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Dentine phosphoproteins in gingival crevicular fluid during root resorption

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SUMMARY External apical root resorption is a common, yet unexplained, phenomenon associated with orthodontic treatment. Available methods of clinical evaluation are radiographic. Biochemical assays offer the advantage of being non-invasive, as well as being diagnostic and potentially prognostic. The hypotheses are firstly that during the process of root resorption, organic matrix proteins are released into the gingival crevicular fluid (GCF) and, secondly, that there is a difference in the levels of these proteins between a group of patients with mild root resorption and a control group.

GCF was collected from the permanent central incisors of untreated subjects (controls, $n = 20$), primary second molars with half of the root resorbed (primary group and positive controls, $n = 20$) and permanent central incisors with mild root resorption in patients undergoing active orthodontic treatment (orthodontic group, $n = 20$). Dentine phosphoproteins (DPP) were measured in the GCF using an enzyme-linked immunosorbent assay developed with DPP isolated from human first premolars and an antibody against rat incisor DPP.

The primary group showed the highest levels of DPP in the GCF compared with the orthodontic ($P = 0.296$) and control ($P = 0.001$) groups. The orthodontic group showed elevated levels relative to the control group ($P = 0.046$). It is concluded that root resorption can be studied using a biochemical immunoassay and that this method can provide quantitative measurement of DPP in GCF.

Introduction

External apical root resorption is a common, yet unexplained, phenomenon associated with orthodontic tooth movement (Brezniak and Wasserstein, 1993a,b, 2002; Vlaskalic *et al.*, 1998; Killiany, 1999; Mah *et al.*, 2000). Fortunately, most patients with root resorption experience it to a mild degree, an amount that does not compromise the dentition. However, there are some patients who experience severe root resorption and its presence becomes a considerable factor in the long-term prognosis of the dentition. To-date, the only diagnostic methods for detecting root resorption are radiographic. While these methods offer ease of use, accessibility and are definitive, many limitations exist. Problems of technique, standardization, limited points of view, and radiation exposure remain. Approximately 60–70 per cent of the mineralized tissue is lost prior to radiographic detection (Andreasen *et al.*, 1987; Chapnick, 1989). Moreover, these methods are static and cannot indicate if the process of root resorption has arrested or is ongoing. A longitudinal series of radiographs is often used. However, radiation exposure limits their frequency, providing only a few points of clinical data. Given these limitations, there is an indication for more sensitive, safer, and more prognostic diagnostic methods for detecting root resorption.

Composition of dentine

The composition of dentine is approximately 70 per cent mineral, 10 per cent water and 20 per cent organic matter, as a percentage of the wet weight. The organic matter consists of approximately 80 per cent type I collagen. Historically, three dentine-specific non-collagenous proteins have been recognized: AG1 (dentine matrix protein 1, DMP1), dentine phosphophoryn (DPP), accounting for approximately 50 per cent of dentine non-collagenous proteins (Dimuzio and Veis, 1978), more recently called dentine phosphoprotein, and dentine sialoprotein (DSP, accounting for 5–8 per cent of dentine non-collagenous proteins) (Butler and Ritchie, 1995). However, it has recently been shown that the two latter proteins, DPP and DSP, are products of the same mRNA transcript and, hence, are portions of one expressed protein, now known as dentine sialophosphoprotein (Ritchie and Wang, 1996).

Gingival crevicular fluid (GCF)

