Altered Diacylglycerol Level and Metabolism in Neutrophils from Patients with Localized Juvenile Periodontitis

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Diacylglycerol, a physiological activator of protein kinase C, was elevated nearly twofold in unstimulated peripheral blood neutrophils from patients with localized juvenile periodontitis compared with cells from normal individuals. These cells also showed an enhanced and prolonged elevation of diglyceride in response to *N*-formylmethionylleucylphenylalanine. The metabolism of a cell-permeant diacylglycerol by diglyceride kinase was significantly decreased, because of a fivefold or higher elevation in the apparent K_m of cellular diglyceride kinase.

Diacylglycerol has been implicated in the regulation of a variety of cellular functions, including growth and differentiation (38, 52), neutrophil activation (the respiratory burst and degranulation) (40), and chemotaxis and chemokinesis (29). Biochemical aberrations in the diacylglycerol (DAG)-protein kinase C (PK-C) pathway are expected to affect the regulation or function of some or all of these processes; it is possible that cellular defects seen in clinical conditions in which function is altered can be traced to altered signal transduction pathways.

One such condition is localized juvenile periodontitis (LJP). Clinically, the condition is characterized by periodontal infection with *Actinobacillus actinomycetemcomitans* in most cases and severe, early-onset molar and incisor bone loss (1). LJP is an apparently inherited (77) condition found most commonly in the black population. Peripheral neutrophils from patients with LJP frequently demonstrate defective function, in particular chemotaxis (13, 42, 74), which is reportedly independent of active infection or treatment state (74).

Neutrophils, the primary cells involved in the inflammatory response, are capable of directed migration to a site of inflammation. This migration of neutrophils may be in response to chemotactic substances elaborated directly by bacteria or by chemotactic factors derived from complement (44, 47). The accumulation of neutrophils in the connective tissue and junctional epithelium of the periodontium is seen characteristically as a feature of chronic periodontal disease (53). The neutrophil is thought to function by clearing infecting microorganisms and other noxious substances. Several conditions in which neutrophil function is impaired also occur with an increase in severe periodontal disease. This is seen in conditions such as cyclic neutropenia (17), Chediak-Higashi syndrome (28, 69), chronic granulomatous disease (19), lazy leukocyte syndrome (19, 50), hyperimmunoglobulinemia E syndrome (31), and diabetes mellitus (30, 51). These observations suggest that decreased neutrophil chemotaxis in LJP may be contributory to the pathogenesis of this disease process.

The focus of this report is to further characterize the

nature of the chemotactic defect seen in patients with LJP, specifically to elaborate the mechanisms surrounding the transduction of the signal for chemotaxis. The primary event involved in initiating these events is thought to be the binding of specific ligand to neutrophil cell membrane receptors. This binding is followed by a transmembrane transduction of the signal, leading to an organization of the cell's orientation and contractile elements and thus leading to locomotion toward higher concentrations of chemotactic factor and ingestion of foreign particles. Human neutrophils are capable of responding to a variety of chemical agents that stimulate them to migrate to a site of infection. Cianciola et al. (13) found significantly reduced chemotaxis in nine patients with LJP compared with that in controls. Subsequently, many other laboratories have independently reported that approximately 75% of patients with LJP have impaired chemotaxis. Characterization of this defect revealed a decreased ability to migrate in response to a chemotactic gradient and a decreased number of receptors for the chemotactic factors *N*-formylmethionylleucylphenylalanine (FMLP) and C5a (75, 76). The majority of patients exhibited abnormal neutrophil chemotaxis that was intrinsic to the cells as evidenced by the fact that it was unaffected by serum (26, 42) and unaffected by conventional treatment of the periodontal disease (74).

We have investigated diacylglycerol (DG) levels and metabolism in unstimulated neutrophils from patients with LJP and unaffected individuals.

