Association of an Abnormality of Neutrophil Chemotaxis in Human Periodontal Disease with a Cell Surface Protein

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Localized juvenile periodontitis (LJP) is characterized by severe, early-onset, molar and incisor bone loss; neutrophil chemotaxis disorders; and a high prevalence of Actinobacillus actinomycetemcomitans infection. LJP is further characterized by significant familial aggregation of the disease. Recent work in our laboratory has demonstrated the selective depletion of a surface glycoprotein of 110,000 Mw (GP110) from LJP neutrophils by using surface labeling with [3H]formaldehyde and autoradiography. The function of GP110 is unknown; however, it does not appear to be a chemotactic factor receptor. Rather, it is bound by a monoclonal antibody (NCD-1) that recognizes a neutrophil differentiation antigen and which itself alters neutrophil chemotactic and secreting functions. To quantify GP110 on LJP and normal neutrophils, fluorescein-labeled NCD-1 was bound to neutrophils and the amount of fluorescence was evaluated by using cytofluorography. Our results indicate that there is a quantifiable reduction (40%) of GP110 on the surface of LJP and GJP neutrophils, compared with controls. Other patients with neutrophil defects express normal quantities of GP110, suggesting disease specificity. Our data suggest that GP110 may be a useful disease marker for LJP and may provide a useful probe for the study of neutrophil chemotactic function and dysfunction.

Most patients with abnormalities of neutrophil chemotaxis have abnormal cellular responses. These abnormalities can be classified in terms of functional defects of adherence, deformability, random migration, chemokinesis, or chemotaxis (11). In certain cases, the abnormality has been defined in terms of a specific biochemical defect (10).

Localized juvenile periodontitis (LJP) is a disease of the supporting structures of the teeth characterized by rapid, early-onset alveolar bone loss localized to first molars and incisors (2). Typically, 70 to 80% of LJP patients demonstrate an associated defect of neutrophil chemotaxis (3, 4, 15, 22). There is a significant familial aggregation of LJP and associated neutrophil abnormalities (12), and the chemotaxis disorder typically precedes the disease and remains after treatment of the clinical condition (19, 22). Investigation of the molecular basis of the neutrophil defect in LJP revealed a decrease in the number of available receptors for the chemotactic peptides C5a and N-formylmethionylleucyl-phenylalanine (FMLP) (11, 12).

In this study, we report the investigation of the surface protein composition of LJP neutrophils as compared with that of normal controls. Generalized juvenile periodontitis (GJP) patients exhibiting neutrophil chemotactic abnormalities were evaluated as well. Our results indicate a selective depletion of a surface glycoprotein of 108,000 to 110,000 daltons (GP110) specifically in LJP and GJP patients with neutrophil chemotactic abnormalities but not in other patients with chemotaxis dysfunction. A monoclonal antibody to this glycoprotein has been produced (6) and has been used to quantify the expression of protein on the neutrophil surface. Our data suggest that GP110 may be a useful disease marker for LJP and may provide a useful probe for the study of neutrophil chemotactic function and dysfunction.

MATERIALS AND METHODS

Subjects. LJP patients were located from among the patients of the Emory University School of Dentistry. LJP was diagnosed clinically and radiographically and characterized by alveolar bone loss localized to first molars and incisors in clinically healthy patients under 25 years of age. Other comparison groups, which included patients with recurrent infections, diabetes mellitus, and other forms of periodontal disease, were found in hospitals affiliated with Emory University. Patients with recurrent infections were children (age, 3 to 11 years) with undefined illnesses characterized by recurrent pyogenic infections and repeated, chronic upper-respiratory infection who exhibited neutrophil chemotaxis abnormalities as their only laboratory test abnormality. Diabetes mellitus patients were adolescent IDDM type I patients who exhibited severe, early-onset periodontal disease as a complication of their diabetes. GJP was diagnosed clinically and radiographically and defined as generalized severe alveolar bone loss on more than 14 teeth (no localization) in clinically healthy patients under 25 years of age.

Normal, healthy controls were located among the student and staff population of Emory and demonstrated no clinical or radiographic evidence of periodontal disease other than mild gingivitis.

