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Osteoclast differentiation and recruitment during early stages of experimental tooth movement in rats


Osteoclasts are derived from macrophage-lineage precursors. ED1 is an antibody that can recognize this lineage of cells. Matrix metalloproteinase 9 (MMP9) is essential for the migration of osteoclasts and their precursors during osteoclastogenesis. The aim of this research was to investigate differentiation and recruitment of osteoclasts during the early phase of experimental tooth movement in rats. The upper three molars of Wistar rats at one side were moved mesially, using Ni–Ti coil springs of 10 cN, for 6, 12, 24, 36, 48, 72, 96, and 120 h. The contralateral sides served as controls. Immunohistochemical staining using ED1 and MMP9 antibodies was performed. ED1+ and MMP9+ mononuclear and multinuclear cells were counted and statistically analysed. After force application, the number of ED1+/MMP9− multinuclear cells first increased in the bone marrow. At compressed areas, the number of ED1+ multinuclear cells decreased; this was followed by an increase in the number of ED1−/MMP9+ mononuclear and multinuclear cells. At tension areas, the number of ED1+/MMP9− multinuclear cells decreased while the number of ED1+ mononuclear cells remained stable. It was concluded that force application induces osteoclast differentiation within the bone marrow. These osteoclasts probably migrate subsequently into the compressed PDL. Pre-existing osteoclasts disappear at the tension areas while the number of mononuclear macrophage-lineage cells remains stable.

Orthodontic tooth movement is mediated by alveolar bone resorption at the compression areas of the periodontal ligament (PDL) and by bone deposition at the tension areas. During orthodontic tooth movement, bone resorption can be initiated at the periodontal surface of the alveolar bone. However, if hyalinization of the PDL occurs, bone resorption starts within the bone marrow, leading to undermining bone resorption (1). The only cells responsible for bone resorption, namely osteoclasts, are derived from haematopoietic stem cells, particularly from the macrophage lineage precursors. Thus, all macrophage-lineage mononuclear cells can be taken as potential osteoclast precursors. ED1 is a monoclonal antibody (mAb) against an antigen that is a rat homolog to human CD68. It has a high affinity and specificity for macrophage-lineage cells, including osteoclasts. Therefore, ED1 can be used as a marker for all potential osteoclast precursors as well as for all osteoclasts (2). It is well known from in vitro studies that the differentiation of these precursors to functionally active multinuclear cells is under the regulation of several cytokines, including macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-kappa B ligand (RANKL), and osteoprotegerin (OPG) (3–6). In late precursors and functional osteoclasts, matrix metalloproteinase-9 (MMP9) is highly expressed (7). In vitro studies have shown that MMP9 is essential for the degradation of collagen matrices, facilitating the migration of osteoclasts and their precursors through the extracellular matrix (ECM) to sites of bone resorption (8–11). Matrix metalloproteinase-9-deficient animals show a delay of osteoclast recruitment, and inhibitors of MMP9 suppress osteoclast migration and bone resorption in developing calvaria and probably also in long bones (12–17). A special situation exists during orthodontic tooth movement, where bone resorption is induced upon mechanical stimulation of the periodontal tissues. Although the appearance of osteoclasts at the compression areas of the PDL after orthodontic force application has been described by many authors (18–24), their origin is still controversial: they may be recruited either from the bone marrow (23) or from precursors within the PDL (25). Also, the possible role of MMP9 in the migration and recruitment of osteoclasts and their precursors in the early phases of orthodontic tooth movement is still unknown, and no immunohistochemical study on the spatial and temporal presence of MMP9 in the PDL has yet been reported.
Assuming that osteoclast differentiation during orthodontic tooth movement follows the same pathways as osteoclasts from calvarial or long bones, it can be hypothesized that in the normal PDL and/or alveolar bone marrow, macrophage-lineage mononuclear cells that do not express MMP9 are present. These cells will start to differentiate, after orthodontic force application. They become committed osteoclast precursors and start to synthesize MMP9 to enable migration through the ECM. In the case of direct bone resorption, these committed precursors may migrate through the ECM of the PDL to the bone surface, fuse, and form multinuclear osteoclasts. In the case of undermining bone resorption, however, either pre-existing osteoclasts in the bone marrow will start bone resorption, or osteoclast precursors in the bone marrow will start to synthesize MMP9, fuse, migrate, and attach to the endosteal surface to form active osteoclasts.