The shortcomings of current clinical indices of assessment of periodontal disease have led to the development of more precise means of determining active disease, prediction of sites of future deterioration, and response to treatment. The gingival crevice contains a variety

of biomolecules derived from bacterial and host cells, which have been shown to indicate the metabolic status of various tissues of the periodontium. These biomolecules are now finding value as diagnostic or prognostic markers of periodontal health (Embery and Waddington, 1994). Biochemical markers in GCF which may relate to existing or predicted tissue regions undergoing metabolic change are derived from bacterial or host cell-derived products. Among the former are endotoxin, amines, butyrate, and a variety of enzymes and their inhibitors, such as trypsin-like proteases and bacterial collagenase. Arising from host cells are leucocytic hydrolase enzymes, lactoferrin, and lysozyme. These appear to be useful inflammatory markers and can be distinguished from products of connective tissue breakdown, which include collagenous and non-collagenous products, such as collagen peptides, osteonectin, fibronectin and proteoglycans. Attention has also been directed to elements of the immune response, such as immunoglobulins, complement, eicosanoids, and cytokines [for a review see McCulloch (1994)]. Alkaline phosphatase, an enzyme associated with osteoblasts, has also been studied (Binder *et al.*, 1987).

Analyses of GCF in the orthodontic literature are relatively few. Interleukin-1β, interleukin-6, tumour necrosis factor-α, β_2-microglobulin (Uematsu *et al.*, 1996a) and transforming growth factor-β_1 (Uematsu *et al.*, 1996b) have been measured in the GCF of teeth undergoing movement. Other factors that have been studied include proteoglycans (Waddington *et al.*, 1994; Waddington and Embery, 2001), acid and alkaline phosphatases (Insoft *et al.*, 1996), pH (Miyajima *et al.*, 1991) and more recently cAMP-dependent protein kinase subunit (RII) (Burke *et al.*, 2002). Other studies have analysed molecules in the GCF in relation to the nature of orthodontic movement. Interleukin-1β and interleukin-1β receptor antagonist were measured and compared with the velocity of tooth movement (Iwasaki *et al.*, 2001). Components of bone matrix, osteocalcin and pyridinium cross-links have been studied with respect to different stages of orthodontic treatment (Griffiths *et al.*, 1998). The relationship of heavy forces from rapid palatal expansion and interleukin-1β and β-glucuronidase has also been investigated (Tzannetou *et al.*, 1999). These studies suggest that GCF can be a very useful anylate in studying the biology of tooth movement and this approach can open many new avenues for orthodontic research.

The loss of root structure generally proceeds in a sequential manner to the loss of cementum, prior to the dissolution of dentine. Cementum breakdown products in the GCF have been studied, but they were detected in both control and treatment groups, rendering them unsuitable as markers for root resorption (Liu *et al.*, 2000). During orthodontic tooth movement, focal areas of cementum are resorbed and subsequently repaired (Owman-Moll *et al.*, 1995). Therefore, proteins of cementum may not be indicative of the permanent loss of root structure. Although small areas of dentine resorption have been shown to repair, larger areas and that of the apex do not repair, thus making the loss of dentine a significant part of the permanent loss of root structure. DMP1 has been evaluated using Western blot analysis. However, this protein was found in both control and treatment groups (Liu *et al.*, 2000). Given this and its minority content in dentine, DMP1 does not appear to be useful as a marker for root resorption. DPP has been detected in the GCF of patients with varying degrees of root resorption using Western blot analysis (Srinivasan *et al.*, 1998) and although this method does not provide quantitative information, it is suggestive that DPP can be a useful marker for root resorption.

The concept of GCF analysis may be adapted for the detection of root resorption, providing that breakdown products of this process, much like those of periodontal disease, are present in the gingival fluid. A biochemical assay could potentially offer advantages of (1) sensitivity, (2) non-invasiveness, (3) no radiation exposure, (4) information on the stage of resorptive activity and severity, (5) possibly identifying at-risk individuals, (6) reducing the time between clinical onset and usual clinical diagnosis, (7) predicting subsequent clinical course and prognosis, (8) implementing alterations in therapy, (9) assessment of the actual response to treatment alterations.

In order to proceed with developing a biochemical assay, the following hypotheses must be tested. First, that during the process of root resorption, organic matrix proteins are released into the nearby gingival crevice, and second, that there is a difference between the levels of DPP in GCF of primary, orthodontic and control groups. Therefore, the first aim of this study was to determine if dentine breakdown products from the process of root resorption are released into the GCF and if they can be measured using biochemical methods. The second aim was to determine if there is a significant difference between the amounts of DPP in the GCF of resorbing primary teeth, and permanent teeth undergoing root resorption during orthodontic treatment, and controls.

