Matrix metalloproteinase -2, -8, -9, and -13 in gingival crevicular fluid of short root anomaly patients

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SUMMARY The aim of the present observational study was to identify and characterize matrix metallo-proteinase (MMP) -2, -8, -9, and -13 in gingival crevicular fluid (GCF) of patients with short root anomaly (SRA). GCF samples collected from affected maxillary central incisors and premolars of five SRA patients and five systemically and periodontally healthy controls were analysed using the zymographic technique for gelatinase A and B (MMP-2 and -9) and by Western blot for collagenase -2 and -3 (MMP-8 and -13).

SRA GCF revealed MMP-9 (30 per cent of the total gelatinolytic activity), of which 18 per cent was in 90 kDa proform and 12 per cent in 71–82 kDa active form. Moreover, high-molecular weight complexes (37 per cent) and low-molecular size fragmented (33 per cent) gelatinolytic enzymes were detectable. No MMP-8 or -13 immunoreactivities existed. These results may suggest that activation and complex formation of MMP-9 is characteristic of SRA GCF. From the findings it may be assumed that the GCF of SRA teeth has low collagenolytic resorptive or pathological activity.

Introduction

Idiopathical short roots, mainly identified in premolars and maxillary incisors, occur in a rare developmental dental condition, i.e. short root anomaly (SRA). Symmetrically affected tooth pairs with rounded apices instead of the usual finely tapering point are characteristic of SRA (Lind, 1972; Jakobsson and Lind, 1973). The condition has a genetic background and is related to hypodontia (Lind, 1972; Apajalahti *et al.*, 1999). In heritable SRA, the diagnosis is verified when some family members display similar short-rooted teeth, and when systemic causes for root shortening can be excluded (Apajalahti *et al.*, 1999). Apajalahti *et al.* (2002) have demonstrated a prevalence of SRA close to 1.3 per cent in systemically healthy Finnish young adults, involving females significantly more often than males.

The turnover and remodelling of the extracellular matrix (ECM) and basement membrane are essential in many physiological processes and in the progression of several tissue destructive inflammatory and malignant diseases (Hanemaaijer *et al.*, 1997; Lindy *et al.*, 1997; Konttinen *et al.*, 1998). Matrix metalloproteinases (MMPs) are a family of at least 20 genetically distinct but structurally related Zn^{2+}-dependent ECM or cell surface-associated enzymes produced by various cell types, of which up-regulation and activation are often associated with tissue destructive pathological conditions such as rheumatoid arthritis (Hanemaaijer *et al.*, 1997; Lindy *et al.*, 1997; Konttinen *et al.*, 1998), periodontitis (Sorsa *et al.*, 1994; Ingman *et al.*, 1996; Golub *et al.*, 1997), and tumour invasion (Johansson *et al.*, 1997).

Collagenase-2 (MMP-8) is suggested to be one of the key mediators of inflammatory tissue destruction associated with periodontitis and peri-implantitis (Sorsa *et al.*, 1994; Ingman *et al.*, 1996; Golub *et al.*, 1997). MMP-8 is stored in a latent form in specific granules of polymorphonuclear leukocytes (PMNs) and is primarily thought to be regulated by its selective granular release from triggered PMNs at the sites of inflammation. Additionally, MMP-8 expression by non-PMN lineage cells has been demonstrated, such as gingival and periodontal ligament (PDL) fibroblasts, endothelial cells, odontoblasts and dental pulp cells, rheumatoid synovial fibroblasts and endothelial cells *in vitro* (Hanemaaijer *et al.*, 1997; Palosaari *et al.*, 2000), and in human gingival sulcular epithelial cells *in vivo* (Tervahartiala *et al.*, 2000).

Collagenase-3 (MMP-13), characterized by broad substrate specificity, is related to pathological conditions such as head and neck, and other carcinomas (Johansson *et al.*, 1997), osteoarthritis, and rheumatoid arthritis (Lindy *et al.*, 1997). Elevated MMP-13 levels in GCF seem to reflect bone-type collagen degradation in adult periodontitis *in vivo* (Golub *et al.*, 1997).