Neutrophil chemotaxis: isolation of neutrophils and chemotaxis. The chemotaxis assay, as it is done in our laboratory, has been described in detail elsewhere (25). Briefly, peripheral venous blood was separated by dextran sedimentation and Ficoll-Hypaque centrifugation. Care was taken to prevent spontaneous modulation of neutrophil surface components by carefully controlling temperature variables as described by Fearn and Collins (7). Further, control experiments were done to determine whether GP110 is inducible or up-regulated under permissive conditions (see below). Neutrophils were suspended in an assay medium consisting...
of Gey balanced salt solution supplemented with 2% bovine serum albumin, at a concentration of 2.5 × 10⁶ cells per ml. The cell suspension was placed in the upper compartment of a modified Boyden chamber separated by a 5-μm-pore-size micropore filter (Sartorius Membranfilter, GmbH, Gottingen, Federal Republic of Germany). The lower compartment contained the synthetic chemotactic peptide FMLP (2 × 10⁻⁸ M) or endotoxin-activated serum (1:10 dilution of normal human serum). Chemotaxis was evaluated by counting the number of neutrophils that accumulated on the distal surface of the filter after a 60-min incubation. Ten high-power fields (400×) were counted for each of triplicate filters. Statistical differences between patients and controls were determined by analysis of variance. All assays were done on at least two occasions, and often more. Results presented are mean values for all tests. All tests were done before the clinical treatment of patients was begun.

Reducive methylation of surface protein. Reductive methylation of surface proteins was accomplished by using an adaptation of the method of Jentoft and Dearborn (13). Optimal conditions have been established in our laboratory and are as follows. Neutrophils (2 × 10⁷ cells) were incubated in phosphate-buffered saline (PBS), pH 7.5, at 37°C with [¹⁴C]formaldehyde (specific activity, 40 to 60 mCi/ml) and sodium cyanoborohydride for 1 h. After incubation, the cells were washed 3 times with PBS and suspended in 200 μl of PBS. This procedure is preferred over acetylation or other methylation procedures that make use of sodium borohydride because the net charge of the molecules is unchanged and the labeling can be done at neutral pH, thereby preventing elimination of O-glycosidically linked glycoproteins. Also, the mild conditions of these techniques are less likely to disrupt cells.

Membrane isolation and SDS-PAGE: extraction of neutrophil membrane components and SDS-PAGE. Extraction of membrane components and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to establish patterns characteristic of neutrophil surface protein. Plasma membranes were isolated by the method of Wright et al. (27). This method has the advantage of high membrane yield with minimal disruption of granules. Disopropylfluorophosphate was used as a protease inhibitor (1). Enzyme markers for subcellular fractions to determine the purity of membrane preparations were used as previously described. SDS was used to solubilize membrane proteins and glycoprotein. Samples were then subjected to one-dimensional electrophoresis as previously described (14) by using 7.5% gels, in the presence and absence of 2-mercaptoethanol. Molecular-weight standards were prepared and calculated by the method of Weber and Osborn (26). Gels were stained with Coomassie blue. Autoradiography was done as previously described.

Quantification of GPⅠⅠⅡ0 with NCD-1 and cytofluorography. The EPICS cytofluorograph (Coulter Electronics, Inc., Hialeah, Fla.) and the FACS (Becton Dickinson and Co., Paramus, N.J.) were used to quantify binding of NCD-1 to normal and defective neutrophils from all patient groups. Pelleted neutrophils were incubated for 30 min at 4°C with a fluorescein isothiocyanate-labeled NCD-1 preparation which was filtered with Centricon Microconcentraters (Amicon Corp., Lexington, Mass.) before use to eliminate unbound fluorescein isothiocyanate. Neutrophils were then washed in PBS containing 0.2% sodium azide and fixed in paraformaldehyde. Labeled cells were analyzed by cytofluorography with a laser light at 480 nm wavelength maintained at constant intensity by using both forward and right-angle light scattering (10). Specificity of NCD-1 labeling was evaluated by inhibition of fluorescein isothiocyanate-labeled NCD-1 binding by unlabeled NCD-1. In addition, other membrane probes such as monoclonal antibodies to iC3b receptor (OKM-1; Ortho Diagnostics, Inc., Raritan, N.J.) and C3b receptor (DIAKO Corp., Santa Barbara, Calif.) were used as controls. Fluorescence was quantified as median channel number of fluorescence intensity.