Materials and methods

Protein extraction

Caries-free premolars ($n = 32$) extracted for orthodontic purposes from patients aged 10–15 years were collected. Immediately following extraction they were placed in a sealed vial containing phosphate-buffered saline and protease inhibitors (1.0 M E-amino-*n*-caproic acid, 1.0 M

phenylmethylsulphonyl fluoride, 1.0 M benzamidine HCl, 1.0 M n-ethylmaleimide, 1.0 M aldrithiol) and stored at –20°C. The enamel and cementum were removed with a high speed handpiece and a diamond cutting burr under water cooling, leaving only dentine, which was crushed into small particles in liquid nitrogen. The dentine particles were dialysed with a 3 kDa exclusion membrane against a guanidine solution (4 M), EDTA (0.5 M), in Tris–HCl (50 mM, pH 7.4) for 4 days with daily changes of the solution. Initial isolation of DPP was performed following the $CaCl_2$ precipitation method of Kuboki *et al.* (1979) and thereafter systematically purified (Fujisawa *et al.*, 1984).

Collection of GCF

This study was conducted with the approval of the Institutional Board Review and informed consent from patients and parents was obtained. Inclusion criteria for this study included good general health, absence of medication, excellent oral hygiene, and no evidence of caries, abscesses or gingivitis. GCF was collected using the method of Offenbacher *et al.* (1986). In brief, the tooth was gently washed with water, dried, and isolated with cotton rolls to prevent saliva contamination. A paper collection strip (Periopaper, Harco, Tustin, CA, USA) was inserted 1 mm into the gingival crevice for 30 seconds. After 1 minute a second collection was undertaken. Both strips were immediately sealed in a microcentrifuge tube and stored at –70°C for later analysis.

GCF was collected from the permanent central incisors of untreated patients (controls, n = 20, ages 12–16 years, 12 females, eight males), primary second molars with half of the root resorbed (primary group, n = 20, ages 9–12 years, 15 females, five males) and permanent central incisors with radiographic evidence of mild root resorption (1–3 mm) in patients undergoing active orthodontic treatment (orthodontic group, n = 20, ages 12–16 years, 13 females, seven males).

DPP determination by enzyme-linked immunosorbent assay (ELISA)

GCF was completely eluted from the periopaper by centrifugal filtration with aliquots of buffer [50 mM phosphate buffer, pH 7.2, containing protease inhibitors; amino-*n*-caproic acid (50 mM), benzamidine-HCl (2.5 mM), N-ethyl maleimide (0.5 mM), and phenylmethylsulphonyl fluoride (0.3 mM)]. Briefly, 100 µl of the above buffer was applied to the two strips and the tube centrifuged at 15 000 g for 5 minutes. This process was repeated twice to ensure complete removal of all proteins from the periopaper. Previous studies have shown that 83–91 per cent of the proteins are recovered after the second wash; Uematsu *et al.* (1996a). The supernatants from the two strips were combined for a total volume of 300 µl and protein determination was estimated using the method of Bradford (1976), with bovine serum albumin as the standard. The samples were then stored at –30°C for further analysis.

ELISA for DPP

The ELISA technique was based on the method of Rennard *et al.* (1980) developed with DPP isolated from human first premolars and an antibody against rat incisor DPP. All samples and standards were assayed in duplicate. DPP values in the samples were recorded as ng/mg derived from the protein standard curve. Data are reported as the mean ± standard error of the mean. GCF collections were assayed for protein content and the ELISA values for DPP were adjusted for DPP/mg protein. Statistical analysis between treatment groups was performed using a Student's *t*-test. P values below 0.05 were required for the differences to be accepted as statistically different.

