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Dentin Sialoprotein and Phosphoprotein Induce Neutrophil Recruitment: A Mechanism Dependent on IL-1β, TNF-α, and CXC Chemokines

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Abstract. Dentin is a reservoir of several potentially active molecules, and dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are the two major noncollagenous proteins. It has been established that dentin molecules are released as a consequence of osteoclast action during the resorption process. Along with osteoclasts, inflammatory cells seem to play an important role at sites of root resorption. Although the role of dentin molecules in dentinogenesis is well known, their role in pathological processes associated with dentin matrix dissolution is unclear. Recent studies have suggested that dentin components may function as chemotactic and activator signals for inflammatory cells at these sites. Herein we present evidence that demineralized dentin crude extract, DSP, and DPP induced dose- and time-dependent neutrophil migration into the peritoneal cavity of mice and that this activity was inhibited by dexamethasone, but not by indomethacin or MK886. The blockade of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) receptors inhibited neutrophil accumulation. The neutrophil migration was also diminished in the absence of the chemokines cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2), but not in the absence of macrophage inflammatory protein-1α (MIP-1α). These results demonstrate that dentin induces neutrophil migration via the synthesis of IL-1β, TNF-α, and chemokines and they suggest that dentin matrix proteins may have an active role in inflammatory cell recruitment during pathological processes associated with dentin and bone matrix dissolution.

Key words: Neutrophil chemotaxis — Dentin sialoprotein — Dentin phosphoprotein — Cytokines — Matrix proteins

Dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) are the two major noncollagenous proteins (NCPs) of the extracellular matrix (ECM) of dentin comprising 50% and 5% to 8% of NCPs respectively [1, 2]. Although originally thought to be found only in dentin, these two ECM proteins are now known to be expressed at low levels by bone and other tissues [3]. These two proteins, encoded by the same gene, are initially produced as one large, precursor protein called dentin sialophosphoprotein DSPP). Thus, proteolytic processing is necessary to convert DSPP to DSP and DPP ([4], review).

Similar to bone, dentin represents a significant storage site for growth factors including transforming growth factor (TGF)-β super family members, such as TGF-β, bone morphogenetic proteins (BMPs), and others [5, 6]. Cells of the dentin–pulp complex are capable of synthesizing these growth factors, which are subsequently sequestered into the dentin matrix and released following tissue injury, potentially stimulating repair mechanisms [6, 7]. The presence of these growth factors has been described, as have their receptors and, more recently, their gene expression profile in health and disease [8, 9]. Furthermore, dentin has also been found to contain metalloproteinases (MMPs) [10]. These enzymes are capable of cleaning components of the extracellular matrix and may regulate the availability of signaling molecules from dentin. MMPs activities may be controlled by tissue inhibitors of metalloproteinases (TimPs) since these enzymes are generally found to be concomitantly expressed [11]. In contrast to the considerable number of studies on dentin proteins and growth factors in dentinogenesis, the role of these entities in pathological processes affecting the developed tooth has received less attention. However, there is evidence that during the resorption process, dentin molecules are released as a consequence of osteoclast action on the root surface [12].

Along with osteoclasts, neutrophils are important cells involved in bone periapical resorption [13], acting as a source of cytokines at these sites [14]. On the

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other hand, the involvement of this leukocyte in the root resorption process has not yet been thoroughly investigated. There are a few studies suggesting that neutrophils could also participate in this event, since they are present in the active phase of pathophysiologic conditions associated with tooth resorption [14, 15]. Moreover, these cells are a source of cytotoxic mediators (e.g., oxygen and nitrogen free radicals, and proteolytic enzymes) and of cytokines [16], which activate surrounding cells, including osteoclasts [17].

The recruitment of neutrophils to periodontal inflammatory sites and their activation is regulated by chemotactic factors derived from dental hard tissue breakdown, such as TGF-β [18] and osteopontin [19], by bacteria and their products and by inflammatory chemotactic mediators released by resident cells [20–25]. The principal chemotactic mediators are eicosanoids, multifunctional inflammatory cytokines, chemokines and nitric oxide (NO) [20–25]. TNF-α and interleukin (IL)-1β, potent stimulators of neutrophil infiltration, may act as final mediators or through the induction and secretion of eicosanoids and chemokines [20, 22–25]. The chemokine family includes small peptides known to mediate the recruitment of leukocytes, and these can be distinguished depending on whether the first two cysteines are separated (CXC) or not (CC) by an intervening amino acid [20]. Macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC), members of the CXC chemokine subfamily and macrophage inflammatory protein (MIP-1α) and RANTES (regulated upon activation, normal T expressed and secreted), members of the CC group, possess recognized chemotactic activity for neutrophils [22, 23, 25].

Despite the evidence that, during the resorption process, dentin molecules are released, it is not yet accepted that these substances contribute to tooth resorption. Consistent with this, it was observed that traumatic root resorption was significantly inhibited in mice immunized with dentin [26]. Furthermore, experimental studies have highlighted the fact that dentin induces the migration of periodontal ligament cells [27], macrophages and neutrophils [28]. In this study, we investigated if dentin proteins, DSP and DPP, induce neutrophil migration into the peritoneal cavity, which is a model of acute inflammation that allows the simple and accurate quantification of migrating cells. In addition, we addressed the pathways involved in the DSP and DPP-induced neutrophil recruitment. We found that DSP and DPP-induced neutrophil migration occurs via induction of IL-1β, TNF-α, KC, and MIP-2 release. The possible involvement of these proteins in pro-inflammatory pathways at sites of pathological exposure of dentin is hypothesized.