Enzyme-linked immunoelectrotransfer blot. The resolved SDS-polyacrylamide gel was electroblotted into a nitrocellulose membrane by using a modification of the methods of Towbin et al. and Tsang et al. (20, 21). Briefly, the gel and membrane were sandwiched between Scotch-Brite pads in a Bio-Rad Transblot System (Bio-Rad Laboratories, Richmond, Calif.). The system was run for 4 h at 4°C to 0.35 A by using a transfer buffer of 25 mM Tris hydrogen chloride–12 mM glycine at pH 8.3 with 20% methanol. At the end of the transfer run, the membrane was removed and cut in half. Part of the wet membrane was stained for protein with 0.2% Coomassie R-250 in 40% methanol and 10% acetic acid for 10 min and then destained with 20% methanol–2% acetic acid solution. The other half was placed in a plastic dish and washed for 10 min with Tween–Trisbuffered saline (TTBS) consisting of 20 mM Tris, 150 mM NaCl, pH 7.5, and 0.05% Tween 20. Unsatubated sites on the membrane were blocked with a 3% gelatin-TTBS solution for 30 min with slow shaking at 37°C and then rinsed twice for 10 min with TTBS. A 1:100 dilution of the first antibody (NCD-1) was added in 1% gelatin-TTBS, incubated overnight with shaking, and then rinsed twice with TTBS. A 1:2,000 dilution of the second antibody (goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate) was incubated with the membrane for 2 h at room temperature and then rinsed with two 10-min rinses of TTBS. The wet membrane was then developed with diaminobenzidine tetrahydrochloride (50 mg/100 ml of PBS, pH 7.2) plus 30 μl of 30% H₂O₂ (added just before use) for 10 min or until brown bands appeared. The membrane was rinsed with water to stop the reaction and photographed while wet. Patterns obtained were compared to the Coomassie-stained membrane. The membranes were dried flat and stored in the dark. Membrane preparations from LJP and control neutrophils were run simultaneously.

RESULTS

Neutrophil chemotaxis. The chemotactic response of peripheral blood neutrophils was measured in the Boyden chamber assay by using endotoxin-activated serum and FMLP as chemotaxants. Each patient was compared to a healthy control for each test, and all patients were tested at least two times on different days. Twelve of the LJP patients demonstrated depressed chemotaxis, and four exhibited normal chemotaxis. Three patients with a history of recurrent infections exhibited depressed chemotaxis, as did three diabetic patients and five patients with GJP (Table 1).

Labeling of neutrophil surface protein. Neutrophils from LJP patients and normal controls were surface labeled by using reductive methylation with [¹⁴C]formaldehyde and sodium cyanoborohydride. Membranes were isolated, and protein was separated by SDS-PAGE. Labeled protein was visualized by autoradiography. Comparison of gels revealed identical patterns with the exception of a depletion of a band at 108,000 to 110,000 daltons in lanes containing LJP neutrophils (Fig. 1). This observation was confirmed by densitometric scanning (Fig. 2; Table 2).

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TABLE 1. Polymorphonuclear leukocyte chemotaxis

<table>
<thead>
<tr>
<th>Condition of patients in group (no. of patients)</th>
<th>Cells/HPF ± SEM</th>
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<tbody>
<tr>
<td>LJP (12)</td>
<td>19.4 ± 4.9b</td>
</tr>
<tr>
<td>Rec. infec. (3)</td>
<td>24.7 ± 5.6b</td>
</tr>
<tr>
<td>Diabetes (3)</td>
<td>34.4 ± 3.6b</td>
</tr>
<tr>
<td>LJP* (4)</td>
<td>44.1 ± 9.6</td>
</tr>
<tr>
<td>Control (15)</td>
<td>46.9 ± 7.8</td>
</tr>
</tbody>
</table>

a Neutrophil chemotaxis for each patient was measured in the Boyden chamber assay by using FMLP as the chemoattractant (2 × 10−8 M). An age- and sex-matched control was used for each assay. The mean ± standard error is expressed for each group. Comparable results were obtained with endotoxin-activated serum. Rec. infec., Patients with a history of severe recurrent infections.

b P < 0.05 (analysis of variance).

c Patients without neutrophil chemotaxis abnormality.

Immunoblot with NCD-1. NCD-1, a monoclonal antibody which recognizes a neutrophil differentiation antigen of 110,000 daltons (5), binds to the glycoprotein band that migrates to the same molecular weight as the depleted protein on LJP neutrophils. This was demonstrated by using an immunoblotting technique, as is demonstrated in Fig. 3 in membrane preparations from a control patient and an LJP patient. NCD-1 bound only to a protein band of similar migrating properties to the glycoprotein depleted on LJP neutrophils. Further, in LJP membrane preparations, the GP110 band stained with less intensity.

