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Expression levels of endothelin-1, endothelin-2, and endothelin-3 vary during the initial, lag, and late phase of orthodontic tooth movement in rats

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SUMMARY Endothelins (ET)-1, ET-2, and ET-3 are one group of cytokines likely to be released during orthodontic tooth movement (OTM). Therefore, the expression of ET levels was investigated to determine the importance and involvement of isopeptides during the several phases of OTM.

Thirty-two male Wistar rats (12–13 weeks old) were divided into four groups of eight: control, 14, 28, and 42 day groups. Tooth movement was induced by a closed-coil spring inserted between the upper left first molar and the upper incisors. The distance between the teeth was measured on days 0, 2, 7, 14, 21, 28, 35, and 42 using a digital calliper. The rate of tooth movement was calculated. The animals were sacrificed on days 14, 28, and 42 and gene expression levels of all three ET were determined using reverse transcription polymerase chain reaction. Statistical analysis was performed using two-way analysis of variance, Bonferroni's correction, and paired *t*-tests.

The distance between the teeth decreased in all appliance groups ($P < 0.001$). The rate of tooth movement was 0.20 ± 0.02, 0.03 ± 0.01, and 0.06 ± 0.02 mm/day between days 0–2, 3–21, and 22–42, respectively. On day 14, gene expression levels for ET-1 ($P < 0.05$) and ET-3 ($P < 0.001$) increased compared with day 0. On day 28, a downregulation of ET-3 was observed when compared with day 0 ($P < 0.001$). On day 42, ET-1 ($P < 0.001$) and ET-3 ($P < 0.01$) gene expression levels were strongly upregulated, while ET-2 gene expression level was downregulated ($P < 0.01$) when compared with day 0. ET-1 and ET-3, but not ET-2, are involved in all three phases of OTM, and ET-1 seems to be the predominant form in the late phase of OTM.

Introduction

Endothelins (ET) are a family of peptide cytokines that include ET-1, ET-2, and ET-3. ET-1 is the principal and most studied isoform in humans (Miyauchi and Masaki, 1999; Rich and McLaughlin, 2003). ET are involved in many physiological and pathophysiological processes and probably also in orthodontic tooth movement (OTM; Drevenšek *et al.*, 2006; Sprogar *et al.*, 2008).

Dependent on force characteristics, OTM comprises three phases: an initial phase, which lasts 1–2 days after force application; a lag phase, which lasts 20–30 days after force application and a late phase, which lasts as long as a force is applied to the teeth. During the initial phase, several changes cause the release of many different hormones, growth factors, cytokines, and other chemical messengers, which modulate OTM (Krishnan and Davidovitch, 2006). ET could be one group of these cytokines since it has been shown that ET-1 is released in the periodontal ligament (PDL) microvasculature after 3 hours continuous loading of a rat molar (Sims, 2001). During the lag phase, in which only minor tooth movement is present, necrotic tissue that has formed due to hypoxia in the initial phase is removed by cells, which functioning is influenced by ET, namely macrophages, foreign body giant cells, and osteoclasts (Ehrenreich *et al.*, 1990; Alam *et al.*, 1992). Therefore, ET could also be involved in the lag phase of OTM. During the late phase, rapid bone formation on the tension side, mediated by osteoblasts and bone resorption on the pressure side, mediated by osteoclasts, are the predominant processes (Reitan, 1967; Rygh, 1976; Krishnan and Davidovitch, 2006). ET-1 has been found in both major types of bone remodelling cells: in osteoblasts (Kitten and Andrews, 2001) as well as in osteoclasts (Sasaki and Hong, 1993). Furthermore, ET-1 increases osteoblast differentiation, proliferation and activity and, therefore, bone formation (Tatrai *et al.*, 1992; Nelson *et al.*, 1999, von Schroeder *et al.*, 2003; Yin *et al.*, 2003; Guise and Mohammad, 2004; Clines *et al.*, 2007). The findings on the influence of ET on bone resorption are inconsistent. Some researchers found that ET, mainly ET-1, increase bone resorption (Tatrai *et al.*, 1992; Sprogar *et al.*, 2008), while others concluded the opposite effect (Alam *et al.*, 1992).

