CD38 Expression in Neutrophils From Patients With Localized Aggressive Periodontitis

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Background: Localized aggressive periodontitis (LAGP) is associated with neutrophil dysfunction including decreased chemotaxis and reduced calcium entry. It has been suggested that CD38 is involved in chemotaxis. Little is known, however, about the relationship of CD38 and LAGP patients. In this study, we focused on the level of CD38 expression between LAGP and normal subjects and examined the involvement of CD38 in abnormal neutrophil chemotaxis of LAGP patients.

Methods: Neutrophils from 12 normal subjects and 12 LAGP patients were isolated from peripheral venous blood. Membrane-associated proteins were extracted from cells with or without N-formylmethionine leucyl-phenylalanine (fMLP) stimulation. CD38 expression was measured using Western blotting. Band density was measured using an imaging densitometer.

Results: There was no statistical difference between normal subjects and LAGP patients in resting CD38 expression (basal level). However, the fMLP-stimulated neutrophils exhibited a significant decrease of CD38 expression in LAGP subjects compared to normal subjects. The decrease of CD38 was positively correlated with the defect in chemotactic migration to fMLP.

Conclusion: These data suggest that the lower expression of CD38 in neutrophils may be related to altered neutrophil function in LAGP. J Periodontol 2005;76:1960-1965.

KEY WORDS
Calcium; CD38; chemotaxis; neutrophil; periodontitis.

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(ADP) ribosyl cyclase enzyme, it is believed that CD38 may be necessary for calcium sensitive biologic responses in a variety of biologic systems. CD38 is believed to be an exclusive ectoenzyme located on the plasma membrane, with its ADP ribosyl cyclase activity situated in the extracellular carboxyl domain. This cyclase activity is able to convert nicotinamide adenine dinucleotide (NAD\(^+\)) into the metabolite known as cyclic ADP-ribose (cADPR), which is a potent calcium mobilizing agent that can act independently of IP\(_3\).\(^7\) The calcium mobilizing properties of cADPR have been implicated in neutrophil chemotaxis to formylpeptide receptor-like 1 (FPRL1) ligands via a calcium-induced calcium release mechanism.\(^5\) In a previous study, we reported that fMLP and IL-8 decreased CD38 protein without changing CD38 mRNA and increased CD38 in supernatants, suggesting that CD38-mediated chemotaxis to fMLP is characterized by proteolytic cleavage of CD38.\(^8\)

Localized aggressive periodontitis (LAGP) is characterized by incisor and first molar bone loss with interproximal attachment loss\(^9\) and is associated with several abnormalities of neutrophil function. Based on recent findings, it has been suggested that the pathogenesis of LAGP is associated with excessive neutrophil function leading to neutrophil-mediated tissue damage.\(^10\) In other words, the neutrophil abnormalities in LAGP are the result of a chronic hyper-activated or primed state of the LAGP neutrophil. These findings are consistent with earlier reports of reduced chemotaxis by neutrophils in LAGP\(^11,12\) because primed neutrophils exhibit reduced motility but secrete greater quantities of superoxide and granule contents. To understand the mechanisms that are related to the onset and outcomes of these abnormal neutrophil functions in LAGP, previous and recent work from our group has demonstrated several signal transduction anomalies. Neutrophils from LAGP patients show reduced calcium entry,\(^13\) defective calcium influx factor,\(^14\) and abnormal protein kinase activity C.\(^15\) among other abnormalities.\(^16-20\)

Taken together with prior evidence, we hypothesized that the decrease in CD38 expression in LAGP neutrophils may be involved in reduced chemotaxis of LAGP patients. To clearly understand the mechanism of reduced chemotaxis in LAGP neutrophils, we sought to investigate if CD38 is involved in reduced chemotaxis of LAGP patients. In this report, the CD38 reduction in neutrophils from patients with LAGP is described.

