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Changes in MMPs and inflammatory cells in experimental gingivitis

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Summary. In periodontal disease, extensive disorganization of the extracellular matrix promotes the loss of adhesion between the teeth and periodontium. A previous study suggested a reduction in the area occupied by collagen in the gingiva, during the first week of periodontal disease induction, however, the remaining fibers were more compact and thicker. Therefore, it was decided to investigate which of the MMP-2, -9, -14 and RECK, an MMP inhibitor, were involved in these modifications taking place in early gingivitis induced by ligature. The results of gene expression analysis indicated no changes for RECK. MMP-14 showed a reduction at 7 days of inflammation, and there was an immediate increase in MMP-2 gene expression and enzymatic activity, apparently by the stimulation of resident cells such as fibroblasts. A peak of MMP-9 expression 5 days after ligature followed after the peak of enzymatic activity found two days earlier. This pattern was consistent with the kinetics of macrophage and neutrophil recruitment. Immunohistochemistry suggested that MMP-9 was produced by both resident and inflammatory cells. Based on this evidence, it is suggested that extracellular matrix remodeling is related to MMP-2 and -9 production and activation. This allowed us to conclude that the host inflammatory response represents a significant factor for the advance of periodontal diseases.

Key words: Matrix metalloproteinases, Inflammatory cells, Gingivitis, Periodontal disease, Experimental model

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Introduction

Periodontal diseases represent a group of lesions affecting human dentition, which might result in the loss of teeth (Pihlstrom et al., 2005). Extensive modifications result from an unbalanced immune response against infectious agents in the microbial dental plaque. In this process the production and activation of a group of enzymes named matrix metalloproteinases (MMPs) can be recognized, which are able to increase tissue remodeling, in addition to other non-specific proteases, various chemokines and nitric oxide (Reynolds et al., 1994; van der Zee et al., 1997; Okada and Murakami, 1998; Ozmeric, 2004).

The MMP family comprises approximately 25 enzymes whose activity depends on zinc and calcium ions. They can be found in secreted form or anchored to the plasma membrane, and are normally produced as zymogens. Activation usually occurs after removal of the pro-domain by proteolysis. It has been found that the MMPs regulate various inflammatory or tissue remodeling processes, and it has been suggested that they take part in the early evolution of the immune system (Sternlicht and Werb, 2001). Diverse categories of diseases are associated with increases in MMP expression, justifying its classification as an important inflammatory marker. Moreover, it is possible to observe a reduction in the expression of MMPs by inhibitory molecules. RECK is one such inhibiting protein, and was described as a key regulator of MMP-2, -9 and -14 activity during vasculogenesis and tumor progression (Takahashi et al., 1998; Oh et al., 2001).

Some gingival microorganisms, such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, are capable of producing proteases that damage the host tissue, especially collagen fibers in gingival connective tissue. However, such microbial proteases were suggested to have a secondary participation in collagen degradation, which seems to

depend mostly on enzymes produced by the host cells (Lee et al., 1995; Madianos et al., 2005; Garlet et al., 2006). The evidence of the importance of MMPs in periodontal destruction are consistent, and they have been supported by a number of studies. Among the evidence, it is important to mention that gingival cells produce high levels of collagenases in culture; there is a predominance of active collagenase in the fluid of periodontal pockets; and MMP mRNAs are expressed by cells obtained from periodontal injuries (Gibson and Fullmer, 1966; Golub et al., 1990; Ryan and Golub, 2000). Each cell type in periodontal tissue is capable of producing a different battery of MMPs when stimulated by cytokines, derivatives of arachidonic acid or growth factors. Gingival fibroblasts were described as efficient sources of collagenases. Furthermore, neutrophils, macrophages and epithelial cells can produce a great variety of MMPs which, once activated, will decrease epithelial adhesion to the connective tissue (Mäkelä et al., 1994; Hannas et al., 2007).

Considering the importance of MMP activity in gingival physiology and bearing in mind that the early stages of gingivitis involve extensive remodeling of both the epithelium and extracellular matrix (Silva et al., 2008), the aim of the present work was to study expression of MMP-2, -9, -14 and RECK, as well as the location of MMP-2 and -9 in adult rat gingivae, and to determine whether they would be involved in the progression of ligature-induced gingivitis in rats.

Materials and methods

Animals

Sixty adult male Wistar rats weighing approximately 120 g each were used in this study. The animals were maintained under specific pathogen free conditions, housed in groups of 5 in propylene cages, fed a standard laboratory diet and given tap water *ad libitum*. The procedures were in accordance with the ethical regulation established by the Brazilian College of Animal Experimentation and approved by the Animal Experimentation Ethics Committee of the State University of Campinas.

Early gingivitis induction and material collection

Animals were randomly divided into 12 groups of 5 individuals each. Four groups were used in each of the 3 protocols performed (RNA extraction, protein extraction and morphological analyses), in total 20 animals per experiment.

