

The influence of type I diabetes mellitus on the expression and activity of gelatinases (matrix metalloproteinases-2 and -9) in induced periodontal disease

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Background and Objective: Periodontal disease corresponds to a group of lesions that affect the tooth-supporting tissues present in the dental follicle. Although bacterial plaque is important, the immune response also contributes to the destruction of periodontal tissues. Diabetes mellitus is closely associated with the development, progression and severity of periodontal disease because it not only affects extracellular matrix organization but also the tissue response to inflammation. The objective of the present investigation was to study the influence of diabetes on experimental periodontal disease by evaluating the degradation of extracellular matrix through the analysis of matrix metalloproteinase (MMP)-2 and MMP-9 expression and activity, using immunofluorescence, zymography and real-time reverse transcription-polymerase chain reaction.

Material and Methods: Wistar rats were divided into normal and diabetic groups and evaluated 0, 15 and 30 d after the induction of periodontal disease by ligature.

Results: MMP-2 and -9 were detected in epithelial cells, in the blood vessel endothelium and in connective tissue cells. The same profile of enzymatic expression of MMP-2 and -9 was observed in normal and diabetic animals, with a peak in activity at day 15 of inflammation. However, in diabetic animals, MMP-2 gelatinolytic activity was reduced after the inflammatory stimulus, whereas that of MMP-9 was increased. MMP-2 gene expression decreased with inflammation in both normal groups and groups with diabetes. In contrast, MMP-9 expression increased in normal animals and decreased in diabetic animals after inflammation.

Conclusion: The results suggest the involvement of MMP-2 and -9 in the dynamics of periodontal disease and that variation in their expression levels results in differences in tissue organization and wound healing in normal and diabetic animals.

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A common consequence of the inflammatory response to bacterial dental plaque is periodontal disease, which promotes progressive destruction of the periodontal tissues (1,2). Bacteria produce proteolytic enzymes that damage the periodontal tissues, but this activity is not sufficient to cause periodontal disease. It is believed that the host immune response to bacterial dental plaque and its products determines disease susceptibility by increasing the secretion of inflammatory cytokines (3). Degradation of periodontal tissue seems to result from the uncontrolled release of chemokines and/or proteases, in addition to their activators and inhibitors, by resident cells in the proximity of the infection or by recruited inflammatory cells (4–10).

Among the proteases, matrix metalloproteinases (MMPs), which are characterized by their dependence on metal ions (usually zinc or calcium) and their capacity to degrade extracellular matrix proteins such as collagen, elastin, laminin, fibronectin and proteoglycans during different physiological and pathological processes, are major factors involved in the tissue remodeling/destruction in periodontal disease (11,12). Among MMPs, gelatinases (MMP-2 and MMP-9) have been extensively studied because they are responsible for the proteolytic degradation of extracellular matrix and contribute to tumor invasion and metastases as a result of their capacity to degrade type IV collagen, laminin and other basement membrane components (13–15), including cancers of the oral mucosal membrane (16–18). The proteolytic activity is a key regulator of chemokine activity during inflammation. In this respect, interleukin-8, a neutrophil chemoattractant, induces the secretion of MMP-9 (19), which, in turn, potentiates the biological activity of interleukin-8 by at least 10-fold. This results in efficient amplification of neutrophil influx to the sites of inflammation (20).

By increasing the organism's susceptibility to bacterial and opportunistic infections (21), diabetes mellitus is a prognostic factor for periodontal disease. Diabetes mellitus is a chronic disease mainly characterized by the insufficient production of insulin or by

an inadequate response to it, which leads to alterations in the metabolism of proteins, carbohydrates and fatty acids (22–24). Studies have shown that the levels of expression of MMP-2 and MMP-9 are increased in diabetics (20,25). Diabetes might also disturb the balance between MMPs and their inhibitors that is essential for wound healing. In fact, excess proteolysis might be the cause of delayed healing of dermal ulcers (26), as well as being responsible for the occurrence of deeper and more frequent periodontal pockets in diabetic patients (27,28).

