

Original Article

CCL2 expression and its correlation with CCL4/CCR5/NF- κ B pathway in patients with periodontal disease

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Abstract: Periodontal disease (PD) is characterized by continuous leukocyte infiltration. CCL2 plays an important role in periodontal pathogenesis. In this study, we studied the possibility of a pathway involved in the production of CCL2 in peripheral blood (PB) and gingival tissues of chronic periodontitis (CP) and aggressive periodontitis (AgP) patients based on our previous work about chemokine signaling pathway leading to production of CCL2 in rheumatoid arthritis (RA) patients. Total RNA was isolated from PB leukocytes and gingival tissue in periodontitis patients and healthy controls. Real-time PCR was used to determine CCL4, CCR5, NF- κ B, and CCL2 expression. Serum level of CCL2 was assessed by enzyme-linked immunosorbent assay. The production of CCL2 in gingival tissue was analyzed by immunohistochemistry. The expression of CCL2 mRNA in CP and AgP patients was greatly decreased than those in healthy controls, both in gingival tissue and on PB leukocyte. The mRNA expression levels of CCL4, CCR5 and NF- κ B in the patients with different periodontal diseases were higher than those in the Healthy group. However, in spite of the higher expression of NF- κ B on AgP PB leukocytes, and more intense CCR5 expression in the CP gingival tissue, the differences were not statistically significant. Serum CCL2 levels were elevated in both CP and AgP patients. Histological examination of CP patients revealed extensive CCL2 expression in mild inflamed gingival tissue. These data provide evidence that the CCL4/CCR5/NF- κ B pathway may be involved in CCL2 expression on PB leukocytes of clinically CP patients.

Keywords: CCL2, leukocytes, chronic periodontitis, aggressive periodontitis, gingival tissue

Introduction

Periodontal diseases are chronic inflammatory diseases of the attachment structures of the teeth. They are triggered by periodontopathogens and the clinical outcome is highly influenced by the host local immune response [1]. The inflammatory reaction is a key step in the development of the immune response, and the immune protection depends on the nature of the migrating cells. Infiltration of mononuclear cells is a common feature of many forms of chronic inflammation including periodontitis [2]. In this situation, chemokines are thought to play important roles in the immunopathogenesis of periodontal diseases. The altered chemotactic behavior was observed in patients with periodontitis [3].

Monocyte chemoattractant protein-1 (CCL2) and macrophage inflammatory protein-1 β (CCL4) are well defined CC chemokines. High levels of CCL2 have been reported in the gingival crevicular fluid of patients with both chronic periodontitis (CP) and aggressive periodontitis (AgP) [4]. The Monocyte chemoattractant protein activity of the crevicular fluids of adult periodontal patients increases with an increase in severity of this disease, also suggesting that CCL2 could be involved in the mechanism of monocyte recruitment from the circulating pool into periodontal tissues. CCL2 induces chemotaxis of circulating monocytes and is different from several other chemoattractants in that it is relatively specific for monocytes [5]. In addition, it has been demonstrated that CCL2 is synthesized in inflamed gingiva by vascular

endothelial cells and mononuclear phagocytes [5]. CCL2 has also been implicated in the pathogenesis of various systemic diseases, such as granulomatous disease, rheumatoid arthritis, heart disease, bone trauma and asthma [5]. CCL4 might play an important role in the host response by recruiting inflammatory cells into the foci of active inflammation and by inducing the release of other cell mediators. It exhibits a chemotactic characteristic for the cluster of differentiation 4 (CD4+) T cells [6] and has been shown to be more prevalent in diseased periodontal tissues and GCF compared to healthy sites [7]. CCL4 is also specifically increased in several inflammatory diseases such as rheumatoid arthritis (RA) [8]. CCR5 has been expressed on monocytes/macrophages. CCL4 chemokine is said to be ligand for CCR5. The CCR5 expressed on the type 1 helper T cell has been shown to induce alveolar bone loss in a mouse model of experimental periodontitis [9]. CCR5 was described as a higher periodontal disease severity mediator in humans and mice [10], but the mechanisms by which CCR5 determines periodontal disease outcome remain unknown.

CCL2, CCL4 and CCR5 have been shown to be involved in inflammatory immune responses. However, the mechanism of interaction of these molecules in an inflammatory milieu, which helps perpetuate inflammation in periodontitis, is not understood; the cellular mechanisms mediating CCL2 production are completely unknown. Multiple signaling mechanisms have been reported to be involved in the intracellular activation of CCL2 gene expression in periodontal tissue by a various stimuli [11]. Rheumatoid Arthritis (RA) and chronic and aggressive periodontitis are chronic inflammatory disorders characterized by deregulation of the host inflammatory response. Both diseases share risk factors and have pathological pathways in common [12]. We previously showed that the chemokine signaling pathway (CCL4/CCR5/c-Jun and c-Fos/CCL2) was involved in CCL2 expression in collagen-induced arthritis (CIA) rat and RA patient tissues [13, 14]. The present study was designed to estimate CCL2, CCL4 and CCR5 levels in periodontal health and disease as well as to investigate the signaling mechanisms involved in the CCL2 induction in different forms of human periodontal disease.