While it has been shown that ET-1 is involved in the late phase of OTM in rats (Sprogar *et al.*, 2008), there is no evidence of the involvement of ET-2 or ET-3. Therefore, the aim of this study was to determine whether ET-2 and

ET-3 are involved in OTM and, furthermore, since ET-1 is the predominant isopeptide in other processes, whether it also predominates in all phases of OTM. Therefore, the expression levels of ET-1, ET-2, and ET-3 during the initial, lag, and late phases of OTM in rats were investigated.

Materials and methods

Animals and study protocol

The investigation was approved by the Veterinary Administration of the Republic of Slovenia (No. 323-02-234/2005/2) and complied with the guiding principles in the 'Care and Use of Animals'.

The study was performed on 32 male Wistar rats (330–350 g, 12–13 weeks old). The animals were housed in groups of four in polycarbonate cages under normal laboratory conditions at a constant temperature (24–25°C) and humidity with a 12 hour circadian cycle. They were fed with soaked standard laboratory rat chow diet (Krka, Novo mesto, Slovenia) and water *ad libitum*.

The animals were divided into four groups (n = 8): control, 14, 28, and 42 day groups. In the last three groups, a closed-coil spring (25cN, wire diameter 0.15 mm; GAC International, Bohemia, New York, USA) was placed between the upper left first molar and the maxillary incisors. Before placing the appliance, the animals were anaesthetized as described previously (Drevenšek *et al.*, 2006; Sprogar *et al.*, 2007). The closed-coil spring was attached to the upper left first molar with a stainless steel ligature wire (diameter 0.25 mm; Dentaurum, Ispringen, Germany) and to the upper incisors by a surgical steel wire (4-0, multifilament, W310, Ethicon; Johnson&Johnson, New Brunswick, New Jersey, USA). To improve fixation of the appliance, a 0.5 mm hole was made using a hard metal burr (HM 1, 204, 005, Meisinger, Neuss, Germany). The hole was drilled through the approximal tooth surfaces, perpendicular to the longitudinal axis of the incisors at the gingival level. The steel wire was inserted through the hole and bent on the approximal surface of the right incisor. Light curing bonding material (Tetric flow, Ivoclar Vivadent, Schaan, Lichtenstein) was used to protect the soft tissues from the sharp wire ends (Sprogar *et al.*, 2007). The superelasticity of the coil spring ensured a constant force (25 cN) during activation (Drevenšek *et al.*, 2006).

Measurements

In the control group and in the 42 day group, the distance between the most mesial point of the upper left first molar and the most palatal point of the ipsilateral incisor at the gingival level was measured. Measurements were undertaken on days 0, 2, 7, 14, 21, 28, 35, and 42 using a digital calliper (Wilson & Wolpert, Utrecht, The Netherlands; Sprogar *et al.* 2007). During this procedure, the animals were anaesthetized. All measurements were carried out twice by two investigators (AH and ŠS) independently within a period of a few minutes. Interexaminer reliability was tested using the intraclass correlation coefficient (ICC) and a paired t-test was used to assess the systematic bias. Tooth movement was calculated by subtracting the distance between teeth on each day (2, 7, 14, 21, 28, 35, and 42) from the distance between the teeth on day 0. The rate of tooth movement was calculated by subtracting the distance between the teeth on each day from the distance between the teeth on the previous day and dividing this difference by the number of days between these two measurements (2, 5, or 7 days).