Quantification of GP110 with NCD-1. The amount of NCD-1 expressed on the surface of peripheral blood neutrophils from all patient groups was quantified by cytofluorography, by using median channel number to quantify fluorescence. Two dilutions of antibody were used. The results of these experiments appear in Table 3. The quantity of GP110 expressed on the surface of neutrophils of LJP patients with chemotactic defects was significantly lower than that of normal (determined by analysis of variance). Likewise, the neutrophils from GJP patients with chemotactic defects expressed significantly less GP110. LJP patients without chemotactic defects did not exhibit a reduction of GP110 expression, nor did any of the other patients exhibiting neutrophil chemotactic defects. Additionally, expression of other defined receptors (C3b, iC3b) on the surface of LJP neutrophils was normal (Table 4) on neutrophils in which NCD-1 expression was reduced.

Experiments were done to control for degranulation of neutrophils as a result of isolation technique and cell manipulation. In these experiments, blood was placed on ice immediately after collection and all isolation procedures were done at 4°C. At 4°C, the median channel number value for saturation of NCD-1 binding was 41.1 ± 3.7. At 22°C, the

FIG. 1. Autoradiograms of labeled membrane preparations from LJP and normal neutrophils. Lanes: 1, LJP; 2, control; 3, LJP; 4, control. Molecular-weight standards are indicated on the right. K, 1,000.

FIG. 2. Densitometric scans of the gels shown in Fig. 1 were done by using an LKB Laser densitometer. The LJP and control lanes were identical except for the region of 110,000 daltons. Peak values from densitometric gel scans of LJP and control membrane preparations were compared by using bands of equal intensity as a standard. The 200K (200,000-molecular-weight) bands from LJP and control preparations were identical; therefore, other peak values are expressed as the ratio of peak height to reference peak height (see Table 2). The 110K band is markedly reduced in the LJP preparation. Other major bands show slight variation: 90K is decreased in LJP, 80K is identical, and 50K is increased in LJP. The ratios of major bands to band 110K are indicated in Table 2, demonstrating the relative reduction of GP110 in LJP.

TABLE 2. Peak ratios for densitometric gel scans

<table>
<thead>
<tr>
<th>Mol wt band ratio</th>
<th>Control</th>
<th>LJP</th>
</tr>
</thead>
<tbody>
<tr>
<td>110/200</td>
<td>1.0</td>
<td>0.45</td>
</tr>
<tr>
<td>90/200</td>
<td>0.81</td>
<td>0.74</td>
</tr>
<tr>
<td>80/200</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>50/200</td>
<td>0.61</td>
<td>0.71</td>
</tr>
<tr>
<td>90/110</td>
<td>0.81</td>
<td>1.60</td>
</tr>
<tr>
<td>80/110</td>
<td>0.63</td>
<td>1.36</td>
</tr>
<tr>
<td>50/110</td>
<td>0.61</td>
<td>1.57</td>
</tr>
</tbody>
</table>

* See Fig. 2.
FIG. 3. Immunoblots of neutrophil surface components were done to evaluate the identity of the band deleted in LJP preparations (GP110) and the antigen which binds NCD-1. By using two separate membrane preparations, one from an LJP patient and one from a normal subject, of equal protein concentration, it is clear that NCD-1 binds to an antigen that comigrates with GP110. Also, the staining intensity of the LJP preparation (lane 3) is markedly less than that of the control preparation (lane 2). Molecular-weight markers are indicated on the left. Lane 1 represents the autoradiogram of normal surface-labeled neutrophils.

The median channel number was 45.6 ± 1.8. It was, therefore, concluded that under the conditions of cell isolation, temperature was not a variable.

In a separate set of experiments, the inhibition of chemotaxis by NCD-1 was evaluated. It has been previously reported that NCD-1 inhibits normal neutrophil chemotaxis to FMLP and C5a (5). In these experiments, the inhibition of LJP chemotaxis by NCD-1 was evaluated. Dose-response experiments (Fig. 4) revealed that LJP neutrophils were not inhibited by NCD-1 at concentrations up to 10 μg.