Materials and Methods

Animals and Reagents

All the experimental procedures involving the use of animals were reviewed and approved by the institutional animal welfare committee. Male BALB/c, C57BL/6, $p55^{-/-}$, and MIP-1α-deficient mice 6 to 8 weeks old, weighing 20 to 25 g, were housed in the animal facility of the Department of Pharmacology and Immunology, Faculty of Medicine of Ribeirão Preto, University of São Paulo. Breeding pairs of mice with targeted disruption of the TNF-α receptor $p55^{-/-}$ gene and MIP-1α-deficient mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Because dentin proteins were obtained from rats, some experiments were performed in male Wistar rats 6 to 8 weeks old, weighing 200 to 250 g. All experiments were performed twice using 5 animals per group; except for experiments with MIP-1α-deficient mice, in which 3 animals per group were used.

Dentin Proteins and Extracts

The extraction and separation of DSP (53 KDa) and DPP (38 KDa) from rat incisor dentin were performed by using standard procedures as described [1, 2]. Demineralized human dentin crude extracts (cExt) were obtained as described [29]. Approval was obtained from the institutional ethics committee for the use of human teeth. Keyhole limpet hemocyanin (KLH) (8–9 MDa) and ovalbumin (OVA) (45 KDa), both from Sigma, tested at 10 μg/mL, were used as control proteins.

Leukocyte Migration

cExt, DSP, and DPP (0.1, 0.3, and 1 μg/cavity) were injected into the peritoneal cavity, and leukocyte migration was evaluated at intervals from 3 to 48 h Protein controls, KLH, and OVA at 10 μg/mL, were similarly injected intraperitoneally and neutrophil migration was assessed 6 hours, later. At the indicated times, the peritoneal cavities were washed twice with a total of 3 and 10 mL of phosphate-buffered saline (PBS), respectively for mice and rats. Approximately 95% to 98% of the injected volume was recovered. The pooled exudates were counted in a Coulter® A^CT cell counter (Coulter Corporation, Miami, Florida, USA) and centrifuged onto microscope slides using a cytospin centrifuge (Shandon Lipshaw Inc., Pittsburgh, Pennsylvania). The slides were air-dried and stained by using the May-Grünwald-Giemsa method for differential counting. In some experiments recombinant murine IL-1β (1 ng per cavity; R & D Systems, Minneapolis, MN, USA) and leukotriene B_4 (LTB_4; 25 ng per cavity) were used as controls.

In Vitro Neutrophil Chemotaxis

For *in vitro* neutrophil chemotaxis experiments, neutrophils were purified from human venous blood by density gradient centrifugation on Percoll. Neutrophil chemotaxis was assayed using a 5-μm pore size polycarbonate membrane (Millipore, Bedford, MA, USA) in micro-Boyden chambers (Neuro Probe, Cabin John, MD, USA). The chamber was incubated in a humidity-controlled unit at 37°C for 1 hour. Cells on the upper surface were removed by scraping, and cells adherent to the lower side of the membrane were fixed in methanol and stained by using May-Grünwald-Giemsa for counting. The number of cells in 6 high-power fields was counted, and the result was expressed as the mean number of neutrophils per field. *N*-formylmethionyl-leucyl-phenylalanine (fMLP) (10^{-6} M) (Sigma, St. Louis, MO, USA), LTB_4 (10^{-8} M) (Sigma), and recombinant human IL-8 (10 ng) (NIBSC 89/520; National