Rats were anesthetized with 80 mg/Kg ketamine hydrochloride and 10 mg/Kg xylazine hydrochloride. Ligature was set using a No. 10 cotton thread tied bilaterally around the first mandibular molars (Johnson, 1975). The animals were sacrificed 3, 5 and 7 days after ligature. Control rats had no ligatures and they were sacrificed at the end of the experiments.

The animals were killed by CO_2 inhalation. After visual examination of the gingiva, fragments of the gingival mucosa adjacent to the first molar were removed with scalpel blades and processed in accordance with the different protocols used in this work.

Oligonucleotides

Synthetic oligonucleotides were designed using the software Gene Runner (Gene Runner Version 3.05, Hastings Software Inc., Hastings, NY, USA). The mRNA sequences were obtained from the NCBI public database (http://www.ncbi.nlm.nih.gov/) and the expected amplicons were supposed to have 151 base pairs (Table 1).

Total RNA extraction, reverse transcription and real-time RT-PCR

RNA was extracted from tissues (5 animals per group, total of 40 fragments) with the RNeasy Mini kit (Qiagen, Austin, TX, USA). After quantification and analysis of integrity on 1.5% agarose gel, reverse transcription was performed using 50 ng RNA from each sample in accordance with the instructions of the MMLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA).

The complementary DNA (cDNA) was quantified and real time PCR was carried out in an ABI Prism 7000 Sequence Detection system, equipped with an SYBR Green PCR Master Mix fluorescence quantification system (Applied Biosystems, Foster City, CA, UK) for amplicon quantification. The reaction mixture contained 150 ng cDNA, 10 pmol of each forward and reverse primers and 12.5 μL SYBR Green, and was adjusted with Milli-Q water to a final volume of 25 μL. The

Table 1. Synthetic oligonucleotides and reference sequences from the NCBI public database.

Primers	Primer forward (5'-3')	Primer reverse (5'-3')	NCBI access
ß-actin	tcctgtggcatccatgaaacta	ccagggcagtaatctccttctg	NM_031144.2
MMP-2	tgcgcttttctcgaatccat	aagtgagaatctcccccaacac	NM_031054.1
MMP-9	tctctgggcgcaaaatgtg	atacgttcccggctgatcag	NM_031055.1
MMP-14	ctgtcccgataagcccaga	gggtatccgtccatcacttg	NM_031056.1
RECK	agaggtcaggctttaaaccacttg	gaacggaagcatggctaacac	XM_233371.3

reaction consisted of 40 cycles of 15 s at 95°C for cDNA denaturation, 1 min at 60°C for annealing, and 1 min at 75°C for elongation, followed by 10 min at 75°C to finish the reaction. The relative level of gene expression was calculated in accordance with the instructions of the User's Bulletin (P/N 4303859) from Applied Biosystems, using ß-actin in the sample as a reference, by the cycle threshold (Ct) method. Briefly, Ct is the point at which the exponential increase in the signal (fluorescence) crosses an arbitrary signal level (usually 10 times the background). The mean Ct values of triplicate measurements were used to calculate the expression of the target gene, with normalization to an internal control (ß-actin), using the ΔCt equation. Finally, the fold increase was calculated using the $2^{-\Delta\Delta Ct}$ equation. The fold increase represents changes in gene expression when the experimental conditions are compared with the control (non inflamed tissue). According to the criteria adopted by Hu and colleagues (2006), the differences were considered to be significant when the gene expression was increased or reduced at least two times. This means that the differential gene expression was associated with a value of fold increase higher than 2.0 or lower than 0.5. Negative controls without RNA and without reverse transcriptase were also performed. All the primer sets were tested and their efficiencies were greater than 90%. The experiments were done in triplicate and repeated 3 times.

Sequencing of the amplified fragments

The specificity of real-time RT-PCR was confirmed by sequencing the amplified fragments. The PCR products were sent to Dr Elida Paula Benquique Ojopi's group at the Department of Psychiatry of the University of São Paulo, who performed the analysis. After PCR and analysis of the reaction products on 2% agarose gel, the PCR products were precipitated with 75% isopropanol and 70% ethanol, before being transferred to the ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystem, Foster City, CA, USA). Sequencing was performed using the Big Dye reagent and the same primers as described above, but in separate reactions. The sequencing data were delivered to the present study group, who compared them with the sequences from the NCBI public database using the software CLUSTAL W (European Bioinformatics Institute, Hinxton, CB, UK [http://www.ebi.ac.uk/clustalw/]).

Zymography

Gingival fragments (5 animals per group, total of 40 fragments) were triturated in a solution containing 50 mM Tris-HCl pH 7.4, 0.2 M NaCl, 0.1% Triton, 10 mM $CaCl_2$ and 1% protease inhibitor cocktail (Sigma Chemical Co., Saint Louis MO, USA) for protein extraction. Total protein was quantified according to the method of Bradford using bovine serum albumin (Sigma Chemical Co.) as standard. The zymography essays were performed on 7.5% polyacrylamide electrophoresis gels containing 0.1% gelatin and using 20 μg protein per sample. After running, the gel was washed with a solution containing 2.5% Triton X-100 at room temperature and incubated overnight in 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl and 0.03% sodium azide at 37°C. Finally, the gels were stained with Coomassie Brilliant blue. The protein bands corresponding to gelatinolytic activity could be observed after washing the gels with a solution containing 30% methanol and 10% acetic acid. The gel was evaluated by band densitometry using the SCION IMAGE software (Scion Corporation, Frederick, MD, USA [http://www.scioncorp.com/pages/scion_image_windows.htm]). Each sample was analyzed individually and the experiments were repeated three times.