Semi-quantitative reverse transcription polymerase chain reaction

All animals in the 14, 28, 42 day, and control group were killed by an intraperitoneal injection of anaesthetic and CO_2. Tissue samples of the alveolar bone containing all three left maxillary molars and their PDL were excised, frozen in liquid nitrogen, and mechanically powdered. The total RNA content was isolated from 100–150 mg of each of the powdered sample using TRIzol reagent (Invitrogen, Carlsbad, California, USA), according to the manufacturer's protocol. Prior to reverse transcription, RNA was treated with RNase-free DNase I (Fermentas, Vilnius, Lithuania). Approximately 500 ng of DNA-free RNA was reverse transcribed into cDNA in a 20 µl reaction using Improm II reverse transcriptase (Promega, Mannheim, Germany) and random hexamer primers (Promega), according to the manufacturer's instructions. Polymerase chain reaction (PRC) with Gotaq Green master mix (Promega) was performed using 1 µl of the reverse transcription reaction in a 25 µl PCR reaction with 15 nmol of the transcript specific primers (Invitrogen): 5′-GCTCCTGCTCCTCCTTGAT-3′ and 5′-CTCGCTCTATGTAAGTCATG-3′ for ET-1; 5′-ggccatccctgcatactcta-3′ and 5′-ctagggaaggggaaccagaG-3′ for ET-2; and 5′-GCACTTGCTTCACTTATAAG-3′ and 5′-CAGAAGCAAGAAGCATCAGT-3′ for ET-3. The internal normalization gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using 3 nmol of primers 5′-TCCCTCAAGATTGTCAGCAA-3′ and 5′-AGATCCACAACGGATACATT-3′. Thermal cycling conditions comprised 32 cycles, each consisting of 30 seconds at 94°C, 1 minute at 55°C, and 30 seconds at 72°C. The number of cycles (32) was empirically determined as the optimal cycle number within the linear range of amplification by measuring the concentration of PCR products after 28, 30, 32, and 34 cycles. Ten microlitres of PCR products were resolved in 1.5 per cent agarose gels containing ethidium bromide. The band intensities were quantified using the TotalLab gel analysis program (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) after image acquisition from ultra violet-illuminated gels by MiniBis (DNR BioImaging Systems, Jerusalem, Israel).

Statistics

Descriptive statistics (mean and standard error) were calculated for each parameter (tooth movement, rate of tooth movement, and gene expression levels) for all animals in all groups. Interexaminer reliability was tested with the ICC and a paired *t*-test was used to assess systematic bias. Within and between groups comparisons were made for gene expression levels using the analysis of variance and Bonferroni's correction in GraphPad Prism 4.00 (GraphPad Software, San Diego, California, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Measurements

Systematic bias, which showed a value of $P > 0.88$ was determined using a paired *t*-test. The ICC was found to be 0.93 ± 0.02. Since reliability was within the standards, the mean value of the four measurements was used for further statistical analysis.

In the control group, the distance between the teeth increased from days 0 to 42 ($P < 0.001$). While in the 42 day group, it decreased from days 0 to 42 ($P < 0.001$). Changes in the distances significantly differed between the control group compared with the 42 day group on days 7, 14, 21, 28, 35, and 42 ($P < 0.001$). The rate of tooth movement was significantly faster in the initial phase compared with the lag and late phases ($P < 0.001$) and was also significantly faster in the late phase compared with the lag phase ($P < 0.05$; Figure 1).

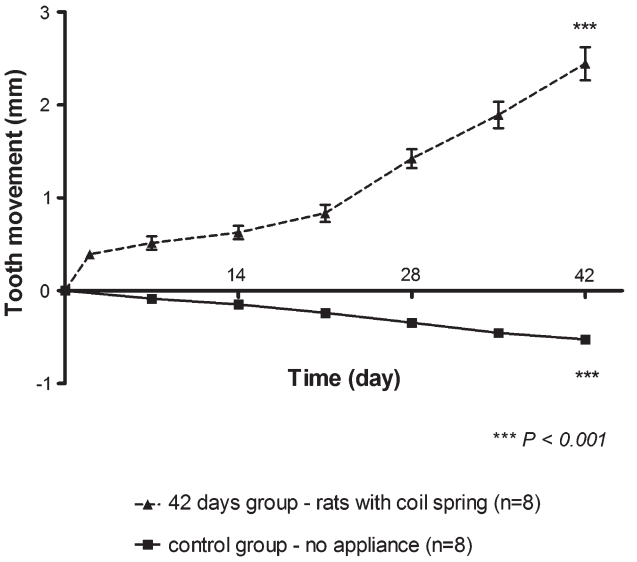

Figure 1 Tooth movement and rates of tooth movement ± mean and standard error in rats in the control (solid line) and the 42 day (dotted line) group in all three phases of orthodontic tooth movement. Filled triangles: 42 days group—rats with coil spring ($n = 8$). Filled squares: control group—no appliance ($n = 8$). Initial phase ≈ days 0–2 (0.20 ± 0.02 mm/day); lag phase ≈ days 3–21 (0.03 ± 0.01 mm/day); and late phase ≈ days 22–42 (0.08 ± 0.02 mm/day).