Results

DPP measured in GCF by ELISA revealed that resorbing primary molars had the highest levels of dentine proteins (11.7 ± 4.1 µg/mg) while the orthodontic group (9.3 ± 4.7 µg/mg) had less and the control group (5.4 ± 4.1 µg/mg) had the lowest levels (Figure 1). Large differences were noted between the control group and the primary group (P = 0.001), statistically significant differences were seen between the control group and the orthodontic group (P = 0.046), but no significant differences were seen between the primary group and the orthodontic group (P = 0.296). All groups were comprised of adolescents and had a tendency for more

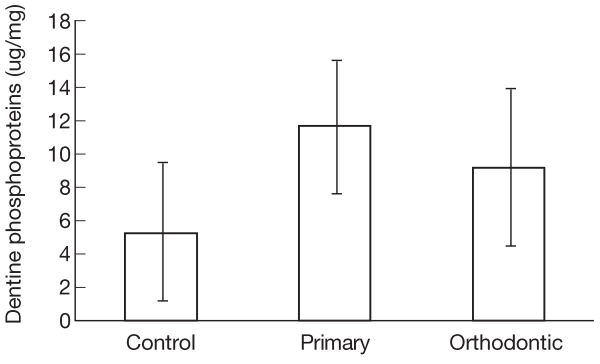

Figure 1 Enzyme-linked immunosorbent assay determination of dentine phosphoproteins in gingival crevicular fluid (GCF). Samples of GCF were obtained from: central incisors of untreated patients (control); second primary molars with half of the root resorbed (primary group) and permanent central incisors with mild root resorption in patients undergoing active orthodontic treatment (orthodontic group). The results are presented as the mean ± standard error of the mean.

females than males. This trend did not produce a discernible effect on the results.

Mineralizing odontoblast cell cultures were analysed for the presence of DPP as a function of time and extent of mineralization (Whelan, 1999). The expression and accumulation of DPP increased with increasing mineralization from day 4 (after the cells were placed in mineralizing media) to day 16, following closely the pattern of calcium deposition and accumulation (Figure 2).

Discussion

A comparison between the three clinical groups showed that the highest amount of DPP was present in the GCF of the primary group, the positive control. Clearly exfoliation of a primary molar involves extensive and complete root resorption and this result was anticipated. The orthodontic group contained permanent incisors with mild radiographic evidence of root resorption, which was relatively less than that of the primary group and hence had lesser amounts of DPP in the GCF. In this study, it was presumed that the composition of dentine from primary and secondary teeth was comparable. While there are microscopic studies describing the structure of primary and secondary dentine, there are no reports available that characterize the DPP of human primary dentine. Perhaps this is related to the number of teeth required to yield sufficient material to study. The presence of DPP in the untreated group (negative control) was not anticipated and is more difficult to explain, as these teeth were not undergoing any clinically visible tooth structure changes. This could be a function of the sensitivity of the ELISA method. The antibody used in this study was developed with rat DPP and while there is homology with human DPP, there are also some differences. Indeed, it is the current goal of many research groups to develop a specific antibody for human DPP, but this has been a significant challenge due to protein folding and extensive post-translational modifications that result in a molecule which is effectively shielded by numerous phosphate and carbohydrate groups. These groups are commonly found on other proteins and are not particularly antigenic, making it very difficult to produce an antibody against DPP. Also, the presence of DPP in the untreated group could be suggestive of more subtle changes taking place at a structural level. DPP is seen to change from the early stages of deposition to maturation (Chang *et al.*, 1996) and these measurements may reflect basal turnover of dentine proteins in maturation of the root that is akin to bone remodelling wherein immature, woven bone is later replaced with mature lamellar bone. Odontoblasts and odontoclasts could be working in a similar manner to the osteoblasts and osteoclasts of bone to form, resorb, remodel and maintain dentine. The control group contained individuals aged 12–16 years, a time by which the apices of the maxillary incisor teeth are formed. It is not known if further dentine remodelling occurs, but studies have shown that dentine is not a homogenous tissue and that its protein components change with age as the root matures (Clarkson *et al.*, 1998). This finding may shed light on the previous report of finding DMP1 in both control and treatment groups (Srinivasan *et al.*, 1998). The Western blot analysis used by those investigators would not have differentiated between basal levels in the controls and other levels in the treatment group.