Neutrophil and macrophage counts

To count the neutrophils, gingival fragments (5 animals per group, total of 20 gingival fragments) were fixed for 12 h with 10% formaldehyde in PBS, washed, dehydrated and embedded in JB4 historesin (Leica, Nussloch, HD, Germany). Four-micrometer sections were obtained and stained with hematoxylin and eosin. The neutrophils were identified on the basis of their typical nuclear morphology, with a multi-lobed configuration. In these counts, cells cut close to the surface could not be identified, but this bias was preserved for all experimental groups, since tissue section thickness was kept the same and no variation in neutrophil size is expected to occur under the present conditions.

To count the macrophages, gingival fragments (5 animals per group, total of 20 gingival fragments) were immersed in Tissue Tek resin (Sakura Finetck, Torrance, CA, USA), frozen in liquid nitrogen and stored at -80°C. Cryostat (Microtome cryostat, Model HM505E, Microm International, Walldorf, HE, DE) sections (7 μm thick) were fixed with 4% paraformaldehyde. Endogenous peroxidase was blocked by incubating the sections with 3% hydrogen peroxide, and the nonspecific binding sites were blocked with a commercial block solution (Pierce, Rockford, IL, USA). The specimens were then incubated with mouse monoclonal anti-rat CD163 antibody, a marker of the macrophage lineage (Serotec, Kidlington, OX, UK) diluted 1:200, followed by treatment with a goat anti-mouse IgG conjugated with horseradish peroxidase (Serotec) diluted 1:500. Color development was carried out with 0.05% 3,3'-diaminobenzidine (Sigma Chemical Co.) and 0.003% hydrogen peroxide. The cell nuclei were counterstained with methyl green. Controls consisted of specimens in which the primary antibody incubation step was omitted.

Counts were made using a 10x ocular connected to a light microscope (Olympus CBA, Olympus America Inc., Center Valley, PA, USA) equipped with a 100x objective (in a total area of 0.2 mm^2). Fifteen microscopic fields of the connective tissue from each

animal were counted (contained in the area defined in Fig. 1), in total 75 fields per experimental group.

Immunofluorescence

Tissue fragments (5 animals per group, total of 20 gingival fragments) were immersed in Tissue Tek resin (Sakura Finetck, Torrance, CA, USA), frozen in liquid nitrogen and stored at -80°C. Cryostat (Microtome cryostat, Model HM505E) sections (7 μm thick) were fixed with 4% paraformaldehyde. Nonspecific binding sites were blocked with a commercial block solution (Pierce, Rockford, IL, USA). The specimens were then incubated with rabbit polyclonal anti-human MMP-2 or MMP-9 antisera (Chemicon, Temecula, CA, USA) diluted 1:100, followed by treatment with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma Chemical Co.) diluted 1:40. The cell nuclei were counterstained with 4',6-diamidino-2-phenylindole DAPI (Sigma Chemical Co.) before mounting in glycerol/phosphate-buffered saline (3:1) containing 1,4-diazabicyclo[2.2.2]-octane (Sigma Chemical Co.) as anti-fading agent. The sections were observed under fluorescence microscopy (Nikon 50i microscope, Nikon Corporation Co., Kawasaki, Kanagawa-ken, JP) equipped with mercury and halogen lamps. The images were acquired using a camera (Nikon DNX 1200F CCD, Nikon 50i microscope, Nikon Corporation Co.) and the proper software (Nikon ATC-1 software, Nikon Corporation Co.). Controls consisted of specimens in which the primary antibody incubation step was omitted. The specificity of the antibodies against MMP-2 and MMP-9 were assessed by Western blotting.

Statistical analysis

Statistical analysis was performed by Analysis of Variance (ANOVA) (a priori) and Tukey (a posteriori), using the SYSTAT 10 software (SYSTAT Version 10, Systat Software Inc., San Jose, CA, USA), to compare the different days of periodontal disease induction. Differences were considered to be statistically significant with a p value < 0.05.

Results

Gingivitis induction

Gingivitis induction due to ligature produced an inflammatory response in the gingival tissue. Clinical signs such as edema, redness and increased spaces between the gingiva and the tooth were apparent after 7 days of gingivitis induction.

MMPs and RECK mRNA levels

Analysis of RECK mRNA expression showed no significant difference between the control and diseased samples. MMP-2, -9 and -14 expression was modulated by inflammation (Fig. 2). MMP-2 expression was significantly increased 5 days after ligature and continued to increase up to 7 days, reaching a 6-fold higher expression when compared with the control. The increase in MMP-9 mRNA occurred by the 3rd day after ligature and remained high until 7 days of inflammation, with a maximum increase at 5 days, when the MMP-9 mRNA levels reached a 10-fold higher expression when compared with the control. On the other hand, MMP-14 showed a significant decrease at 7 days with an approximately 3-fold reduction. Expression of the endogenous control ß-actin showed no differences.