The cleavage products of interstitial-type collagens resulting from MMP-8 and -13 action denature spontaneously at body temperature to gelatin and are susceptible to further degradation by gelatinase A and B (MMP-2 and -9) (Murphy *et al.*, 1980). Gelatinases are further capable of cleaving, especially type IV collagen and other basement membrane constituents, and can thus regulate basement membrane remodelling (Reynolds, 1996). Increased levels of gelatinases, especially PMN

gelatinase B (MMP-9), have been found in the GCF of subjects with periodontitis, but they are also abundantly present in the GCF of clinically healthy patients (Ingman *et al.*, 1994).

To our knowledge, the presence and molecular forms of MMPs have not been studied in SRA. Thus, the aim of the present observational study was to identify and characterize the possible presence of MMP-2, -8, -9, and -13 in GCF of SRA patients, in order to address in more detail the molecular mechanisms of periodontal remodelling associated with short-rooted teeth in this rare condition.

Subjects and methods

Patients

GCF was collected from five SRA patients (three females, two males) with a mean age of 26 years, at the Institute of Dentistry, University of Helsinki, Finland. A family series with SRA has been collected previously and family members examined (Apajalahti *et al.*, 1999). Diagnosis of SRA was first based on familial occurrence: one or two first-degree relatives of the patients had the same anomaly. Root length was estimated on panoramic radiograms. The roots were the same length or shorter than the crowns of the maxillary incisors and premolars bilaterally. The patients had no systemic diseases and were not on any continuing medication. None of the patients had undergone orthodontic treatment. On clinical examination, gingival tissues around the short-rooted teeth were found to be clinically healthy when assessed for gingival bleeding (Ainamo and Bay, 1975) and probing depth (Lindhe and Nyman, 1985). No signs of periodontal disease were evident on the panoramic radiographs. The control group (four females, one male; mean age 24 years) comprised systemically healthy dental students with clinically healthy gingiva and no radiographically detectable signs of periodontal disease. None of the patients had used antibiotics within the preceding 6 months. The study was carried out with the informed consent of the patients, and was approved by the Ethics Committee of the Institute of Dentistry, University of Helsinki.

Gingival crevicular fluid collection

GCF samples were collected from two SRA teeth, the maxillary central incisors or premolars, from each SRA patient as well as from the control patients. The surfaces of the teeth were dried gently and kept dry with cotton rolls. Two filter-paper strips were placed at the gingival margin into the sulcus for 3 minutes. The absorbed fluid was eluted from each strip into 25 µl of 0.2 M NaCl–1.0 mM $CaCl_2$–50 mM Tris–HCl, pH 7.5, and stored at –20ºC until used for enzyme assays.

Western blotting

The molecular forms of MMP-8 and -13 in the GCF of SRA patients and controls were analysed by the Western blot method. The samples were treated with Laemmli buffer, pH 6.8, and heated for 5 minutes at 100°C. Low-range pre-stained SDS–PAGE standard (Bio-Rad, Richmond, CA, USA) served as a molecular weight marker. The samples were separated on 8–10 per cent SDS–PAGE and then electrophoretically transferred to nitrocellulose membranes. To block non-specific binding sites on the nitrocellulose membranes, the membranes were diluted in 3 per cent gelatine in 10 mM Tris–HCl, pH 7.5–0.05 per cent Triton X-100–22 mM NaCl (TTBS) for 1 hour at 37ºC, after which the membranes were washed with TTBS four times, for 15 minutes each. The membranes were then incubated with mouse monoclonal anti-human MMP-13 (1:150 dilution in TTBS; Oncogene, Cambridge, MA, USA) and rabbit polyclonal anti-human MMP-8 (1:500 dilution in TTBS), as described by Hanemaaijer *et al.* (1997), at 22°C for 24 hours. After four 15-minute washes with TTBS, the membranes were incubated with goat anti-mouse IgG for MMP-13 and goat anti-rabbit IgG for MMP-8 alkaline phosphatase conjugates (1:500 dilutions in TTBS, Sigma, St Louis, MO, USA) for 1 hour. After four washings with TTBS for 15 minutes, one 15-minute wash with 10 mM Tris–HCl, pH 7.5–22 mM NaCl and one 15-minute wash with alkaline phosphatase buffer (100 mM NaCl–5 mM $MgCl_2$–100 mM Tris, pH 9.5), the immunoblots were visualized by the addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate diluted to *N-N*-dimethyl formamide (Sigma) in 100 mM Tris–HCl–5 mM $MgCl_2$–100 mM NaCl, pH 9.5.

