Altered serum levels of the osteoclast-specific TRACP 5b isoform in Chinese children undergoing orthodontic treatment

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SUMMARY. Orthodontic tooth movement is dependent upon the ability of mechanical forces to induce remodelling activity within the tooth-supporting alveolar bone. In view of the importance of bone resorption in mediating tooth movement, the aim of this study was to establish if alterations in the osteoclast-specific bone marker tartrate-resistant acid phosphatase (TRACP) 5b could be detected in the sera of patients undergoing orthodontic treatment. The sample consisted of 14 subjects (10 girls and 4 boys) aged 10.5–16.5 years (mean 12.6 years) being treated with fixed appliances and a distalizing headgear. Venous blood samples (3 ml) were collected from the cubital vein pre-treatment (T0) and 2, 4, and 6 months into treatment (T1–T3); serum TRACP 5b levels were quantified using a solid-phase immunofixed enzyme activity assay. When the data were pooled and treated cross-sectionally, a significant increase in immunoreactive TRACP 5b was detected at 2 months (T1) indicating increased bone resorptive activity. However, when the serum profiles of individual patients were recorded longitudinally, a very different pattern emerged, not all patients following the same trend. This is not surprising given normal anatomical variation and differences between the patients in age, gender, and mechanotherapy. Designed as a pilot to demonstrate ‘proof of principle’, this study is the first to show that the TRACP 5b isoform can be detected in the sera of patients undergoing orthodontic treatment. It further suggests that serum bone marker measurements offer a simple and minimally invasive method for correlating the findings of laboratory and animal experimentation with clinical data.

Introduction

The presence of bone markers such as acid and alkaline phosphatase (ALP) in serum has been widely utilized in clinical medicine as surrogate measures of bone turnover in metabolic bone diseases. Minimally invasive, requiring no more than a blood sample, serum markers have proved indispensable in diagnosis and the tracking of disease progression, with the added benefit of being able to predict bone loss in the early stages of osteoporosis or osteopenia before the actual loss of bone becomes apparent in bone scans (Alatalo et al., 2003).

Several degradation products have been used as laboratory markers of bone metabolism to date: these include urinary pyridinoline (Alvarez et al., 1995; Weaver et al., 1997; Szulc et al., 2000), urinary hydroxyproline (Alvarez et al., 1995; Weaver et al., 1997), serum telopeptide carboxy-terminal propeptide of type I collagen (CTx; Alvarez et al., 1995; Pi et al., 2006), and urinary CTx (Alvarez et al., 1995; Weaver et al., 1997; Szulc et al., 2000; Pi et al., 2006). The metalloenzyme tartrate-resistant acid phosphatase (TRACP) has also been widely used as a biochemical marker for bone resorption (Alvarez et al., 1995; Weaver et al., 1997; Szulc et al., 2000). Because TRACP is found in other tissues besides bone, an important advance has been the identification of TRACP 5b, an osteoclast-specific isoform, which has removed any ambiguity regarding its source. The commercial availability of specific human and rat solid-phase immunofixed enzyme activity assays has further provided a simple and accurate method for measuring TRACP 5b in bodily fluids and culture supernatants for both the laboratory and the clinical investigator.

TRACP (formerly abbreviated as TRAP) is an iron-containing 35 kDa glycoprotein that hydrolyses phosphomonoesters. It is produced as a monomer by osteoclasts and is cleaved by cathepsins to create a disulfide-linked dimer that acts on bone matrix phosphoserine-containing proteins in lysosomal vesicles. Following phosphate cleavage, TRACP is released from the cell where it serves as a marker of bone resorption. Osteoclasts and activated macrophages express high amounts of TRACP, which they secrete into the circulation (Yaziji et al., 1995). In humans, total serum TRACP is not a good marker of bone resorption because two isoforms of the enzyme are found; TRACP 5a derived from macrophages and dendritic cells containing sialic acid residues (Janckila et al., 2002) and osteoclast-derived TRACP 5b which is not glycosylated (Halleen et al., 2000). Human TRACP 5b is secreted and released by osteoclasts as a catalytically active enzyme that

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is inactivated in the circulation by the formation of complexes with α2-macroglobulin and calcium—this means that all catalytically active TRACP 5b molecules in serum are freshly liberated from osteoclasts (Ylipahkala et al., 2003). In rat serum, only the TRACP 5b isoform is present (Alatalo et al., 2003).

