the percentage of “nonviable” cells in sections of PMNs of controls and of PLS patients by using light microscopy (Fig. 1D). It showed that a significantly higher percentage of PLS patient’s cells was affected ($P < 0.05$).

Processing of hCAP-18. By using enzymes and antimicrobial peptides, PMNs are very well equipped to handle bacteria in an anaerobic environment such as the periodontal pocket. LL-37 is an important antimicrobial peptide in PMNs, and its precursor hCAP-18 is stored spatially separated from the PMN-derived serine proteinases. Upon degranulation and exocytosis of the contents of the secretory granules in the extracellular environment, proteinase 3 has been demonstrated to process hCAP-18 into LL-37 (65). Although PLS patients have normal levels of serum hCAP-18 (data not shown), the lack of proteinase 3 activity in PLS patients may affect its processing to LL-37. We studied the extracellular processing of endogenous hCAP-18 in the PMNs of PLS patients of family A and compared this with their parents. We stimulated PMNs to degranulate by the use of various secretagogues (65). After 15 min of incubation, supernatants were collected and analyzed by Western blotting for the presence of hCAP-18, intermediate fragments (not fully processed hCAP-18 fragments), and LL-37.

The Western blot analysis clearly showed that hCAP-18 (at 18 kDa) was present in the exocytosed material of PMNs of the PLS patients and their parents (Fig. 2A). Since ionomycin is known to be the more potent secretagogue (65), the net yield of hCAP-18 was the highest. The striking result on the Western blot, however, was the yield in LL-37. Whereas the parents (AC1 and AC2) demonstrated demarcated bands at 4 kDa, most likely being LL-37, the PLS patients (AP1 and AP2) showed lower levels of LL-37. In the case of the weaker secretagogues PMA and FMLP, increased exposure times were necessary to visualize them. Another notable observation on the Western blot was the more pronounced presence of intermediate, not fully processed hCAP-18 fragments in the samples of the patients. Densitometric analysis of these bands confirmed that the PMNs of PLS patients had produced less LL-37 than the PMNs of their parents and that their intermediate frag-
ments were more pronounced (Table 1). Previously, we have shown that PLS patients have no proteinase 3 activity (16). To confirm our previously reported findings, we measured proteinase 3 activity in exocytosed material of the PMNs stimulated with ionomycin (Fig. 2B). The parents (AC1 and AC2) demonstrated proteinase 3 activity, whereas the patients (AP1 and AP2) were negative.

Serine proteinases are crucial in the anaerobic killing of *A. actinomycetemcomitans*. PLS patients are often infected with periodontitis-associated pathogens, *A. actinomycetemcomitans* in particular. We demonstrated that PLS patients had, next to the absence of serine proteinase activity and the reduced amounts of proteins themselves, also reduced levels of LL-37 and that PMNs were incapable of effectively neutralizing the leukotoxin produced by *A. actinomycetemcomitans*. Since the high leukotoxic activity of *A. actinomycetemcomitans* results in a reduced killing efficiency by PMNs (37), it is possible that PLS patients are even less efficient in killing this pathogen. In the following experiments we determined whether a high leukotoxic activity of *A. actinomycetemcomitans*, together with reduced levels of serine proteinase activity and reduced levels of LL-37 in PMNs of PLS patients, might cause difficulties in dealing with this particular pathogen in an anaerobic environment. The incubation of *A. actinomycetemcomitans* with the PMNs of PLS patients (AP1 and AP2) and their parents (AC1 and AC2) showed that there is a delay in killing for the PLS patients (Fig. 3). After 2 h of incubation, this delay seemed more pronounced in that the PMNs of the parents had killed 82% of the bacteria, whereas the PMNs of PLS patients had killed only 61% of the bacteria. Unfortu-
nately, there was no material for other PLS patients available to verify these data.

**DISCUSSION**

The results presented here provide new information about the biological mechanisms underlying the pathogenesis of a severe form of early-onset periodontitis as evidenced in patients suffering from Papillon-Lefèvre syndrome (PLS). Although periodontitis in PLS is often considered to be related to defective PMN functioning, no conclusive evidence has come forward in support of this assumption. The present results suggest that PMNs from PLS patients have difficulty in coping with *A. actinomycetemcomitans* in an anaerobic environment. Our results thus provide evidence for a mechanism to link a monogenetic defect in PMN functioning with one of the well-known periodontitis associated pathogens in humans. The microbiological status of PLS patients suggests that a majority of them are infected with *A. actinomycetemcomitans* (1, 5, 7, 13, 17, 18, 20, 22, 33, 41, 42, 46, 53, 60, 67, 70, 71, 74, 75, 76). This is in line with our finding that their resistance to this pathogen may be compromised.