Materials and methods

Patients and specimens

A total of 52 individuals were recruited in the study. All subjects were recruited from the Department of Periodontology, School of Dentistry, Tianjin Medical University, Tianjin and the Department of Periodontology, Tianjin Stomatological Hospital, Tianjin between 2014 and 2015. Complete medical and dental histories were taken from all subjects. None of the subjects had a history of systemic disease and none had received antibiotics or other medicines or periodontal treatment within the past 4 months. None of the women were pregnant, lactating and postmenopausal. The participants who had never smoked or had quit smoking for at least 6 months were included. The diagnosis of periodontitis from the study participants were defined in accordance with the classification agreed at The American Academy of Periodontology in 1999 [15]. In brief, the diagnoses, which are generalized aggressive periodontitis (GAgP), chronic periodontitis (CP) and healthy groups, were made based on the relevant dental history and clinical records. The subjects were classified as follows.

CP group

1) age \geq 35 years, 2) attachment loss (AL) \geq 5 mm on more than one tooth, and 3) more than three sites of probing depth (PD) $>$ 6 mm on more than one tooth distributed in each quadrant, but with no evidence of rapid progression.

GAgP group

1) eight or more teeth with AL \geq 5 mm, 2) PD $>$ 6 mm, and 3) at least three affected teeth that were not first molars or incisors.

Healthy group

No evidence of AL and PD at more than one site $>$ 3 mm, clinical inflammation, sulcular bleeding and radiographic evidence of bone loss.

Determination of periodontal status

To determine the clinical periodontal status, all subjects had a clinical periodontal examination including the measurement of probing depth

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and clinical attachment loss. Attachment level and pocket depth measurements were taken at 6 sites per tooth by a single calibrated investigator. The calibrated examiner performed all the clinical assessments using William's periodontal probe. Measurements were made at the mesiobuccal, buccal, distobuccal, distolingual, lingual and mesio-lingual positions. Dichotomous measurement of supragingival plaque (PI) and bleeding on probing (BOP) were also performed at 6 sites per tooth.

Collection of serum samples

Blood samples were obtained from 22 patients (n=9 for GAgP group and n=13 for CP group, respectively). For eighteen (n=9 for GAgP group and n=9 for CP group, respectively) of the twenty-two corresponding paired samples, an additional set of peripheral blood leukocytes was used for analyses of gene expression. Control peripheral blood leukocytes and control sera were obtained from a group of 9 healthy individuals who were matched with the GAgP group (n=9) and CP group (n=13) for sex and mean age.

Collection of gingival tissues

Gingival tissue was obtained from 21 subjects undergoing periodontal surgery for disease and nondisease related reasons. Samples of inflamed gingival tissues were obtained from 13 patients with chronic periodontitis. All subjects in this group had previous oral hygiene instruction and scaling and root planning prior to surgery, but continued to demonstrate bleeding on probing. The control group comprised 8 periodontally healthy subjects from whom biopsies of gingival tissue were taken during surgical procedures for wisdom teeth removal.

Each specimen was divided into two samples which were approximately in similar sizes. One sample was stored in liquid nitrogen and used for quantitative real-time PCR, whereas the other sample was stored in 10% formalin solution for immunohistochemistry and histopathological assessment. The experimental protocol was approved by the Human Ethics Review Committee at our institution, and signed consent was obtained from each patient.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from gingival tissue using the TRIZOL reagent (10296010; Invitro-

gen, Carlsbad, CA, USA). The leukocytes were prepared from 10 ml of EDTA anticoagulated peripheral blood (PB) and were washed with phosphate-buffered saline (PBS), then lysed with TRIZOL LS reagent. The total cellular RNA was purified according to the instruction manual. The reverse transcription reaction was performed with Superscript III first-strand kit (Invitrogen, 18080-051) according to the manufacturer's instructions. Messenger RNA (mRNA) expression was determined by real-time PCR using SYBR Green Master Mix under standard thermocycler conditions (4367659; Applied Biosystems, Foster City, CA, USA). Data were collected and quantitatively analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). A human GAPDH gene was used as an endogenous control for sample normalization. Results were presented as the fold expression relative to that of GAPDH. PB leukocyte samples (healthy, CP and GAgP) were analyzed by one-way analysis of variance (ANOVA) with LSD-t test. Student's t test was used to evaluate chemokine/cytokine production by gingival tissue between the CP and control groups. PCR primers were as follows: for human GAPDH, forward 5'-ATTGCCCTCAACGACCACT-3' and reverse 5'-ATGAGGTCCACCACCCTGT-3'; for human CCL2, forward 5'-CTTCTGTGCTGCTGCTCAT-3' and reverse 5'-CGGAGTTGGGTTTGGCTTGTC-3'; for human CCL4, forward 5'-CTGTGCTGATCCAGTGAATC-3' and reverse 5'-TCAGTTCAGTCCAGGTCATACA-3'; for human CCR5, forward 5'-TTCTGGGCTCCCTAACATT-3' and reverse 5'-TTGGTCCAACCTGTTAGAGCTA-3', and for human NF- κ B, forward 5'-TCTGGCGCAGAAGTTGGGT-3' and reverse 5'-CCAGAGACCTCATAGTTGT-3'.