It was the purpose of this study to investigate this hypothetical cascade of events and, specifically, the sequential expression of MMP9 in all macrophage-lineage cells, including osteoclasts, during the early phase of experimental tooth movement.

**Material and methods**

**Experimental design**

Forty, 6-wk-old male Wistar rats were used in this study. The animals were acclimatized for at least 1 wk before the start of the experiment and were housed under normal laboratory conditions, with powdered laboratory chow (Sniff, Soest, the Netherlands) and water available ad libitum. The rats were divided into eight groups, with five animals in each group. A split-mouth design was used to control for individual variance. The maxillary three molars on one randomly chosen side were moved as one unit to the mesial by means of a coil spring. The contralateral maxillary molars served as controls. Ethical permission for the study was obtained according to the guidelines of the Board for Animal Experiments of the Radboud University Nijmegen.

The orthodontic appliance has been described extensively elsewhere (26). Briefly, a preformed stainless-steel ligature wire enclosing all three molars was bonded on the experimental maxillary sides (Clearfil SE BOND; Kuraray Europe, Düsseldorf, Germany). As a result, the three molars served as controls. Ethical permission for the study was obtained according to the guidelines of the Board for Animal Experiments of the Radboud University Nijmegen.

The orthodontic appliance has been described extensively elsewhere (26). Briefly, a preformed stainless-steel ligature wire enclosing all three molars was bonded on the experimental maxillary sides (Clearfil SE BOND; Kuraray Europe, Düsseldorf, Germany). As a result, the three molars could be moved together as one unit. For anchorage, a transverse hole was drilled through the alveolar bone and both maxillary incisors at the mid-root level. A stainless-steel ligature wire was put through the hole and a custom-made 10 cN Sentalloy close coil spring (GAC, New York, NY, USA) was activated and attached to the molar block and the incisor anchorage to deliver a constant mesial force. The force-level calibration was performed in an earlier study of our group. The superelastic properties of the springs and the delivered force were tested in a laboratory set-up at 38°C. Five springs were randomly chosen from a package of 80. Over 150 measurements were made to test each spring. The springs were shown to deliver a reproducible force of 10 ± 2 cN over a range of 3–15 mm activation. Using this design, tipping movement is almost completely prevented and the three molars together move almost bodily to the mesial. All treatments described above were performed under general anaesthesia [FFM-mix (fluanisone 6.8 mg kg⁻¹, fentanyl 0.1 mg kg⁻¹, and midazolam 3.4 mg kg⁻¹), 2.8 ml kg⁻¹ intraperitoneally].

After 6, 12, 24, 36, 48, 72, 96, and 120 h of force application, groups of five rats were killed by inhalation anaesthesia (5% isoflurane for induction and 2-3% for maintenance) and perfusion with 4% freshly made paraformaldehyde in phosphate-buffered saline (PBS). The maxillae were dissected and fixed in 4% paraformaldehyde for 24 h, then decalciﬁed with 10% (w/v) ethylene diamine tetra-acetate (EDTA) and embedded in parafﬁn.

**Selection of sections for evaluation**

Serial parasagittal sections of 5-µm thickness were cut from parafﬁn-embedded tissue blocks, mounted on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany), and stained with haematoxylin and eosin for general tissue survey. For immunohistochemical evaluation, sections that contained at least the radicular pulp of three roots of the maxillary molars were selected. For each antibody (MMP9 or ED1), a minimum of three sections, at least 25 sections apart, from each control or experimental side were stained.

