Circulating Promyelocytes and Low Levels of CD16 Expression on Polymorphonuclear Leukocytes Accompany Early-Onset Periodontitis

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Early-onset periodontitis (EOP) is characterized by rapidly progressive alveolar bone loss, chemotactic defects of neutrophils, and significant familial aggregation. We found immature myeloid lineage cells, defined as promyelocytes, in the peripheral blood in patients with EOP. A hematological examination of peripheral blood cells showed normal reference values regarding cell proportions. Flow cytometry revealed significantly lower expression of CD16, a glycosylphosphatidylinositol (GPI)-anchored protein, on peripheral neutrophils in patients compared with those in age- and sex-matched healthy controls, whereas the levels of CD11a and CD11b expression were similar. The chemotactic response of neutrophils was lower toward not only formylmethionyl-leucyl-phenylalanine but also complement fragment C5a than that of healthy controls. The expression of another GPI-anchored protein, CD14, was equally expressed by controls and patients. Therefore, the low level of CD16 expression was not due to the incomplete synthesis of the GPI anchor. GPI anchors of CD16 on neutrophils from controls and patients were both partially resistant to phosphatidylinositol-specific phospholipase C. The presence of promyelocytes in peripheral blood, low expression of CD16, and low chemotactic response of neutrophils suggest that patients with EOP have an abnormal maturation system in myeloid lineage cells in the bone marrow, which may be associated with the onset and course of EOP.

Material and Methods

Reagents. Mono-Poly resolving medium was obtained from ICN Biomedic Co. (Costa Mesa, Calif.). Bacillus cereus phosphatidylinositol (PI)-specific phospholipase C (PIPLC) was purchased from Boehringer Mannheim (Indianapolis, Ind.). Bovine serum albumin (BSA), formyl-methionyl-leucyl-phenylalanine (FMLP), and complement fragment C5a were purchased from Sigma Chemical Co. (St. Louis, Mo.). Antibodies were purchased as follows: monoclonal antibodies (MAbs) to CD11a conjugated with fluorescein isothiocyanate (Bear1) from Coulter and Immunotech (Miami, Fla.), and MAbs to CD11b conjugated with phycocyanin (PE) (HI111) from Pharmingen (San Diego, Calif.). MCA-4 was kindly provided by Dr. R. G. Schmid (Department of Anatomy, Tohoku University School of Medicine, Sendai, Japan). The antibodies were used at a concentration of 10 μg/ml.

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to CD16 conjugated with PE (Leu-11c) and CD14 conjugated with PE (Leu-M3) from Becton Dickinson (Mountain View, Calif.).

**Patients and healthy controls.** Six patients with EOP, diagnosed on the basis of previously determined criteria (2) at the Department of Endodontics and Periodontics, Tohoku University School of Dentistry, were included in this study. The patients, who were Asians, consisted of two men and four women (mean age, 27.5 years; range, 20 to 32 years), all of whom had received periodontal treatment. Two patients were sisters, and the other four were unrelated individuals. The conditions after treatment were as follows: (i) general bone levels were located in one-third to two-thirds from the cement-enamel junction, (ii) lamina dura of most alveolar bone processes was clear, (iii) inflammatory sites were hardly observed, and (iv) the depth of most periodontal pockets was less than 3 mm due to pocket elimination by surgical treatment. The healthy control group consisted of four Asian men and two Asian women (mean age, 28.2 years; range, 25 to 27 years) with no attachment loss. All patients and healthy controls participating in this study had taken no medications that would affect the oral microflora or the inflammatory and/or immune responses during the last 3 months and were essentially in good health.

**Isolation of human neutrophils.** Neutrophils from heparinized (10 U/ml) peripheral venous blood were isolated by density gradient centrifugation on Mono-Poly resolving medium (300 × g for 30 min at room temperature) (22). The fraction containing neutrophils was harvested and washed twice with phosphate-buffered saline (PBS) at 4°C. Neutrophils were resuspended (2 × 10^6 cells/ml) in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 1% BSA and placed on ice until use (within 60 min). The viability of neutrophils was morphologically above 95%.