Characterization of the amplified fragments

The construction of dissociation curves after the real time reactions showed specific amplifications for all the

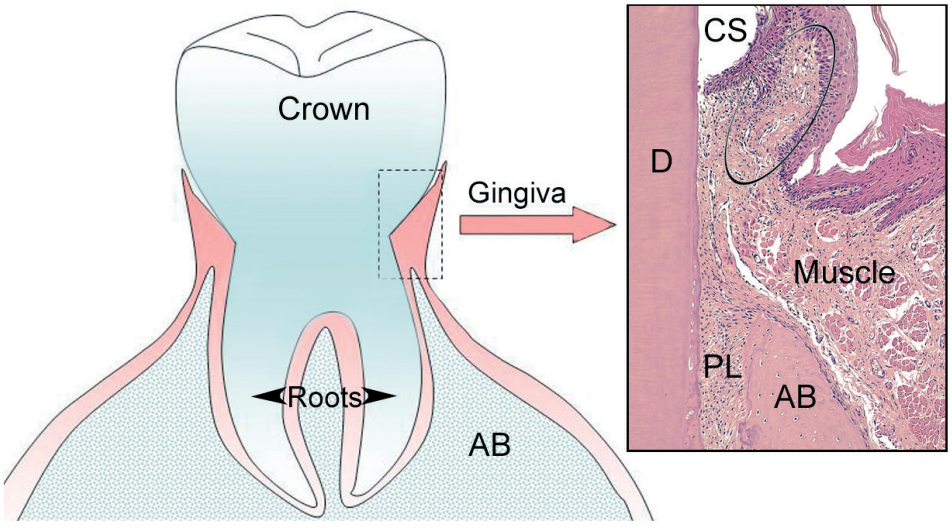

Fig. 1. Schematic drawing of a tooth and adjacent tissues, indicating the area of the gingival employed in this study. The inset corresponds to a hematoxylin-and-eosin stained section of the corresponding area, indicating the area considered for cell counts and analysis of the immuno-histochemistry, and sampled for measurements of gelatinase activity and mRNA content (ellipse). AB, alveolar bone; D, dentin; CS, crevicular space; PL, periodontal ligament.

genes studied. Sequencing confirmed the specificity of the reactions for all mRNAs, demonstrating a minimal similarity index of 93% when comparing the sequences with the NCBI notations (Table 2).

Gelatinolytic activity

Zymography using gelatin as substratum indicated changes in the activity of MMP-2 and -9 (Fig. 3A). To improve the gelatinolytic activity evaluation, an index comparing all experimental times with respect to the control was established.

Three forms of MMP-2 (latent, intermediate and active) were found in the periodontal tissue extracts (Fig. 3B), with a progressive increase in MMP-2 activity up to 7 days of inflammation for all of the 3 forms, especially the active one. Latent MMP-2 showed an approximate 4-fold increase after 7 days of inflammation. Intermediate MMP-2 showed an approximate 2-fold increase after 5 and 7 days of inflammation. The active form of MMP-2 showed an approximate 7-fold increase by day 5 and a 9-fold increase after 7 days of inflammation.

Three forms of MMP-9 (latent lipocalin-conjugated, latent and active) were also found (Fig. 3C). The amount of both of the latent forms showed a transient increase

Table 2. Dissociation temperatures and similarity index when comparing the amplicons with the NCBI notations.

Primers	Dissociation temperature (°C)	Similarity (%)[a]	NCBI access
ß-actin	81	100 / 100	NM_031144.2
MMP-2	83	93 / 93	NM_031054.1
MMP-9	85	99 / 97	NM_031055.1
MMP-14	83	100 /100	NM_031056.1
RECK	77	100 / 98	XM_233371.3

[a]: Similarity index for the amplicons from the forward and the reverse primers, respectively.

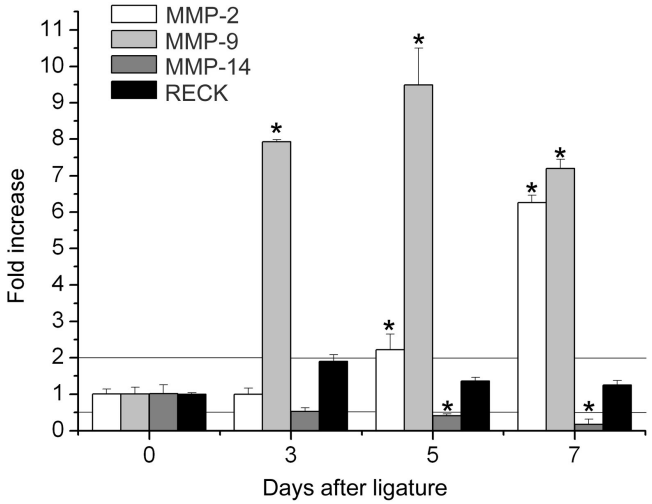

Fig. 2. Real-time PCR analysis. Changes in the gene expression for MMP-2, -9, -14 and RECK in the diseased gingival tissue. The values for fold increase were calculated based on the tissue without inflammation. ß-actin was used as endogenous control. The asterisks indicated two-fold variations above or below the control levels.