Gelatine zymography

The GCF samples were analysed by zymography, with SDS–PAGE gels containing 1 mg/ml type I gelatine as substrate (Sigma). Before electrophoresis, the samples were incubated for two hours at 22ºC, after which the samples were loaded into 10 per cent gels. Molecular weight markers were low-range pre-stained standards (Bio-Rad). After electrophoresis, the gels were washed for 30 minutes in 50 mM Tris–HCl, 2.5 per cent Tween 80 and 0.02 per cent NaN_3, pH 7.5, and then for 30 minutes with the same buffer supplemented with 1 µM $ZnCl_2$ and 5 mM $CaCl_2$. The gels were then incubated overnight in 50 mM Tris–HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.02 per cent NaN_3, pH 7.5, at 37ºC and then stained with Coomassie Brilliant Blue R250 and destained as described by Ingman *et al.* (1994).

The intensities of different molecular weight forms of MMPs were evaluated with the Bio-Rad Model GS-700 Imaging Densitometer equipped with the Molecular Analyst/PC program.

Results

### Gelatinases (MMP-2 and -9) and collagenases (MMP-8 and -13) in GCF samples

The gelatine zymogram of all the SRA GCF samples revealed both high- and low-molecular weight gelatinolytic proteinases (Figure 1a). The gelatinase forms detected in GCF of SRA were high-molecular weight complexes (120 kDa) (37 per cent of the total gelatinolytic activity), low-molecular size (<50 kDa) species (33 per cent), and gelatinase B (MMP-9) (30 per cent), of which 18 per cent was in 90 kDa proform and 12 per cent converted to active 71–82 kDa species. Control GCF samples revealed gelatinase B (MMP-9) in 90 kDa proform (14 per cent), but not in active 71–82 kDa form. Other molecular species of gelatinases detected in the control group included high-molecular weight complexes (59 per cent) and low-molecular size fragments (27 per cent) (Figure 1b). No 62–72 kDa gelatinase A (MMP-2) species were found in the SRA and control GCF samples. No MMP-8 and -13 immunoreactivities were detectable with the Western blot technique (not shown) in the SRA and control GCF samples.

Discussion

In the present observational study, zymographic analysis of the GCF of SRA patients revealed gelatinolytic proteinases of high- and low-molecular size, but with no MMP-8 or -13 immunoreactivities. The major species detected in SRA GCF represented 92 kDa gelatinase B (MMP-9) that had undergone activation associated with fragmentation and complex formation (Ingman *et al*., 1994; Westerlund *et al*., 1996). The presence of these molecular forms of MMP-9 in SRA GCF is also characteristic of active periodontitis GCF (Gangbar *et al*., 1990; Westerlund *et al*., 1996), suggesting ongoing MMP-9-dependent proteolytic processes in the periodontium of SRA teeth. Differing from periodontitis and gingivitis GCF (Ingman *et al*., 1994; Golub *et al*., 1997), no 72 kDa MMP-2 and collagenases (MMP-8 and -13) were detectable in SRA GCF. These results suggest that activation and complex formation of 92 kDa MMP-9 is characteristic of SRA GCF.

A prevalence of only 1.3 per cent for SRA has been found in Finland (Apajalahti *et al*., 2002); thus, due to the low prevalence of this rare anomaly, only five SRA patients could be included in the study group.