**Neutrophil chemotaxis assay.** Chemotaxis was assayed as described previously (21), with some modifications. Cell suspensions in RPMI 1640 containing 1% BSA (2 × 10^6/300 μl) were distributed into chemotactic chambers (Chemotaxis-cell; Kurabo Co., Osaka, Japan) fitted with a 0.48-cm²-area polycarbonate filter (3-μm pore size). The filters separated the cells from the lower compartment (24-well multwell plate; Nunc, Roskilde, Denmark) containing 1 ml of RPMI 1640 containing 1% BSA with various concentrations of FMLP and C5a as chemotactic factors. After incubation for 30 min at 37°C, the filters were fixed with absolute methanol for 5 min, placed into neutral buffered formalin for 5 min, and stained with hematoxylin. The migrated cells on the bottom of the filters were counted under a microscope (magnification, ×400). In each experiment, at least five fields in duplicate filters were counted.

**Flow cytometry analysis of neutrophils.** Heparinized peripheral blood cells (PBCs) were prepared for flow cytometry by using NH₄Cl lysis. Peripheral blood was diluted 10× with lysis solution (150 mM NH₄Cl-0.1 mM EDTA-10 mM potassium bicarbonate in distilled water at pH 7.3); then erythrocytes were lysed for 10 min at room temperature and washed twice with PBS. Cell suspensions (10^6 cells/sample) were incubated with saturated MAh for 30 min at 4°C, washed twice with 0.1% Na2O.1% BSA in PBS, and fixed with 1% paraformaldehyde in PBS. Flow cytometry (fluorescence-activated cell sorting [FACS]) was performed on a FACSScan (Becton Dickinson). The granulocyte population was gated on the basis of forward and right-angle scatters. Data were collected for 15,000 events, which were stored in list mode and then analyzed with Lysis II software (Becton Dickinson).

**PIPLC treatment.** Cells from heparinized peripheral blood treated with lysis solution were incubated (10^6 cells/ml) in RPMI 1640 supplemented with 0.2 mM glutamine for 1 h at 37°C with or without 5 U of PIPLC per ml in microcentrifuge tubes (BioMedical Equipment Co., Tokyo, Japan) and then washed twice with PBS (42).

**Hematological characteristics.** To examine the hematological characteristics of patients, peripheral blood samples were applied to an automatic blood analyzer (Sysmex SF-3000; TOA Medical Electronics Co., Ltd., Kobe, Japan). Leukocytes were classified by using low- and high-angle scatter in two types of scattergrams, and erythrocytes, platelets, and leukocytes were counted by using a direct current system.

**Histological detection of PBCs.** PBCs (10^5 cells) were cytosponged onto glass slides, dried, and processed for Giemsa (Merck Japan Co., Ltd., Tokyo, Japan) staining.

**Statistical analysis.** Statistical significance between any two groups was analyzed by Student's t test.

**RESULTS**

Circulating promyelocytes in the peripheral blood of patients with EOP. The morphology of peripheral blood from patients with EOP was assessed by Giemsa staining. In general, PBCs are composed of granulocytes, monocytes, and lymphocytes, which were all mature cells in normal subjects. In addition to mature blood cells, however, bone marrow progenitor cells were frequently observed in the peripheral blood of patients (Fig. 1A). These cells were large, and they had a nucleus with multiple nucleoli and dispersed chromatin pattern (Fig. 1B). The cells contained azurophilic granules, and their cytoplasm was basophilic (Fig. 1B). These morphological characteristics are the hallmark of promyelocytes. These cells were observed in all patients examined (n = 6) but hardly observed in healthy controls, and there was a significant difference (P < 0.005) between the two groups (percentages standard deviation [means ± SD] were [6.5 ± 1.5] × 10^-3 [patients] and [0.5 ± 0.5] × 10^-3 [controls]). These results suggested an abnormal hematopoietic mechanism, especially regarding myeloid lineage cells, in these patients. Chromatin condensed apoptotic neutrophils were not detected.

**Hematological examination.** To determine whether hematological characteristics differed between healthy controls and patients, cell numbers and proportions of leukocytes, erythrocytes, and platelets were determined in peripheral blood samples. No obvious differences were identified in terms of normal ranges of cell numbers or the proportions of neutrophils, basophils, eosinophils, monocytes, and lymphocytes (data not shown).