Enzyme-linked immunosorbent assays (ELISAs)

Serum level of CCL2 was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Catalog no. 4338807, BioLegend, Inc., USA), according to the manufacturer's instructions. The plate was read at 450 nm in an ELISA plate reader (Multiskan MK3, Thermo Scientific, MA, USA). Concentrations of CCL2 in each sample were extrapolated from standards. The detection limit of the CCL2 assay was 1.6 pg/ml. The range of detection level for CCL2 was from 7.8 to 500 pg/ml. Differences between the three study groups (healthy, CP and GAgP) were determined with one-way analysis of variance (ANOVA). When ANOVA showed statistical

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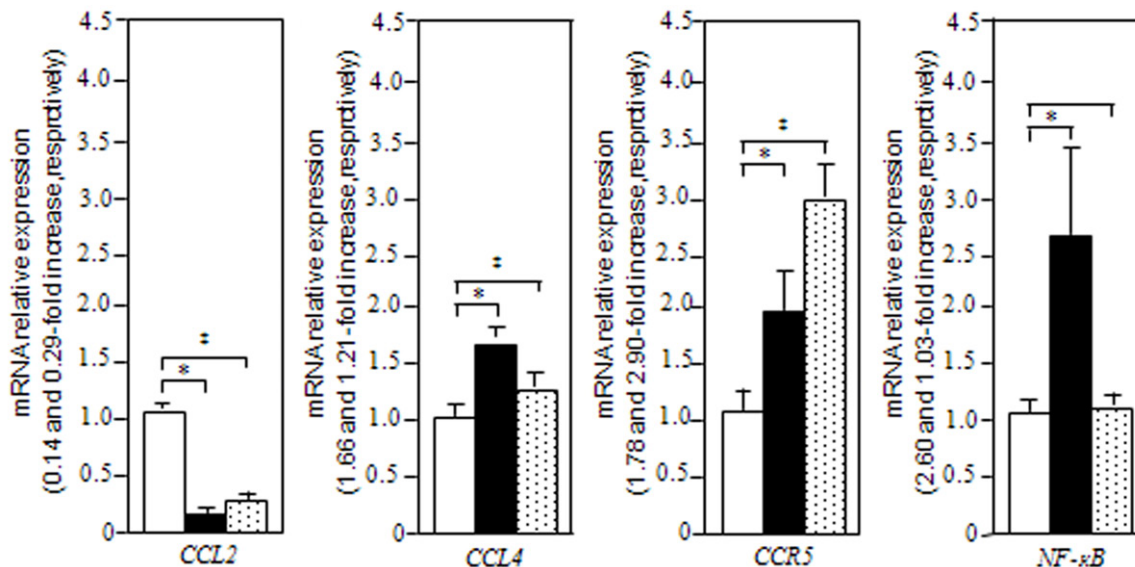


Figure 1. Real-time PCR analysis of CCL2, CCL4, CCR5, and NF-κB mRNA levels on CP and GAgP PB leukocytes. The relative mRNA expression levels were normalized to the levels of GAPDH. The significance of differences was determined using analysis of variance (ANOVA)/LSD-t test. *+P < 0.05, open bars healthy controls (n=9); solid bars CP patients (n=9); coarse stippling bars GAgP patients (n=9).

significance, LSD-t test was performed to explore the differences between any two groups.

Immunohistochemical analysis

The paraffin sections were prepared for routine histological and immunohistochemical examination. For immunostaining, all sections were treated in a microwave oven four times with citrate buffer (pH 6.0) for 5 min at 960 W. Sections were incubated overnight at 4°C with the primary polyclonal rabbit antibody (LifeSpan BioSciences, Inc., Seattle, WA, USA), namely anti-CCL2 in dilutions 1:50. Subsequently, sections were incubated with biotinylated goat anti-rabbit IgG (H+L) (Jackson) followed by horseradish peroxidase streptavidin (Vector) for 1 h at room temperature. Color was developed using 3,3'-diamino benzidine tetrahydrochloride, and sections were counterstained with Mayer's hematoxylin.

