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Granulocyte chemotactic protein 2 (gcp-2/cxcl6) complements interleukin-8 in periodontal disease

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Background and Objective: Mucosal inflammatory responses are orchestrated largely by pro-inflammatory chemokines. The chemokine granulocyte chemotactic protein 2 (CXCL6) is involved in neutrophil recruitment and migration. Previous studies have shown that granulocyte chemotactic protein 2 is up-regulated during mucosal inflammation (e.g. in inflammatory bowel disease), similarly to the functionally and structurally related chemokine interleukin-8. Nevertheless, unlike interleukin-8, a role of granulocyte chemotactic protein 2 in gingival inflammation has not been yet demonstrated. In this study we aimed to evaluate the expression of the chemokine granulocyte chemotactic protein 2 in clinically healthy vs. diseased gingival tissues and to explore possible correlations with clinical and microbiological markers of periodontitis.

Material and Methods: Gene expression in 184 'diseased' and 63 'healthy' gingival tissue specimens from 90 patients with periodontitis was analyzed using Affymetrix U133Plus2.0 arrays. The expression of granulocyte chemotactic protein 2 was further confirmed by real-time reverse transcription–polymerase chain reaction, western blotting and enzyme-linked immunosorbent assay, while the localization of granulocyte chemotactic protein 2 in gingival tissues was analyzed by immunohistochemistry. Plaque samples from the adjacent periodontal pockets were collected and evaluated for 11 species of periodontal bacteria using checkerboard DNA–DNA hybridizations.

Results: Among all known chemokines, GCP-2 expression was the most up-regulated (3.8-fold, $p < 1.1 \times 10^{-16}$), in 'diseased' vs. 'healthy' tissue as compared to a 2.6-fold increased expression of interleukin-8 mRNA ($p < 1.2 \times 10^{-15}$). Increased expression of granulocyte chemotactic protein 2 correlated with higher levels of 'red' and 'orange' complex pathogens and with increased probing depth, but not with attachment loss. Immunohistochemistry showed that granulocyte chemotactic protein 2 was expressed in gingival vascular endothelium.

Conclusion: The level of expression of granulocyte chemotactic protein 2 correlates with the severity of periodontitis and appears to act as a hitherto unrecognized functional adjunct to interleukin-8 in diseased gingival tissues.

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Neutrophils play a pivotal role as a first line of cellular defense against pathogens in periodontal homeostasis, whilst defects of neutrophil function, and also hyperactive neutrophils, are associated with severe periodontal disease (1). Neutrophils are recruited to an inflammatory site by a gradient of specific chemokines, which represent a family of chemotactic cytokines produced by different cell types, including epithelial and endothelial cells, in response to activation by microbial metabolic products or pro-inflammatory cytokines (2).

Human granulocyte chemotactic protein-2 (CXCL6) is a CXC chemokine with a conserved Glu–Leu–Arg (ELR^+) motif (3). Similarly to other ELR^+ CXC chemokines, such as interleukin-8 (CXCL8) and ENA-78 (CXCL5), granulocyte chemotactic protein 2 possesses potent chemotactic and pro-angiogenic properties (4). Granulocyte chemotactic protein 2, similarly to interleukin-8, activates target cells by binding to CXC chemokine receptors (CXCR)-1 and (CXCR)-2 (5). Both receptors are expressed by neutrophil granulocytes, but not by other blood-derived cells, such as lymphocytes or monocytes.

Interleukin-8 is constitutively expressed in periodontal health and it mediates neutrophil recruitment to the gingival tissues adjacent to the periodontal crevice and into the gingival crevicular fluid, maintaining a subclinical inflammatory response to the ubiquitous microbiota of the dental plaque (6,7). In periodontal disease, the expression of interleukin-8 is strongly up-regulated and correlates with disease activity (8,9), whereas a regulated expression of the functionally related granulocyte chemotactic protein 2 has not yet been described (10). However, a role for granulocyte chemotactic protein 2 has been demonstrated in other mucosal chronic inflammatory conditions, such as inflammatory bowel disease (11,12) or chronic rhinosinusitis (13,14).