**Phenotypic analysis of peripheral neutrophils.** We examined the expression of various cell surface markers, i.e., inte-
grin β2 family, LFA-1 (CD11a), Mac-1 (CD11b), and FcγRIII (CD16), on neutrophils by flow cytometry. Erythrocytes were lysed, and then PBCs stained with each MAb were analyzed for the expression of these molecules on a granulocyte population that consisted almost entirely of neutrophils. The expression of LFA-1 and Mac-1 on neutrophils did not significantly differ between controls and patients (Fig. 2 and Table 1).

The intensity of CD16 expression on neutrophils significantly differed between two groups (Fig. 2 and Table 2). Almost all neutrophils from controls expressed high levels of CD16 (Fig. 2). Neutrophils from patients were classified into three groups according to the intensity of CD16 expression: bright (III), low (II), and negative (I). The mean percentages of the cells in the gated fractions (I, II, and III) of controls were 8.3, 18.6, and 73.1%, respectively, and those of the gated fractions in patients were 43.0, 31.1, and 25.9%, respectively (Table 2).

**Chemotactic ability of neutrophils from patients.** CD16 and FMLP receptors on neutrophils are functionally linked during FMLP-induced chemotaxis (30). We therefore examined the chemotaxis of neutrophils toward FMLP. We purified neutrophils from peripheral blood by density gradient centrifugation and then assayed chemotaxis by using various concentrations of FMLP. Figure 3A shows the chemotactic responses to 1, 10, and 100 nM FMLP of neutrophils from controls (n = 6) and those from patients (n = 6). Neutrophils from patients displayed a significantly impaired chemotactic response to FMLP compared with that from controls.

Anti-CD16 MAb inhibits the chemotactic response of neutrophils to FMLP but does not affect other chemotactic factors, including C5a, leukotriene B4, interleukin-8 (IL-8), and platelet-activating factor (30). Therefore, we examined the response of neutrophils to other chemotactic factors. Figure 3B shows that the chemotactic response of neutrophils to C5a was impaired in patients compared with controls.

**Downregulation of CD16 expression is not due to impaired synthesis of the GPI anchor moiety.** Although the CD16 expressed on NK cells and macrophages is a transmembrane form (FcγRIIIA), on neutrophils it is linked to the cell membrane via the GPI moiety (FcγRIIIB) (28, 51). CD14 is another GPI-anchored protein expressed on neutrophils (62). Flow cytometry revealed that 90% of the neutrophils from controls and patients expressed CD14, and the expression profiles of the two groups were the same (Fig. 4). These results indicated that synthesis of the GPI anchor itself was normal in neutrophils of patients and that the reduced expression of CD16 on neutrophils in patients was not due to incomplete synthesis of the GPI anchor.

**Sensitivity of the GPI anchor of CD16 to PIPLC.** The release of a protein from a cell membrane surface by bacterial PIPLC is the initial indicator of the presence of a GPI anchor (36). However, some GPI anchors are resistant (25) or partially resistant (35, 53) to hydrolysis by bacterial PIPLC. Furthermore, some GPI anchors become resistant after mitogenic stimulation (44, 52). We therefore examined the sensitivity of the GPI anchor of CD16 expressed on neutrophils from controls and patients.

Purified peripheral neutrophils from controls and patients were cultured for 1 h at 37°C with or without PIPLC, and then

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**TABLE 1. Expression of CD11a and CD11b on neutrophils**

<table>
<thead>
<tr>
<th>Source</th>
<th>Median channel fluorescence ± SD</th>
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<tbody>
<tr>
<td></td>
<td>CD11a</td>
</tr>
<tr>
<td>Patients</td>
<td>127.5 ± 16.6</td>
</tr>
<tr>
<td>Controls</td>
<td>126.6 ± 9.8</td>
</tr>
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</table>

* Each group contained six subjects.

