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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Papillon-Lefèvre Syndrome: Correlating the Molecular, Cellular, and Clinical Consequences of Cathepsin C/Dipeptidyl Peptidase I Deficiency in Humans

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A variety of neutral serine proteases are important for the effector functions of immune cells. The neutrophil-derived serine proteases cathepsin G and neutrophil elastase are implicated in the host defense against invading bacterial and fungal pathogens. Likewise, the cytotoxic lymphocyte and NK cell granule-associated granzymes A and B are important for the elimination of virus-infected cells. The activation of many of these serine proteases depends on the N-terminal processing activity of the lysosomal cysteine protease cathepsin C/dipeptidyl peptidase I (DPPI). Although mice deficient in DPPI have defects in serine protease activation in multiple cellular compartments, the role of DPPI for human serine protease activation is largely undefined. Papillon-Lefèvre syndrome (PLS) is a rare autosomal recessive disease associated with loss-of-function mutations in the DPPI gene locus. In this study, we established that the loss of DPPI activity is associated with severe reduction in the activity and stability of neutrophil-derived serine proteases. Surprisingly, patients with PLS retain significant granzyme activities in a cytotoxic lymphocyte compartment (lymphokine-activated killer) and have normal lymphokine-activated killer-mediated cytotoxicity against K562 cells. Neutrophils from patients with PLS do not uniformly have a defect in their ability to kill *Staphylococcus aureus* and *Escherichia coli*, suggesting that serine proteases do not represent the major mechanism used by human neutrophils for killing common bacteria. Therefore, this study defines the consequences of DPPI deficiency for the activation of several immune cell serine proteases in humans, and provides a molecular explanation for the lack of a generalized T cell immunodeficiency phenotype in patients with PLS. 


Papillon-Lefèvre syndrome (PLS) is a rare autosomal recessive disease that affects one to four persons per million (1, 2). It is characterized by erythematous palmoplantar hyperkeratosis and early-onset periodontitis that leads to the loss of primary and secondary teeth in most patients. In addition to these cardinal features, increased susceptibility to various types of infections has been reported in a small percentage of PLS patients (3–6), but these individuals are not known to be unusually susceptible to viral infections.

In 1999, two groups simultaneously reported that loss-of-function mutations in the dipeptidyl peptidase I (DPPI) gene are strongly correlated with PLS manifestations (7, 8). Virtually all PLS patients have a severe reduction in the activity of DPPI, a lysosomal cysteine protease that is required for the activation of many granule-associated serine proteases in the mouse, including neutrophil-derived cathepsin G (CG), neutrophil elastase (NE), proteinase 3 (PR3) (9), the cytotoxic lymphocyte-associated proteases known as granzymes A and B (10), and mast cell chymase (11).

It is well established that DPPI-deficient mice have defects in serine protease activities in multiple hematopoietic lineages. Although a recent study reported that PLS is associated with deficiency of serine proteases in polymorphonuclear neutrophils (PMNs) (12), the functional consequences of this deficiency, and the role of DPPI in other cellular compartments remain largely undefined. Based on the phenotypes seen in DPPI-deficient mice, all patients with PLS should have generalized immunodeficiency as a consequence of the loss of activation of these serine proteases. For example, CG and NE have been proposed to play a direct role in neutrophil responses against invading pathogens. Mice deficient for NE or CG are more susceptible to common bacterial infections (including infections with *Staphylococcus aureus* and *Escherichia coli*) (13–15), whereas mice deficient for both CG and NE are also more susceptible to certain fungal infections (13, 15). In addition, cytotoxic lymphocytes derived from mice deficient for both granzymes A and B have a severe defect in their ability to induce target cell death in vitro (16). These mice have attenuated graft-vs-host disease (16, 17), and display a decreased ability to clear certain viral infections in vivo (18, 19). Patients with PLS would be expected to have a hematophagocytic syndrome (20, 21) if their granzymes were inactive in the absence of DPPI.

However, the study of the natural history of human patients with PLS has failed to detect a generalized T cell immunodeficiency phenotype. In this report, we describe the apparent reason why: Humans deficient for DPPI can still activate granzymes and have well-preserved cytotoxic lymphocyte function. In contrast, DPPI deficiency is associated with a severe reduction in the activity and
stability of human neutrophil-derived serine proteases. The loss of function of these proteases is not associated with severe infections in all PLS patients, indicating that serine proteases are not the primary tool used by neutrophils to clear common bacterial pathogens.