MATERIALS AND METHODS

Subjects. All patients were obtained from the patient population of Emory University School of Postgraduate Dentistry, Emory Dental Research Center, Emory Clinic, and affiliated hospitals. Informed consent was obtained from all subjects or, in the case of minors, from their parents or legal guardians. A complete medical history and battery of clinical laboratory tests including SMA-22, prothrombin time and urinalysis was completed for all patients. Health questionnaires which elicited information regarding tobacco use, drug and alcohol consumption, illnesses, and blood loss were administered to patients and controls on each day of neutrophil function testing; for females, additional informa-

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tion regarding birth control medication, menstruation, and pregnancy was gathered.

The periodontal disease patient group was composed of individuals diagnosed with LJP. Patients with LJP were defined as young patients (under 30) exhibiting alveolar bone loss localized to molars and incisors and not more than two additional teeth (≤ 14 teeth including 12 molars and incisors). The control group consisted of healthy individuals from laboratory and dental school populations who had no radiographic evidence of bone loss or evidence of periodontal disease other than mild gingivitis. Patients and controls were age, sex, and race matched for most experiments. Patients with LJP who exhibited defective chemotaxis were chosen for this study.

Isolation of neutrophils and neutrophil functional assays. (i) **Isolation of neutrophils.** Neutrophils were isolated as previously described (74). Briefly, neutrophils were separated from heparinized venous blood by Ficoll-Hypaque centrifugation, washed two times in phosphate-buffered saline after lysis of erythrocytes with NH_4Cl buffer, and suspended in assay medium appropriate to the assay being performed. Chemotaxis and superoxide generation assays were performed simultaneously with samples from the same batch of cells whenever possible.

(ii) **Chemotaxis.** The chemotaxis assay, as it is routinely performed in our laboratory, has been described in detail elsewhere (74). In summary, neutrophils were suspended in an assay medium consisting of Gey's balanced salt solution supplemented with 2% bovine serum albumin at a concentration of 2.5×10^6 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, Göttingen, Germany). The lower compartment contained the synthetic chemotactic peptide FMLP. 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 ($400\times$) were counted for each set of triplicate filters. Statistical differences between patients and controls were determined by analysis of variance.

Lipid extraction and quantitation of *sn*-1,2-diglyceride. After incubation, cells (10^7 cells per datum point) were extracted by the method of Bligh and Dyer (9); cell samples (0.8 ml) were mixed with 3 ml of chloroform-methanol (1:2, vol/vol). Chloroform and 1 M NaCl (1 ml each) were added and mixed by vortexing. After centrifugation, the chloroform phase was removed and analyzed for DG within 48 h. Prior to analysis, samples were kept at -20°C to minimize acyl group migration. DG was quantified by using the method of Preiss et al. (54) by enzymatic conversion of DAG to [^{32}P]phosphatidic acid ([^{32}P]PA) by using *Escherichia coli* diglyceride kinase, followed by thin-layer chromatography (TLC) resolution and counting. The TLC system to be used readily resolves PA from ceramide phosphate, which run at R_f values of 0.40 and 0.19, respectively. 1-*O*-Alkyl-2-acylglycerol (prepared by treatment of 1-*O*-hexadecyl-2-oleoylphosphatidylcholine with phospholipase C) is also a substrate for the diglyceride kinase, and the phosphorylated product comigrates with diacylphosphatidic acid. We therefore treated half of the samples with *Rhizopus* lipase, which hydrolyzes 1-acyl glycerols and not the alkyl-acylglycerol, to determine total DAG. Moreover, the principle enzyme activity can be determined by the DAG/alkyl-acylglycerol rates since phospholipase C hydrolyzes phosphatidylinositol-4,5-diphosphate (PIP_2), yielding DAG, and phospholipase C hydrolyzes phosphatidylcholine, yielding 50% DAG and

FIG. 1. Diacylglycerol levels in unstimulated neutrophils from normal donors versus patients with LJP. Neutrophils from unaffected donors and patients with LJP were isolated and incubated as described previously (41, 73). Cells (10^7 in 0.8 ml of phosphate-buffered saline) were equilibrated at 37°C for 5 min with shaking in plastic test tubes (13 by 100 mm). Lipids were extracted by transfer of the sample to chloroform-methanol according to the method of Bligh and Dyer (9). The total 1,2-DG was assayed by the method of Preiss et al. (55), with modifications (77). The data shown are from 18 normal donors and 10 donors with LJP. Each datum point represents the average of three to six analyses for each donor.