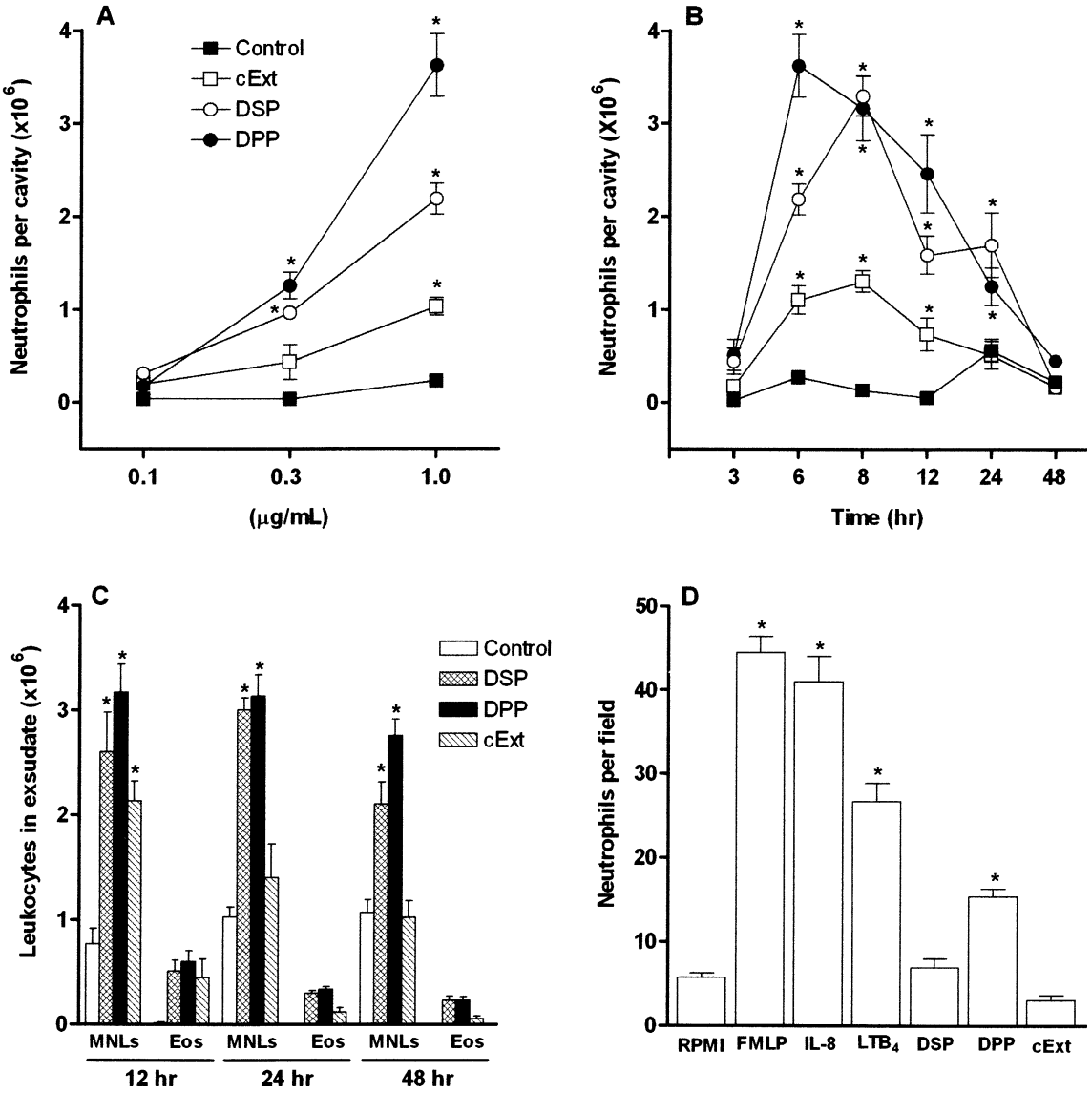

Fig. 1. Dose-dependence (panel A) and time-course (panel B) of neutrophil migration induced by dentin proteins. Mice were injected intraperitoneally with PBS (control) DSP, DPP, and cExt at the indicated doses, and neutrophil migration was determined 6 hours later. The time-course of neutrophil migration was determined by injection of PBS (control), DSP, DPP, and cExt (1 μg/cavity). Leukocyte differential counts were performed at 12, 24, and 48 hours after DSP, DPP, and cExt challenge (panel C). The cells were defined as mononuclear leukocytes (MNLs) and eosinophils (Eos). For all experiments results are representative means ± SEM. $*P < 0.05$ compared to control. *In vitro* neutrophil chemotaxis induced by DSP and DPP was performed as described in "Materials and Methods," fMLP (10^{-6} M), LTB_4 (10^{-8} M), IL-8 (10 ng), and RPMI were used as controls. $*P < 0.05$ compared to RPMI (panel D).

Institute for Biological Standards and Control, London, UK) were positive controls and RPMI medium (Gibco, Grand Island, NY, USA) alone was the negative control. DPP was tested at 10 μg/mL, 100 μg/mL, and 1 mg/mL. DSP and cExt were tested at 1 mg/mL.

Anti-Inflammatory Drugs and Antibodies

The protocols for anti-inflammatory drug treatments were performed as described previously [30, 31]. The animals were treated 1 hour before stimuli with subcutaneous (s.c.) dexamethasone phosphate (1 mg/kg; Sigma), or with leukotriene synthesis inhibitor (MK886; 1 mg/kg, orally; Merck Sharp & Dohme B.V., Haarlem, The Netherlands), or 30 minutes before with a cyclooxygenase inhibitor (indomethacin, 5 mg/kg, s.c.; USP lot HLD-006), or 15 minutes before with a human IL-1 receptor antagonist (IL-1ra; 20 mg/kg, i.v., NIBSC 92/644). The control mice received an equivalent volume of PBS at the same time that the test mice received the anti-inflammatory drugs.

The sheep antiserum anti-mouse TNF-α (35 μL per cavity; NIBSC H92/B8), anti-KC, anti-MIP-2 antibodies (Peprotech Inc., Rocky Hill, NJ, USA) and control rabbit nonimmune serum (DAKO, Glostrup, Denmark), both at 800 ng/cavity, were co-administered i.p. with DSP and DPP (1 μg/cavity) and neutrophil migration was assessed 6 hours later.

Cell Culture

Macrophages were harvested by washing the peritoneal cavities with PBS. Cells were cultured in RPMI with 5% fetal bovine serum (Hy-Clone, Logan, Utah, USA), penicillin (100 U/mL) and streptomycin (1 mg/mL) (Sigma), plated at 10^7 cells/mL/well and allowed to adhere. After 24 hours, unadhered cells were removed by washing with 3 mL of sterile PBS and adhered macrophages (>97%) were stimulated with DSP, DPP, and cExt at 100 ng/mL for 24 hours at 37°C in an atmosphere of 5% CO_2. All experiments were performed twice in triplicate.

Enzyme Linked Immunosorbent Assay (ELISA)

The concentrations of cytokines in supernatants from DSP-, DPP-, and cExt-stimulated macrophages and in peritoneal exudates were determined using sandwich ELISA following the manufacturer's instructions. The mouse antibodies anti-IL-1β, TNF-α, MIP-1α, MIP-2, and RANTES were from R & D Systems and anti-KC was from Peprotech. The concentration of each cytokine was calculated from a standard curve.

Nitrite Levels

The secretion of NO was evaluated by measuring NO_2^- accumulation in culture supernatants by the Griess reaction [32]. Briefly, 50 μL of sample was mixed with an equal amount of Griess reagent containing 1% sulfanilamide (Sigma) and 0.1% naphthylethylenediamine dihydrochloride (Sigma) in 2% phosphoric acid. Absorbance was measured at 540 nm and nitrite concentration was determined using a standard curve of sodium nitrite (Merck).

Statistical Analysis

Numerical values are means ± standard error of mean (SEM). The data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's post test. Statistical significance was considered to be achieved at $P < 0.05$.