Elevated TRACP 5b activity has been detected in patients with a number of diseases, including postmenopausal osteoporosis (Halleen et al., 2001, 2002), Paget’s disease (Halleen et al., 2001), Albers-Schönberg disease (type II autosomal dominant osteopetrosis; Alatalo et al., 2004), and bone metastases resulting from prostate, breast, and other forms of cancer (Halleen et al., 2001; Salminen et al., 2005). Reduced serum levels have also been shown to be correlated with changes in bone mineral density and other markers of bone turnover in early postmenopausal women on hormone replacement therapy (Halleen et al., 2002), in monitoring the clinical performance of the bisphosphonate alendronate in a similar study group (Hannon et al., 2004; Nenonen et al., 2005) and in patients with multiple myeloma undergoing autologous stem cell transplantation and bisphosphonate treatment (Terpos et al., 2004).

Classical histological studies conducted on animal models and human volunteers in Scandinavia during the first half of the last century, established the essential role of osteoclast recruitment and activation in mediating bone resorption at sites of perceived compression during orthodontic treatment (Sandstedt, 1904, 1905; Reitan, 1951). More recently, TRAP-positive osteoclasts and multinucleate giant cells have been identified in the periodontal ligament (PDL) and bone of rat and mouse tooth movement models (Keeling et al., 1993; Brudvik and Rygh, 1994; Gu et al., 1999; Andrade et al., 2009; Braga et al., 2011), and the number shown to be correlated with the rate of movement (Ren et al., 2005; Tomizuka et al., 2007). For obvious reasons, it is neither practical nor ethically justifiable to biopsy the periodontal tissues of orthodontic patients for the purpose of investigating the mechanobiology of tooth movement. However, the use of serum markers as surrogate or indirect measures of osteoblast and osteoclast function, does offer a simple and practical approach to correlating the findings of animal experimentation with clinical data. With this in mind, the aim of the present study was to verify for the first time if the osteoclast-specific TRACP 5b isoform could be detected in the sera of patients undergoing various forms of orthodontic treatment.

**Materials and methods**

**Study population**

This study was approved by the Institutional Review Board of the University of Hong Kong to recruit patients who were to start fixed appliance orthodontic therapy in combination with a distalizing headgear (IRG Reference Number: UW 07-109). The sample included 14 healthy subjects (10 girls and 4 boys) aged 10.5–16.5 years (mean 12.6 years) chosen from the 17 patients enrolled by MacLaine et al. (2010) to evaluate the effects of orthodontic treatment on systemic inflammatory markers and who had sufficient serum remaining for additional analyses. Children in this age range have unerupted third molars and any remaining growth potential greatly facilitates molar correction. All patients had a Class II division 1 malocclusion, except for one who had a Class I (Table 1). Headgear

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Classification</th>
<th>Extractions</th>
<th>Appliance and headgear type</th>
<th>Canine retraction mechanics</th>
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<td>1</td>
<td>F</td>
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<td>Frictionless</td>
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<td>Sliding mechanics</td>
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<tr>
<td>3</td>
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<td>F</td>
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<td>—</td>
<td>Cervical pull</td>
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</tr>
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<tr>
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<tr>
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<tr>
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<td>12.4</td>
<td>Class I</td>
<td>—</td>
<td>TPA</td>
<td>Combination HG</td>
</tr>
</tbody>
</table>

Appliance: 0.022 × 0.028“ slot pre-adjusted edgewise brackets (Roth prescription).
compliance was assessed using self-reporting time charts. All patients were ethnic Chinese.

Exclusion criteria included patients with upper respiratory tract infections, asthma, obesity, smokers or a systemic disease capable of affecting the expression of TRACP 5b. Also, excluded were patients on long-term medication, those with generalized gingivitis, localized or generalized periodontitis, and those with poor oral hygiene. Children were only allowed to participate if a parent/guardian agreed to attend each visit.

Baseline 3ml blood samples (T0) were taken from the cubital vein of each subject prior to the commencement of orthodontic treatment. Each subject had topical Eutectic Mixture of Local Anesthetics (EMLA) cream (APP Pharmaceuticals, Schaumberg, Illinois, USA) composed of lidocaine 2.5 per cent and prilocaine 2.5 per cent applied to the cubital fossa area, which was left for 40 minutes. Alcohol was used to clean the area before drawing each blood sample. Three further 3ml blood samples were taken at 2 (T1), 4 (T2), and 6 months (T3) into treatment. These blood samples were collected 1 week after the subject’s routine orthodontic appointment.