50% alkyl-acylglycerol. Known quantities of *sn*-1,2-dioleoylglycerol were carried through the same procedures to construct a standard curve. The presence of a long chain base in incubations had no effect on the analysis (41).

Quantitation of cellular [^{32}P]PA generation. Isolated neutrophils were suspended in buffer (pH 7.4) containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 136 mM NaCl, 4.9 mM KCl, 0.33 mM CaCl_2 , and 5.5 mM glucose (buffer B). Neutrophils (5×10^8 cells in 2 ml) were preincubated with [^{32}P]NaH₂PO₄ (1 mCi) at 37°C for 1 h on a shaking water bath. Cells were washed twice by centrifugation and resuspension and finally resuspended in buffer B to a concentration of 6.25×10^6 cells, divided into treatment groups, and incubated with agonists. Aliquots (0.8 ml) were withdrawn at indicated time points, extracted, and subjected to TLC separation, autoradiography, and quantification as described above for diglyceride, except that 1% HClO_4 was included during extraction. PA identity and quantitation were confirmed by using a second TLC solvent system consisting of chloroform-methanol-water (50:25:6, vol/vol/vol) (25). Authentic phospholipid standards (PA, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and cardiolipin) were cochromatographed to allow identification and to confirm adequate separation of lipids.

Prelabeling of cells with *myo*-[2- ^3H]inositol. Isolated neutrophils were resuspended to 10^8 cells per ml with medium 199 (GIBCO) containing 2% fetal calf serum plus 40 mM HEPES buffer (pH 7.4), *myo*-[2- ^3H]inositol (40 $\mu\text{Ci/ml}$) was added, and cells were incubated at 37°C for 2.5 h on a shaking water bath. A 5×10^5 -fold molar excess of unlabeled *myo*-inositol (1 mM) was added, and the incubation was continued for an additional 1 h. Cells were washed twice by centrifugation ($600 \times g$) and resuspended in Hanks' balanced

salt solution containing 20 mM HEPES, 10 mM LiCl, and 1 mM unlabeled *myo*-inositol to a final concentration of 1.5×10^7 cells per ml. The cells were then incubated at 37°C with agonist. Incubations (1 ml) were terminated with chloroform-methanol (1:1, vol/vol) and 0.5 ml of 2.4 N NaOH and used for quantification of inositol phosphates, while the organic phase was washed again with 2.4 N HCl and analyzed for inositol-containing lipids.

Quantitation of [3 H]inositol phosphates and [3 H]inositol-containing lipids. Inositol phosphates were analyzed following incubations of [3 H]inositol-prelabeled cells essentially as described by Downes and Michell (22). No attempt was made to resolve isomeric forms. The washed chloroform phase from extracted, [3 H]inositol-prelabeled cells was dried and resolubilized in 100 μ l of chloroform containing 5% methanol. A 20- μ l aliquot was spotted onto a 1% oxalate-impregnated Silica Gel H TLC plate (Analtech), and the plate was developed with chloroform-methanol-4 N ammonium hydroxide (90:70:20, vol/vol/vol) containing 2 mM cyclohexylene-dinitrotetraacetic acid (60). Positions corresponding to standard phosphatidylinositol 4,5-bis-phosphate, phosphatidylinositol 5-phosphate and phosphatidylinositol (run in adjacent lanes) were scraped from the plate and quantitated by scintillation counting as above.