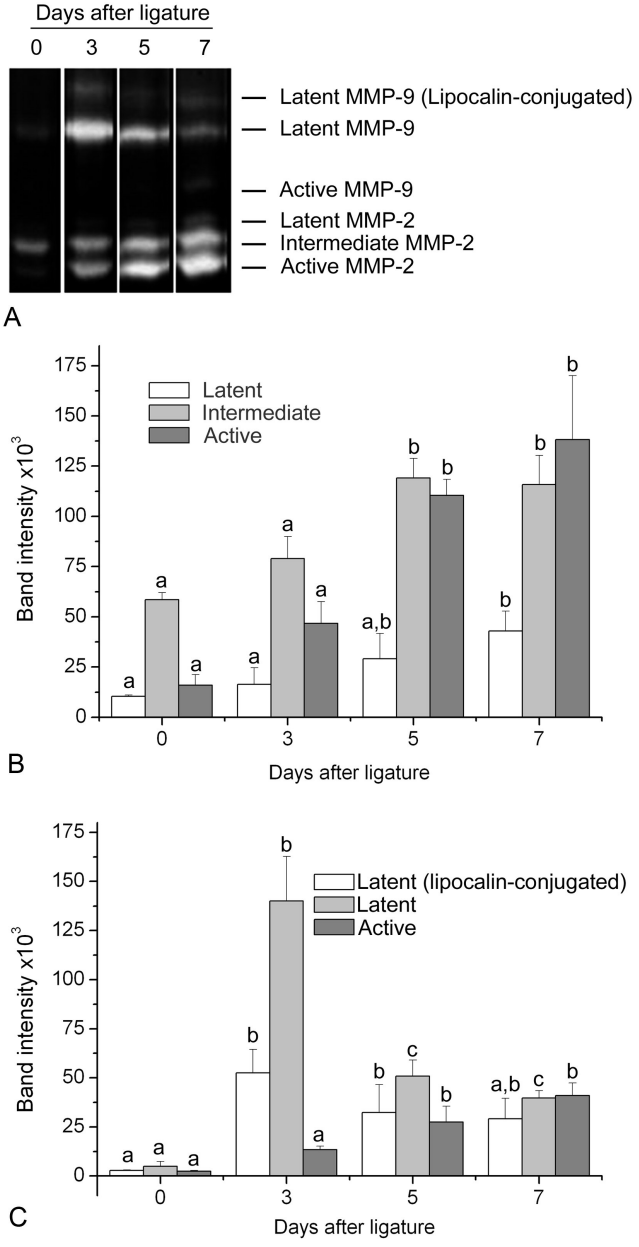

Fig. 3. Gelatinolytic activity for MMP-2 and -9 in the diseased gingival tissue. **A.** Zymography gel with the bands corresponding to MMP-2 and MMP-9. MMP-2 **(B)** and MMP-9 **(C)** activity in gingival tissue in response to ligature. Different letters indicate statistical differences for a given enzyme between the groups ($p<0.05$; Tukey).

after 3 days of inflammation while the active form showed a progressive increase up to 7 days of inflammation. The latent lipocalin-conjugated form showed an approximate 18-fold increase by day 3 and 11-fold by day 5, returning to the basal level after 7 days of inflammation. The latent isolated form showed an approximate 29-fold increase by day 3, a 10-fold increase by day 5 and an 8-fold increase after 7 days of inflammation. The active form showed an approximate 11-fold increase by day 5 and a 16-fold increase after 7 days of inflammation.