In this study, we sought to evaluate the expression of granulocyte chemotactic protein 2 in clinically healthy vs. diseased gingival tissues and to explore possible correlations with clinical and microbiological markers of periodontitis.

Material and methods

The design and procedures of the study were approved by the Columbia University Medical Center Institutional Review Board.

Subjects

A total of 90 subjects with moderate to severe periodontitis (63 with chronic periodontitis and 27 with aggressive periodontitis) were recruited among the patients referred for periodontal therapy to the Clinic for Post-doctoral Periodontics, Columbia University College of Dental Medicine. Eligible patients were (i) at least 13 years old; (ii) had a minimum of 24 teeth present; (iii) had no past history of systematic periodontal therapy other than occasional prophylaxis provided by the referring general dentist; (iv) had received no systemic antibiotics or anti-inflammatory drugs for at least 6 mo; (v) harbored a minimum of four teeth with radiographic bone loss; (vi) did not suffer from diabetes mellitus; (vii) did not suffer from any of the systemic conditions or genetic disorders that entail a diagnosis of 'Periodontitis as a manifestation of systemic diseases'; (viii) were not pregnant; and (ix) were not current users of tobacco products or of nicotine-replacement medication. Signed informed consent was obtained prior to enrollment in the study.

Clinical examination and procedures

All participants underwent a full-mouth examination of the periodontal tissues at six sites per tooth, using a manual probe. The examination included assessments of the presence/absence of dental plaque and bleeding on probing, and linear measurements of probing pocket depth and clinical attachment level.

Identification of donor sites and harvesting of gingival tissue samples was performed as described previously (15). In brief, a 'diseased' interproximal papilla showed bleeding on probing, probing pocket depth $\geq$ 4 mm and clinical attachment level $\geq$ 3 mm, whilst a 'healthy' papilla demonstrated no bleeding on probing, probing pocket depth $\leq$ 4 mm and clinical attachment level $\leq$ 2 mm. All tissue specimens were collected during periodontal surgery. Each patient contributed one to three 'diseased' tissue samples (184 samples in total) and 63 patients contributed one 'healthy' tissue sample.

Subgingival plaque samples were obtained from the adjacent periodontal pockets and analyzed for 11 periodontal species of bacteria (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Campylobacter rectus*, *Micromonas micros*, *Eikenella corrodens*, *Veillonella parvula* and *Actinomyces naeslundii*) using checkerboard hybridizations as described previously (16,17).

mRNA quantification

Total RNA from 184 'diseased' and 63 'healthy' gingival tissue specimens was extracted, amplified, reverse transcribed, labeled and hybridized to AffymetrixU133Plus2.0 arrays (Affymetrix, Santa Clara, CA, USA), as described previously (15). Independent confirmation of the microarray data obtained was performed by conducting quantitative real-time polymerase chain reaction (PCR) analyses on tissue samples from five patients who showed a strong differential expression for granulocyte chemotactic protein 2 mRNA in 'diseased' gingival samples. Three patients contributed a pair of 'healthy' and 'diseased' tissue samples each, while two patients contributed 'diseased' tissue samples only. The Taqman Gene Expression Assays Hs00237017_m1 and Hs99999905_m1 were used for granulocyte chemotactic protein 2 and glyceraldehyde-3-phosphate dehydrogenase, respectively (Applied Biosystems, Foster City, CA, USA). Three technical replicates per sample and gene were performed.