RESULTS

Intracellular diglyceride. Diglyceride mass was quantified in unstimulated peripheral blood neutrophils from 18 normal donors and from 10 individuals diagnosed as having LJP (Fig. 1). All patients with LJP exhibited neutrophil chemotaxis dysfunction. Average DG in normal donors was 189 ± 27 pmol/ 10^7 cells, whereas the average level in neutrophils from patients with LJP was significantly elevated to 342 ± 28 pmol/ 10^7 cells ($P < 0.001$). The usual mass assay for diglycerides detects contributions not only from 1,2-DAG but also from 1-*O*-alkyl 2-acylglycerol (40, 72). 1-*O*-alkyl-linked species are reportedly PK-C inhibitors rather than activators (20), making this distinction important. We therefore utilized a modification (72) of the assay to quantify both 1,2-DAG and 1-*O*-alkyl-2-acylglycerol. Nearly 90% of the diglyceride in cells from three donors with LJP was DAG (about 300 pmol/ 10^7 cells), whereas the ether-linked diglyceride level was indistinguishable from that in neutrophils from four unaffected donors (40 ± 5 and 45 ± 5 pmol/ 10^7 cells, respectively).

The chemotactic peptide (FMLP) caused a rapid and transient increase in DG (Fig. 2) which peaked at 30 s and returned to near basal levels by about 1 min, which is similar to results described in previous reports (32, 61, 73). In neutrophils from two donors with LJP, both the basal and FMLP-stimulated levels of DG were elevated substantially above those seen in normal cells (Fig. 2). The FMLP-stimulated diglyceride peaked slightly later (30 to 60 s) and appeared to persist longer (beyond 5 min) than in normal cells. Thus, despite the elevated diglyceride level, FMLP activation mechanisms are intact in the cells from patients with LJP.

Phosphoinositide turnover. FMLP is known to stimulate hydrolysis of phosphoinositides, with consequent release of inositol 1,4,5-trisphosphate which then triggers the release of calcium into the cytosol (39). By using cells from normal donors or patients with LJP prelabeled with [3 H]inositol, we quantified (73) release of [3 H]inositol phosphates (IP_3 , IP_2 , and IP) in response to FMLP. In two experiments with paired control cells and cells from patients with LJP, no

FIG. 2. Time course for production of DAG in FMLP-stimulated neutrophils from unaffected donors versus patients with LJP. Cells were prewarmed at 37°C for 5 min and then stimulated at zero time with FMLP (1 μ M). Aliquots (10^7 cells per datum point) were removed at the indicated times, transferred to chloroform-methanol (1:2), and analyzed for DG as described for Fig. 1. For each donor, incubations were carried out in quadruplicate. Data from five unaffected donors were averaged, and data from two patients with LJP are shown individually. Error bars represent standard errors of the means. Where not shown, error bars did not exceed the size of the symbol.

differences were seen in the uptake of [3 H]inositol into phospholipids or in FMLP-stimulated release of IP_3 , IP_2 , or IP (data not shown).

DG kinase activity. Reduced activity of DG kinase could also cause elevated DAG levels. This enzyme, present in a variety of cells (37), phosphorylates DAG to produce PA. In platelets, this pathway was demonstrated, by using the cell-permeant dioctanoylglycerol (diC8), to be a major route of metabolism of DAG (7, 8). The metabolisms of diC8 by normal neutrophils and neutrophils from patients with LJP are compared in Fig. 3. In three experiments, dioctanoyl-[32 P]PA production was diminished by 50 to 70% in neutrophils from patients with LJP at concentrations of diC8 from 5 to 200 μ M.

A saturation curve of rate versus concentration from a separate experiment is shown in Fig. 4. The apparent K_m for diC8 in normal cells was 13 μ M and was elevated more than fivefold (72 μ M) in cells from patients with LJP. The V_{max} was decreased by only 15%. Thus, the major defect in this case was in the K_m . This experiment was repeated with five patients with LJP, yielding similar results. These data demonstrate a change in neutrophils from patients with LJP in the function of DAG kinase.