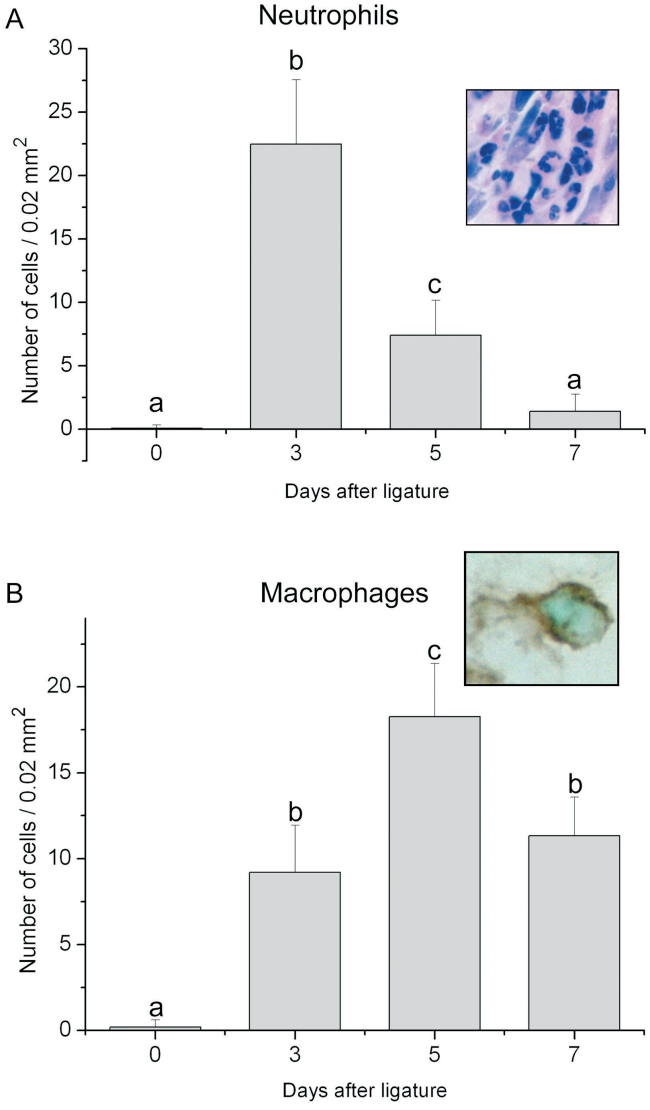

Fig. 4. Migration of inflammatory cells to the diseased gingival tissue. **A.** Dynamics of neutrophil infiltration during disease progression. Different letters represent different values ($p<0.05$; Tukey). **B.** Dynamics for macrophage infiltration during inflammation progression. Different letters indicated statistical differences between the groups ($p<0.05$; Tukey).

Inflammatory cell migration

A significant increase in the number of inflammatory cells in the gingival tissue with periodontal disease induction was observed. There was a migratory profile characterized by the accumulation of neutrophils, especially after 3 days of inflammation (Fig. 4A). An accumulation of macrophages was observed 2 days later, at day 5 (Fig. 4B).

Location of MMP-2 and MMP-9

MMP-2 and -9 were detected in gingival epithelial cells, with evident labeling of the basal cell layer. These MMPs were also present in connective tissue cells, probably inflammatory cells and fibroblasts.

MMP-2 was more evident in the inflamed tissue between 5 and 7 days. In the connective tissue, the stained cells were fusiform and presented an expanded cytoplasm, probably indicating an increase in fibroblast activity (Fig. 5).

MMP-9 was observed especially at days 3 and 5 in the basal epithelial cells, connective tissue cells, and more conspicuously, inside the blood vessels. The cells in the connective tissue were activated fibroblasts or macrophages. Inside blood vessels the cells presented the characteristic rounded morphology of polymorphonuclear cells (Fig. 5).

Although the immunofluorescence pattern was preserved throughout the experimental period, the number of stained cells increased in the course of inflammation, indicating a higher amount of MMP-2 and -9 in the inflamed tissue, a finding confirmed by immunoblots for MMP-2 and -9 (data not shown).

Discussion

The results of this study indicate the involvement of MMP-2 and -9 in the early tissue modifications associated with gingivitis.

The use of cotton thread for mandibular molar ligation, as described by Johnson (1975), was efficient, inducing periodontal disease in the rat model. This ligature favored the formation of bacterial dental plaque with sequential Gram-positive and Gram-negative populations (result not shown) and induced an inflammatory response with edema, redness, and increased spaces between the gingival and the tooth, reproducing many aspects of the human disease (Payne et al., 1975).

MMP-2 and -9 showed transient increase in mRNA content and enzyme activity during the progression of gingivitis. It is currently thought that leukocyte migration to the inflammation site significantly alters the production, secretion and/or activation of MMPs. The neutrophils are the host's first line of defense. They represent 50-70% of the total leukocyte population and are the first cells to leave the circulation and reach the affected tissue. At the inflammation site, the neutrophils

become activated and release a series of elements stored in their pre-formed granules, including some MMPs (Westerlund et al., 1996). Macrophages arrive later at the inflammatory site, and are capable of responding to a number of signals (Kolaczkowska et al., 2006).

MMP-2 presented a continuous increase up to 7 days of inflammation. According to the enzyme location, it was concluded that its major source is resident cells, such as epithelial cells, fibroblasts and/or macrophages at the inflammatory site. However, the most significant increase in mRNA expression, proteolytic activity and intensity of immunostaining for MMP-2 starts at 5 days of inflammation, coincident with a peak in macrophages in the tissue. Thus, macrophage recruitment and the