Statistical analysis

Comparisons between three groups were performed using one-way ANOVA followed by a LSD-t test. Comparisons between 2 groups were performed using student's t tests. All experiments were performed at least twice with comparable results. Data are presented

as means ± standard deviation (SD). The significance levels were set to P < 0.05. These calculations were carried out using a statistical Software Package Program (SPSS version 12.0, Software Package Programme, Chicago, Illinois, USA).

Results

CCL2, CCL4, CCR5, and NF-κB genes expression in gingival tissues and peripheral blood of the periodontitis patients

The mRNA levels of CCL2, CCL4, CCR5 and NF-κB were determined using real-time PCR. The real-time PCR results were converted to mRNA, and fold changes were calculated. Our data show that CCL4 expression on the leukocytes in CP (1.66-fold, P < 0.05; **Figure 1**) and GAgP PB was higher than in the control group PB (**Figure 1**). CCL4 expression was significantly up-regulated in the gingival tissue of CP patients as compared with their expression in normal gingival tissue (**Figure 1**). We also detected CCR5 expression was significantly increased on leukocytes in both CP and GAgP PB compared with its expression in normal PB (**Figure 1**). CCR5 was more intensely expressed in CP gingival tissue than in controls, although there was absence of statistical significance (**Figure 2**).

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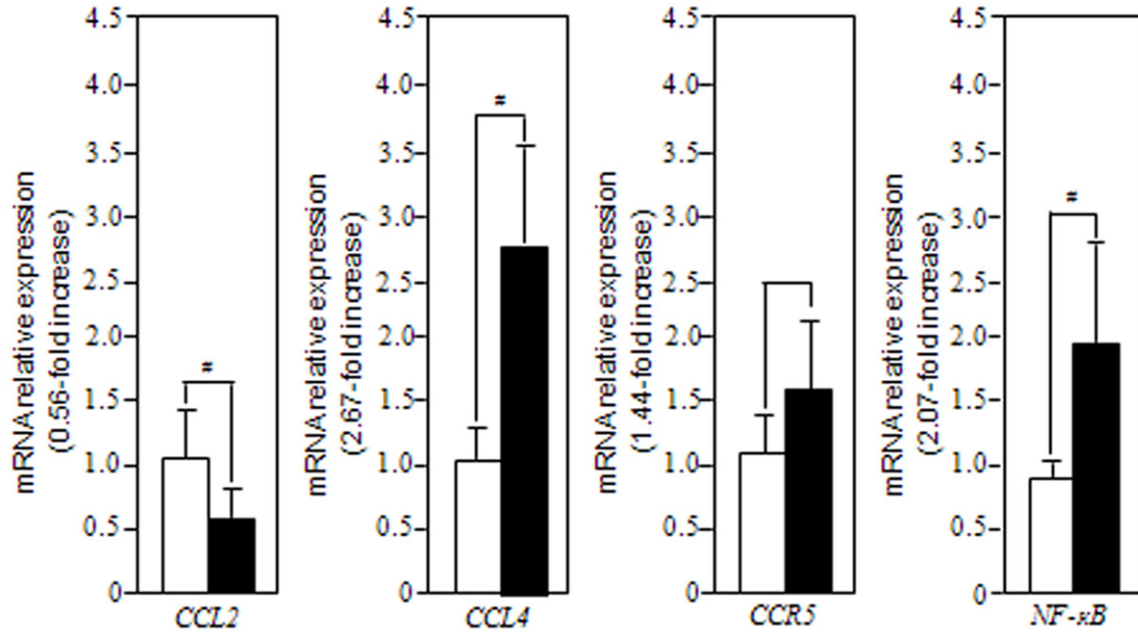


Figure 2. Real-time PCR analysis of *CCL2*, *CCL4*, *CCR5*, and *NF-κB* mRNA levels in CP gingival tissue. The relative mRNA expression levels were normalized to the levels of *GAPDH*. The significance of differences was determined using Student's t-test. #*P* < 0.05, open bars healthy controls (n=8); solid bars CP patients (n=13).

Table 1. Summary of *CCL2*, *CCL4*, *CCR5*, *NF-κB* gene expression in Real-time PCR

Gene name	Relative mRNA expression in CP gingival tissue (CP/control)	Fold	Relative mRNA expression on CP PB leukocytes (CP/control)	Fold	Relative mRNA expression on GAgP PB leukocytes (GAgP/control)	Fold
<i>CCL2</i>	(0.59 ± 0.25)/(1.06 ± 0.40)	0.56#	(0.15 ± 0.01)/(1.01 ± 0.11)	0.14*	(0.29 ± 0.03)/(1.01 ± 0.11)	0.29#
<i>CCL4</i>	(2.73 ± 0.72)/(1.02 ± 0.20)	2.67#	(1.66 ± 0.09)/(1.00 ± 0.09)	1.66*	(1.22 ± 0.12)/(1.00 ± 0.09)	1.21#
<i>CCR5</i>	(1.58 ± 0.47)/(1.10 ± 0.30)	1.44	(1.81 ± 0.36)/(1.01 ± 0.17)	1.78*	(2.95 ± 0.28)/(1.01 ± 0.17)	2.90#
<i>NF-κB</i>	(1.88 ± 0.70)/(0.91 ± 0.11)	2.07#	(2.62 ± 0.74)/(1.00 ± 0.09)	2.60*	(1.03 ± 0.09)/(1.00 ± 0.09)	1.03