DISCUSSION

In this article, we report a naturally occurring elevation of DAG in chemotactically defective neutrophils from patients with LJP. This elevation appears to be caused by decreased DG kinase activity in these cells, which is associated with a fivefold increase in enzyme K_m . Elevated DAG has been seen in other pathological conditions. *ras*-transformed cells show a two- to threefold elevation (56), and the elevation is

FIG. 3. Dioctanoyl-[^{32}P]PA formation from added diC8 in neutrophils from unaffected donors and patients with LJP. Cells from normal donors and patients with LJP (5×10^8 cells/2 ml in HEPES-buffered saline) were preincubated for 1 h at 37°C with gentle shaking with [^{32}P]NaH $_2$ PO $_4$ (1 mCi; specific activity, 1,000 Ci/mol) and then reisolated to remove excess label, as described in reference 73. Cellular uptake of ^{32}P was the same within experimental error in cells from both normal donors and patients with LJP. In addition, the formation of ^{32}P -phospholipids (chloroform phase) during the preincubation period was the same in both cell populations, indicating that the ATP pool had been radiolabeled to a similar extent. Labeled cells ($10^7/0.8$ ml for each incubation) were equilibrated at 37°C for 5 min prior to the addition of various concentrations of diC8. At the indicated times, incubations were terminated by transfer to 3 ml of chloroform-methanol (1:2, vol/vol). Lipids were extracted (9), and diC8-[^{32}P]PA was separated by TLC, visualized by autoradiography, and quantified as described (73). Endogenous [^{32}P]PA (R_f , 0.38) and diC8-[^{32}P]PA (R_f , 0.24) were readily separated. The left and right panels show results with neutrophils from unaffected controls and patients with LJP, respectively. Added diC8 concentrations were as follows: 5 μM (●), 10 μM (○), 25 μM (▲), 100 μM (□), and 200 μM (■). The results shown are representative of results for three experiments. Error bars represent the standard errors of three incubations from one cell preparation. CPM, counts per minute.

thought to be due to phospholipase C- or D-dependent hydrolysis of phosphatidylcholine (58). Livers of choline-deficient rats show elevated DAG, which may be linked to the liver cancers associated with this diet (10); elevated diglyceride in this case is presumably due to perturbed phosphatidylcholine biosynthesis. The present studies provide a new example of pathologically elevated DAG and the first example of decreased metabolism by diglyceride kinase as a mechanism.

The observed functional change in diglyceride kinase is

consistent with either a mutant enzyme or a change in the enzyme's regulation. DAG kinase exists in several isoforms differing in molecular weight, immunochemical characteristics, molecular species specificity, and tissue distribution and can exist in both soluble and membrane-associated forms (for a review, see reference 37). Forms of the enzyme can in some cases be phosphorylated, by both PK-C and PK-A, with variable effects on function. In neutrophils, activation of PK-C results in translocation of DAG kinase activity from cytosol to membrane but does not alter the K_m

FIG. 4. Representative kinetics of cellular diglyceride kinase activity in neutrophils from donor with LJP and an unaffected individual. Preincubation conditions and addition of short-chain DAG were as described for Fig. 3, except that a higher range of diC8 concentration was used (up to 500 μM). The rate, v , refers to diC8-[^{32}P]PA formed at 1 min after the addition of diC8. Each point represents the average of three incubations. The K_m was calculated from a nonlinear least-squares fit of the data according to the method of Duggleby (24). For neutrophils from a patient with LJP, $K_m = 72 \mu\text{M}$ and $V_{\max} = 21 \mu\text{M}/\text{min}/10^7$ cells; for neutrophils from the unaffected individuals, $K_m = 13 \mu\text{M}$ and $V_{\max} = \mu\text{M}/\text{min}/10^7$ cells.

of the enzyme (36). Thus, it seems unlikely that the altered K_m seen in the present studies can be accounted for by this mechanism. The elucidation of the nature of postreceptor signal transduction in a variety of tissues has led to the recognition of the association between the changes in Ca^{2+} induced by receptor binding and the turnover in phosphoinositides (32, 48, 49) and other phospholipids. This observation was central to the further characterization of the role of second messenger molecules in a variety of cell types, including the neutrophil.

