Activation of cyclic AMP response element-binding protein (CREB) in aggressive periodontal disease

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Objectives: To report a novel observation of neutrophil signal transduction abnormalities in patients with localized aggressive periodontitis (LAP) that are associated with an enhanced phosphorylation of the nuclear signal transduction protein cyclic AMP response element-binding factor (CREB). **Method and Materials:** Peripheral venous blood neutrophils of 18 subjects, 9 patients with LAP and 9 race-, sex-, and age-matched healthy controls, were isolated and prepared using the Ficoll-Hypaque density-gradient technique. Neutrophils (5.4×10^6/mL) were stimulated with the chemoattractant FMLP (10^{-6} mol/L) for 5 minutes and lysed. Aliquots of these samples were separated by SDS-PAGE (60 µg/lane) on 9.0% (w/v) polyacrylamide slab gels and transferred electrophoretically to polyvinyl difluoride membranes. The cell lysates were immunoblotted with a 1:1,000 dilution of rabbit-phospho-CREB antibody that recognizes only the phosphorylated form of CREB at Ser^{133}. The activated CREB was visualized with a luminol-enhanced chemoluminescence detection system and evaluated by laser densitometry. **Results:** In patients with LAP, the average activation of CREB displayed an overexpression for the unstimulated peripheral blood neutrophils of 80.3% (17.5-fold) compared to healthy controls (4.6%). **Conclusion:** LAP neutrophils who express their phenotype appear to be constitutively primed, as evidenced by activated CREB in resting cells compared to normal individuals. The genetically primed neutrophil phenotype may contribute to neutrophil-mediated tissue damage in the pathogenesis of LAP. *(Quintessence Int 2009;40:857–863)*

Key words: host defense mechanisms, localized aggressive periodontitis, nuclear transcription factor CREB, primed LAP neutrophil phenotype, signal transduction abnormalities

Localized aggressive periodontitis (LAP) is a destructive periodontal disease entity characterized by bone loss around molar and incisor teeth. There are several hypotheses for possible etiologic mechanisms, including an innate or induced functional LAP neutrophil deficiency in host resistance to bacterial infection, an increased receptor-mediated respiratory burst activity, or an enhanced adhesion to endothelial cells.[1–4]

LAP neutrophils display altered regulations of key cellular functions involving a variety of signal transduction pathways. Defective plasma membrane calcium channels[5,6] and a decreased diacyglycerol kinase (DG) activity[7,8] resulting in an enhanced intracellular accumulation of diacylglycerol levels are reported to be associated with LAP. Increased diacylglycerol levels as important

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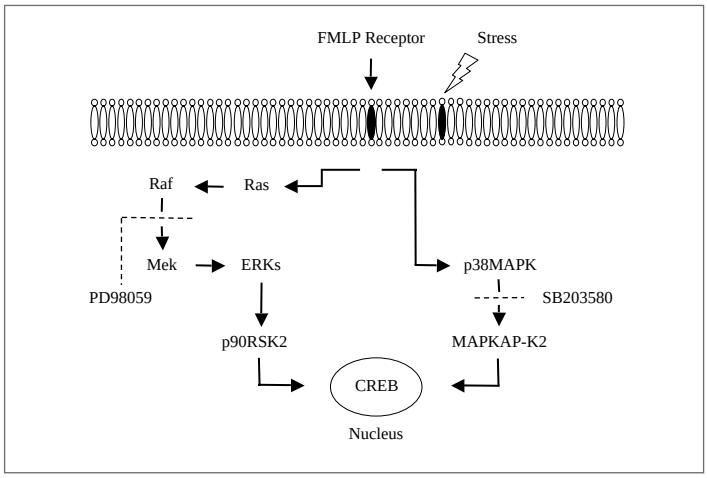