Immunoblot analysis

Total gingival tissue protein was prepared by homogenization and lysis of

frozen biopsy tissues in modified RIPA buffer [50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.25% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P-40, 0.1% (w/v) sodium dodecyl sulfate, 1% (v/v) Triton X-100 (all from Sigma-Aldrich, St Louis, MO, USA)] containing Protease Inhibitor Cocktail III (Calbiochem, San Diego, CA, USA) on ice. Lysates were cleared by ultracentrifugation, and the total protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Samples were heat denatured in 4 × Laemmli buffer, size-separated by electrophoresis on a 12% sodium dodecyl sulphate gel, blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) and blocked with blocking buffer [phosphate-buffered saline containing 0.05% (v/v) Tween-20 and 5% (w/v) nonfat dry milk]. Blots were incubated with murine monoclonal anti-(human granulocyte chemotactic protein 2) (MAB333, clone 60910; R&D Systems, Minneapolis, MN, USA) at a 1:250 dilution, overnight at 4°C. Immunodetection was performed using biotinylated rabbit anti-mouse IgG (Amersham Pharmacia Biotech), streptavidin-conjugated horseradish peroxidase (Amersham Pharmacia Biotech) and enhanced chemiluminescence (Amersham Pharmacia Biotech). Densitometric analysis was performed using the software IMAGEJ 1.38x (NIH, Bethesda, MD, USA).

Immunohistochemistry

'Healthy' and 'diseased' specimens of gingival tissue, obtained as described above, were embedded in OCT Tissue Tek (Sakura Finetek, Torrance, CA, USA) and snap frozen in isopentane/liquid nitrogen. Five-micrometer-thick cryostat sections were prepared and dried on coated object slides (Superfrost plus; Micron, Walldorf, Germany) for 30 min at 20°C. Fixation of specimens was carried out with absolute acetone at −20°C for 10 min and endogenous peroxidase activity was blocked by application of 2% hydrogen peroxide in methanol. The slides were subsequently incubated with murine monoclonal anti-(human granulocyte chemotactic protein 2) (MAB333, clone 60910; R&D Systems) or the appropriate mouse isotype control (dilution: 1:100 in phosphate-buffered saline) for 3 h at 20°C. Immunoreactivity was visualized using horseradish peroxidase-coupled secondary antibodies and diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin and examined by light microscopy.

Measurement of granulocyte chemotactic protein 2 by enzyme-linked immunosorbent assay

The level of granulocyte chemotactic protein 2 in gingival tissue lysates was determined using a Quantikine human CXCL6/granulocyte chemotactic protein 2 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), according to the manufacturer's protocol.

One 'healthy' and one 'diseased' gingival tissue specimen from five consecutively recruited patients was homogenized and lysed as described above. The absolute amount of protein was quantified using a Bradford assay, adjusted to a total protein concentration of 1 mg/mL and used undiluted for the assay. The assay was performed with two technical replicates.

Statistical analysis

For gene expression analyses, R version 2.3.1 (Linux OS) or SAS for PC version 9.1 (SAS Institute, Cary, NC, USA) were used. Expression data were first normalized and summarized using the log scale robust multi-array analysis (18) with default settings. Differential expression was assayed using a standard mixed-effects linear model approach, in which patients were conditioned as random effects to account for the within-mouth correlation of granulocyte chemotactic protein 2 levels resulting from multiple gingival tissue samples being collected from each patient. Using this approach we explored the association between granulocyte chemotactic protein 2 mRNA levels (dependent variable) and the following independent variables (fixed effects): gingival tissue status ('healthy' vs. 'diseased', as described above); probing pocket depth; clinical attachment level; bacterial colonization level with each of the aforementioned 11 species of bacteria; age; gender; and race/ethnicity. All results reported herein stem from univariate models, with the exception of probing pocket depth and clinical attachment level, which were modeled simultaneously to obtain the independent association between either probing pocket depth or clinical attachment level and granulocyte chemotactic protein 2 expression. Granulocyte chemotactic protein 2 expression fold change was computed by dividing the average raw expression values among the comparison group of the independent variable by the average expression in the reference group (i.e. average expression in 'diseased' tissue samples divided by average expression among 'healthy' samples). Therefore, fold change values represent relative differences in RNA levels.