Ca^{2+} -mobilizing agonists have been shown to increase DAG in cells (11, 54, 55, 62). The time course of DAG generation, however, differs markedly from that of IP_3 , which is consistent with the idea that DAG is formed from other sources besides PIP_2 (27, 56). Analysis of DAG generated by Ca^{2+} -mobilizing agonists revealed one pool enriched in stearic and arachidonic acids, suggesting derivation from inositol phospholipids, and another pool of DAGs composed predominantly of palmitic, stearic, oleic, linoleic, and arachidonic acids, suggesting another source (11). The supposition that DAG has a source other than PIP_2 is based on the observation that accumulation of DAG is an order of magnitude greater than that of *myo*-inositol phosphates (11). The likely source of additional DAG is phosphatidylcholine (6, 35, 59). The observation of normal IP_3 levels and elevated DAG in LJP support this supposition.

DAG produced in the plasma membrane is believed to be further metabolized to PA because of the action of DG kinase. Translocation of DAG kinase from the cytosol to the membrane is induced by DAG and chemotactic peptides in neutrophils (36). Another possible route of DAG degradation is hydrolysis by DAG lipase, but it is not known to what extent this enzyme is found in the plasma membranes of most cells. PA accumulates faster than DAG, and changes in fatty acid composition of PA precede those in DAG (11). These observations are consistent with the idea that DAG is not the major or only source of PA.

Incubation of cells with GTP analogs causes an increase in PA accompanied by a decrease in phosphatidylcholine and release of choline (11, 45). The fatty acid composition of the PA is also consistent with phosphatidylcholine as a source independent of DAG (35). These results suggest that a major mechanism by which PA is produced during Ca^{2+} -mobilizing agonist action is by the G-protein-mediated action of a phospholipase D, the major substrate of which is phosphatidylcholine. There is also evidence of phorbol ester-stimulated activation of phospholipase D (45). The biologic function of the large amounts of PA remains to be elucidated. However, the large increase in DAG in LJP appears to be independent of phospholipase D activity.

Early observations indicated that Ca^{2+} -mobilizing agonists stimulated the phosphodiesteratic breakdown of PIP_2 (21, 63), resulting in production of IP_3 (2–5), which caused the release of Ca^{2+} from intracellular stores (67). The increase in IP_3 with agonists is detectable within a few seconds and generally precedes or is coincident with the increase in cytosolic Ca^{2+} (12, 43, 57, 70, 71) with most, but not all, agonists (61, 62). The action of IP_3 is short-lived, as it is rapidly degraded by soluble and membrane-bound phosphomonoesterases (23, 33, 34, 35, 36, 64, 66) to *myo*-inositol, which can be reincorporated into PI by CDP-DAG *myo*-inositol transferase in the endoplasmic reticulum and recycled to the plasma membrane (48). Neutrophils from patients with LJP do not demonstrate intracellular Ca^{2+} increases compared with normal neutrophils (68), in spite of normal Ca^{2+} release from intracellular stores (20) and nor-

mal production of IP_3 . These data suggest an increase in membrane Ca^{2+} channels after stimulation of the neutrophils from donors with LJP.

Mammalian tissues contain a variety of phospholipase C activities with different substrate specificities. In neutrophils, the substrate specificity of phospholipase C is PIP_2 and PIP. The regulation of neutrophil membrane associated-phosphoinositide phospholipase C is by G protein (14–16, 65). In almost all cell types, the membrane-associated phospholipase-hydrolyzing PIP_2 is completely dependent on calcium for activity (14, 46, 78).

The elevated DAG may account for other abnormalities reported for neutrophils from donors with LJP. Evidence for a role for PK-C in chemokinesis and chemotaxis has been presented (40). Thus, the presence of elevated DAG might be expected to interfere with these processes. Investigations are currently under way to determine whether the DG kinase defect in LJP represents a central mechanism which may explain the multiple abnormalities seen in LJP.

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