Fig 1 Pathway for the activation of CREB during stimulation of human neutrophils with FMLP. Occupation of the FMLP receptor upregulates the activity of intracellular-located mitogen-activated protein kinases p38MAPK and MAPKAP-K2. This mechanism initiates the phosphorylation of CREB at Ser^{133} as an upstream event in the IL-1 beta and cyclooxygenase-2 activation. The transformation of p38MAPK to MAPKAP-K2 induced by stress and ultraviolet radiation can by downregulated by the pyridinyl imidazole inhibitor SB203580 *(dashed line)*. A second activation pathway for CREB is the upregulation of extracellular-regulated kinases (ERKs) that initiate p90RSK. Phosphorylation of CREB induced by growth factors and phorbol esters is inhibited by PD98059 *(dashed line)*.[16] (Raf) activator of MED/Erk kinase pathway; (Ras) subcellular membrane binding protein; (Mek) mitogen-activated extracellular signal-regulated kinase.

messengers in lipid signaling and decreased DG activity may lead to essential changes in the physical properties of the bilayer membrane structure of the LAP neutrophil. The molecular basis for neutrophil abnormalities in LAP neutrophils includes a decreased number or alteration of chemotaxis-related surface receptors for FMLP *, C5a, interleukin (IL)-8, and the surface glycoprotein GP110.[9] The reduced ability to respond to a protein kinase C-dependent stimulus together with the exposition to a bacterial challenge, ie, *Aggregatibacter actinomycetemcomitans*, characterizes the partially activated "primed state" of LAP neutrophils.[10] Recently, results of an impaired endogenous 15-lipoxin (LX) activity from LAP donors with lower amounts of the 15-hydroxyeicosapentaenoic acid (15-HETE) substrate generated by LAP neutrophils could not be confirmed.[11,12]

Intracellular activation pathways are associated with an enhanced phosphorylation of specific proteins that initiate nuclear signaling. On the nuclear level, cell activation is known to be associated with the activity of the 47-kDa (47 kilodalton) master transcription factor CREB (cyclic adenosine monophosphate [AMP] response element-binding protein). Recently, the signal transduction pathway for CREB was demonstrated in macrophages.[13] Stimulation of the macrophage by lipopolysaccharides (LPS), FMLP, or proinflammatory cytokines upregulates the activity of intracellular-located mitogen- and stress-activated protein kinases. This mechanism initiates the phosphorylation of CREB as an upstream event in the IL-1 beta and cyclooxygenase-2 (COX-2) activation that occurs to produce compounds in the inflammatory disease process.[14,15]

The phosphorylation of CREB, acting altogether with other signaling proteins located in the nucleus, is considered to be a regulatory mechanism for enzyme activity in the cytosolic compartments of the neutrophil. In unstimulated neutrophils, the kinetics of the CREB-initiated pathway is controlled by 38-kDa mitogen-activated protein kinases (p38MAPK). The transformation of p38MAPK to MAPK-activated protein kinase 2 (MAPKAP-K2), an enzyme that lies immediately downstream of p38MAPK, can be downregulated by the pyridinyl imidazole inhibitor SB203580.* In stimulated neutrophils, an additional activation pathway for CREB is the upregulation of extracellular-regulated kinases (ERKs) that initiate p90RSK.* Phosphorylation of CREB induced by growth factors and phorbol esters is inhibited by PD98059.*[16]

Recently, CREB as a nuclear signal messenger molecule was identified to stimulate the scavenger receptor class B type I promoter activity in the ovary.[17] The functional mechanisms of CREB have been reported for brain cells[18] and human neutrophils.[16,19] However, they have not yet been identified in neutrophils from patients with localized periodontal disease. The focus of this report is to

*FMLP = N-formyl-L-methionyl-L-leucyl-L-phenylalanine; SB203580 = 4-(4-fluorophenyl)-2-[4-(methylsulfinyl)phenyl]-5-(4-pyridyl)imidazol; P90RSK = 90-kDa ribosomal S6 kinase (also referred to as MAPKAP-K1); PD98059 = (2-(2'-amino-3'-methoxyphenol)-axanapthalen-4-one).

identify the 47-kDa CREB in LAP neutrophils as key signal transduction protein for the chemotactic defect in individuals with LAP. A novel observation is reported of neutrophil signal transduction abnormalities in patients with LAP associated with CREB that undergoes phosphorylation in unstimulated LAP neutrophils.