Materials and Methods

Subjects

PLS subjects were identified using a web site (http://hematology.wustl.edu/registry) generated by a study approved by the Washington University School of Medicine Institutional Review Board. All subjects and parents provided informed consent to participate, and completed a questionnaire regarding their medical and family history. All patients were examined by at least one of us to confirm physical findings.

DNA extraction and detection of mutations

Genomic DNA was extracted from peripheral venous blood using the PureGen kit (Genta Systems, Minneapolis, MN). Each exon (1–7) of the DPPI gene was amplified independently by PCR (Qiagen, Valencia, CA) using previously published flanking, intronic primers (7, 8). Unincorporated primers and nucleotides were removed by digestion with exonuclease 1 (Amersham Biosciences, Piscataway, NJ) and shrimp alkaline phosphatase phosphatase (Roche Molecular Biochemicals, Indianapolis, IN). Both DNA strands of the PCR products were sequenced directly using the same primers used for PCR (BigDye, version 2, Terminator Cycle Sequencing; Applied Biosystems, Foster City, CA). Mutations were detected by comparison with GenBank entry U79415 for DPPI and BLAST 2 sequence queries (www.ncbi.nlm.nih.gov/BLAST/).

Neutrophil isolation

Neutrophils were isolated from heparinized whole blood by Ficoll-Hypaque density gradient centrifugation according to manufacturer’s recommendations (Sigma-Aldrich, Saint Louis, MO) followed by hypotonic lysis of erythrocytes. The percentage of neutrophils was >95% as determined by Wright-Giemsa staining.

Culture of lymphokine-activated killer (LAK) cells

Lymphocytes from heparinized whole blood were cultured in high-dose recombinant human (rh)IL-2 for 8–9 days (1000 U/ml rhIL-2; R&D Systems, Minneapolis, MN) as previously described (10).

Enzymatic activity

Neutrophil and LAK cell pellets were resuspended in lysis buffer (1 M NaCl, 50 mM Tris (pH 8.0), 1 mM EDTA, 0.2% Triton X-100) at 10^7 cell/ml, briefly sonicated, and cleared by centrifugation. Ten microliters of each cell lysate (equivalent to 10^5 cells) were used to determine the specific enzymatic activity. All reactions were performed in duplicate. DPPI activity was determined by the hydrolysis of glycyl-l-arginine-7-amino-4-methylcoumarin (Bachem, Torrance, CA) as previously described (10). Hydrolysis was detected using a fluorescent plate reader (Bio-Tek Instrument, Winooski, VT) at 340-nm excitation and 460-nm emission. CG activity was determined using the substrate S-succinyl-Ala-Ala-Pro-Pho-pNA, NE activity was determined using the substrate S-methoxy succinyl-Ala-Ala-Pro-Val-pNA, and PR3 activity was determined using the substrate N-t-butoxy carbonyl-Ala-Ala-Nva-thiobenzyl ester, as previously described (9). Granzyme A activity was determined by the hydrolysis of N-α-benzoyl oxy carbonyl-l-lysine thiobenzyl ester, and granzyme B activity was determined by the hydrolysis of N-t-butoxy carbonyl-l-Ala-Ala-Asp-thio benzyl ester as previously described (10).

Western blot analysis

Cell pellets were directly lysed in SDS sample buffer (25 mM Tris (pH 7), 10% glycerol, 1% SDS, 10% 2-ME), and equivalent amounts of protein were resolved on a 10% SDS-PAGE gel. Specific proteins were probed using the following Abs: mouse anti-human CG (Novoceastra, Newcastle upon Tyne, U.K.), rabbit anti-human NE (Calbiochem, San Diego, CA), mouse anti-human PR3 (Lab Vision Corporation, Fremont, CA), mouse anti-human granzyme A (Research Diagnostics, Flanders, NJ), mouse anti-human granzyme B (Research Diagnostics), and rabbit anti-human β-actin (Sigma-Aldrich) followed by appropriate HRP-conjugated secondary Abs.