Gene expression levels of ET-1, ET-2, and ET-3 during OTM

Reverse transcription PCR analysis showed that the expression of ET-1, ET-2, and ET-3 genes varied considerably during the time course of force application (Figure 2). For all three transcripts tested, time course-dependent gene expression profiles were observed after normalization to the expression level of the housekeeping gene GAPDH. The basal levels of ET-1 and ET-3 transcripts in the control group (day 0) were almost the same, while ET-2 was significantly higher when compared with ET-1 and ET-3 ($P < 0.001$). A subsequent consensus increase during the following 14 days of force application was further observed for ET-1 and ET-3 but not for ET-2 gene expression level (Figure 2), bringing all three transcripts to almost the same level. A strong downregulation of ET-3 and a non-significant downregulation of ET-1 and ET-2 levels were observed on day 28 of force application. Therefore, on day 28, the ET-2 transcript level remained significantly higher compared with both ET-1 and ET-3 transcript levels ($P < 0.001$). Even more evident change in gene expression for all three ET was observed after 42 days of force application. On day 42, a strong upregulation of ET-1 and ET-3 genes was observed. The ET-1 transcript level rose sevenfold compared with the level on day 28, thus exceeding the expression level at any previous time point (Figure 2). The expression level of ET-1

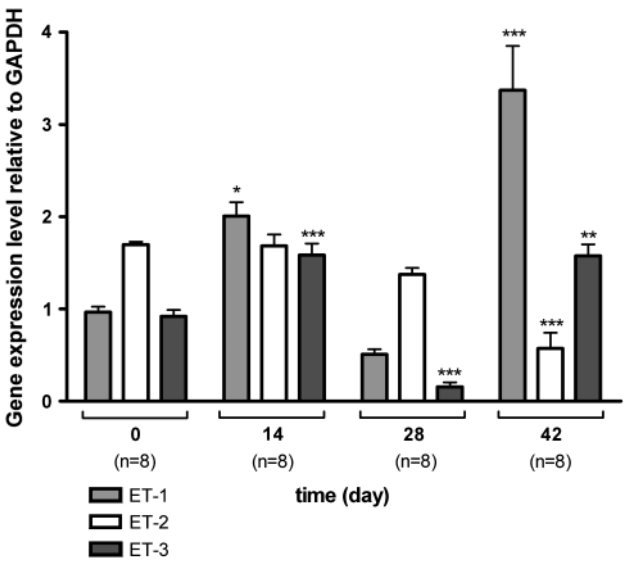

Figure 2 Gene expression levels of endothelins (ET)-1, ET-2, and ET-3 in alveolar bone and the periodontal ligament during orthodontic tooth movement on days 0, 14, 28, and 42. The relative expression levels of the indicated genes were performed after normalization against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

on day 42 was also significantly higher compared with ET-2 and ET-3 transcript levels ($P < 0.001$ and $P < 0.01$, respectively). In contrast, the ET-2 transcript level was significantly downregulated and reached the lowest level compared with previous time points (Figure 2).

Discussion

OTM is modulated by many chemical messengers (Krishnan and Davidovitch, 2006), among which may be ET. Until now, only the involvement of ET-1 in bone modelling has been studied, using *in vitro* and *ex vivo* models. However, the involvement of ET-2 and ET-3 in these processes is not known nor is it clear whether ET-1 is the predominant isopeptide form of the ET family in all phases of OTM. Studies examining the role of ET at the molecular level using *in vivo* models could provide a deeper insight into the process of OTM, thereby identifying the important pharmacological targets for modulating this process. The present data showed that ET-1 and ET-3 could have a parallel role in OTM since their gene expression levels demonstrated a similar pattern. ET-2 appeared to have no significant role during OTM in this model (Figure 2).