METHOD AND MATERIALS

Subjects

Eighteen patients, 9 subjects with LAP and 9 race , sex , and age-matched healthy individuals, were recruited from the Dental Research Clinic, Department of Periodontology and Oral Biology, Goldman School of Dental Medicine, Boston University and affiliated health centers, in accordance with a protocol approved by Boston University Medical Center Internal Review Board for human subjects. The general health of the subjects was good. Patients with LAP were diagnosed according to clinical and radiographic criteria: age of onset in the circumpubertal period (< 13 years old) with alveolar bone loss localized around the permanent first molars and incisors. Informed consent was obtained from all subjects before peripheral venous blood was drawn. Health questionnaires were administered that elicited information regarding tobacco use, drug and alcohol consumption, medication, and any systemic condition that might have affected the inflammatory periodontal disease process on the day of neutrophil testing; for females, additional information regarding birth control medication, menstruation, and pregnancy was gathered. All subjects denied taking medication for 2 weeks before venipuncture.

Isolation and preparation of neutrophils

Freshly isolated neutrophils were obtained the morning of each experiment. For each patient, 45 mL of peripheral venous blood was collected into Vacutainer cell preparation tubes (Becton Dickinson) containing 25 units/mL of heparin. Neutrophils were isolated using the Ficoll-Hypaque density-gradient technique.[20] Briefly, 5 mL of whole blood was overlaid on successive layers of 3 mL MonoPoly (Flow Laboratories) and 1 mL Histopaque 1077 (Sigma Chemical) and centrifuged for 30 minutes at 500 × *g*. The polymorphonuclear neutrophil–rich layer was collected, and cells were washed with phosphate-buffered saline (PBS; 13.3 mmol/L sodium hydrogen phosphate; 6.7 mmol/L potassium dihydrogen phosphate, pH 7.2; 0.15 mol/L sodium chloride). Contaminating erythrocytes were lysed by a 10-minute incubation in ice-cold ammonium chloride buffer (155 mmol/L ammonium chloride; 10 mmol/L potassium bicarbonate; 120 μmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4). Purified neutrophils were washed and resuspended in PBS. The amount of neutrophils was calculated with the hemacytometer technique (Reichert Scientific Instruments). Viability is usually 99% or greater (trypan blue exclusion test), and contamination with mononuclear cells was under 1% (Wright-Giemsa differential staining, Gugol blue staining kit [Fisher Scientific]).

Standardization and validation

The optimal concentration of freshly isolated human neutrophils to activate phospho-CREB was evaluated utilizing 0.6×10^6, 1.2×10^6, 3.0×10^6, and 5.4×10^6 cells/mL PBS. The time for the optimal activation of phosphorylation was determined by stimulating human neutrophils with either 1.0 μmol/L FMLP for 15 seconds, 30 seconds, 1 minute, 3 minutes, 5 minutes, 7 minutes, 10 minutes, and 15 minutes or a 15-second and 15-minute treatment with 0.25% (v/v) di-methyl-sulfoxide (DMSO).

Immunoblotting/detection of activated CREB

Half of the neutrophils (3.0×10^6/mL) were stimulated with the chemoattractant FMLP (1.0 μmol/L) for 5 minutes, lysed, and centrifuged[21]; the other half were left unstimulated with 0.25% (v/v) DMSO. Aliquots of these samples were separated by SDS-PAGE (60 μg/lane) on 9.0% (w/v) polyacrylamide slab gels and transferred electrophoretically to poly-di-vinyl filter (PDVF) transfer membranes (Millipore). Activated pCREB was assayed by Western blotting. The cell lysates