Results

Leukocyte Recruitment

DSP, DPP, and cExt induced a dose- (Fig. 1a) and time-dependent (Fig. 1b) neutrophil migration that peaked at 6 hours for DPP and at 8 hours for DSP and cExt, both at 1 μg per cavity. From this point, the neutrophil migration declined and returned to control levels 48 hours later (Fig. 1b). cExt induced significantly lower neutrophil extravasation than did dentin proteins (Fig. 1a, b). The proteins used as controls (ovalbumin, with a molecular weight similar to that of dentin proteins, and KLH with higher molecular weight), tested at 10 μg per cavity, did not induce significant neutrophil migration after 6 hours (control, 0.048 ± 0.041; KLH, 1.10 ± 0.64; and ovalbumin, 0.89 ± 0.51×10^6 cells/cavity) compared to DSP (2.18 ± 0.28×10^6 cells/cavity) and DPP (3.62 ± 0.58×10^6 cells/cavity). A similar pattern of DSP- and DPP-induced neutrophil accumulation was observed in rats (control, 6.87 ± 1.83; DSP, 16.34* ± 8.73; and DPP, 19.67* ± 5.99×10^6 cells/cavity; $n = 5$, $*P < 0.01$) excluding the possibility that the phenomenon reported here results from a species cross-reactivity.

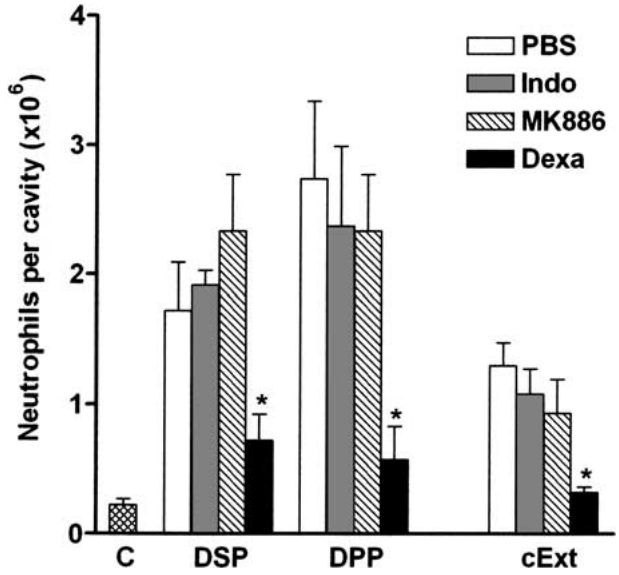

Fig. 2. Effect of anti-inflammatory drugs on DSP-, DPP-, and cExt-induced neutrophil migration. BALB/c mice were pretreated 1 hour before with PBS (s.c.); Indo (indomethacin, 5 mg/kg; s.c.); dexa (dexamethasone, 1 mg/kg; s.c.) or MK886 (1 mg/Kg; orally) and neutrophil migration was assessed 6 hours after injections of PBS (C), DSP, DPP, and cExt (1 μg/cavity). Results are representative means ± SEM. $*P < 0.05$ compared to mice pretreated with PBS.

Differential analysis revealed that the leukocyte infiltration in response to cExt, DSP, and DPP challenge comprised >80% polymorphonuclear leukocytes 6 hours after injections, whereas <20% of cells were monocytes and eosinophils, suggesting that neutrophils constituted the majority of leukocytes at this time (data not shown). However, the numbers of monocytes and eosinophils increased from 12 hours and persisted to the final time-point analyzed at 48 hours (Fig. 1c).

In vitro, DPP at 10 and 100 μg/mL exhibited neutrophil chemotaxis similar to that of control medium (data not shown). At 1 mg/mL, DPP, but not DSP or cExt, induced significant neutrophil chemotaxis compared with control medium. DPP-induced neutrophil chemotaxis was significantly lower compared with the positive controls fMLP, IL-8, and LTB_4 (Fig. 1d).

Effect of Anti-Inflammatory Drugs

The neutrophil migration triggered by dentin proteins and crude extract was significantly inhibited by dexamethasone. However, the treatment with MK886 or indomethacin, described as effective agents at the doses used [30, 31], was ineffective in modifying neutrophil migration (Fig. 2).

Production of Cytokines, Chemokines, and NO by DSP-, DPP-, and cExt-Stimulated Macrophages

DSP- and DPP-treated macrophages displayed a similar production of cytokines, except for TNF-α, the