For all experiments not involving microarrays, statistical analyses were performed using GRAPHPAD PRISM 5 (GraphPad, San Diego, CA, USA) for unpaired, two-tailed *t*-tests. Mean values and standard error values are reported for all quantitative assessments. Differences between chemokine concentrations or real-time PCR cycles were considered significant if *p*-values were < 0.05.

Analysis of granulocyte chemotactic protein 2 protein expression using western blotting and immunohistochemistry was performed at least in triplicate, with similar results obtained on each occasion.

Results

The mean age of patients was 42 years (range 13–76 years), 50% were female, and the ethnicity was as follows: 76% Hispanic, 15% Black, 6% White (15) and 3% unknown unreported. According to the criteria of the 1999 International Workshop (Classification of Periodontal Disease and Conditions), 70% of the patients had chronic periodontitis and 30% had aggressive periodontitis.

Table 1. Microarray analysis of chemokine and chemokine receptor mRNA expression in periodontal disease

Probe	Gene	Description	Fold change	*p*-value
206336_at	CXCL6	Chemokine (C-X-C motif) ligand 6	3.85	1.1E-16
217028_at	CXCR4	Chemokine (C-X-C motif) receptor 4	3.56	1.1E-16
204470_at	CXCL1	Chemokine (C-X-C motif) ligand 1	3.45	1.1E-16
209201_x_at	CXCR4	Chemokine (C-X-C motif) receptor 4	3.07	1.1E-16
211919_s_at	CXCR4	Chemokine (C-X-C motif) receptor 4	2.90	1.1E-16
202859_x_at	IL-8	interleukin 8	2.57	1.2E-15
205242_at	CXCL13	Chemokine (C-X-C motif) ligand 13	2.42	7.9E-14
211506_s_at	IL-8	interleukin 8	2.26	1.9E-12
209924_at	CCL18	Chemokine (C-C motif) ligand 18	2.18	1.1E-16
214146_s_at	PPBP	Chemokine (C-X-C motif) ligand 7	2.11	7.0E-14
32128_at	CCL18	Chemokine (C-C motif) ligand 18	2.08	2.4E-15
203666_at	CXCL12	Chemokine (C-X-C motif) ligand 12	2.05	1.1E-16
209774_x_at	CXCL2	Chemokine (C-X-C motif) ligand 2	1.90	2.6E-12
203936_s_at	MMP-9	Matrix metalloproteinase 9	1.81	1.8E-13
210072_at	CCL19	Chemokine (C-C motif) ligand 19	1.78	5.6E-10
209687_at	CXCL12	Chemokine (C-X-C motif) ligand 12	1.76	1.4E-15
208335_s_at	DARC	Duffy blood group, chemokine receptor	1.71	1.1E-16
205098_at	CCR1	Chemokine (C-C motif) receptor 1	1.66	1.1E-16
207850_at	CXCL3	Chemokine (C-X-C motif) ligand 3	1.61	6.1E-11
214974_x_at	CXCL5	Chemokine (C-X-C motif) ligand 5	1.60	8.8E-07
1405_i_at	CCL5	Chemokine (C-C motif) ligand 5	1.59	6.4E-11
206337_at	CCR7	Chemokine (C-C motif) receptor 7	1.59	9.0E-12
1555759_a_at	CCL5	Chemokine (C-C motif) ligand 5	1.56	1.3E-12
205099_s_at	CCR1	Chemokine (C-C motif) receptor 1	1.48	5.2E-14
205114_s_at	CCL3	Chemokine (C-C motif) ligand 3	1.48	7.2E-09
206390_x_at	PF4	Chemokine (C-X-C motif) ligand 4	1.45	3.4E-13
206366_x_at	XCL1	Chemokine (C motif) ligand 1	1.44	1.1E-16
214567_s_at	XCL1	Chemokine (C motif) ligand 1	1.42	4.4E-13
204655_at	CCL5	Chemokine (C-C motif) ligand 5	1.41	1.1E-08
205392_s_at	CCL14	Chemokine (C-C motif) ligand 14	1.40	2.9E-09
204103_at	CCL4	Chemokine (C-C motif) ligand 4	1.39	1.6E-10
214038_at	CCL8	Chemokine (C-C motif) ligand 8	1.35	4.1E-07
207794_at	CCR2	Chemokine (C-C motif) receptor 2	1.31	3.6E-12
220565_at	CCR10	Chemokine (C-C motif) receptor 10	1.30	1.4E-11
219161_s_at	CKLF	Chemokine-like factor	1.28	2.1E-11
223451_s_at	CKLF	Chemokine-like factor	1.24	5.3E-11
211434_s_at	CCRL2	Chemokine (C-C motif) receptor-like 2	1.23	2.5E-11
206126_at	BLR1	Chemokine (C-X-C motif) receptor 5	1.15	3.9E-07
210133_at	CCL11	Chemokine (C-C motif) ligand 11	1.15	6.8E-07
210548_at	CCL23	Chemokine (C-C motif) ligand 23	1.13	1.1E-07
224027_at	CCL28	Chemokine (C-C motif) ligand 28	0.90	8.7E-08
220351_at	CCRL1	Chemokine (C-C motif) receptor-like 1	0.71	4.4E-16
222484_s_at	CXCL14	Chemokine (C-X-C motif) ligand 14	0.59	3.7E-14
218002_s_at	CXCL14	Chemokine (C-X-C motif) ligand 14	0.56	5.1E-15
237038_at	CXCL14	Chemokine (C-X-C motif) ligand 14	0.55	1.1E-16