Bacterial killing assay

E. coli and S. aureus were grown in tryptic soy base, and mid-log phase bacteria were incubated with purified neutrophils as described (15) with the following modifications: 10^7 PMNs were resuspended in DMEM supplemented with 20% autologous serum and mixed with 10^9 S. aureus or E. coli in a total volume of 0.5 ml and incubated at 37°C. At the indicated time, aliquots were taken, and serial dilutions were spread on agarose plates, and CFU were determined after overnight incubation. To arrive at the mean and SD for each individual control and patient, all killing studies were performed in duplicate; four aliquots were independently counted at each time point. The number of CFU enumerated at time 0 was defined as a value of 100%.

LAK killing assay

A flow-based cytotoxic assay was performed as previously described (22) with the following modifications: K652 cells were transfected with pEGFP-C1 (BD Clontech, Palo Alto, CA), and stable clones were selected using G418 (1 mg/ml). A stable clone expressing a high level of GFP was used in the killing assays. K652 target cells expressing GFP were incubated with varying numbers of day 8–9 LAK cells in triplicate for 6 h. After incubation, cells were stained with 2 μg/ml 7-aminoactinomycin D (7AAD; Molecular Probes, Eugene, OR) for 10 min, washed, and analyzed by flow cytometry, during which cells were collected for 60 s per sample. Effector and target cells were distinguished on the basis of GFP expression. Live and dead cells were differentiated by 7AAD uptake. The percentage of remaining live target cells was calculated using the following formula: (number of live target cells in the presence of effector cells/number of live target cells in medium) × 100.

Statistical analysis

Wilcoxon’s rank sum test for unpaired variable (two-tailed) was used to compare differences between groups. Values of p < 0.05 were considered significant.

Results

Clinical presentation of PLS patients

Three patients met the diagnostic criteria for PLS. The first patient (Pt1) was an adopted 13-year-old Panamanian male whose family history was unavailable. The second (Pt2) and third (Pt3) patients, a 42-year-old female and 17-year-old female, were Caucasians from North America, and were both the products of non-consanguineous matings. All three PLS patients presented with early-onset periodontitis and hyperkeratosis. Interestingly, when Pt1 was treated for a positive tuberculosis skin test with six-drug therapy, his skin lesions improved remarkably, only to return upon the cessation of antibiotic therapy. Pt1 was also diagnosed with retinitis pigmentosa, a condition not reported in any other PLS patients. Pt2 had a significant history of recurrent infections with S. aureus, reporting multiple skin infections, a sinus infection, a subepithelial abscess, and a left frontal brain abscess. Pt3 had no history of infections at the time of presentation.

Identification of DPPI mutations in PLS patients

DNA sequencing confirmed that Pt1 was homozygous for a 910T→A missense mutation, leading to a Y337X change in a highly conserved region in exon 7. This patient has apparently been previously reported (23). Pt2 was a compound heterozygote, who carried a 96T→G (Y32X) mutation, leading to an early stop codon in exon 1 on one allele, and a 815G→C (R272P) missense mutation in a highly conserved region in exon 6 on the other allele. A patient with identical mutations was previously reported (24). Pt3 carried one previously described 748C→T mutation leading to an early stop codon in exon 5 (23). Sequencing of the other allele did not reveal a mutation in the coding region of the gene (Fig. 1A). However, we could not rule out a mutation in intronic sequences that could lead to abnormal splicing of the transcript, or a mutation in nearby regulatory sequences that could reduce gene expression.

DPPI activity in PLS patients

DPPI activity in the peripheral blood neutrophils of all three PLS patients was severely reduced (2.4, 1.1, and 0.5% of normal levels
Summary of mutations in the DPPI locus and DPPI activity in PLS patients. A. Schematic representation of the coding regions of the DPPI gene. The boxes depict the seven exons, with the amino acid numbers shown at the end of each exon. The mutations are indicated with arrows. Both Y32X and R250X result in premature stop codons, whereas the other changes lead to missense mutations. Asterisks indicate the sites of catalytic triad. B and C. Shown is DPPI activity in neutrophils (PMN) and LAK cells, respectively. Control levels represent the average activity of healthy controls and the patients. Neutrophils derived from PLS patients have 1% of the CG activity of healthy control neutrophils (Fig. 1, A). NE and PR3 activity was similarly reduced (6.7 ± 1.2 and 7.5 ± 2.5% that of normal for NE and PR3, respectively; Fig. 2, B and C).