#Statistically significant difference to the healthy controls (*P* < 0.05; student's t-test; n=13). *Statistically significant difference to the healthy controls (*P* < 0.05; one-way ANOVA with LSD-t test; n=9). #Statistically significant difference to the healthy controls (*P* < 0.05; one-way ANOVA with LSD-t test; n=9). RT-PCR, real time polymerase chain reaction; mRNA, messenger RNA; CP, chronic periodontitis; GAgP, generalized aggressive periodontitis; PB, peripheral blood.

Our results have also shown that *NF-κB* is highly expressed both on leukocytes and in the gingival tissue of CP patients (*P* < 0.05; **Figures 1** and **2**). On the other hand, in aggressive periodontitis (AgP) patients the intensity of *NF-κB* mRNA expression on leukocytes was higher (**Figure 1**), in spite of a lack of statistical significance.

It is worth noting that the CP gingival tissue exhibits significantly lower *CCL2* expression compared with controls (**Figure 2**), the *CCL2* was also significantly down-regulated on CP PB leukocytes than control leukocytes (**Figure 1**), as determined by quantitative RT-PCR. The relative mRNA levels of these four genes and fold changes on PB leukocytes and in gingival tis-

sue from the real-time PCR are summarized in **Table 1**.

CCL2 levels in serum of CP and GAgP patients

To determine whether PB from both patient groups (i.e. generalized aggressive periodontitis and chronic periodontitis) contained significant quantities of *CCL2* compared to normal volunteers, serum *CCL2* levels were determined by ELISA using 13 CP samples and 9 GAgP samples. Serum levels of CP *CCL2* ranged from 134.52 pg/ml to 208.31 pg/ml (mean 173 ± 26 pg/ml). The range of detection level for *CCL2* in GAgP serum samples was from 101.31 pg/ml to 185.34 pg/ml (mean 135 ± 33 pg/ml). In contrast normal sera obtained

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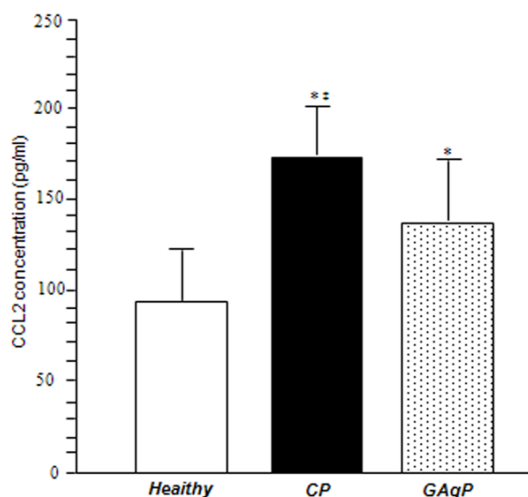


Figure 3. Concentrations of CCL2 protein in serum, assessed by ELISA technique. The significance of differences was determined using analysis of variance (ANOVA)/LSD-t test. *Significant difference from healthy group. †Indicates a significant difference between generalized aggressive periodontitis and chronic periodontitis group. ** $P < 0.05$, open bars healthy controls (n=9); solid bars CP patients (n=13); coarse stippling bars GAgP patients (n=9).

from 9 volunteers contained significantly less CCL2 (54.77 pg/ml to 123.87 pg/ml) (mean 92 ± 28 pg/ml). ANOVA showed that differences in the level of CCL2 among these groups were statistically significant at $P < 0.05$. Pairwise comparisons showed that the differences were statistically significant between CP and control and between GAgP and control groups (**Figure 3**).

Expression of CCL2 in inflamed gingival tissues

Experiments utilizing a polyclonal antibody CCL2 were carried out to determine whether gingival tissues from CP patients expressed CCL2 protein in vivo. The samples were placed into 3 groups according to the gingival inflammatory cell counts (monocyte + lymphocyte). Paraffin sections from normal, mild, and highly inflamed gingival tissues were examined by incubation with CCL2 antibody. Virtually no CCL2+ cells could be detected in an extensive inflammatory infiltrate in the gingival tissue from a CP patient. Numerous CCL2+ cells signal increased in minimally inflamed CP gingival tissue. Little or no CCL2 expression was shown in normal gingival tissue from healthy patients showed a low degree of inflammation.