MMP-9 is a permissive factor for further insulin degradation, which is probably associated with the vascular complications resulting from the lack of this hormone, and high glucose levels also contribute to the activation of latent MMP-9 secreted by leukocytes. In addition, MMP-9 also promotes the proliferation of T cells, thus further contributing to inflammation in type I diabetics (20).

In a previous morphological study we showed that diabetes causes degeneration of the dermal papilla, increased migration of inflammatory cells and accumulation of collagen in gingival connective tissue (29).

Considering that diabetes mellitus is associated with the aggravation of periodontal disease and that gingival connective tissue is primarily affected, the aim of the present investigation was to study how periodontal disease is influenced by diabetes mellitus by determining the pattern of gelatinase expression and function. We used zymography, immunofluorescence and real-time reverse transcription-polymerase chain reaction (RT-PCR) to analyze the activity, location and expression of MMP-2 and MMP-9, respectively, in normal and streptozotocin-treated rats with experimentally induced periodontal disease.

Material and methods

Animals

Seventy-eight adult male albino Wistar rats (*Rattus norvegicus*), weighing \approx 120 g (Centro Multidisciplinar para Investigaç o Biol gica, Universidade Estadual de Campinas, SP, Brazil),

were used in the experiments (13 animals per experimental group). Animals were placed in propylene cages and kept under specific pathogen-free conditions on a 12-h light–dark cycle, with water and granulated rodent chow (Labina/Purina, Cascavel, Brazil) available *ad libitum*. The experimental procedures were performed in accordance with the ethical guidelines established by Col gio Brasileiro de Experimenta o Animal and were approved by the Ethics Committee on Animal Experimentation of the Institute of Biology, Universidade Estadual de Campinas (protocol number 649-1).

Induction of diabetes

Diabetes mellitus was induced with the diabetogenic agent, streptozotocin (65 mg/kg of body weight; Sigma Chemical Co., St Louis, MO, USA) (30) diluted in 0.3 mL of 1 M citrate buffer, pH 4.4. The drug was injected intraperitoneally once a week for 3 wk. Glycosuria was measured with a Glucotest strip (Roche Diagnostics, Mannheim, Germany), and glycemia was evaluated at regular intervals using the Accu-Chek Active monitoring system (Roche Diagnostics), according to the manufacturer's instructions. Normal animals were inoculated with equivalent volumes of citrate buffer using the same route and doses and submitted to the same tests.

Induction of periodontal disease by ligature

Rats were anesthetized with 80 mg/kg of ketamine hydrochloride and with 10 mg/kg of xylazine hydrochloride. Periodontal disease was induced with no. 10 cotton thread tied bilaterally around the first lower molar (31). Diabetic rats were subjected to ligature 10 d after the third streptozotocin injection.

Experimental design and tissue sampling

The rats were randomly divided into two groups (normal and diabetic; $n = 39$ in each group), of which 13 rats from each group were killed at 0-, 15- and 30-d time-points after ligature. Controls were normal rats not subjected to ligature.

The animals were killed by CO₂ inhalation. Immediately after death, fragments of the gingival mucosa adjacent to the first molar were removed with scalpel blades and frozen in liquid nitrogen or processed for light microscopy.

Immunofluorescence

Tissue fragments (taken from four animals in each group) were immersed in Tissue Tek[®] resin (Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen and stored at -80°C. Cryostat (Microm model HM505E, Walldorf, Germany) sections (7 µm thick) were fixed with 4% paraformaldehyde. Nonspecific binding sites were blocked with a commercial blocking solution (Pierce, Rockford, IL, USA). The specimens were then incubated with rabbit polyclonal antihuman MMP-2 and MMP-9 sera (Chemicon, Temecula, CA, USA), diluted 1 : 100, followed by treatment with goat antirabbit IgG conjugated with fluorescein isothiocyanate (Sigma) diluted 1 : 40. The cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (Sigma) before mounting in glycerol/phosphate-buffered saline (3 : 1) containing 1,4-diazabicyclo[2.2.2]-octane (Sigma) as an antifading agent. The sections were observed under a Nikon 50i microscope (Kawasaki, Japan) equipped with mercury and halogen lamps. The images were acquired using a Nikon DNX 1200F CCD (Kawasaki, Japan) camera and the ATC-1 program (Cambridge, MA, USA). Controls consisted of specimens in which the primary antibody incubation step was omitted. The specificities of the antibodies against MMP-2 and MMP-9 were assessed by western blotting.