Laboratory analysis

Serum separator tubes were used to collect the blood, in which the samples were left for 30 minutes to clot. The tubes were then centrifuged at 2300 r.p.m. for 15 minutes and the serum from the upper layer decanted into 0.2 ml aliquots and stored at −70°C until all samples had been collected. Samples were assayed in duplicate for TRACP 5b using a solid-phase immunofixed enzyme activity assay [SB-TR201A: Immunodiagnostic Systems (IDS)Ltd, Boldon Business Park, Tyne and Wear, UK] according to the manufacturer’s instructions. Briefly, the plates were coated with monoclonal anti-TRACP antibodies to which calibrators, controls, and samples were added, binding any TRACP 5b to the antibodies. Releasing agent was then added to the wells. After incubation and shaking at 850–950 r.p.m. for 60 minutes, any unbound substances were washed away. P-nitrophenol phosphate (pNpp) was added as substrate and the wells incubated a second time. Sodium hydroxide was added to stop the reaction and the optical density read at 405 nm with a VICTOR 3 Multilabel Plate Reader (PerkinElmer, Waltham, Massachusetts, USA).

Statistical analysis

Data were analysed with statistical analysis computer software (IBM SPSS Statistics 19.0.0: IBM Corporation, Somers, New York, USA). As the normality (Kolmogorov–Smirnov test) of the TRACP 5b data appeared valid, paired t-tests were used to examine differences between baseline and the three time periods. The level of significance was set at P < 0.05. Given the small number of participants and different treatment protocols (Table 1), no distinction was made between the extraction and non-extraction cases in analysing the data. Furthermore, in view of the findings of Chen et al. (2005) that no significant gender differences in serum TRACP 5b or ALP levels existed in normal Chinese children at any age, no distinction was made between boys and girls.

Results

Each patient had blood samples taken at all time points, and all 14 patients reported a minimum of 8 hours headgear wear per day (range 8–14 hours) according to their time charts. When the data were pooled and treated cross-sectionally, immunoreactive TRACP 5b was found in the sera of all subjects (Figure 1), but over the 6 month time course of the study period, significantly increased levels of enzyme activity could only be detected at (T1), 2 months after the commencement of orthodontic treatment.

When the serum profiles of individual patients were recorded longitudinally, a very different pattern of TRACP 5b activity emerged in which the patients could be broadly divided into two distinct groups. Those recording an increase in serum TRACP 5b activity at 2 months, totalling eight in number (Figure 2), and a further group of six in which there was a decrease (Figure 3); the subsequent level of immunoreactive TRACP 5b detected in these patients at 4 and 6 months, however, tended to be somewhat variable. The remaining two patients (5 and 13) demonstrated an essentially linear pattern of activity (Figure 3).

Of the eight patients who demonstrated increases in serum TRACP 5b activity at 2 months, more than half showed reduced levels at 4 months (Figure 2). The most dramatic increase was seen in patient 2 at 6 months, who was undergoing space opening with nickel–titanium springs on a rectangular wire. The activity of TRACP 5b detected in patients 4 and 8 ignored the overall trend (Figure 3); patient 4 showed an almost linear trend until headgear wear was
Elevated levels of interleukin-1

Discussion

stopped and then increased TRACP 5b activity at 6 months. Patient 8 had a transpalatal arch to enhance posterior anchorage, unlike patient 14, in whom it was used for molar de-rotation.

The largest reduction in TRACP 5b at 6 months was detected in patient 8, whose upper second molars were included in the appliance. Patient 7 also showed a striking reduction at the same time point while using cervical pull headgear during the alignment phase of treatment. Marked reductions in TRACP 5b serum activity to below baseline levels were detected in five patients (patients 7, 8, 11, 12, and 14) at 6 months. The serum profiles of patients 5 and 13 were almost linear despite different treatment mechanics.

Discussion

Elevated levels of interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), IL-2, and other so-called immunoregulatory cytokines in the sera of patients with periodontal disease have been established for more than 20 years (McFarlane et al., 1990; McFarlane and Meikle, 1991). While the presence of cytokines in gingival crevicular fluid during tooth movement has been widely reported (Grieve et al., 1994; Lowney et al., 1995; Uematsu et al., 1996), fueling the idea that tooth movement is an inflammatory process, little consideration has been given to the use of serum markers to monitor tissue remodelling during orthodontic treatment. Indeed, only one has been published to date, showing that orthodontic tooth movement does not significantly alter serum levels of the inflammatory markers C-reactive protein, TNF-α, or IL-6 (MacLaine et al., 2010).