DISCUSSION

The data reported in this paper correlate the reduced expression of an antigenic determinant on the surface of neutrophils with a disease state in which neutrophil function is impaired. In a previous study (21), it was demonstrated that the antigenic determinant is a differentiation antigen closely linked to the cellular function of chemotaxis. Interestingly, all the patients with neutrophil chemotactic abnormalities examined, only patients with juvenile periodontitis (localized or generalized) exhibited reduced expression of this surface protein.

The assessment of surface proteins on SDS-polyacrylamide gels by densitometry is difficult and, at best, semi-quantitative. For this reason, fluorescein-labeled monoclonal antibody to the glycoprotein and cytofluorography were used. The densitometric patterns for controls were qualitatively reproducible from subject to subject and day to day, as were the SDS-polyacrylamide gels (Fig. 1). LJP patterns were similarly reproducible between patients. Patterns similar to LJP were also obtained with the GJP patients. The LJP patients with normal neutrophil chemotaxis, however, did not exhibit a depletion at 110,000 daltons and were indistinguishable from controls. The data suggest a correlation between GP110 depletion and functional abnormalities. These observations were further supported by immunoblot experiments as illustrated in Fig. 3. NCD-1 monoclonal antibody to GP110 is observed to produce a diminished band for the LJP patient neutrophil preparation.

Clinical data and in vitro neutrophil observations have suggested that the neutrophil abnormalities in LJP are genetically determined and inherited (12). This hypothesis is further supported by the data presented here and elsewhere in that the observation of defective chemotaxis and GP110 depletion was found to be independent of patient age and clinical status. Furthermore, the similar findings in LJP and GJP support the concept of a close relationship of the pathogenesis of two distinct clinical conditions.

The leukocyte function antigen glycoprotein complex has been described as an integral requirement for adherence and the functional integrity of neutrophils, including chemotaxis (8, 16–18). Regarding the relationship of leukocyte function antigen expression and neutrophil function in patients with periodontal disease, Page et al. (R. C. Page, P. Beatty, and T. C. Waldrup, J. Periodontal Res., in press) have reported that patients with generalized prepubertal periodontitis exhibit leukocyte function antigen deficiency, corresponding to

<table>
<thead>
<tr>
<th>Condition of patients in group (no. of patients)</th>
<th>Median channel no. ± SEM at NCD-1 dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:10</td>
</tr>
<tr>
<td>LJP (12)</td>
<td>42.5 ± 5.6b</td>
</tr>
<tr>
<td>Rec. infec. (3)</td>
<td>68.0 ± 9.6</td>
</tr>
<tr>
<td>Diabetes (3)</td>
<td>67.3 ± 2.1</td>
</tr>
<tr>
<td>GJP (5)</td>
<td>33.5 ± 1.7b</td>
</tr>
<tr>
<td>LJP* (4)</td>
<td>64.8 ± 9.1</td>
</tr>
<tr>
<td>Control (15)</td>
<td>65.8 ± 3.7</td>
</tr>
</tbody>
</table>

a The expression of GP110 on the neutrophil surface of each patient was quantified by using fluoresceinated NCD-1 and cytofluorography. Values are expressed as median channel number for fluorescence intensity. An age- and sex-matched control was used for each assay. The mean ± standard error is expressed for each group. Rec. infect., Patients with a history of severe recurrent infections.

b P < 0.05 (analysis of variance).

c Patients without neutrophil chemotaxis abnormality.

Receptors for iC3b and C3b were quantified for defective LJP and control neutrophils by using cytofluorography. There was no difference detected in either the number of fluorescent cells or the fluorescence intensity (median channel number).
neutrophil that the LJP neutrophil differs in many ways from the normal neutrophil. The selective depletion of a differentiation antigen recognized by the monoclonal antibody NCD-1 provides insight into the possible mechanism for these differences. It is not clear what the function of this antigen is. However, based upon studies of the neutrophil precursor cell line, HL-60 (21), the cumulative data would suggest an error in stem cell maturation in the LJP patient. This concept is further supported by the observation that there is no internalization of GP110 during phagocytosis (21).

In conclusion, the LJP neutrophil and NCD-1 continue to be useful probes for the examination of the mechanisms of neutrophil function. Studies are currently under way to determine the efficacy of NCD-1 for the epidemiologic screening of patients at risk for development of LJP. Finally, the biochemical and biological characteristics of P110 expression on LJP neutrophils will be elucidated to further characterize the molecular basis of neutrophil chemotaxis depression in LJP.

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