Data are shown for all chemokines, chemokine receptors and selected functionally related genes with a *p*-value below the Bonferroni threshold of 9.14×10^{-7}. Multiple *Affymetrix* probes may map to the same gene. Fold change is defined as the ratio of gene expression in disease over expression in health. Thus, a fold change of < 1.0 indicates a down-regulation in disease vs. health.

mRNA for granulocyte chemotactic protein 2 is up-regulated in periodontitis

Microarray data demonstrated that, among all chemokines, granulocyte chemotactic protein expression was the most highly regulated [3.8-fold (standard error of the mean, 1.12), $p < 1.1 \times 10^{-16}$] in 'diseased' vs. 'healthy' gingival tissues. In comparison, a 2.6-fold (standard error of the mean, 1.14) increased expression of interleukin-8 mRNA ($p < 1.2 \times 10^{-15}$) in 'diseased' vs. 'healthy' gingival tissues was observed. Table 1 summarizes microarray-based mRNA expression data for all chemokines, chemokine receptors and selected functionally related genes with a *p*-value below the Bonferroni threshold of 9.14×10^{-7}.

Confirmatory real-time reverse transcription PCR showed a mean difference of 5.64 cycles (standard error of the mean, 1.39) between 'healthy' and 'diseased' samples, resulting in a $2^{5.64} = 49.8$-fold increased expression of granulocyte chemotactic protein 2 mRNA (Fig. 1).

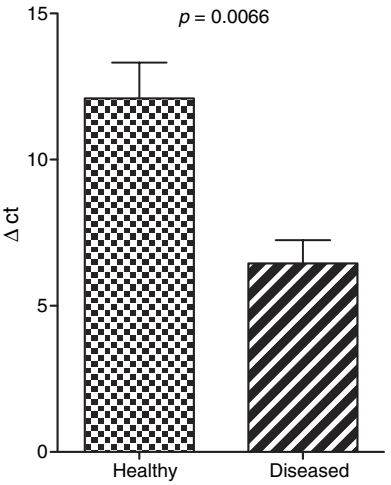

Fig. 1. Real-time reverse transcription–polymerase chain reaction analysis of granulocyte chemotactic protein 2 mRNA in 'healthy' and 'diseased' gingival tissues. The results confirm microarray expression data for granulocyte chemotactic protein 2 mRNA in five patients, each contributing two gingival specimens. The observed mean difference of 5.62 ± 1.39 cycles corresponds to a mean up-regulation of granulocyte chemotactic protein 2 in 'diseased' vs. 'healthy' gingival tissue of 49.8-fold. Δct, no of PCR cycles where the reports dye in sufficiently high to cross a threshold value; normalized against GAPDH as an endogenous control.