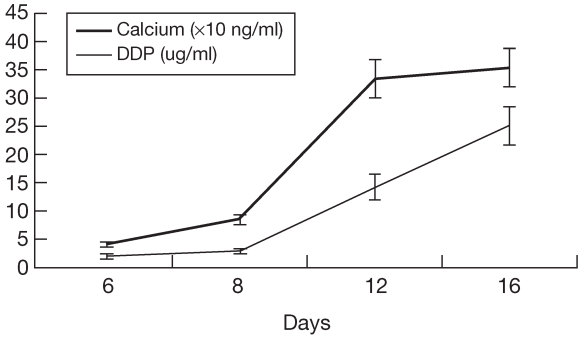

Figure 2 Calcium and dentine phosphoprotein measurements in calcifying odontoblast culture. The results are presented as the mean ± standard error of the mean.

A significant limitation of this research and others that utilize GCF to study root resorption is the inexact science of radiographic determination of root loss. The images cannot provide information on the extent, state of activity, three-dimensional location, and nature of the process—whether it is pathological as in root loss associated with orthodontic treatment or physiological as in root loss with exfoliation of primary teeth. Additionally, it is not known if root resorption is a linear process or if it occurs with periods of greater and lesser activity. These considerations could explain the wide range of values found in the primary and orthodontic groups. In certain medical situations, a biopsy can provide valuable information on the state of disease and nature of activity. However, this is not a possibility in the study of root resorption. Therefore, longitudinal studies are the only means available to provide more information and correlate the measurements to the biological state, albeit they are still constrained by the limitations of radiographs.

It has been shown that odontoblast cell cultures exhibit the presence of DPP from day 4 in mineralizing media with incremental increases through to the 16th day (Whelan, 1999). As odontoblasts mature to a certain stage they become productive, secreting cells. The DPP levels were compared with calcium levels in odontoblast cell culture, another indication of mineralization. The DPP level corresponded to the calcium levels, indicating that the odontoblast cells started deposition of organic matrix and mineralization

at approximately day 4 and continued as they matured. These findings further support the applicability of the developed ELISA for analytical determination of DPP.

The development of a sensitive, non-invasive bioassay for root resorption would benefit the science and practice of orthodontics in many ways. As a research tool, it would be useful to answer questions such as when root resorption occurs, if there is a threshold beyond which the loss of dentine products results in the permanent loss of root structure, what are the optimum force levels, which types of force lead to root resorption, and which clinical parameters are associated with root resorption. DPP is an excellent biomarker of root resorption as it is the majority organic, non-collagenous constituent of dentine, and is likely to be more indicative of the permanent loss of root structure compared with cementum proteins. Cementum undergoes active resorption and repair during tooth movement (Owman-Moll *et al.*, 1995), and the net loss of cementum in permanent root resorption may be overshadowed by this extensive activity. While dentine also has the capability to repair following resorption, larger dentine defects and those at the apex do not. Furthermore, its involvement is indicative that the root resorption process is more involved. If this technology is developed to the point where it is easily implemented in the clinic, much like the bioassays in osteoporosis and other metabolic disorders, the orthodontist may use this method to manage root resorption better. Early detection would lead to intervention and thereby limit a common complication of orthodontic treatment.

Conclusions

DPP is released into the GCF and can be measured. DPP seems to be most abundant in the GCF of exfoliating teeth. Lesser amounts were found in the GCF of teeth undergoing apical root resorption in association with orthodontic treatment and the lowest amounts were found in the GCF of control teeth. These findings suggest that biochemical assays hold promise for the detection of DPP and the management of external apical root resorption. However, further studies are required to develop more sensitive assays, correlate the measurements with clinical findings and produce a practical test for clinical use.

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