Zymography

Gingival fragments (three animals per group) were triturated in a solution of 50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 0.1% Triton, 10 mM CaCl₂ and 1% protease inhibitor cocktail (Sigma) for protein extraction. Total protein was quantified according to the method of Bradford (32) using bovine serum albumin (Sigma) as a standard. The zymog-

raphy assays were performed on 7.5% polyacrylamide electrophoresis gels containing 0.1% gelatin and using 20 µg of protein per sample. After electrophoresis, the gels were washed with 2.5% Triton X-100 at room temperature and incubated overnight in a solution of 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 were 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 program. Each sample was analyzed individually and the experiments were repeated three times.

Oligonucleotides

The MMP-2, MMP-9 and α -actin (an endogenous reaction control whose expression is not altered in the presence of periodontal disease) mRNA sequences were obtained from the National Center for Biotechnology Information public database. Synthetic oligonucleotides were designed using the GENE RUNNER AND ABI PRISM 7000 Sequence Detection System software (Amersham Bioscience, Warrington, UK) and used as primers for real-time RT-PCR (Table 1).

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

RNA was extracted from tissues (six animals per group) using the RNeasy Mini[®] kit (Qiagen, Austin, TX, USA). After RNA quantification and analysis of RNA integrity on a 1.5% agarose gel, reverse transcription was performed using 0.63 µg of RNA from each sample, according to the instructions of the Molone Murine Leukaemia Virus Reverse Transcriptase kit (Invitrogen,

Carlsbad, CA, USA). The cDNA was quantified in an ultraviolet spectrophotometer by determining the absorbance at 260 nm, and the 260/280 nm absorbance ratio was calculated. Real-time PCR was carried out in an ABI Prism 7000 Sequence Detection system equipped with a SYBR Green PCR Master Mix[®] fluorescence quantification system (Applied Biosystems, Warrington, UK) for amplicon quantification. The reaction mixture contained 150 ng of cDNA, 10 pmol of forward and reverse primers and 12.5 µL of SYBR Green, and was adjusted with Milli-Q water to a final volume of 25 µL. The reaction cycle consisted of 40 cycles of 15 s at 95°C for cDNA denaturing, 1 min at 60°C for annealing and 1 min at 75°C for elongation, followed by 10 min at 75°C to finalize the reaction. The relative level of gene expression was calculated according to the instructions of the User's Bulletin (P/N 4303859) from Applied Biosystems, using α -actin in the sample as a reference, by the cycle threshold method. Briefly, cycle threshold is the point at which the exponential increase in the signal (fluorescence) crosses a somewhat arbitrary signal level (usually 10 times higher than the background). The mean cycle threshold values of triplicate measurements were used to calculate the expression of the target gene, with normalization to an internal control (α -actin), using the Δ Ct formula, according to the User's Bulletin (Applied Biosystems). Negative controls without RNA and without reverse transcriptase were also included. The experiments were run in triplicate and repeated three times.

Sequencing of the amplified fragments

The specificity of real-time RT-PCR was confirmed by sequencing the

Table 1. Synthetic oligonucleotides used as primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number (NCBI)
α -Actin	tctgtggcatccatgaaacta	ccagggcagtaatctctctg	NM_031144.2
MMP-2	tgcgctttctcgaatccat	aagtgagaatctcccccaacac	NM_031054.1
MMP-9	tctctgggcgcaaaatgtg	atagttcccggctgatcag	NM_031055.1

MMP, matrix metalloproteinase.

NCBI, National Center for Biotechnology Information.

amplified fragments. After PCR and analysis of the reaction products on 2% agarose gels, the PCR products were precipitated with 75% isopropanol and 70% ethanol before being transferred to the ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed using the Big Dye[®] reagent and the same primers as described above, but in a separate reaction.