Association between demographic, clinical or bacteriological characteristics and expression of granulocyte chemotactic protein 2 mRNA

Age, race and gender were not related to the expression levels of granulocyte chemotactic protein 2. No significant difference in expression levels of the chemokine could be detected between subjects with aggressive and chronic periodontitis. A 1-standard deviation increase of *P. gingivalis*, *T. forsythia*, *C. rectus* and *P. intermedia* was associated with an approximate 1.55-fold increase in granulocyte chemotactic protein 2 expression (all p-values < 0.001), whereas *T. denticola* ($p < 0.0001$) and *M. micros* ($p < 0.01$) were each associated with an approximate 1.4-fold increase in expression. A 1-mm increase in probing pocket depth was associated with a 1.33-fold increase in granulocyte chemotactic protein 2 expression, which was independent of clinical attachment level. Conversely, clinical attachment level was not associated with granulocyte chemotactic protein 2 expression after accounting for probing pocket depth level.

Granulocyte chemotactic protein 2 expression is increased in periodontitis

Immunoblot analysis (Fig. 2) showed stronger expression of granulocyte chemotactic protein 2 in 'diseased' than in 'healthy' gingival tissue samples. The higher expression of granulocyte chemotactic protein 2 in periodontitis was confirmed and quantified by ELISA measurements (Fig. 3).

In frozen sections of gingival tissue, a pronounced immunoreactivity for granulocyte chemotactic protein 2 was observed in endothelium in 'diseased' gingival tissue, whilst only weak staining was observed in 'healthy' tissue (Fig. 4). In a similar manner, sections from 'diseased' tissues showed a stronger diffuse staining for granulocyte chemotactic protein 2 than 'healthy' tissue sections (Fig. 4).

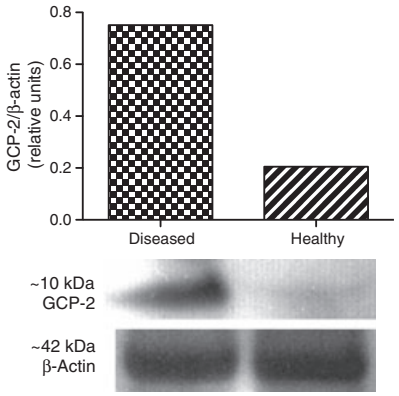

Fig. 2. Western blot analysis of granulocyte chemotactic protein 2 protein in 'healthy' and 'diseased' tissue samples. Stronger expression of granulocyte chemotactic protein 2 was observed in 'diseased' gingival tissues compared with healthy gingival tissues. The band for granulocyte chemotactic protein 2 was detected at approx. 10 kDa, and equal loading was demonstrated by β-actin probing. Densitometric analysis revealed a 3.6-fold up-regulation of granulocyte chemotactic protein 2 in 'diseased' tissue. The analysis was performed in three consecutively recruited patients, each contributing one 'diseased' and one 'healthy' gingival tissue specimen. GCP-2, secreted chemokine.