Western blot analysis using specific anti-human CG, NE, and PR3 Abs revealed near absence of these three proteases in neutrophil lysates derived from all three patients (Fig. 2D). Because these cells were directly lysed in SDS denaturing sample buffer, it is unlikely that the absence of protein is due to random degradation after cell lysis, especially because normal neutrophils contained abundant amounts of full-length proteases. We have previously shown that murine DPPI +/− neutrophils contain significantly lower amounts of immunoreactive CG in the presence of equivalent levels of steady-state mRNA (9). Results from this study confirm that the unprocessed form of human neutrophil serine proteases is likewise more susceptible to intracellular degradation.

Granzyme activity in PLS patients

We have previously shown that DPPI is essential for the activation of granzymes A and B in murine cytotoxic lymphocytes. Cytotoxic lymphocytes derived from DPPI-deficient mice contain normal amounts of granzymes A and B, but these proteases retain prodipeptides at the N terminus and are completely inactive (10). To evaluate whether these findings are also true for humans, we assayed the granzyme A and B activity in LAK cells. LAK cells were obtained by culturing PBMC in high-dose IL-2 for 8–9 days, which is known to generate an expanded population of highly cytolitic lymphocytes that express large amounts of perforin and granzymes. We found that N-α-benzyloxycarbonyl-L-lysine thio-benzyl ester esterase activity (a measure of granzyme A activity) in LAK cells was reduced by ~50%, and aspase activity (a measure of granzyme B activity) was reduced by 30–40%, in all three patients (Fig. 3, A and B). The residual enzymatic activity correlated well with the reduced amount of immunoreactive granzyme A seen on Western blot analysis (Fig. 3C). In Pt1 and Pt2, a slightly larger form of granzyme B was apparent, along with the mature, fully processed form (Fig. 3C).

Microbicidal activity of neutrophils from PLS patients

To examine whether reduced serine protease activity is associated with a defect in neutrophil function, we tested the in vitro microbicidal activity of neutrophils purified from PLS patients. Using experimental conditions outlined in Materials and Methods, the neutrophils of Pt1 exhibited normal killing activity against both E. coli and S. aureus (Fig. 4). In contrast, the neutrophils of Pt2 displayed a significant defect in their ability to control both Gram-positive and Gram-negative bacteria (Fig. 4). Of note, Pt2 had previously presented with recurrent S. aureus infections. Although the neutrophils of Pt3 could control the bacterial load at early time points, her cells exhibited a significant defect in their ability to kill S. aureus at later time points (Fig. 4B).

Cytotoxicity of LAK cells from PLS patients

We tested the ability of LAK cells from PLS patients to kill susceptible target cells. The K562 target cell line stably expressed...
GFP, which allowed us to easily identify the target cell population by flow cytometry (22). Varying numbers of day 8–9 LAK cells from controls or PLS patients were incubated with GFP-expressing K562 target cells. After 6 h of incubation, cells were harvested, stained with 7AAD (a marker of apoptotic cells), and analyzed by flow cytometry. Target cell death defined by this assay correlated well with standard $^{51}$Cr or $^{125}$I-iodo-2'-deoxyuridine release assays (22). LAK cells from all three patients had no significant defect in their ability to induce K562 cell death (Fig. 5).

Discussion

In this study, we showed that human DPPI deficiency is associated with a severe reduction in the levels and activities of the neutrophil-derived serine proteases CG, NE, and PR3. Surprisingly, we found that DPPI is not essential for the activation of granzymes A and B in humans, although it is required for the optimal activation and stability of these enzymes. Furthermore, we provide evidence that the human neutrophil-derived serine proteases do not represent the dominant mechanism used by neutrophils for the killing of common bacteria.