Statistical analysis

Statistical analysis was performed by two-way analysis of variance using the MINITAB 14 software (Madrid, Spain). The *F*-test was used to determine differences between the diabetic and nondiabetic groups relative to the different days of induction of periodontal disease. A *p*-value of < 0.05 was considered to be statistically significant.

Results

The induction of periodontal disease caused by ligature produced an inflammatory response in the gingival tissue, presenting clinical signs such as edema, redness and loss of adhesion to the tooth. These were seen in the experimental diabetic and normal groups, but appeared to be aggravated in diabetics.

Location of MMP-2 and MMP-9

In the gingival tissue of normal and diabetic animals, MMP-2 and -9 were clearly stained in epithelial cells and basal layer cells (Fig. 1). Furthermore, these MMPs were present in the vessel endothelium and connective tissue cells, probably in inflammatory cells and fibroblasts. Although this immunofluorescence pattern was observed throughout the experimental period, the number of stained cells increased with inflammation, resulting in a higher amount of MMP-2 and -9 in inflamed tissue (Fig. 1), a finding confirmed by western blots for MMP-9 (data not shown).

Gelatinolytic activity

Latent (72 kDa), intermediate (68 kDa) and active (62 kDa) forms of

MMP-2 were found in the periodontal tissue extracts and their amount was higher in normal rats than in diabetic rats (Fig. 2). The amount of the three MMP-2 forms showed a transient increase after 15 d of inflammation in both normal and diabetic rats.

To improve the accuracy of evaluation of gelatinolytic activity, an index comparing the results at all experimental time-points with respect to the control was established. Latent MMP-2 showed a significant transient increase after 15 d of inflammation and a significant decrease after 30 d of inflammation. Diabetic rats showed a significant decrease in the amount of latent MMP-2 compared with controls (Fig. 2A). A significant transient increase of the intermediate form of MMP-2 was observed by day 15 of inflammation in normal rats, which returned to control levels by day 30. Diabetes caused a significant decrease in intermediate MMP-2 in noninflamed gingiva. Inflammation caused a significant transient increase in the amount of intermediate MMP-2 in diabetics (Fig. 2B).

A significant transient increase of active MMP-2 was also observed during inflammation, followed by a return to control levels. Diabetes caused a marked 92% decrease in active MMP-2 in noninflamed gingiva. Although the significant transient increase observed for nondiabetics was also noted in diabetic animals, the levels of MMP-2 in nondiabetic rats remained extremely low (Fig. 2C).

The profile of MMP-9 (82 kDa) activity was similar to that of MMP-2, with a transient increase observed after 15 d of inflammation. MMP-9 activity was significantly lower in diabetic rats. However, during inflammation, diabetic rats presented an MMP-9 activity \approx 13-fold higher than that of the noninflamed diabetic rats (Fig. 2D).

MMP-2 and MMP-9 mRNA levels

MMP-2 mRNA expression (Fig. 3A) showed no significant difference between normal and diabetic rats. However, significant decreases took place during inflammation.

MMP-9 gene expression was markedly (156%) higher in diabetic rats

compared with the controls. Inflammation had different effects on MMP-9 expression in normal and diabetic rats. Whereas a significant increase was observed only after 30 d of inflammation in normal rats, inflammation caused a marked reduction of MMP-9 expression in diabetic rats (Fig. 3B).

Discussion

There is a good correlation between diabetes mellitus and the prevalence and severity of periodontal disease (10,21,27,33–35). In addition, type I diabetes mellitus has been associated with diverse alterations in the immune response, inflammation and extracellular matrix synthesis (20,36).