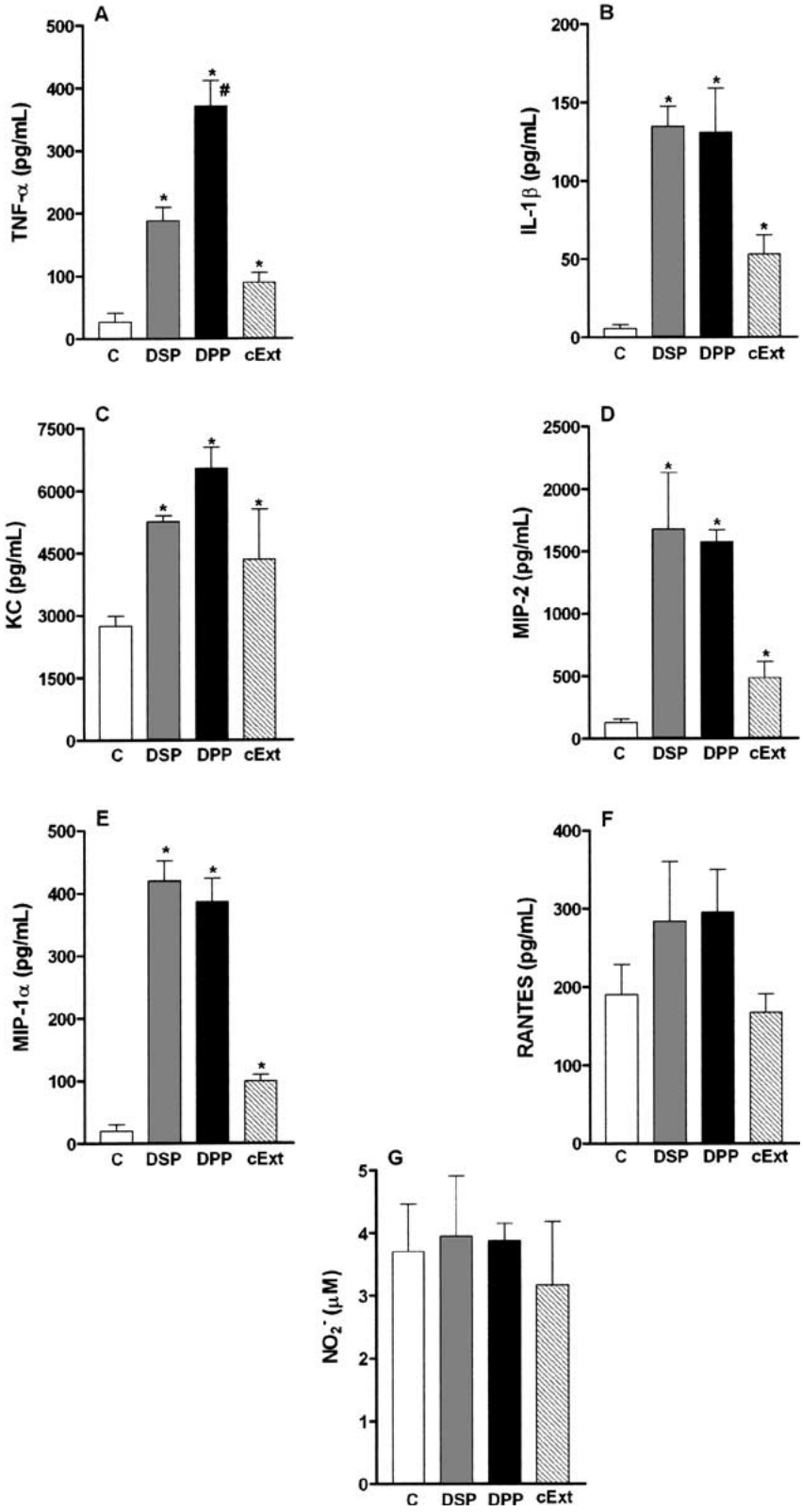

Fig. 3. Production of TNF-α, IL-1β, KC, MIP-2, MIP-1α, RANTES, and NO_2^- by DSP, DPP, and cExt-challenged macrophages. Peritoneal macrophages (10^7 cells/well) were incubated for 24 hours with PBS (C), DSP, DPP, and cExt (100 ng/mL), and, supernatants were assayed for cytokine and nitrite production as described in "Materials and Methods." Data are the mean ± SEM of triplicates. $^*P < 0.05$ versus control values; $^{\#}P < 0.05$ comparing two proteins.

levels of which were greater for DPP (Fig. 3a). cExt was less effective than DSP and DPP in the induction of all tested mediators. Larger amounts of KC were produced, followed by MIP-2, MIP-1α, TNF-α, and IL1-β (Fig. 3b through e). RANTES and nitrite production was not affected by dentin (Fig. 3f, g). Consequently, the participation of RANTES and NO in neutrophil recruitment was ruled out and the role of

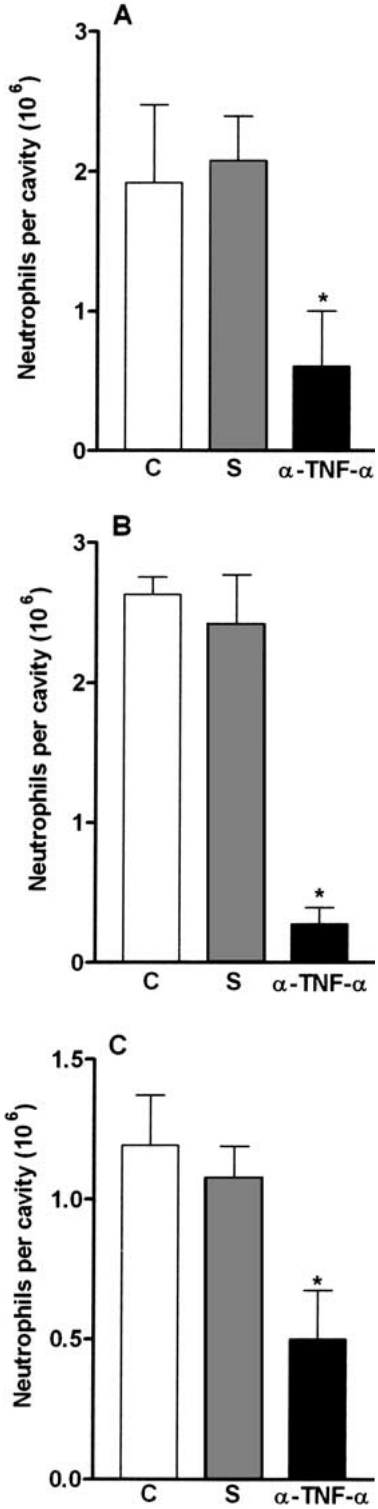

Fig. 4. Effect of TNF-α antibodies on neutrophil migration induced by DSP (panel A), DPP (panel B), and cExt (panel C). Mice were treated with DSP, DPP, and cExt (1 μg/cavity) co-administrated with rabbit normal serum (S) (800 ng/cavity) or anti-TNF-α antibody (35 μL/cavity), and neutrophil migration was assessed 6 hours later. $*P < 0.05$ compared to control.

IL1-β, TNF-α, KC, MIP-2, and MIP-1α was investigated.