The periodontal pocket is unique: it forms an anaerobic environment with a more or less specific bacterial flora (50). The anaerobic environment forces PMNs to use compounds other than those generated via oxygen, for which PMNs are well adapted. They release, for instance, increased levels of...
TABLE 1. Densitometric analysis of the immunoreactive bands of a Western blot of hCAP-18/LL-37 of exocytosed material of PMNs isolated from PLS patients and their parents of family Aa

<table>
<thead>
<tr>
<th>Secretagogue</th>
<th>hCAP-18</th>
<th>Intermediate-size fragments</th>
<th>LL-37</th>
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<tbody>
<tr>
<td></td>
<td>Parents</td>
<td>Patients</td>
<td></td>
</tr>
<tr>
<td>FMLP</td>
<td>46.0 ± 0.1</td>
<td>65.9 ± 5.4</td>
<td>15.4 ± 3.7</td>
</tr>
<tr>
<td>PMA</td>
<td>48.1 ± 4.7</td>
<td>61.0 ± 0.3</td>
<td>13.6 ± 0.1</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>40.5 ± 0.1</td>
<td>46.7 ± 1.1</td>
<td>10.3 ± 0.7</td>
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aSee Fig. 2A.

The total amounts of immune reactive protein were determined by densitometry. Individual bands for hCAP-18, intermediate fragments, and LL-37 are expressed as percent values relative to the total immunoreactive protein (100%). The total immunoreactive protein was expressed per secretagogue (FMLP, PMA, and ionomycin).

Serine proteinases (12). Extensive research has revealed multiple roles of serine proteinases in inflammation and host defense, including a role in the neutralization of toxins. Our present data demonstrate that the PMNs of PLS patients are unable to degrade the leukotoxin of A. actinomycetemcomitans, which is the prime virulence factor for this pathogen (19, 77). Leukotoxin selectively kills human leukocytes (77) and increases the release of proinflammatory interleukin-1β from human macrophages (40). Our observation that the PMNs from PLS patients are more susceptible to lysis by exogenously added leukotoxin supports earlier studies showing that serine protease-mediated degradation of leukotoxin is essential for the neutralization (34). The importance of this leukotoxin in the pathogenesis of periodontitis is illustrated by the high prevalence of early-onset periodontitis in a group of children in Morocco (30) who all carried a strain of A. actinomycetemcomitans producing an extremely high level of leukotoxin. Early-onset periodontitis is a relatively rare phenomenon; it suggests that dealing with the leukotoxin seems to be crucial, particularly in children to adequately cope with an A. actinomycetemcomitans infection.

Serine proteinases, and more specifically protease 3, have also been suggested to play an essential role in the processing of hCAP-18 into the antimicrobial peptide LL-37 (65). We demonstrated that the PMNs of PLS patients release reduced levels of LL-37. Since LL-37 possesses antimicrobial activity against A. actinomycetemcomitans (69), inhibition of processing and release of this antimicrobial peptide may result in reduced bacterial clearance (14). This is supported by the observation that the absence of LL-37 in Morbus Kostmann is associated with a high prevalence of A. actinomycetemcomitans and severe periodontal disease (56). Sorensen et al. (65) have demonstrated that depletion of protease 3 from exocytosed material of PMNs results in a marked decreased processing of hCAP-18 to LL-37. By using a natural human model deficient in serine protease activity (PLS) (16, 54), we have clearly shown the presence of processed hCAP-18 in exocytosed material of PMNs, despite the absence of serine protease activity. Therefore, these data suggest that, in the absence of PMN serine protease activity, hCAP-18 can be processed by less-efficient mechanisms. It is known that hCAP-18 in seminal fluid, which is derived from the epithelium of the epididymis, is processed by the aspartic protease gastricin, resulting in ALL-38 (66). Although the LL-37 release by stimulated PMNs from PLS patients migrated at the predicted size on SDS-PAGE gels, we cannot formally exclude the possibility that PLS PMNs release an alternatively processed form of hCAP-18. Alternative processing of hCAP-18 is further suggested by differences that were noted in the intensity of the intermediate-size fragments. Furthermore, we have indirect evidence to suggest that the processing of hCAP-18 in PMNs of PLS patients is associated with enzymes located in the azurophilic granules, since in PMNs from patients the rate of processing was found to diminish with less-potent azurophilic granule secretagogues (11, 65). The question of which enzymes process hCAP-18 in PMNs of PLS patients remains to be elucidated.

Finally, serine proteinases possess antimicrobial activity through direct enzymatic action and/or through peptides derived from the enzymes (3). In addition, research with mouse models has indicated that serine proteinases are required for adequate killing of pathogens such as S. aureus and K. pneumoniae (4, 48, 57, 57). The relatively high prevalence of A. actinomycetemcomitans illustrates the notion that PLS patients may have serious trouble in coping with A. actinomycetemcomi-
tans infections. This microorganism is mainly killed by PMNs via the oxygen independent pathway, e.g., antimicrobial peptides and serine proteinases (51), components that seem to fail via the oxygen independent pathway, e.g., antimicrobial peptidases. A. actinomycetemcomitans is mainly killed by PMNs.

Our knowledge of the key players in periodontal disease is still very limited. Over the last few decades it has become apparent that certain pathogens may be important in the development of the disease (38). The role of the host in the defense against these pathogens has only recently become the subject of research. The findings presented here support the notion that severe early-onset periodontitis in PLs is likely to be caused by malfunctioning of the defense against periodontal pathogens, A. actinomycetemcomitans in particular. It demonstrates, similarly to the Kostmann syndrome, a direct link between an inheritable defect in PMN functioning with difficulty in coping with a specific periodontitis-associated pathogen. Furthermore, it may provide clues in general to understand what exactly causes individuals to be more susceptible to certain pathogens.

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