Discussion

Our data are the first to report a differential expression of the ELR$^+$ CXC chemokine granulocyte chemotactic protein 2 in periodontally 'healthy' and 'diseased' gingival tissues and in fact demonstrated that granulocyte chemotactic protein 2 is the most strongly up-regulated chemokine among all known chemokines in periodontitis. The levels of expression of granulocyte chemotactic protein 2 in the gingival tissues correlated positively with clinical and microbiological markers of periodontitis, such as probing pocket depth and levels of red/orange complex periodontal pathogens. In analyses accounting for probing pocket depth, a correlation between granulocyte chemotactic protein 2 and clinical attachment level was not found, suggesting that granulocyte chemotactic protein 2 expression reflects current periodontal inflammatory status, rather than a cumulative history of periodontitis.

Whilst interleukin-8 has been shown to be expressed in periodontal pocket epithelium in both periodontal health and disease in order to maintain continuous neutrophil migration into the sulcus/pocket via interactions with CXC neutrophil receptors (7), our data suggest that granulocyte chemotactic protein 2 expression originates from the microvascular endothelium of inflamed gingival tissue. The observed expression pattern of granulocyte chemotactic protein 2 in periodontal inflammation corroborates earlier findings by Gijsbers et al. (11), who detected pronounced expression of granulocyte chemotactic protein 2 in intestinal microvascular endothelium exclusively adjacent to regions with ulcerated or eroded epithelium in specimens from patients with inflammatory bowel disease (11). As periodontitis is also characterized by loss of epithelial integrity in the pocket (19), the observed up-regulation of granulocyte chemotactic protein 2 in the gingival microvascular endothelium

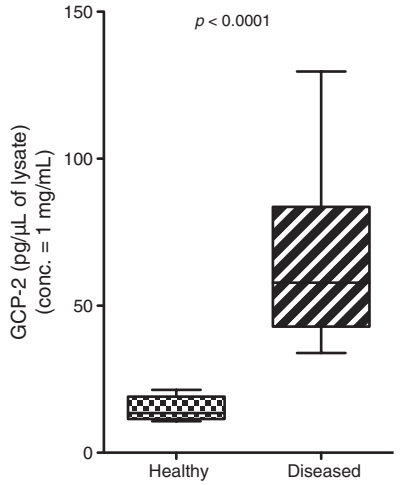

Fig. 3. Enzyme-linked immunosorbent assay (ELISA) analysis of granulocyte chemotactic protein 2 in homogenized 'healthy' and 'diseased' tissue samples. Up-regulation of granulocyte chemotactic protein 2 in 'diseased' gingival tissue was determined by ELISA ($n = 5$ consecutively recruited patients, each contributing one 'diseased' and one 'healthy' gingival tissue specimen). The mean difference of granulocyte chemotactic protein 2 concentrations observed was 58.16 ± 10.17 pg/µL of lysate. GCP-2, granulocyte chemotactic protein 2.

appears to reflect a similar, supplementary neutrophil-recruitment mechanism to the site of tissue injury. It is plausible to suggest that, in situations of gingival health or incipient periodontal infection (i.e. in relatively shallow pockets colonized by a low periodontal pathogen burden) microbial metabolic products stimulate junctional and pocket epithelial cells to release interleukin-8 and recruit a steady stream of neutrophils. In states of more severe disease, characterized by deeper pockets and higher levels of virulent, invading pathogens, the theater of inflammatory warfare shifts from the pocket epithelium to the gingival connective tissue and the vascular endothelium, forming a second line of defense (20). Hence, the enhanced recruitment of neutrophils from the bloodstream to the connective tissue seems to be primarily mediated by granulocyte chemotactic protein 2, as indicated by the increased expression of granulocyte chemotactic protein 2 relative to the expression of interleukin-8. Our data therefore suggest that, with increasing severity of the periodontal lesion, neutrophil recruitment to the vascular endothelium mediated by granulocyte chemotactic protein 2 is complementary to the interleukin-8 activity in the pocket epithelium.