Figure 4 shows adjacent gingival tissue sections from patients with chronic adult periodontitis. Positive cells are predominantly expressed along the basal layer of the junctional epithelium and by endothelial cells, fibroblast, and mononuclear phagocytes in the inflammatory infiltrate (**Figure 4F** and **4K**). **Figure 4B, 4C, 4G, 4H, 4L** and **4M** show a representative area at higher magnification. Slides were negative when the primary antibody was omitted (**Figure 4D, 4I** and **4N**). Histological examination of CP gingival tissues demonstrated a significant increase in chronic gingival inflammation (**Figure 4J** and **4O**). CCL2 staining was either faint or nonexistent in severe inflamed gingival tissues of patients with chronic adult periodontitis (**Figure 4K**). **Figure 4F** shows a mild inflamed gingival tissue. We detected CCL2-positive cells in the basal layers of epithelium. Endothelial cells found in small blood vessels of **Figure 4F** were clearly positive for CCL2 expression. CCL2-expressing monocyte-like cells were also found at the inflamed site. Of note is the number of immunoreactive CCL2 cells decreased with increasing severity of inflammation.

Discussion

Leukocyte migration to the site of inflammation plays an important role in the initiation and perpetuation of periodontal diseases. It is a highly complex process involving many signaling pathways and molecules/receptors. In the present study, we investigated CCL2, CCL4, CCR5 and NF- κ B levels in gingival tissue and peripheral blood (PB) of patients with different periodontal diseases.

The mRNA expression levels of CCL4, CCR5 and NF- κ B in patients with different periodontal diseases were higher than those in the healthy group on real-time PCR analysis.

The expression of NF- κ B ($P=0.67$) on GAgP PB leukocytes and the intensity of CCR5 ($P=0.13$) expression in the CP gingival tissue were somewhat higher in the patients than in controls, but failed to reach the level of significance (**Figures 1** and **2**), possibly owing to the small numbers of patients.

We investigated local and systemic level of CCL2 in patients with CP and GAgP periodontitis at both the protein and mRNA levels. In this

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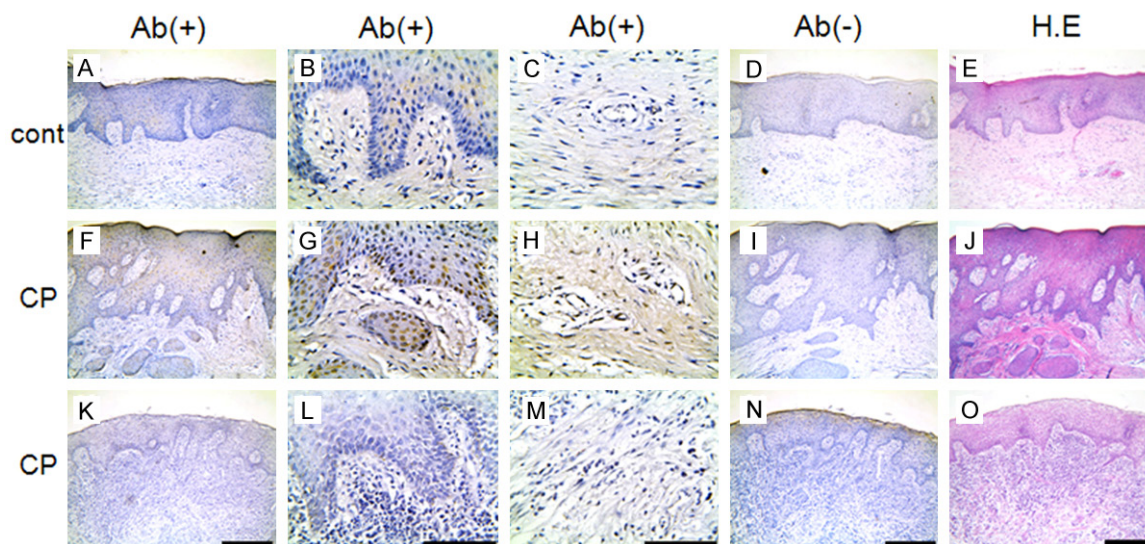


Figure 4. Immunohistochemical study of CCL2 expression in gingival tissue from CP patients. Photomicrographs were taken from healthy controls (A-E), minimally inflamed human gingival tissue from CP patients (F-J), highly inflamed human gingival tissue from CP patients (K-O). (A-C, F-H, K-M) Show CCL2 immunoreactivity (brown staining). (D, I, N) Show background staining with CCL2 antibody omitted (blue staining). (O) Show maximal inflammatory cell infiltration. (Hematoxylin and eosin staining in (E, J, O); avidin-biotin-peroxidase staining in (A-C, F-H, K-M). Original magnification $\times 400$ in (A, D-F, I-K, N and O); $\times 600$ in (B, C, G, H, L and M). bar =100 μ m.

study, serum CCL2 was found to be significantly elevated in chronic and generalized aggressive periodontitis compared with healthy controls (**Figure 3**). The possible reason for increase in serum levels of CCL2 could be either spillover from the GCF or gingival tissues to peripheral circulation or it could be due to systemic inflammatory response to progressive disease in the periodontal pocket.