**MATERIALS AND METHODS**

**Patient Population**

Twelve patients with LAGP were recruited from the patient population of Boston University Goldman Dental School, Clinical Research Center, Boston, Massachussets. The study protocol was approved by the Boston University Institutional Review Board (IRB). Subjects were enrolled between 2003 and 2004. Informed consent was obtained prior to drawing peripheral venous blood. The general health of subjects was good. The patients were diagnosed according to clinical and radiographic criteria, age of onset around circumpubertal period (<13 years old), and alveolar bone loss localized around the first permanent molars and incisors. The 12 normal subjects were healthy individuals who had no radiographic evidence of bone loss or any sign of periodontal disease other than mild gingivitis.

**Isolation of Neutrophils**

Peripheral venous blood was collected into vacutainer tubes containing 25 U/ml heparin. Neutrophils were isolated by Ficoll-Hypaque density centrifugation as previously described.\(^16\) Peripheral blood (4.5 ml) was layered on the separating medium,\(^4\) and the tubes were centrifuged at 500 \(\times\) g for 30 minutes. After the neutrophil fraction was collected and contaminating erythrocytes were lysed, the isolated neutrophils were washed twice with Dulbecco’s phosphate-buffered saline without magnesium and calcium.\(^5\) Cell viability was continuously assessed and 99% of the cells were trypan blue negative during different stages of incubation and stimulation. The preparations were >95% neutrophils.

**CD38 Analysis by Western Blotting**

Neutrophils were incubated with or without 10\(^{-8}\) M fMLP for 60 minutes at 37°C. After stimulation, cells were collected by centrifugation at 13,000 rpm, and cells were lysed in cold buffer (1% Triton X-100, 2 mM EDTA, leupeptin 2 \(\mu\)g/ml, pepstatin 1 \(\mu\)g/ml, and phenylmethylsulphonyl fluoride [PMSF] 50 \(\mu\)g/ml). Protein content was measured by the Bradford method, and samples (50 \(\mu\)g) were resolved on a 10% sodium dodecyl sulfate polyacrylamide gel by electrophoresis (SDS-PAGE) under non-reducing conditions, and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (350 mA; 90 minutes). The membranes were blocked with 5% non-fat dry milk for 90 minutes, and incubated at room temperature for 3 hours with mouse monoclonal anti-human CD38 antibody (2 \(\mu\)g/ml).\(^1\) The membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin G (IgG) in Tris-buffered saline for 1 hour at room temperature. The band density was measured using an imaging densitometer.\(^1\)

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\(^4\) Histopaque 1199 and Histopaque 1077, Sigma, St. Louis, MO.
\(^5\) Sigma.
\(^6\) Santa Cruz Biotechnology, Santa Cruz, CA.
\(^7\) Bio Rad, Hercules, CA.
Chemotaxis Assay

The chemotaxis assay was performed as previously described.20 Neutrophils were suspended in Gey’s balanced salt solution, supplemented with 2% bovine serum albumin at a concentration of $2.5 \times 10^6$ cells/ml. The cell suspension was placed in the upper compartment of a modified Boyden chamber separated by a 5-mm pore size micropore filter, whereas the lower compartment was loaded with either the buffer solution or a chemoattractant solution of fMLP at a concentration of $2 \times 10^{-8}$ M. The cell migration response was evaluated by the enumeration of cells on the distal surface of the filter after a 2-hour incubation in a 37°C humidified air chamber. Three representative high-power microscopic fields ($\times400$) were counted for each of the triplicate filters. When performed in the absence of fMLP in the lower compartment, the assay provided a measure of neutrophil-random migration.

The chemotactic migration of neutrophils from LAgP patients was expressed as a calculated percentage of the chemotactic migration of neutrophils from individual age-, race-, and gender-matched healthy subjects. Statistical differences between LAgP patients and controls were determined by analysis of variance (ANOVA).

Statistical Analysis

Comparisons between groups were analyzed by ANOVA. When statistically significant differences were observed, the difference between the two groups was analyzed by pairwise comparisons using the Bonferroni method. Pearson correlation analysis was performed where appropriate. The difference was considered significant if the $P$ value was <0.05.