Similarly to interleukin-8, granulocyte chemotactic protein 2 triggers the degranulation of gelatinase B/matrix metalloproteinase (MMP)-9 in neutrophils (3), a proteinase that cleaves interleukin-8, ENA-78 and granulocyte chemotactic protein 2 at the N-terminal end. This cleavage results in a potentiation of interleukin-8, and thus a positive feedback (21), and generates a biologically inactive form of ENA-78 (22), but does not affect the biological activity of granulocyte chemotactic protein 2, suggesting that granulocyte chemotactic protein 2 can act as a potent chemoattractant for neutrophils even in an MMP-9-rich environment. Our data confirm the merely limited up-regulation of gelatinase B mRNA in periodontally diseased tissue that was recently reported by another group (23). This is likely due of the fact that neutrophils store gelatinase B in secondary secretory granules for rapid degranulation in an acute phase of inflammation rather than produce this proteinase in the connective tissue (24,25).

Recently, a causative role of continuous and uncontrolled excessive neutrophil recruitment and activation, resulting in 'neutrophil-mediated tissue injury', has been suggested for localized aggressive periodontitis (26,27). Interestingly, we were unable to demonstrate a significant difference in granulocyte chemotactic protein 2 expression between clinically distinct phenotypes of periodontal disease (aggressive vs. chronic periodontitis). Although only 30% of the patients in our sample suffered from aggressive periodontitis, the study was sufficiently powered to detect differences of two-fold or higher in expression of granulocyte chemotactic protein 2 between the two disease entities. In addition, because the vast majority of patients in the study presented with periodontitis of high extent, we did not attempt to evaluate differences in the expression of granulocyte chemotactic protein 2 between localized and generalized

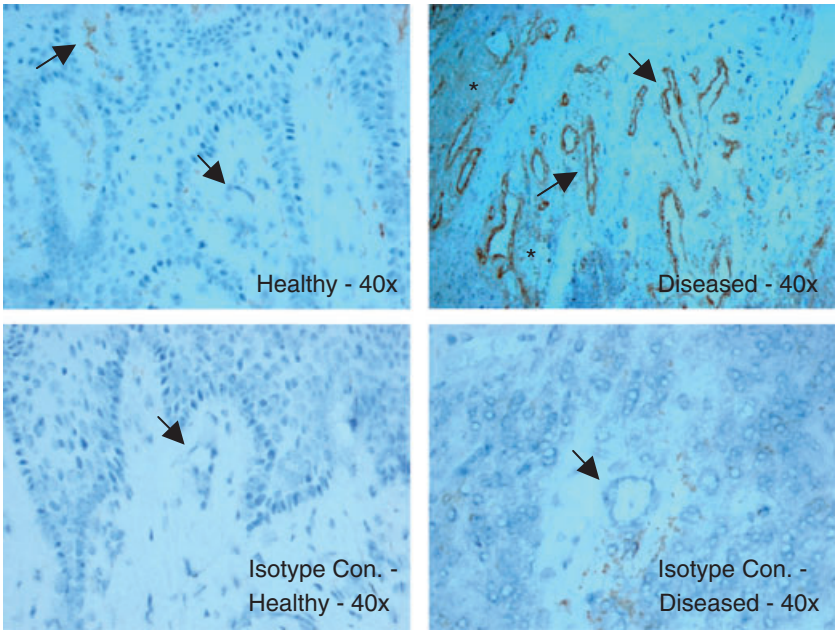

Fig. 4. Immunostaining of granulocyte chemotactic protein 2 in frozen tissue sections. Granulocyte chemotactic protein 2 expression was enhanced in 'diseased' gingival tissues. Granulocyte chemotactic protein 2 immunoreactivity was predominant in endothelial cells of the gingival microvasculature (arrows). Furthermore, a diffuse positive staining of the connective tissue was detected primarily in 'diseased' samples as a typical sign of chemokine secretion (asterisk). Isotype Con., isotype control.

forms of either chronic or aggressive periodontitis.

In summary, our data demonstrated a supplementary role for granulocyte chemotactic protein 2 to the established one of interleukin-8 in enhancing neutrophil recruitment in established periodontitis lesions.

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