Different MMPs, especially MMP-2 and MMP-9, have been implicated in the destruction of periodontal tissue (11). In fact, significant concentrations of MMP-2 and -9 have been demonstrated in both the gingival crevicular fluid and the periodontal tissues of patients and animals with periodontal disease (37–39), and MMP-9 levels in gingival crevicular fluid have been used to determine the stage of periodontitis (40). Other studies have shown that collagen degradation caused by the presence of some MMPs, especially gelatinases and collagenases, can be regulated by periodontal bacteria and their products. Dental plaque and its metabolites, enzymes, toxins, bacterial colonization factors and bacterial components, such as lipopolysaccharide, are able to regulate some proteinases produced by host cells and also to induce the production of cytokines involved in the recruitment of inflammatory cells to the site of inflammation (3,34,41).

The use of cotton thread to make the ligature around the inferior molar, as described by Johnson (31), was efficient in experimental periodontal disease induction, using the rat model. This ligature favored the formation of bacterial plaque and induced an inflammatory response, reproducing human periodontal disease. In the present study, periodontal disease was characterized by the appearance of clinical signs of a gingival inflammatory response, with edema, redness and loss of gingival adhesion to the tooth. In the

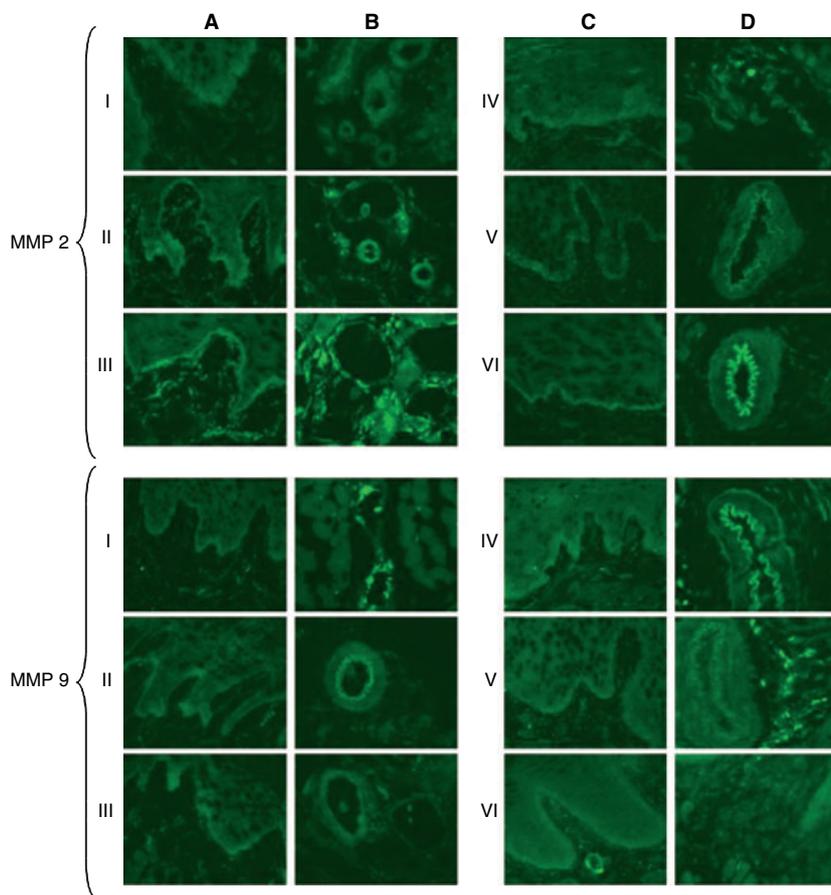


Fig. 1. Immunofluorescence staining for matrix metalloproteinase (MMP)-2 and MMP-9 (green staining) in gingival tissue sections from normal rats (columns A and B) and from diabetic rats (columns C and D). Rows I, control; rows II, normal rats after 15 d of inflammation; rows III, rats after 30 d of inflammation; rows IV, diabetic rats with no inflammation; rows V, diabetic rats after 15 d of inflammation; rows VI, diabetic rats after 30 d of inflammation. Note the stronger green staining in the basal epithelial cell layer, vessel endothelium and connective tissue cells at all experimental time-points for both MMPs. Magnification: columns A and C, 200 \times ; columns B and D, 400 \times .

diabetic animals, aggravation of the response resulted in flaccid tissue, inflamed gingiva with diminished tensile strength and less adhesion to the tooth.