Role of TNF-α and IL-1β in DSP-, DPP-, and cExt-Induced Neutrophil Migration

The TNF-α-antiserum, but not normal serum, significantly reduced the DSP-, DPP-, and cExt-stimulated neutrophil recruitment (Fig. 4). The confirmation of TNF-α involvement was achieved by a significant reduction of neutrophil migration in $p55^{-/-}$ mice challenged with DSP (Fig. 5b) and DPP (Fig. 5c), but not with LTB_4 (Fig. 5a). The similar neutrophil migration in $p55^{-/-}$ and wild-type mice challenged with LTB_4 indicates that the reduction in DSP- and DPP-induced neutrophil migration was not caused by hyporesponsiveness of knockout mice.

The prior treatment of mice with IL-1ra significantly inhibited the neutrophil recruitment induced by DSP, DPP, and cExt. The specificity of the method was confirmed by a marked reduction in neutrophil numbers in the IL-1β-treated group (Fig. 6). Consistent with these results, we detected significant levels of IL-1β (control, 35.28 ± 6.69; DSP, 134.56* ± 18.38; and DPP, 141.66* ± 23.57 pg/cavity; $*P < 0.05$) and TNF-α (control, 42.4 ± 3.9; DSP, 186.99* ± 31.49; DPP, 370.97* ± 56.34 pg/cavity, $*P < 0.05$) in the peritoneal exudates 6 hours after intraperitoneal administration of dentin proteins.

Role of CC and CXC Chemokines in DSP- and DPP-Induced Neutrophil Recruitment

Anti-MIP-2 and anti-KC antibodies, but not normal serum, significantly reduced the neutrophil migration induced by DSP (Fig. 7a) and DPP (Fig. 7b). Compared to wild-type mice, no differences were detected in neutrophil numbers in MIP-1α-deficient mice injected with DPP and cExt. In contrast, in DSP-stimulated MIP-$1\alpha^{-/-}$ mice, a significant increase in neutrophil migration was observed (Fig. 7c).

Discussion

After mineralization, the dentin molecules remain trapped in the mineralized phase, being exposed or released as a consequence of injury to the periodontal ligament, which may result from luxation, orthodontic movement, or infections of tooth and periodontal structures [17]. The exposure of dentin allows osteoclasts to colonize the root surface and start the resorption process. These cells dissolve the mineralized matrix and then endocytose, transport, and continually release the matrix components during resorption [12]. Once released, these molecules may be able to act at resorption sites and function as chemotactic and activator signals for inflammatory cells [28], bone, and periodontal ligament cells [27], and could, theoretically, influence the course of resorption. Consistent with this, the dentin immunoneutralization

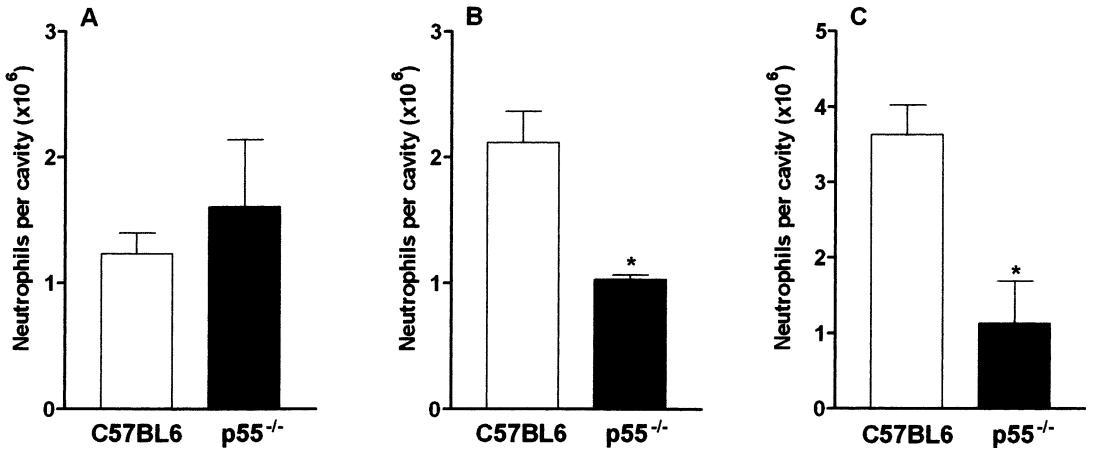

Fig. 5. TNF-α dependence of neutrophil migration induced by DSP and DPP. C57BL/6 and $p55^{-/-}$ mice were injected with LTB_4 (25 ng/cavity) (panel A), DSP (1 μg/cavity) (panel B), and DPP (1 μg/cavity) (panel C). Results are representative means ± SEM. *$P < 0.05$ compared to wild-type mice.

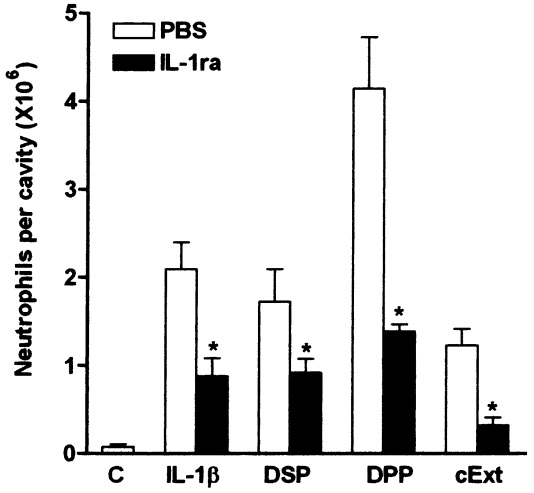

Fig. 6. Effect of IL-1 receptor antagonist on DSP-, DPP-, and cExt-induced neutrophil migration. BALB/c mice were pretreated with PBS or IL-1ra (20 mg/kg, i.v., 30 minutes before) and injected intraperitoneally with recombinant murine IL-1β (1 ng/cavity), DSP, DPP, and cExt (1 μg/cavity). Neutrophil migration was evaluated after 6 hours. Results are representative means ± SEM. *Significantly different from mice pretreated with PBS at $P < 0.05$.

procedure protects mice from traumatic root resorption [26].