The present animal model expressed all three phases of OTM (Figure 1). The initial phase lasted 48 hours and the lag phase approximately 20 days, followed by the late phase. This is in agreement with other studies (Shirazi *et al.*, 2002; von Böhl *et al.*, 2004; Krishnan and Davidovitch, 2006; Yoshimatsu *et al.*, 2006). The proposed model that shows the increases in gene expression levels of ET on day 14 represents the involvement of ET in the initial and later lag phase. Gene expression levels of ET on day 28 represent their involvement in the shift from the lag phase to the late phase, and on day 42 their involvement in the late phase of OTM, during which bone formation on the tension side and bone resorption on the pressure side enabled movement of teeth through alveolar bone.

ET-1 and ET-3, but not ET-2, are most likely involved in the events of the initial and lag phase of OTM since their gene expression levels were significantly upregulated on day 14. It appears that both ET-1 and ET-3 contribute equally to these events since their gene expression levels were similar (Figure 2). ET are released probably due to mechanical loading, ischaemia, and hypoxia (Kourembanas *et al.*, 1991; Schmitz-Spanke and Schipke 2000), which appear immediately after force application and last throughout most of the initial and lag phase of OTM. It has been shown that after acute loading of a rat molar, ET-1 immunofluorescence increased in the microvasculature of the PDL and alveolar bone socket surface (Sims, 2001). During the initial phase in the present study, the rate of tooth movement was high (Figure 1). This rapid movement is the result of the shift of the tooth in its PDL space and early bone resorption (Keeling *et al.* 1993, King *et al.* 1997; Noxon *et al.*, 2001), following which the lag phase starts. The events of the lag phase, where the rate of tooth movement is significantly decreased due to hyalinization (Figure 1), are intense cellular activity and the reestablishment of cell and fibre function (Rygh, 1973). These events are mediated by a variety of cells, among which are fibroblasts, macrophages, osteoclasts, and osteoblasts, which are all under the influence of ET (Ehrenreich *et al.* 1990; Zeballos *et al.*, 1991; Alam *et al.*, 1992; Kitten and Andrews, 2001). Gene expression levels of ET on day 28 showed the involvement of these peptides in the last days of necrotic tissue degradation and the start of the bone modelling process. Since the gene expression levels of ET-1 and ET-2 did not differ from those on day 0 and as the significant downregulation of the gene expression level of ET-3 seems to be a compensatory mechanism activated due to excessive peptide levels from the earlier phases, it seems that none of the ET isopeptides seems to contribute to these events (Figure 2). The late phase is dominated by bone resorption on the pressure side and bone formation on the tension side of the loaded tooth. During that time, the rate of tooth movement increased compared with the lag phase (Figure 1). This is a result of an increase in osteoblast and osteoclast number and activity (Ren *et al.*, 2005, Krishnan and Davidovitch, 2006). These cells mediate bone remodelling and, therefore, enable teeth to move through alveolar bone. ET-1 and ET-3 peptides contribute to the bone modelling processes since their gene expression levels on day 42 were upregulated. Furthermore, it appears that ET-1 dominates that phase of OTM since its gene expression level was significantly higher compared with that of ET-3 (Figure 2). However, it appears that ET-2 has no role in that phase of OTM, due to the relatively small change and downregulation of its gene expression (Figure 2).

Many studies have already shown that ET-1 increases bone formation (Tatrai *et al.*, 1992, Yin *et al.*, 2003; Guise and Mohammad, 2004; Clines *et al.*, 2007). Lately, it has also been suggested that ET-1 increases osteoclastic bone resorption during OTM (Sprogar *et al.*, 2008). No data on the effects of ET-3 on bone modelling are currently available. However, considering the similar expression patterns between ET-1 and ET-3, which could be a result of the involvement of the same transcript factors, it is likely that ET-3 plays a similar role to ET-1 in bone remodelling, increasing both bone formation and resorption.

Conclusions

During the late phase of OTM, when bone remodelling comprises bone formation and bone resorption, ET-1 and ET-3 are the predominant ET isoforms. ET-1 and ET-3 are equally important during the initial and lag phase, but ET-1 predominates the late phase. The role of ET-2 seems to be irrelevant during OTM.

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