Although nearly all PLS patients have severe periodontitis, only 15–20% are predisposed to recurrent infections. Most of these infections are mild skin pyodermas, but occasionally, severe and/or fatal pyogenic abscesses involving internal organs do occur. According to a recent study (3), the most common causative pathogen for pyogenic liver abscesses in PLS patients is S. aureus. Studies in the mouse have clearly demonstrated the importance of CG and NE for the clearance of bacterial and fungal infections (13–15). However, we have shown that, despite the nearly complete inactivation of all three major neutrophil-derived serine proteases, CG, NE, and PR3, in all three PLS patients, these neutrophils are not uniformly defective in microbicidal activity in vitro. Although neutrophil-derived serine proteases may not function as direct effector enzymes in microbicidal killing, they may regulate innate immune responses through other regulatory mechanisms in vivo. CG, NE, and PR3 have been shown to catalyze the release of more active and stable form of the CXC chemokines IL-8 (25) and epithelial cell-derived neutrophil activating protein-78 (26). In addition, NE and CG can cleave endothelial cell-associated adhesion molecules (i.e., ICAM-1 and VCAM-1) (27, 28), thereby potentially influencing PMN transmigration. Moreover, CG can activate the protease-activated receptor 4 on the surface of platelets (29). Activated platelets can, in turn, up-regulate surface expression of integrins on PMNs through platelet-PMN interactions. These mechanisms may contribute to the recruitment of PMNs to combat localized infections in vivo. Hence, in the absence of neutrophil-derived serine proteases, patients with PLS may develop aggressive periodontal disease, even though they have no overt PMN dysfunction. Still, how does one explain the differential susceptibility to bacterial infection between the oral cavity and other tissues? One possible explanation is that humans potentially harbor a mix of bacterial pathogens, both aerobic and anaerobic, in their oral cavities; the gingival crevices may provide a niche for the accumulation and overgrowth of these bacteria. These factors may overwhelm a slightly weakened innate immune response.

Although cytotoxic lymphocytes derived from DPPI-deficient mice are severely deficient for granzyme A and B activity (16), we found that PLS patients retained a significant amount of granzyme A and B activity in their LAK cells. In addition, these LAK cells have no significant defect in their ability to induce target cell death in vitro. We do not yet understand this striking interspecies difference. However, the three PLS patients studied here do not possess complete loss-of-function mutations of the DPPI gene, whereas the murine mutation is a true null. The small amount of residual DPPI activity detected in PLS LAK cells could potentially be generated from the mutant DPPI gene products. Indeed, when DPPI was isolated from the LAK cell lysates of healthy controls and PLS patients by immunoprecipitation, we detected the same level of residual activity (3–4% of normal levels; data not shown).

**FIGURE 4.** Microbicidal activity of neutrophils from patients with PLS. Survival of E. coli (A) and S. aureus (B) in the presence of controls or PLS neutrophils over time. The number of remaining live bacteria was calculated using the formula: (number of colonies at time $T = 0$) $\times 100$. Control values represent the average killing efficiency $\pm$ SD of three separate healthy donors (each measured in quadruplicate) from four independent experiments. For each patient, the mean (also measured in quadruplicate) was statistically compared with the group of means determined for the three different control healthy donors. Asterisks indicate significant differences between controls vs PLS patients at the indicated time point. *, $p < 0.02$; **, $p < 0.01$; ***, $p < 0.001$.

**FIGURE 3.** Granzyme activities in patients with PLS. Peripheral blood lymphocytes from PLS patients and healthy controls were cultured concurrently in high-dose rhIL-2 (1000 U/ml) for 8–9 days. A and B. The relative granzyme A activity (A) and granzyme B activity (B) was measured per 10⁷ LAK cells. Values represent mean of duplicate determinations. C. Western blot analysis confirms that there are various levels and forms of immunoreactive granzyme A and B in PLS patients.
We are indebted to the patients and their families for their participation in and help to explain the surprisingly good prognosis for many of the patients with severe microbicidal defects may benefit from aggressive antibiotic therapy at the first sign of infection. The studies suggest that in vitro screening for microbicidal activity in cell lines with Paps-PLS syndrome and Paps-PLS cells. This explanation is certainly possible, because mice also have a PPPI-like activity that is capable of partially activating granzyme C in the absence of DPPI (10). One possible candidate enzyme is human DPPIV (which is identical with rodent DPPII) (30), an aminopeptidase that has a slightly different substrate specificity, whose activity is fully retained in patients with PLS (data not shown). Regardless, the normal cytotoxicity of LAK cells helps to explain why patients with PLS have no apparent defect in their ability to clear viral infections, and why they are not unusually susceptible to tumor development.

In conclusion, we have defined several of the enzymatic and cellular consequences of severe DPPI deficiency in humans. These studies suggest that in vitro screening for microbicidal activity in patients with PLS may be a valuable adjunct for the care of young patients. Individuals with severe microbial defects may benefit from aggressive antibiotic therapy at the first sign of infection. The data also provide a molecular explanation for the lack of a general immunodeficiency phenotype in humans with DPPI deficiency, and help to explain the surprisingly good prognosis for many of these patients.

Acknowledgments

We are indebted to the patients and their families for their participation in the studies.

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