Our results showed a transient increase in the gelatinolytic activity of MMP-2 and MMP-9 in response to the inflammatory process. In addition, MMP-9 levels were also higher than the control during inflammation. The MMP-9 level was lower in diabetic rats than in control rats. However, inflammation caused a significant increase in MMP-9 activity. The surprisingly high amount and activity of MMP-9 suggests that this enzyme is involved in the mechanism of inflammation. This is in accordance with the previous demonstration of enhanced migration of

inflammatory cells (29), with MMP-9 being constitutively expressed by polymorphonuclear leukocytes and macrophages, whereas MMP-2 preferentially derives from fibroblasts and endothelial cells (11,20).

MMP-2 gene expression was reduced during inflammation in both diabetic and nondiabetic rats, whereas MMP-9 expression was increased. Moreover, MMP gene expression was also affected by chronic inflammation. While MMP-2 was not influenced by diabetes, this condition led to an increase in the expression of MMP-9. Thus, in addition to the accumulation of MMPs mediated by inflammatory cells, local expression also contributes to the increased content and activity of

MMP-9 during inflammation in both normal and diabetic rats.

According to the literature, periodontal disease is a very common chronic subclinical inflammation, which might go unnoticed for long periods. This disease is also more prevalent and more intense in diabetic patients (42) because these patients are more susceptible to opportunistic bacterial infections, probably as a result of generalized circulatory disorders (21).

The higher MMP-9 activity in diabetic animals during inflammation, observed in the present study, is in agreement with the results of previous studies (20,26). In contrast, MMP-2 activity was lower in diabetic animals during inflammation, even though it is secreted by inflammatory cells. The reason for this is currently unknown, but it is probable that either some mediators down-regulate the expression of MMP-2 in diabetics or it reflects an imbalance between this enzyme and its inhibitors.

In the inflamed gingiva, the secretion of interleukin-1 α , interleukin-1 α , tumor necrosis factor- α , interleukin-6, interleukin-8, transforming growth factor- α , many growth factors and prostaglandins by inflammatory cells, fibroblasts and epithelial cells were affected (43). Diabetic individuals show an abnormal response to lipopolysaccharide, which results in an exacerbated secretion of inflammatory mediators such as prostaglandin E₂, interleukin-1 α and tumor necrosis factor- α , and in an inflammatory response induced by the accumulation of anomalous glycoproteins resulting from a reversible nonenzymatic glycation process (advanced glycation end-products). These advanced glycation end-products can bind to macrophage and monocyte receptors, resulting in an increased local and systemic secretion of these mediators, causing more severe periodontitis (34,36).

Interleukin-1 increases MMP synthesis, whereas transforming growth factor- α decreases MMP synthesis. The imbalanced production of these mediators seems to be responsible for the increase in the synthesis and accumulation of extracellular matrix components. Increased production of

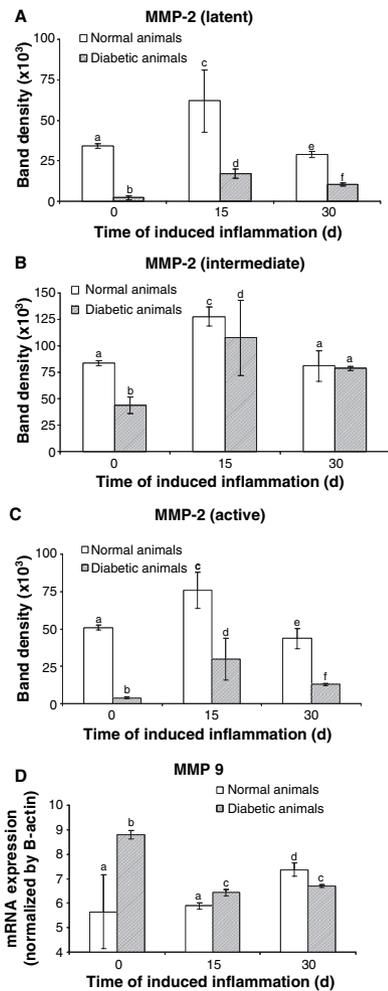


Fig. 2. Band density of the gelatin zymogram. Gelatinolytic activity was detected for both matrix metalloproteinase (MMP)-2 and MMP-9. The relative intensity of the bands obtained for the latent (A), intermediate (B) and active (C) forms of MMP-2 are shown. (D) Relative intensity of the bands obtained for MMP-9. The results represent the mean \pm standard deviation. Different letters indicate statistical significance ($p < 0.05$, analysis of variance and the F -test).