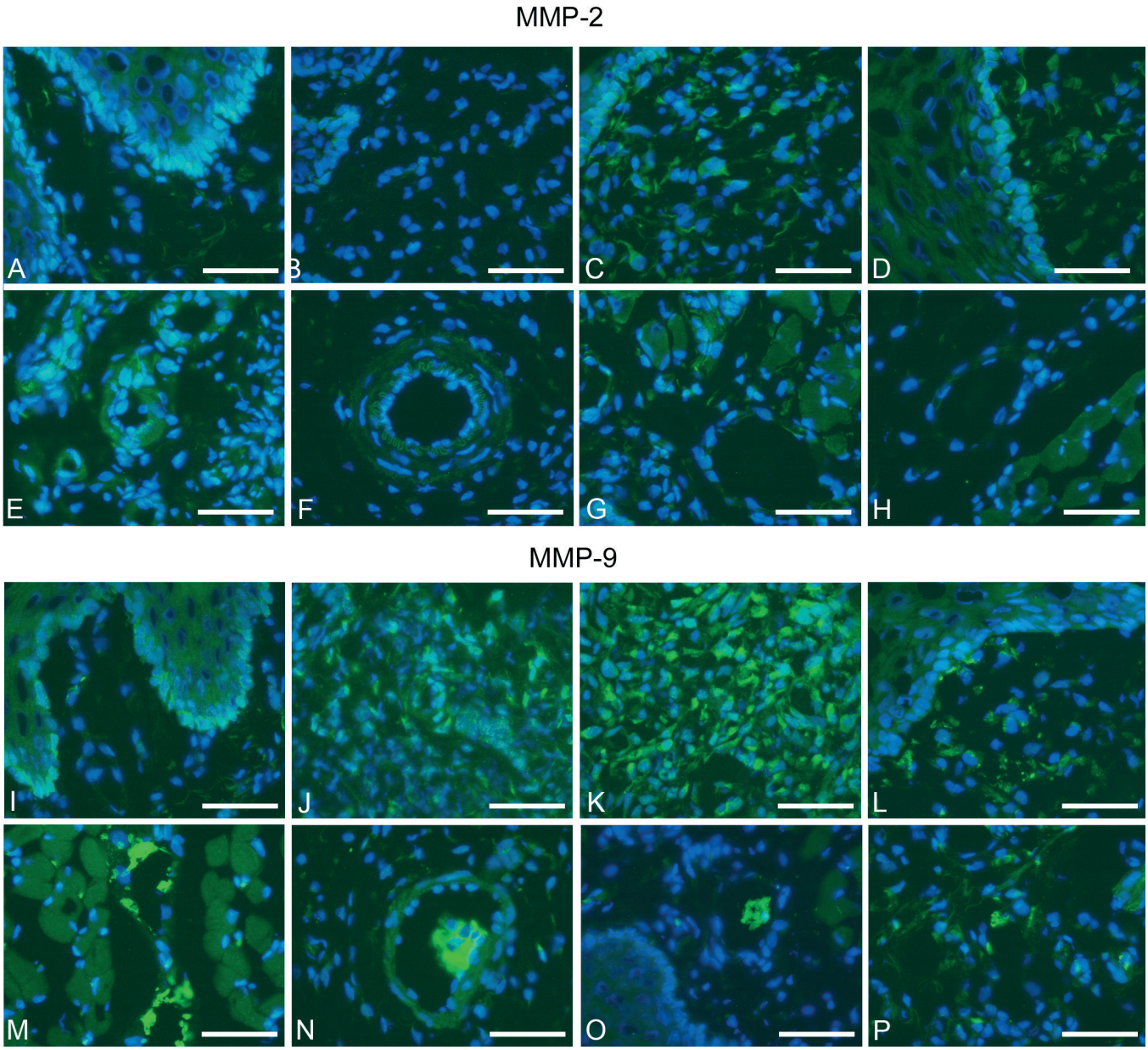

Fig. 5. Immunofluorescence staining for MMP-2 (**A-H**) and MMP-9 (**I-P**) (green fluorescence) in gingival sections showing the epithelial and connective tissues (**A-D** and **I-L**) and blood vessels (**E-H** and **M-P**). **A, E, I and M.** Control animals (Day 0). **B, F, J and N.** Animals after 3 days of ligature. **C, G, K and O.** Animals after 5 days of ligature. **D, H, L and P.** Animals after 7 days of ligature. The nuclei are evidenced by the blue fluorescence of DAPI. MMP-2 staining was stronger in the diseased tissue between 5 and 7 days, especially in the fusiform cells dispersed in the connective tissue. MMP-9 staining was stronger in the diseased tissue between 3 and 5 days in the basal epithelial cells, connective tissue cells, and, more conspicuously, in rounded cells, likely polymorphonuclear cells inside the blood vessels. Bars: 25 μm

increased MMP-2 content seem closely related to each other.

Such an association was not so simple for MMP-9. MMP-9 production is associated with leukocytes, especially neutrophils. In contrast to MMP-2, which is constitutively secreted, MMP-9 undergoes regulated secretion from pre-formed granules in response to proper stimulation (Westerlund et al., 1996; Kolaczkowska et al., 2006). Therefore, it is more realistic to think about changes in MMP-9 expression and activity as a direct result of inflammation. In the present study, maximum enzymatic activity was observed at 3 days and maximum mRNA expression at 5 days after ligature. These findings led us to ask how the amount of protein could precede an increase in gene expression. Two possibilities were considered (i) the cells that secrete MMP-9 protein at day 3 and those that showed an increment in gene expression are not necessarily the same, and/or are responding to two different signaling systems and (ii) the protein could be brought to the inflamed gingival tissue by inflammatory cells, mostly neutrophils, and this preceded the increase in gene expression by different cells two days later. The present study showed that maximum protein activity coincided with the peak of neutrophil migration and the maximum mRNA expression paralleled the peak of macrophage recruitment. Moreover, zymographs showed a MMP-9 band characteristic of the latent form associated with lipocalin. This form is described as neutrophil gelatinase-associated lipocalin (Bu et al., 2006; Westerlund et al., 1996) and it represents strong evidence of significant neutrophil participation in the process of MMP-9 release in gingivitis.