RESULTS

Twelve normal subjects and 12 LAgP patients participated in the study. Table 1 represents demographics and chemotaxis assay. Control subjects (five males and seven females) were 19 to 34 years of age (mean age, 23.3 ± 4.5 [±SD] years). LAgP patients (five males and seven females) were 14 to 35 years of age (mean age, 22.5 ± 6.3 [±SD] years). Neutrophil chemotaxis was analyzed by chemotactic migration to fMLP. The chemotactic migration values of LAgP patients are expressed as an average of the calculated percentages of the chemotactic migration of neutrophils from individual age-, race-, and gender-matched controls. Statistical differences between LAgP patients and controls were determined by analysis of variance (ANOVA).

Protein was extracted from neutrophils stimulated with $10^{-8}$ M fMLP from normal subjects and LAgP patients, and CD38 was measured using Western blotting. CD38 expression was measured by the band density (OD mm). The CD38 expression from normal subjects in a resting condition ranged from 14.3 to 40.0, and CD38 expression from LAgP subjects in a resting condition ranged from 13.1 to 35.7. The CD38 expression in stimulated condition from normal subjects ranged from 9.8 to 28.5, and the CD38 in stimulated condition from LAgP patients ranged from 7.1 to 23 (Table 2).

Figure 1 shows the expression levels of CD38 before and after stimulation by fMLP from control subjects and LAgP subjects. In resting cells, the level of CD38 expression (basal level) was not statistically different between normal subjects and LAgP patients. However, the decrease in CD38 levels was significantly more reduced in LAgP than in control neutrophils (Fig. 1).

The correlation between the CD38 defect and chemotaxis defect of neutrophils from LAgP patients was then analyzed (Fig. 2). The CD38 decrease in neutrophils from patients with LAgP was positively correlated with the defect of chemotactic migration to fMLP ($r^2 = 0.8924$).

DISCUSSION

In this paper, we report a reduction in CD38 expression in chemotactically abnormal neutrophils from LAgP patients. The decreased levels of CD38...
expression correlated well with chemotactic migration to fMLP in each patient in the LAgP population. Thus, the decrease of CD38 expression seems to relate to neutrophil chemotactic migration.

Reduced chemotaxis is one of the neutrophil abnormalities in LAgP patients. Reduced chemotaxis is one of the neutrophil abnormalities in LAgP patients. Various mechanisms have been suggested to explain these observations, including cytoskeleton dysfunction and abnormalities in signal transduction cascade. LAgP signaling abnormalities are known to be associated with increased intracellular diacylglycerol (DAG) levels.

Table 2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control Subjects</th>
<th>LAgP Patients</th>
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<tbody>
<tr>
<td></td>
<td>Resting (A)</td>
<td>fMLP (B)</td>
</tr>
<tr>
<td>1</td>
<td>26.2</td>
<td>18.5</td>
</tr>
<tr>
<td>2</td>
<td>16.3</td>
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<td>12</td>
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Figure 1. Neutrophils were incubated with or without $10^{-8}$ M fMLP for 60 minutes. Samples (50 μg protein per lane) were loaded and analyzed as described in Materials and Methods. Values are means ± SE of 12 subjects.

Figure 2. Correlation between chemotactic migration to fMLP and CD38 expression of neutrophils from patients with LAgP. The chemotactic migration and CD38 expression values of patients are represented by percentages of the responses obtained from the matching controls. The correlation coefficient is shown in the lower right-hand corner.
and decreased PKC activity. Furthermore, Agarwal et al.\(^{21}\) demonstrated that intracellular Ca\(^{2+}\) increases in response to chemotactic factor do not occur in neutrophils from patients with LAgP. Daniel et al.\(^{13}\) further revealed that low levels of intracellular Ca\(^{2+}\) seemed to be caused by a dysfunction of the influx of extracellular Ca\(^{2+}\) in neutrophils from the patients. Therefore, calcium receptors seem to relate to abnormalities. Recently, the ryanodine receptor and CD38 has been identified for fMLP-mediated chemotaxis working through FPRL1.\(^{4,5}\) The involvement of CD38 has been identified for fMLP-mediated chemotaxis and suggesting that downregulation of CD38 levels induced by fMLP, which causes the significance of this issue.

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