transforming growth factor- α and interleukin-6 seems to be characteristic of gingival fibromatosis (43) and this might correlate with the decreased expression of MMPs that occurs during inflammation. The changes in cytokine profiles found in gingival tissues from normal and diabetic rats regulate the differential expression and activity of gelatinases during inflammation and diabetes, thus contributing to the inflammatory response and fibrosis.

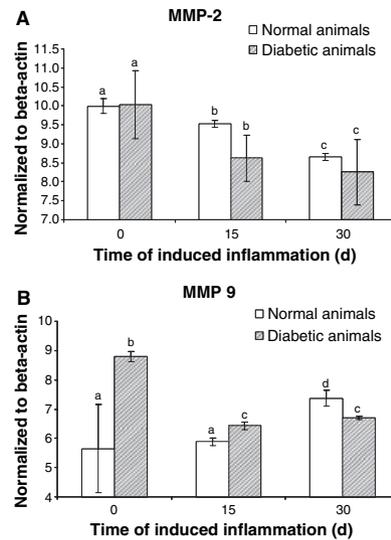


Fig. 3. Analysis of the gene expression of (A) matrix metalloproteinase (MMP)-2 and of (B) MMP-9 by real-time polymerase chain reaction. Beta actin was used as an endogenous control of the reaction. Results represent the mean \pm standard deviation. Different letters indicate statistical significance ($p < 0.05$, analysis of variance and the F -test).

Elevated serum concentrations of MMP-9 and MMP-2 have been observed in diabetic patients (20,25), and MMP-9 is a permissive insulin degradation factor in diabetics after its activation by endogenous trypsin. Overexpression of MMP-9 has been associated with vascular complications, retinopathy, nephropathy and arteriosclerosis in diabetic patients (20). The elevated activity of MMP-9 has been associated with endothelial dysfunction and apoptosis in murine models of diabetes (20). The imbalance between the expression of MMPs and of tissue inhibitor of metalloproteinases (TIMPs) in diabetic animals has been shown to cause excessive proteolysis and a delay in the healing of dermal ulcers, probably as a result of the increased amounts of MMP-1, -2, -3 and -9, interleukin-6 and tumor necrosis factor- α , and the decrease in TIMP1 and TIMP2 was influenced by glycation of the extracellular matrix and by advanced glycation end-product formation (26). It thus seems that the increased MMP-9 activity observed in diabetic rats is related to an altered inflammatory reaction in these animals

to the recruitment of inflammatory cells and/or to an unnoticed spatial accumulation of the enzyme. Accordingly, it has been suggested that the activation of advanced glycation end-product receptors might stimulate the inflammatory response, which, in turn, regulates MMP activity (26).

The present results permit us to conclude the following.

- (i) Periodontal disease is associated with changes in the expression of MMP-2 and MMP-9, with reduced activity being observed after longer time-periods, resulting in the fibrotic process observed in both normal and diabetic rats (29).
- (ii) Diabetes caused reduced MMP-2 and -9 activities with associated higher expression of MMP-9 (but not of MMP-2).
- (iii) The inflammatory reaction in diabetic animals was associated with a transient increase in MMP-2 and MMP-9 activity.
- (iv) However, the reduced activity of MMP-2 observed in diabetic animals was maintained during inflammation, whereas that of MMP-9 increased. As MMP-2 and -9 activities share many regulatory factors, it is apparent that tissue remodeling results from the activity of specific and localized mediators and not from a diffuse response to inflammation.

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