**TABLE 2. Expression of CD16 on neutrophils**

<table>
<thead>
<tr>
<th>Source</th>
<th>Mean % of cells ± SD in fraction</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Patients</td>
<td>43.0 ± 17.0*</td>
</tr>
<tr>
<td>Controls</td>
<td>8.3 ± 4.3*</td>
</tr>
</tbody>
</table>

* Each group contained six subjects.

b For values marked with the same number of symbols, P < 0.005.
The expression of CD16 was assessed by flow cytometry. Figure 5 shows that incubating neutrophils for 1 h without PIPLC induced more CD16 expression in controls (bottom row). In the patient with the highest level of CD16 expression on neutrophils among those tested, CD16 expression was enhanced after incubating neutrophils without PIPLC (Fig. 5, top row). However, the intensity of the expression was still lower than that in healthy controls. In another whose CD16 expression was relatively low, the expression of CD16 significantly changed (Fig. 5, middle row). The bright fraction increased, whereas the negative fraction decreased. These results showed that incubating the neutrophils for 1 h without stimulants induced CD16 expression on neutrophils rapidly in controls as well as patients.

We incubated neutrophils for 1 h with 5 U of PIPLC per ml to hydrolyze the GPI anchor of CD16. The expression of CD16 on neutrophils from healthy controls and patients was partially reduced, indicating that GPI anchors of CD16 on neutrophils from both groups were partially resistant to PIPLC hydrolysis.

**DISCUSSION**

Considerable evidence points to a familial pattern of EOP. Both localized and generalized forms of the disease can occur in the same family (26). Although the exact mode of inheritance is still unclear, a study supporting a genetic disposition for the disease (37) has been reported. The disease might be the result of a complex interplay between genetically determined alterations of the host response and a specific bacterial challenge.

The amount of GP110 (a neutrophil membrane matrix component and differentiation antigen) is reduced on neutrophils in localized juvenile periodontitis (LJP) patients (59), and neutrophils from LJP patients express high levels of type 2 complement receptor, which is normally expressed only on immature neutrophils (26, 47). In this study, we frequently found immature granulocytes, which were identified as promyelocytes at the light microscopic level, in PBCs of all six patients. These patients had received periodontal treatment and showed no obvious periodontal inflammation. These results suggest that the patients with EOP have an intrinsic hematopoietic disorder, especially in hypergranulopoiesis. In support of this notion, the hyperhematopoietic condition induced by an injection of hematopoietic factors such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, or IL-3 mobilizes peripheral blood progenitors from bone marrow (55).

The expression of CD16 on granulocytes correlates well with the maturation of neutrophils. CD16 is first detected on the cell surface at the stage of metamyelocytes and maintained to the stage of segmented mature neutrophils (23, 54). Neutrophils from all six patients expressed lower CD16 levels than those of controls. Neutrophil apoptosis has been associated with a reduction in CD16 expression (19). However, in this study, very few apoptotic neutrophils were evident in patient peripheral blood. These results also suggest abnormal myeloid lineage maturation in patients, and reduced CD16 expression might be due to the incomplete maturation of some peripheral neutrophils.

CD16 expression on neutrophils is reduced in patients with AIDS and AIDS-related complex (10), in thermally injured patients (7), in patients with chronic myeloid leukemia (16), and in preterm neonates (15). In these patients, a granulopoiesis disorder and abnormal immune responses are induced (12, 24, 39, 41). The reduction of CD16 expression on neutrophils during EOP might be closely associated with the initiation of this disease. Åsman and Bergstrom (6) reported that FcγRIII expression was lower in patients with juvenile periodontitis who had received no periodontal treatment. However, in this study, the expression of FcγRIII on neutrophils was not different between patients and healthy controls (6). On the other hand, FcγRIII expression on neutrophils showed no differences in adult periodontitis patients (27).
The anchoring of GPI appears to arise by the attachment of a protein to a preformed lipid precursor. This attachment requires the removal of a relatively short, C-terminal hydrophobic domain. The formation of this linkage appears to occur in the endoplasmic reticulum. After attachment of the protein to the GPI anchor, the protein will be transported via the Golgi complex to the cell surface (36). The impairment of GPI anchor synthesis leads to an inability to express GPI-anchored proteins on the cell surface (60). In this study, the expression of CD14, a GPI-anchored protein on neutrophils, was not significantly different between controls and patients. This finding indicates that the downregulation of CD16 expression does not result from impaired GPI anchor synthesis. Since the GPI anchor has a PI structure, GPI-anchored protein can be released from the cell surface through PI hydrolysis by PIPLC. However, the susceptibility of hydrolysis by PIPLC is diverse. Thy-1 (35) and Qa-2 (53) are partially resistant to PIPLC, probably resulting from variations in the GPI anchor. Substituting the 2-OH on the inositol ring with an ester-linked palmitic acid residue in the GPI anchor may cause resistance to PIPLC in acetylcholinesterase from erythrocytes (46), and the anchor becomes resistant to PIPLC after mitogenic stimulation with Thy-1 (44) and Qa-2 (52). In this study, the PIPLC sensitivity of the GPI anchor moiety in CD16 was partially resistant to PIPLC in controls and patients. Moreover, incubating neutrophils in the medium induced a rapid increase in the surface expression of CD16 (Fig. 5), which was probably due to the translocation of storage compartments to the cell surface. These results suggest that the reduced expression of CD16 results from either pre- or posttranslational abnormalities but not a failure in the biosynthesis of GPI anchors.