Cellular recruitment during root resorption probably relies on the establishment of chemotactic or haptotactic gradients within this microenvironment. However, the identity of the chemoattractants responsible remains to be established. We recently showed that dentin extracts triggered an intense cell migration in a time- and dose-dependent manner, as well as macrophage expression of IL-1β, TNF-α, NO, and hydrogen peroxide (H_2O_2) [28]. In this study, we investigated the ability of dentin proteins and crude extract to attract inflammatory cells into the peritoneal cavity, as a model of acute inflammation. The model of acute peritonitis was chosen to address the pathways involved in dentin-induced neutrophil chemotaxis because of the simplicity in quantifying cell recruitment and in performing pharmacological and immune manipulation. It was observed that dentin proteins, DSP and DPP, as well as crude extracts induced neutrophil recruitment. We focused our efforts on DSP and DPP because they are the major noncollagenous proteins of dentin [1, 2].

The polymorphonuclear leukocyte infiltration was four times higher than that of other leukocyte types 6 hours after DSP, DPP, and cExt injection. Demineralized crude extract was less effective in inducing neutrophil migration when compared with purified proteins. These data indicate that despite the possibility of the presence of other potentially active molecules in the crude extract it is likely that low levels of DSP and DPP mediate the cExt-induced neutrophil migration. The actual amount of DSP and DPP in cExt remains to be determined.

Although the role of neutrophils in dental resorption remains unexplored, these cells have been shown to be present during the active phase of physiologic resorption of deciduous teeth [15], and are key cells involved in tissue destructive events in several inflammatory bone diseases, such as arthritis [33] and periodontal diseases [34]. Moreover, these cells are an important source of cytokines at periapical resorption sites [14]. Furthermore, good evidence for the role of neutrophils in periapical resorption is given by the inhibition of the development of lesions in neutropenic rats [13].

Next, we investigated the mechanism by which DSP, DPP, and cExt induced the neutrophil recruitment. We found that dexamethasone, a glucocorticoid, markedly reduced neutrophil recruitment, suggesting the participation of cytokines and eicosanoids in this process, since this class of drugs inhibits these mediators [30, 31]. The

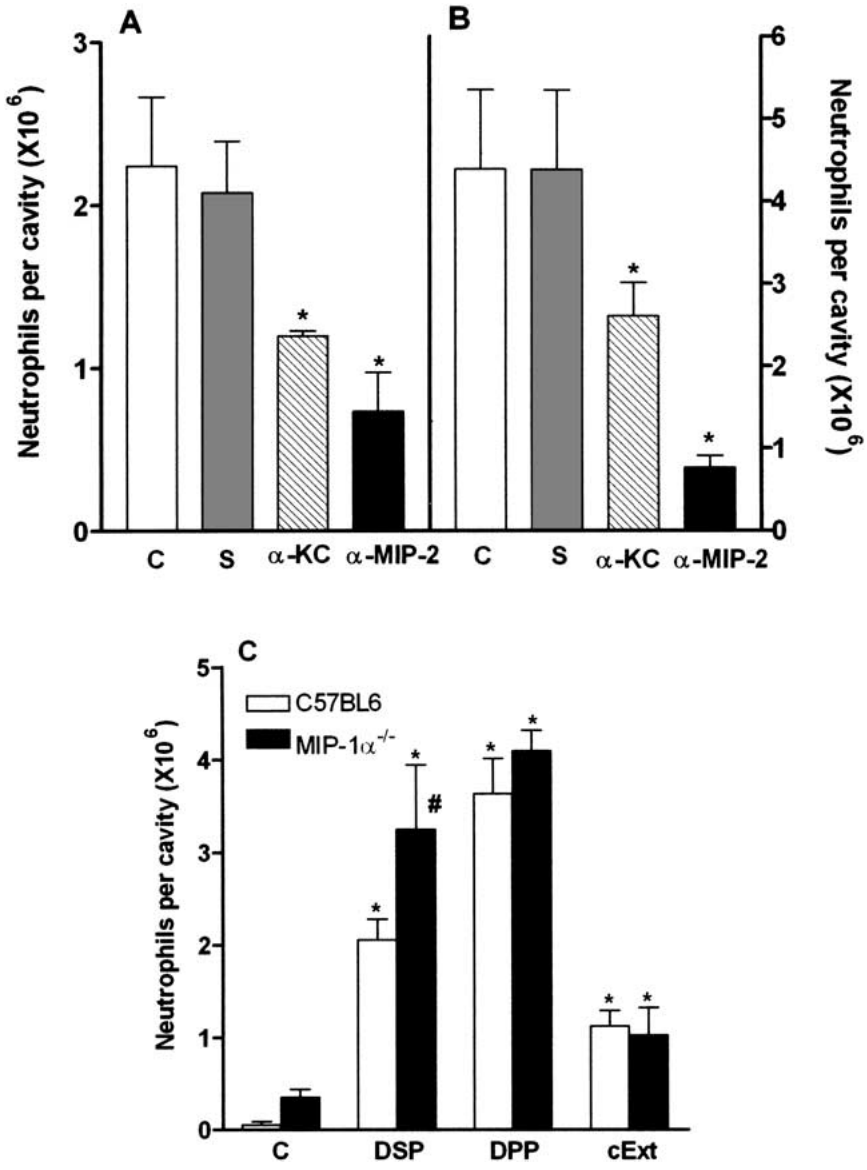

Fig. 7. Effects of CXC and CC chemokines on DSP- and DPP-induced neutrophil migration. Mice were treated i.p with DSP (panel A) and DPP (panel B) (1 μg/cavity) co-administrated with rabbit normal serum (S) (800 ng/cavity) or antibodies directed against KC and MIP-2 (800 ng/cavity). C57BL/6 and MIP-1α-deficient mice (MIP-1α$^{-/-}$) were i.p injected with PBS (C), DSP, DPP, and cExt (1 μg/cavity) (panel C). In all experiments, neutrophil migration was evaluated after 6 hours. Results are representative means ± SEM of two independent experiments with 3 to 5 mice per group. *Significantly different from respective control values at $P < 0.05$. $^{\#}P < 0.05$ compared to wild-type mice.