In order to investigate the CCL2 expression in gingival tissue biopsies from CP patients exhibiting different degrees of inflammation, we performed immunohistochemistry. Of particular interest was the observation that the marked CCL2 expression was detected in minimally inflamed gingival samples, there is little or no CCL2 expression in moderate-to-severe inflamed gingival tissue (**Figure 4**). Moreover, the levels of CCL2 correlated inversely with the degree of gingival inflammation. This could be due to regulatory mechanisms of CCL2 locally. These observations strongly suggest that CCL2 may induce migration of leukocytes in the earlier phases of gingival inflammation, other mediators rather than CCL2 plays a significant role in the recruitment of monocytes/macrophages into the gingival tissues in the later stages of inflammatory process. A high degree of redundancy was observed in chemokine-

receptor interactions, since many chemokines bind to more than one receptor and vice versa [16]. These findings corroborate with the concept of the complexity of the chemokine/chemokine receptor system. A possible relationship between the amount of CCL2 and severity of inflammation has been reported for periodontal disease. It has been previously demonstrated that only few leukocytes expressed CCL2 in the periodontal tissues [2]. In contrast to these results, Yu et al. found that CCL2 was expressed in gingival tissues of patients with mild-to-moderate periodontitis and these levels have been demonstrated to be correlated with the degree of inflammation [5]. It is noteworthy that the expressions of CCL2 mRNA in CP and GAgP patients was greatly decreased than those in healthy controls, both in gingival tissue and on PB leukocyte (**Figures 1 and 2**). The lower CCL2 mRNA concentrations were found, possibly due to the short half-life and instability of its mRNA.

Next, we investigated the signaling pathways involved in the production of CCL2 in different forms of human periodontal disease. It is of interest to demonstrate which transcriptional factor is involved in CCL2 expression. There are several binding sites for a number of transcription factors, including NF- κ B, Sp-1, and AP-1, in

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the 5' region of the CCL2 gene [17]. The present study determined that significantly higher levels of NF- κ B mRNAs are found on PB leukocyte ($P=0.0007$) and in gingival tissue biopsies ($P=0.03$) from patients with CP as compared to healthy subjects. The values of NF- κ B ($P=0.67$) were somewhat higher on GAgP PB leukocyte than in controls, but failed to reach the level of significance. The expression of NF- κ B does not always result in high NF- κ B activity. The transcriptional activity of NF- κ B was only inferred rather than demonstrated. Phosphorylation of DNA binding proteins is also important for DNA binding activity [18]. Earlier studies demonstrated that the activation of NF- κ B (p50/p65) is significant in periodontal diseased tissues [19]. Macrophages play a central role in the proinflammatory cytokine response induced by periodontopathogens and are an important target for lipopolysaccharide (LPS). *Aggregatibacter actinomycetemcomitans* LPS can activate the NF- κ B pathway because it increases the expression and the phosphorylation states of the NF- κ B p65 subunit by macrophages [20]. McDonald et al. [21] mentioned that the effect of LPS towards nuclear NF- κ B activation in neutrophils was rapid and transient. In the absence of serum, a high concentration (100~500 ng/ml) of LPS from *Porphyromonas gingivalis* and *Escherichia coli* enhanced the DNA binding activity of NF- κ B [22]. These observations and our experiments suggest that a potent role of NF- κ B in the transcriptional regulation of CCL2 production in chronic periodontitis.

By the comprehensive microarray-based pathway analysis (ingenuity pathway analysis, IPA), it has been demonstrated that CCL4 upregulated CCL2 expression through CCL4/CCR5/c-Jun and c-Fos/CCL2 pathway in collagen-induced arthritis (CIA) rat and RA patients in an earlier study by the author [13]. In this study, the expressions of CCL4, CCR5, NF- κ B gene and CCL2 protein were markedly up-regulated in both generalized aggressive and chronic periodontitis patients compared to the controls, which paralleled the activation of NF- κ B. However, the expression levels of NF- κ B mRNA on PB leukocytes ($P=0.67$) were somewhat higher in the GAgP patients than in controls, no significant difference could also be observed in the CCR5 levels in the gingival tissue of CP ($P=0.13$) patients and healthy control. *The significant differences* needs to be further investi-