Although preliminary in scope and using the patients sampled by MacLaine et al. (2010), this study is the first to show that alterations in the osteoclast-specific TRACP 5b isoform can be detected in the sera of patients undergoing orthodontic treatment. When the data were pooled and treated in a cross-sectional manner, a significant increase in TRACP 5b activity was detected 2 months after the start of treatment (T1), indicating an increase in bone resorptive activity. However, the mean is a statistical artefact designed to produce order from large amounts of population data, such as height and weight. It was recognized more than 100 years ago that treating growth values cross-sectionally and simply taking the average flattens out individual variation and was the reason why Boas (1892) insisted that longitudinal growth studies were needed to understand the dynamics of human growth (Tanner, 1962). The same applies to studies of orthodontic treatment outcome. Given the small sample size of most orthodontic investigations, very few patients are likely to show the mean or average change—as a consequence, if the data are just analysed cross-sectionally, much valuable information is likely to be lost. In the present investigation, when the data were examined longitudinally, we found that it looked very different, not all patients following the same average trend. This is not surprising given the individual variation in tissue characteristics among persons of a similar age group (Reitan, 1957), as well as differences in mechanotherapy.

Orthodontic tooth movement is a multifaceted and poorly understood process and most studies have focussed on the tissue, cellular, and molecular changes occurring in the PDL and supporting bone resulting from appliance activation (Meikle, 2006). But there is another important question that has received little if any attention that also needs to be addressed: What is the effect of the appliance itself on the metabolism and remodelling dynamics of the tooth-supporting periodontal tissues? The reason for asking this question is because stress-shielding and osteopenia resulting from the implantation of rigid metallic devices into bone is a well-recognized complication of fracture fixation and joint arthroplasty in orthopaedic surgery (Woo et al., 1976; Huiskes et al., 1992; Glassman et al., 2006; Uthoff et al., 2006).

If nothing else, an orthodontic fixed appliance is a metallic device of varying rigidity and might therefore be expected to induce some degree of osteopenia of the tooth-supporting alveolar bone—a statement based on the serendipitous discovery of Milne et al. (2009) that a cross-arch expansion spring bonded to the maxillary molars of 6-week-old rats resulted in a reduction in alveolar bone mass, irrespective of whether the spring was activated or not. Serum ALP levels were found to have declined significantly in both experimental and sham-treated rats over an 8-day time course, confirming the decreased bone formation observed histologically. Three-dimensional finite element analysis of stresses generated in the bone further showed that the orthodontic appliance created a constant loading condition shielding some areas of bone from mechanical stress, the result being a reduction in occlusal loading below the critical threshold required to maintain normal osseous architecture (Milne et al., 2009). More recent work in the...
Figure 3 TRACP 5b activities in serum samples from patients recording a decreased level of the enzyme after 2 months of treatment. Blood samples were taken pre-treatment (T0) and 2, 4, and 6 months following the commencement of orthodontic treatment (T1–T3). Patients 8 and 13 were treated with extractions and the remaining non-extraction.

rat has confirmed the reduction in serum ALP, but no significant differences in serum TRACP 5b activity could be detected between experimental and control groups (Patel, 2010; Meikle, unpublished findings), signifying that bone loss was due to a reduction in bone formation, bone resorption remaining unchanged—in other words, the normal remodelling cycle of bone formation and bone resorption had been uncoupled. Instead of stimulating bone formation, 'growing bone' as Angle (1907) and many of his contemporaries believed, these data imply that an orthodontic appliance may in fact have a negative effect on bone mass.

This rather complicates how one interprets the findings of serum measurements in patients undergoing orthodontic treatment. In addition to the remodelling activity occurring at the bone–PDL interface associated with tooth movement, there is the impact of the appliance itself and its potential for inducing a localized negative skeletal balance within the dentoalveolar bone (Milne et al., 2009). To fully understand the osseous response to orthodontic treatment in patients, will therefore require not only the measurement of serum bone markers in a much larger number of patients with clearly defined treatment protocols but also the application of three-dimensional imaging techniques to visualize and quantitate alterations in the structure of the tooth-supporting trabecular and cortical bone.

Conclusions

This pilot study designed to demonstrate a 'proof of principle' is the first to identify systemically detectable bone resorptive activity in patients undergoing orthodontic treatment, as evidenced by levels of the osteoclast-specific TRACP 5b isoform in their sera. When the data were pooled and treated cross-sectionally, a significant increase in TRACP 5b activity was detected 2 months after the start of treatment (T1). However, when the data were examined longitudinally, the response of individual patients was shown to be highly variable, not all following the same average trend. This is not surprising given normal anatomical variation and differences between the patients in age, gender, and mechanotherapy.

Funding

Research Grants Council; Competitive Earmarked Research Grant (A/C No. 1026968.22311.08003.324.1 to B.M.R).

Acknowledgements

We are particularly grateful to Mr A.P.K. Wong for technical support in the laboratory and Mr S. Yeung for assistance with the statistics.

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