involvement of LTB_4 or prostaglandins can be ruled out because MK886, an LTB_4 synthesis inhibitor, and indomethacin, a cyclooxygenase inhibitor, were ineffective in inhibiting DSP-, DPP-, and cExt-induced neutrophil accumulation. Taken together, these results suggest that dentin-induced leukocyte accumulation occurs predominantly via cytokine production. The absence of cExt- and DSP-induced neutrophil chemotaxis *in vitro* and the fact that DPP did not induce a significant neutrophil migration compared with classical chemotactic factors, such as FMLP, LTB_4, and IL-8, reinforces the conclusion that the mechanisms of neutrophil recruitment induced by both proteins are indirect and result mainly from the endogenous release of inflammatory mediators. This suggestion is reinforced by the observation that cExt, DSP, and DPP are able to induce the release of cytokines, which seem to be the endogenous mediators of dentin-induced neutrophil recruitment. Interestingly, osteopontin, a protein structurally related to DSP, activates osteoclasts [35] and macrophages [19] interacting with the CD44 receptor and the αvβ3 integrin. The possible involvement of these receptors in the interaction of DSP and DPP with macrophages needs to be further investigated.

IL-1β and TNF-α appear to be critical mediators in dentin-induced neutrophil influx because the blockade of TNF-α levels by neutralizing antibodies or the use of TNF-receptor-deficient mice significantly impaired neutrophil accumulation. Likewise, the treatment with IL-1ra reduced neutrophil recruitment. Consistent with the role of IL-1β and TNF-α in dentin-induced neutrophil migration, the concentrations of these cytokines increased markedly in the peritoneum following intraperitoneal injections or *in vitro* after treatment of macrophages with these proteins. In addition to the participation of IL-1β and TNF-α in DSP- and DPP-

induced leukocyte recruitment, it was observed that immunoneutralization with either KC or MIP-2, CXC chemokines that selectively attract neutrophils [20, 25], markedly reduced the neutrophil accumulation, suggesting an additive effect of these chemokines in this phenomenon. On the other hand, it has been shown previously that the combined injection of these antibodies is necessary to attenuate TNF-α-induced neutrophil migration, attributable to the action of different mediators [25].

Despite the high levels of MIP-1α in macrophage supernatants, the DSP-, DPP-, and cExt-induced neutrophil accumulation seems not to be dependent on MIP-1α. DSP-challenged MIP-1α-deficient mice exhibited a significant increase in neutrophil migration, suggesting that additional pathways might be activated by DSP in the absence of this chemokine.

Although we have focused our attention on chemotactic activity for neutrophils, it is important to note that it is possible that dentin may have additional biological activities within the root resorption milieu by regulating macrophage infiltration and function. Moreover, upregulation of cytokine and chemokine production has been implicated in the neutrophil accumulation, and these mediators also participate in the recruitment of other cell types such as monocytes, lymphocytes [20, 22, 23] and osteoclast precursors [36–38]. It is noteworthy that IL-1β, TNF-α, and MIP-1α are important proresorptive factors [36, 38] and their additional supply by dentin-stimulated cells may attract and increase the activity of osteoclasts. Consistent with this suggestion, significant inhibition of root resorption and a decrease in the number of resorbing cells has been observed after neutralization with IL-1 and TNF-α in rats [39].

The biological significance of the pro-inflammatory properties of dentin has not been fully investigated; however, our results support the notion that neutrophils will be recruited in situations where dentin release occurs and, as discussed previously, this may aggravate the dentin resorption. Furthermore, some dentin proteins, such as osteopontin, are able to attract and activate osteoclasts [35], which could also contribute to the maintenance of root resorption. On the other hand, it is important to note that DSPP and also other dentin proteins such as osteopontin, bone sialoprotein, and dentin matrix protein-1 could also downmodulate the inflammatory process owing to their ability to inhibit complement-mediated cell lysis [40].

The mechanisms of dentin resorption involving matrix-released molecules display similarity with those of bone resorption [41]. DSP and DPP are not restricted to dentin, since they also are expressed in bone, although at lower concentrations [3]. Thus at periapical resorption sites, DPP and DSP delivered from bone might exhibit effects similar to those of DSP and DPP from dentin. The participation of metalloproteinases and tissue inhibitors of metalloproteinases in the mechanisms of root resorption has been described in the literature [42, 43]. These enzymes are capable of cleaning components of the extracellular matrix and may regulate the availability of signaling molecules from dentin. The possible involvement of these enzymes in the release or inactivation of DSP and DPP needs to be further investigated.

In summary, this work demonstrates that dentin proteins induce leukocyte chemotaxis via the release of inflammatory mediators, a finding that has not been reported previously. It is plausible that the interaction between dentin and inflammatory cells may function as an additional source of TNF-α, IL-1β, MIP-1α, MIP-2, and KC in the root resorption milieu. These activities might be important in further delineating the possible involvement of DSP and DPP in inflammatory events coupled with their release at root resorption sites. Furthermore, this knowledge will contribute to our understanding of the cellular events important in dental tissue injury and repair.

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