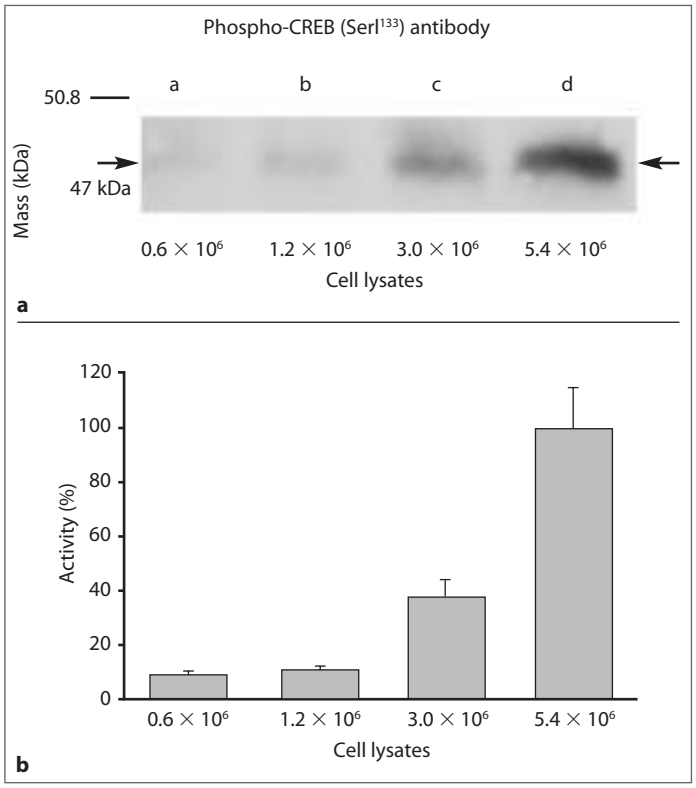

Fig 2 Standardization of freshly isolated neutrophil samples. Activated CREB was monitored by Western blotting with a rabbit-phospho-CREB antibody (1. Ab) that recognizes only the phosphorylated form of the protein. The amount of neutrophils isolated from peripheral blood was 0.6×10^6 cells (lane a), 1.2×10^6 cells (lane b), 3.0×10^6 cells (lane c), and 5.4×10^6 cells (lane d).

were immunoblotted with a 1:1,000 dilution of rabbit-phospho-CREB antibody (1. Ab) that recognizes only the phosphorylated form of CREB at Ser^{133} (p-CREB) (Cell Signaling). The activated CREB was visualized with an luminol-enhanced chemoluminescence detection system (Pierce) that monitored the activity of horseradish peroxidase (HRP) bound to the secondary Ab.[22] The percentage of activity for pCREB in LAP neutrophils and healthy controls was estimated densitometrically (Hoefer Laser-Densitometer GS 300, Hoefer Scientific Instruments). Ab dilutions and conditions for Western blotting differed from a previous report[16] by blocking and incubation in 5% milk, overnight incubation of the 1. Ab at 4°C, and utilizing a 1:20,000 solution of the goat-anti-rabbit IgG (2. Ab) (Cell Signaling).[23] All of the autoradiographic observations were performed on different preparations of cells.

RESULTS

Standardization and validation

In previous reports, the amount of freshly isolated PMNs taken for analysis of PMN compounds ranked between 5.0 and 7.5×10^6 cells.[12,16] An increase was observed from lane a (0.6×10^6 cells) to lane b (1.2×10^6 cells, 1.3-fold), and from lane c (3.0×10^6 cells, 4.4-fold) to lane d (5.4×10^6 cells, 11.4-fold). The optimal concentration of human neutrophils to activate pCREB on SDS-PAGE was evaluated utilizing 5.4×10^6 cells (Fig 2). Recently, a time course for the activation of various signal transduction compounds was established that revealed a maximal activation at about 3 minutes after stimulation of neutrophils from guinea pigs with FMLP.[16] According to the present results in human cells, the activation kinetics in human neutrophils displayed a maximal upregulation after 5 minutes of cell stimulation with FMLP. Following 30 seconds and 1 minute of stimulation, no signal could be detected. At 3 minutes, an increase of activation occurred, with a maximum at 5 minutes. At 7 minutes, the phosphorylation decreased, and it disappeared 15 minutes following FMLP stimulation. The increased amount after stimulating the neutrophils with FMLP for 5 minutes was estimated by densitometry, comparing the height of the band in lane d (5 minutes) with that in lane c (3 minutes, 1.6-fold), lane e (7 minutes, 1.4-fold), and lane f (10 minutes, 2.1-fold) (data not shown).