gated in larger samples. These findings and our observations from a previous study [13, 14] suggest that CCL4 may stimulate CCL2 expression through CCL4/CCR5/NF- κ B/CCL2 pathway on PB leukocytes of clinically CP patients. CCL4 exerts its effects by binding to CCR5 on the surface of the targeted leukocytes. CCR5, once activated, trigger a set of cellular signaling processes that result in activation of phospholipase C (PLC) together with inositol triphosphate (IP3) and diacylglycerol (DAG) formation, intracellular calcium release, protein kinase C (PKC) activation, and proline-rich tyrosine kinase 2 (PYK2) activation. PYK2 regulates the activity of JNK and p38 in the MAPK pathway, leading to enhance the transactivation function of NF- κ B. Expressions of CCL2 were markedly up-regulated at the levels of protein, in parallel with the activation of NF- κ B. Neither p38 nor c-Jun-N-terminal kinase (JNK) directly phosphorylates NF- κ B [23]. However, inhibition of either pathway strongly reduces transcription from both NF- κ B-dependent cytokine promoters and promoters controlled by synthetic NF- κ B binding sites [24]. p38 MAP kinase regulates NF- κ B-dependent transcription in part by TFIIB, and TATA-binding protein (TBP) [23]. The observation that NF- κ B is able to interact directly with the TATA-binding protein of the TFIID complex, supports this possibility [25]. The MAP kinase p38 is involved in NF- κ B-dependent gene expression. This pathway is clearly dissected from IKK-induced NF- κ B activation.

The AP-1 binding site is likely to be one of the important sites for CCL2 production [17]. AP-1 activity is known to be regulated by MAPK pathways [26]. MAP kinase signaling pathways influence AP-1 activity by both increasing the abundance of AP-1 components and by stimulating their activity directly [27]. The proinflammatory factors activate principal MAPK family members, such as extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK), in responsive cell types [28]. The phosphorylation of MAPKs was shown to cause activation of their downstream transcription factors including Elk-1, c-Jun, ATF2 and CREB, which in turn regulated the expression of c-Fos and c-Jun, components of the AP-1 complex [29]. AP-1 can bind to AP-1-binding sites in promoters and may activate the transcription of various target genes in response to cytokines

[27], while AP-1 functions are regulated through changes in the abundance of the Jun and Fos components as well as post-transcriptional modification by protein phosphorylation [30]. It has been shown that proinflammatory factors enhance c-Fos and c-Jun mRNA expression and/or AP-1-DNA binding activity in synovial fibroblasts, skin fibroblasts and gingival fibroblasts [31]. The activation of NF- κ B, AP-1, or the combination of transcription factor NF- κ B and AP-1 upregulates CCL2 gene transcriptions, depending on the types of cell or stimuli [32].

Taken together, these observations suggest that the p38 MAPK/NF- κ B (in the present study), MAPKs/activator protein-1 (AP-1) and inhibitor of κ B (I κ B) kinases (IKKs)/I κ B/nuclear factor- κ B (NF- κ B) cascades may mediate the CCL4 stimulated synthesis of CCL2 on CP PB leukocyte, though it has not been clarified whether these 3 pathways run on the same axis or in parallel. The p38MAPK cascade may act as the upstream signaling for the activation of AP-1 and NF- κ B, which induce CCL2 expression. Based on the PCR and ELISA results showing the significantly higher CCL4, CCR5, NF- κ B mRNA levels and CCL2 protein level in CP PB, we deduced the signaling pathway (CCL4/CCR5/PLC²/PKC/PYK2/p38 MAPK/NF- κ B) of these cytokines in relation to CCL2 in this study.

In the present study, we identify higher serum levels of CCL2 in both generalized aggressive and chronic periodontitis patients compared to the controls. This points toward an increased systemic load of inflammatory mediators in patients with periodontitis. Heightened CCL2 expression has been observed in several chronic inflammatory diseases such as atherosclerosis, osteoarthritis, rheumatoid arthritis, tumors and delayed type hypersensitivity reactions [33]. Thus, any increase in plasma levels of CCL2 in patients with periodontitis who are otherwise clinically healthy may be hypothesized to be a risk indicator for systemic illnesses. Advances in diagnostic research are moving towards methods whereby the periodontal risk can be identified and quantified by objective measures using biomarkers. Patients with periodontitis may have elevated circulating levels of specific inflammatory markers that can be correlated to the severity of the disease [34]. Estimation of serum CCL2 levels could be reliable indicators of periodontal disease activity.

Further research in this direction could also reveal reliable markers to forecast the progression of periodontitis in high-risk groups.

In conclusion, we studied the possibility of a pathway involved in CCL2 expression in different forms of human periodontal disease. We indicate that CCL4 increased CCL2 expression by binding to CCR5 and activating PKC (α , β), PYK2, and leading to MAPK (ERK, JUK and p38 MAPK), which enhanced binding of TBP to the TATA box and TBP's interaction with the p65 subunit of NF- κ B and resulted in CCL2 expression on CP PB leukocyte. To understand the precise signaling mechanism of CCL2 in human periodontitis, and to explain the cascade of events in chemokine-induced leukocyte migration into inflammatory sites, further study of animal models is indispensable. These findings may provide a better understanding of the mechanisms of periodontal pathogenesis. Targeting of CCL2 or its signaling pathways is a potentially novel therapeutic strategy for periodontitis.

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Disclosure of conflict of interest

None.

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