Recently, it has been reported that CD16 and FMLP receptors on neutrophils are functionally linked during FMLP-induced chemotaxis and may share a common signal transduction pathway. The MAb to FcγRIII (3G8) specifically inhibits chemotaxis of neutrophils in response to FMLP but has no effect on C5a, leukotriene B4, IL-8, and platelet-activating factor (30). In this study, neutrophils from patients who expressed low levels of CD16 exhibited a much lower response to FMLP compared with those from controls. However, the response toward C5a was also reduced. This finding indicated that the downregulated chemotaxis response in patients was not specific. Although the $K_d$ values for FMLP as well as C5a binding to control and LJP neutrophils are essentially the same (26, 57, 58), the number of effective binding sites on LJP neutrophils is reduced, resulting in a reduced chemotaxis response. Moreover, the antigenic determinant on GP110, which is less expressed on LJP neutrophils with chemotactic defects (59), is closely linked to chemotaxis responses, and the antibody to GP110 inhibits the chemotaxis response of neutrophil for FMLP (17, 18). Although in a previous study, about 70% of affected patients had chemotactic disorders (26), all six patients examined in this study exhibited lower chemotactic activity and low CD16 expression. These findings suggested that a mechanism by which the chemotaxis response to FMLP is downregulated in EOP patients could be partially attributed to the low level of CD16 expression.

Leukocyte adhesion deficiency, which decreases the expression of adhesion molecules, including CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), and CD11c/CD18 (p150/95), accompanies severe periodontal diseases (3, 4). Several phenotypic analyses of integrin β2 have been reported. Patients with LJP expressed normal level of CD18 on the neutrophil cell surface, but this slowly decreased to a level lower than that of controls.

![Image](http://iai.asm.org/ on August 15, 2017 by guest)
upon stimulation (40). A low expression level of CD11/CD18 in patients with generalized prepubertal periodontitis has been reported (43). Expression of the integrin β2 family, LFA-1, Mac-1, and p150/95 was increased in a patient with LJP (1). In this study, we showed that levels of LFA-1 and Mac-1 expression on peripheral neutrophils were similar in controls and patients. Why various patterns of the integrin β2 family expression are observed on neutrophils from patients is unclear but could be related to conditions such as local infection, disease category, or methodology.

Neutrophils protect against infection, but they are also considered to mediate tissue destruction in inflammatory diseases (61). Important components of these pathologic processes are linked to the neutrophilic release of a complex assortment of oxygen metabolites and proteolytic enzymes, which can destroy normal cells and extracellular matrices. More elastases and oxygen radicals are released from neutrophils after stimulation in patients with juvenile periodontitis than in controls (5, 6). These substances can act as mediators of tissue destruction in immunologic injury caused by neutrophils (61). This evidence suggests that neutrophil functions are closely associated with the onset and course of periodontitis. Hypergranulopoiesis and low CD16 expression on peripheral neutrophils, which might be intrinsic and systemic hematopoietic diseases, may be low CD16 expression on peripheral neutrophils, which might be intrinsic and systemic hematopoietic diseases, may be closely associated with the onset and course of EOP.

Considering that prevalence of the disease varies between racial groups (37, 49), the findings reported here may have a racial component. In addition, the number of subjects needs to be increased before we know how generalizable these findings may be.

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