Detection of activated CREB

The CREB activation evaluated by densitometry from 9 LAP and 9 normal donors is represented in Fig 3. In the unstimulated controls, only a weak activation of 4.6% was detectable. Stimulation with FMLP induces a further phosphorylation of 23.5% (5.1-fold). In contrast, the patients with LAP revealed a statistically significant increase in the activation of CREB (overexpression) for the unstimulated PMNs of 80.3% compared to normal individuals. An additional stimulation of LAP neutrophils with FMLP induces a further moderate activation. This hyperresponse of the LAP neutrophil might be related to the primed neutrophil phenotype (see Fig 3).

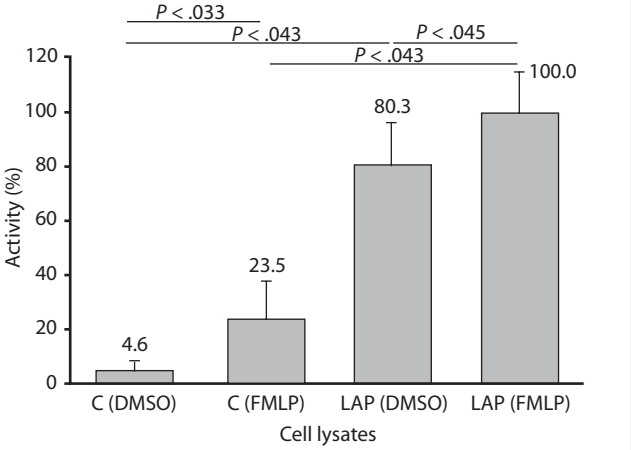

Fig 3 Activation/phosphorylation of CREB in neutrophils from 9 healthy controls (C = normal donors) and 9 subjects with LAP. Cells (10^6 in 400 µL of 7.5 g $MgCl_2$-$CaCl_2$ glucose-enriched buffer) were equilibrated at 37°C for 10 minutes. The 100% values are the activities of CREB in LAP neutrophils stimulated with FMLP (1.0 µmol/L) for 5 minutes.

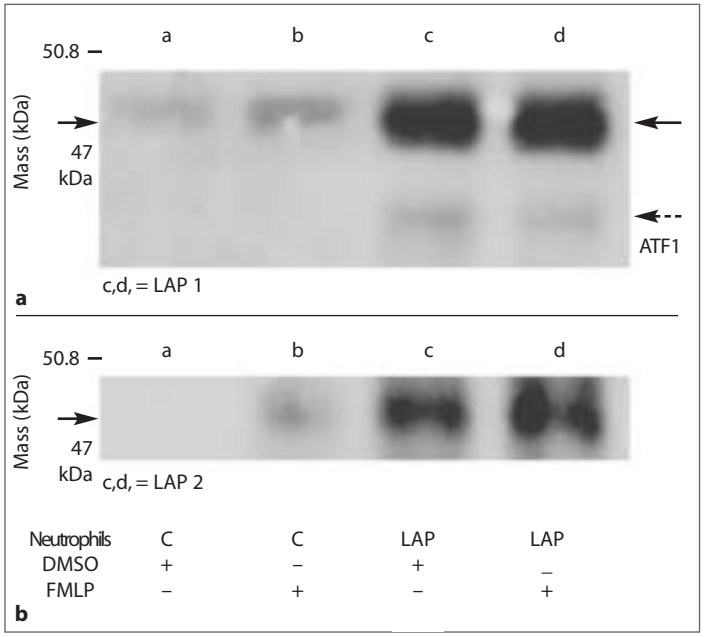

Fig 4 Representative sample of phosphorylation-kinetics of CREB in neutrophils of 2 healthy controls (C = normal donors; lanes a and b) and 2 patients with LAP (lanes c and d). Cells were treated with 0.25% (v/v) DMSO (unstimulated cells). The amount of FMLP used to stimulate cells was 1.0 µmol/L for 5 minutes. The concentrations of protein applied to the gel was 60 µL for lanes a to d. The membrane was blotted with an antibody that recognizes only the phosphorylated form of CREB at Ser^{133} (p-CREB). The blots in lanes a to d display the enhanced activation of CREB and activating transcription factor (ATF1) in PMNs from patients with LAP compared to normal donors. 47-kDa CREB, solid arrows; position of the closely related ATF1, broken arrows.