The pathologically elevated pattern of MMP expression and activation in inflammatory conditions of the PDL has been well demonstrated (Sorsa *et al*., 1994; Ingman *et al*., 1996; Golub *et al*., 1997). In addition to disease-induced matrix remodelling and destruction, elevated collagenase activity has been detected in GCF during orthodontic tooth movement, in which the mechanical force applied is considered to lead to rapid remodelling
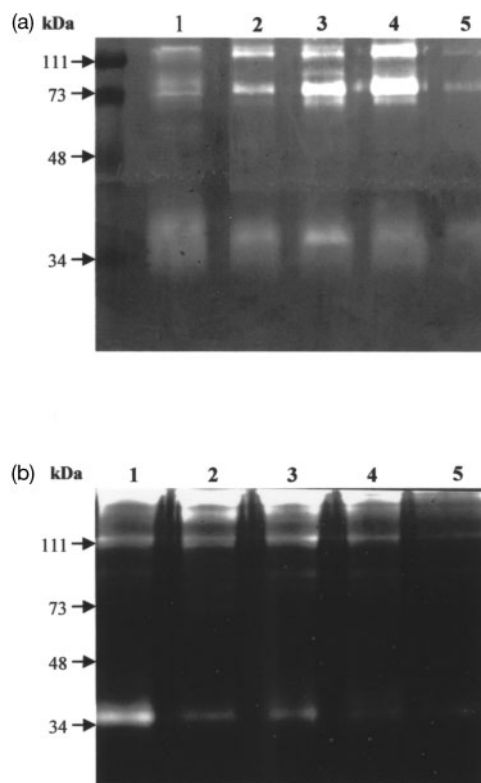
Figure 1 Gelatine zymographs of GCF of SRA (a) and control samples (b). (a) SRA GCF samples in lanes 1–5 show both high- and low-molecular weight gelatinolytic proteinases. MMP-9 is seen both in 90 kDa proform and 71–82 kDa active forms. (b) In contrast to SRA samples, clearly less 90 kDa proform of MMP-9 and hardly any 71–82 kDa active MMP-9 species are seen in GCF of control samples. Mobilities of the molecular weight markers (kDa) are indicated on the left.

of the PDL (Sorsa *et al*., 1992). Moreover, PDL fibroblast-derived MMPs are suggested to respond to mechanical stress and take part in ECM remodelling.

Degradation of PDL precedes root resorption (Rygh, 1977) and is followed by recruitment of resorptive cells, e.g. odontoclasts that remove root structure (Karring *et al*., 1984). *In vitro* animal studies have indicated increased collagenolytic activity in the resorption of the tissue of primary teeth (Okamura *et al*., 1993) and MMP-1 involvement in root resorption (Domon *et al*., 1999). Such root resorption activity has been related in humans to an increase in the amount of MMPs produced by periodontal fibroblast-like cells (Wu *et al*., 1999).

Futher loss of the apical structure of SRA teeth is a common finding during orthodontic treatment (Lind, 1972) and under severe masticatory stress. Maxillary central incisors, those most frequently and characteristically involved in SRA (Lind, 1972; Apajalahti *et al*., 2002), have been regarded as the most sensitive to root resorption (Levander *et al*., 1998). In this respect, SRA may be misdiagnosed as root resorption. In subjects undergoing orthodontic treatment, radiographs are needed

to evaluate pre-treatment root length and morphology in order to allow analysis of possible loss of apical structure. Studies of apical root resorption in orthodontically treated teeth indicate a very high incidence of minor apical changes (Linge and Linge, 1991; Kurol *et al.*, 1996), but the extreme resorption leading to a root length shorter than the crown, characteristic of SRA, is seen in less than 0.5 per cent of orthodontic subjects (Janson *et al.*, 2000).

In order to adapt to changing local conditions, ECM is characterized by continuous and rapid remodelling even under steady state conditions. In the present study, a characteristic feature of SRA GCF was found to be the activation, complex formation, and fragmentation of MMP-9. It thus appears that MMP-9, but not MMP-2, -8, and -13, are more significantly involved in periodontal ECM remodelling of SRA teeth. In this respect, SRA GCF differs from gingivitis and periodontitis GCF (Gangbar *et al.*, 1990; Ingman *et al.*, 1994; Westerlund *et al.*, 1996; Golub *et al.*, 1997). It is noteworthy that MMP-2, -8, and -13, but not MMP-9, can split native triple helical interstitial collagens by producing the characteristic αA (3/4) and αB (1/4) degradation products, and thus can initiate collagenolysis (Weiss, 1989; Konttinen *et al.*, 1998). It may be assumed that there was no significant collagenolytic resorptive or pathological activity in the periodontal tissues of the SRA teeth in the present study group. The *in vivo* findings further support the fact that the roots of SRA patients are developmentally short. The biochemical findings suggest that SRA is a constitutional condition with a characteristic MMP profile in the adjacent GCF.

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