The immunohistochemistry results for MMP-9 also corroborate the hypothesis that migratory cells carry MMPs to the inflamed gingiva. Based on the observation that the labeled cells at 3 days after ligature were rounded and inside blood vessels, it is absolutely coherent to believe in a contribution from polymorphonuclear cells to the significant increase in the total amount of MMP-9. As suggested by the maximum migration of neutrophils at 3 days after ligature, the present findings are definite in pointing to these cells as the major source of "ready-to-use" MMP-9, as they store proteases, which are used to degrade the extracellular matrix during tissue barrier penetration, tissue remnant removal and tissue remodeling (Owen and Campbell, 1995). It is also important to stress that mature neutrophils are not able to synthesize proteases. Therefore, the neutrophil proteases, including MMP-9, are totally pre-formed in the early stages of cell differentiation and stored in cytoplasmic granules (Dewald et al., 1982; Hasty et al., 1986; Boxer and Smolen, 1988; Takahashi et al., 1988). The present results firmly suggest that the large increase in MMP-9 activity at 3 days is derived from migratory neutrophils, their activation and degranulation, in response to both signals produced by the bacterial dental plaque cells and inflammatory mediators released by the gingival cells.

It is also important to note that the MMP-9 mRNA reach the maximum level at 5 days after ligature, coinciding with the peak of macrophage migration. This indicates that such cells might become activated and start to express the MMP-9 gene after being stimulated by inflammatory signals produced by other cells in the gingival tissue (Bartold and Narayanan, 2006). Immunohistochemistry for MMP-9 also showed some cells with intense cytoplasmic staining in the gingival connective tissue at 5 and 7 days after ligature, again suggesting the accumulation of this enzyme inside the cells in later phases of gingivitis.

Another relevant aspect of MMP biology is related to the process of enzyme activation. The present results showed that the active MMP-2 was the most up-regulated form when compared with the intermediary and latent forms, reaching a 9-fold increase 5 and 7 days after ligature. It suggests a continuous production and activation of MMP-2. The continuity of the process indicates the massive production of MMP-2 by resident cells of the gingival tissue, such as fibroblasts, resident macrophages and epithelial cells. The active form of MMP-9 presented a 14-fold increase 7 days after ligature. However, the formation of the active form was followed by a reduction in the latent forms, indicating that a large fraction of the enzyme production was time-restricted. It also corroborates the massive increment of MMP-9 content associated with migratory cells.

The unbalanced production of inflammatory mediators seems to be responsible for the increase in the synthesis and accumulation of extracellular matrix components. As a matter of fact, increased production of TGF-ß and IL-6 seems to be characteristic of gingival fibromatosis, and this is correlated with the decreased expression of MMPs during inflammation. In the present model we have demonstrated that extracellular degradation became less pronounced at 7 days after ligature, resulting in thicker and more compact collagen fibers in the gingival connective tissue (results not shown).

The present results indicate that RECK is not modulated in the early progression of gingivitis, at least for the period evaluated, and using the ligature model. Furthermore, they indicate that MMP-14 was the only enzyme analyzed which showed reduced expression within the first week after ligature. This finding is interesting because MMP-14 (or MT-MMP1) is described as an activator of some proteases (Murphy et al., 1999). Since MMP-14 is bound to the cell surface, it is likely that keeping its expression at low levels is a mechanism for controlling the progression of inflammation.

This scenario might consist of the host response trying to control the inflammatory process, or result from a frustrated attempt to heal. Moreover, a significant reduction in mRNA expression for MMP-2 and -9 in later stages of periodontal disease (15 or 30 days) was also observed, when the arrangement of collagen was characteristic of fibrosis (Silva et al., 2008).

Considering the scarce information about MMP location (Dahan et al., 2001) and the absence of a well characterized gingivitis model, sequential analysis of the ligature models in rats appears to be timely and promising. Taken together, the current data allow one to conclude that (i) gingivitis is clearly associated with transient changes in the expression of MMP-2 and MMP-9; (ii) that these enzymes are produced and/or secreted by resident gingival cells and migrating inflammatory cells, such as neutrophils and macrophages; (iii) that the gelatinolytic activity of MMP-2 and -9, as well as the mRNA expression for MMP-14 are reduced 7 days after ligature, leading to the onset of a fibrotic process; and (iv) that RECK expression is not modulated up to the seventh day of gingivitis induction. An important aspect to consider is that gelatinase activity (MMP-2 and -9) on fibrillar collagens is secondary to cleavage by collagenases. Investigation of the expression and activity of these enzymes is thus required for a better understanding of morphological epithelial and connective tissue modifications in the early phases of gingivitis.

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