As a representative sample, the activation of CREB in neutrophils of 2 patients with LAP compared to controls, both unstimulated with 0.25% (v/v) DMSO (lanes a and c) and stimulated (lanes b and d), is presented in Fig 4. There is a striking difference of the phosphorylation of CREB in LAP neutrophils (lanes c and d) compared to the healthy controls (lanes a and b). Unstimulated LAP neutrophils (lane c) show a marked activation compared to the unstimulated controls (lane a) (see Fig 4). Furthermore, the additional stimulation in LAP neutrophils with 1.0 µmol/L FMLP for 5 minutes slightly increases the activation of CREB (lanes c and d). The DMSO label refers to the solvent system used. DMSO is buffer alone, and FMLP is dissolved in the same buffer (see Fig 4).

DISCUSSION

In this article, a novel observation is presented for LAP neutrophils. In particular, the 47-kDa cyclic AMP response element-binding factor (CREB) that undergoes naturally occurring phosphorylation/activation in unstimulated PMNs from patients with LAP is identified. The overexpression of CREB being located downstream to the reported signal transduction abnormalities[5–7] appears to be an intrinsic event in the genetically primed LAP neutrophil, thus explaining the functional hyperresponse of the cell. CREB is considered to be a substrate for p90RSK2 and MAPKAP-K2 and other intracellular protein kinases, such as protein kinase C (PKC), diacylglycerol, and calcium influx factor, acting further upstream close to the bilayer membrane of the neutrophil.[16]

For other inflammatory, neutrophil-mediated conditions, an enhanced expression of nuclear CREB was reported. These include neutrophil damages in acute inflammatory lung injury, hemorrhage or endotoxemia, acute myeloid leukemia, and inflammatory injuries of intestinal epithelial cell lines.[19,24–26] It has been suggested that the release of inflammatory cytokines such as IL-1, IL-6, and COX-2 is mediated through cAMP-mediated activation of nuclear CREB.[13] This cross-link between the overexpression of CREB in LAP neutrophils with an immediate induction of inflammatory mediators confirms the hypothesis of a neutrophil-mediated inflammatory hyperresponse documented for patients with LAP. In these disease conditions excessive PMN responses may lead to early losses of inflammatory barriers. It is of special interest whether the application of lipid-derived bioactive mediators with anti-inflammatory actions such as aspirin-triggered lipoxin analogues (LX-ATL) are able to inhibit neutrophil recruitment to further protect the periodontally compromised tissues.[12,27]

In LAP, the mode of inheritance has been reported as autosomal dominant.[28] Functional neutrophil abnormalities present in both peripheral blood neutrophils and the lesion site persist beyond elimination of the infecting pathogens and resolution of the inflammatory lesion. There is apparent evidence that LAP neutrophil dysfunctions are not homogeneously distributed. However, while there is a strong relationship between altered neutrophil function and LAP, in only 65% to 75% of subjects with LAP are neutrophil abnormalities are measurably expressed. Fully 25% of clinically diagnosed cases do not reveal the associated chemotaxis defect.[9] This might be due to the variability between the cell genotype and its phenotypic expression. In reports in which no detectable neutrophil defects were found, ie, dissociation of effector mechanisms and receptor density or neutrophil immaturity, the results were explained by genetically associated population differences.[29,30] The difficulties to obtain repeatable laboratory data from LAP neutrophils are related to genetic pleiomorphism of neutrophil defects that are also seen in other inherited diseases with PMN dysfunctions.[31] It is assumed that the overexpression of CREB in LAP neutrophils elicited in this study might be limited to those LAP patients whose peripheral blood PMNs measurably express their phenotype.

CONCLUSION

Our novel findings indicate that certain LAP neutrophils appear to be constitutively primed, as evidenced by activated CREB in resting cells, compared to neutrophils in normal individuals. These data suggest that the genetically primed neutrophil phenotype may contribute to neutrophil-mediated tissue damage in the pathogenesis of LAP, and perhaps in other forms of periodontal disease. Further investigations in a larger patient population group with a detailed sequence analysis of the promoter region encoding for CREB in LAP and normal neutrophils are necessary to confirm this hypothesis.

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