**Immunohistochemistry**

For ED1 staining, the sections were deparafﬁnized and rehydrated through a graded series of ethanol solutions. The sections were treated with citrate buffer (pH 6.0) in a microwave oven for 10 min, then with 1% Trypsin (Difco Laboratories, Detroit, MI, USA) at 37°C for 5 min, and finally with a solution containing H₂O₂ and methanol (1:9, v/v) to inhibit endogenous peroxidase activity. Non-speciﬁc binding of the secondary antibody was minimized by pre-incubation for 1 h in PBS containing 20% normal donkey serum (Chemicon Europe, Chandlers Ford, Hampshire, UK). The sections were exposed sequentially to a monoclonal mouse-anti-rat immunoglobulin (IgG1) to ED1 (MCA341R; Serotec, DPC, Breda, the Netherlands) diluted 1:200 in 2% donkey serum in PBS, at 4°C overnight, biotinylated donkey-anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 h, Vectorstain ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA) for 1 h, and a working solution of 3,3′ diaminobenzidine tetrahydrochloride containing 0.02% H₂O₂ with nickel enhancement (Sigma Chemical, St Louis, MO, USA) for 10 min. Thereafter, the slides were treated with 5% copper sulphate for 5 min, then rinsed and faintly counterstained with haematoxylin. All procedures were carried out in a humid chamber at 20°C except for incubation with the ﬁrst antibody (which was carried out at 4°C). Dark brown granular deposits on the cell membrane indicated positive cells.

For MMP9 staining, the general treatments were the same as for ED1, except that the ﬁrst antibody was a monoclonal mouse anti-human immunoglobulin (IgG) to MMP9 (MAB911; R&D systems, Minneapolis, MN, USA), diluted 1:20 in 2% donkey serum in PBS. For both ED1 and MMP9 staining, negative controls were performed by omitting the ﬁrst antibodies, and sections of healing palatal wounds were used as positive controls. For the validation of MMP9 staining, neighbouring sections were stained with cathepsin K or MMP9 (Fig. 1B,C). Cathepsin K antibody was polyclonal goat–anti-human IgG (sc-6507; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500 in 2% donkey serum in PBS. The secondary antibody for
cathepsin K staining was biotinylated donkey anti-goat IgG (Jackson Immunoresearch Laboratories). The remainder of the staining conditions for cathepsin K were the same as for ED1 and MMP9.

**Measurements**

Four categories of positive cells, namely mononuclear ED1⁺, mononuclear MMP9⁺, multinuclear ED1⁺, and multinuclear MMP9⁺, were counted along the mesial and distal areas of the selected roots according to their locations – bone marrow, bone surface, PDL, and root surface – in both experimental and control sides. The above four compartments are illustrated in Fig. 1. Because the three maxillary molars were moved mesially as one unit, the mesial areas of all roots at the experimental sides were defined as compression areas, whereas the distal areas were defined as tension areas. The bone marrow compartment was taken as a whole because of the difficulty in distinguishing between tension and compression areas in the thin interdental septum. The net effect of the force application was defined as the difference in the number of positive cells between the experimental side and the control side. To standardize the data, root length was measured. The data were presented as the number of positive cells per mm of root length.

Intra-examiner reliability was measured on 10 successive sections, and $R^2$ values of $>0.80$ were obtained for cell counting and root length measurements in all four compartments. The observer was blinded for the time period.

**Statistical analysis**

As most of the data of the control sides showed a non-normal distribution, the Kruskal–Wallis test was used to evaluate time dependency of the number of positive mononuclear or multinuclear cells for each marker (MMP9 or ED1) in these sides. The data for the net effect, which is the difference between experimental and control sides, however, was normally distributed. Therefore, one-way analysis of variance (ANOVA) and Tukey’s multiple-comparison post hoc test were used to analyse the time dependency of the net effect. Student’s $t$-tests were performed with Bonferroni’s correction for multiple comparisons to compare differences between experimental and control groups at selected time-points. To analyse differences between MMP9⁺ and ED1⁺ cell numbers, the Mann–Whitney $U$-test was performed. The significance level was set at $P < 0.05$.

**Results**

**General tissue survey**

In the control samples, only a few resorption lacunae were present on the distal bone surfaces. Root resorption lacunae were occasionally present on the distal surface of the roots. After 6 h of force application, the molars had bodily moved to the mesial within their sockets, with the mesial PDL clearly showing compression over its whole length and the distal PDL showing widening (Fig. 2A). Hyalinization of the compressed PDL was found in all samples of 24 h and longer. After 36 h, undermining resorption started within the adjacent bone marrow in some samples and, after 48 h, it had become a general feature and direct bone resorption started from the bone surface next to the hyalinization areas. Root resorption became apparent at the root surface at the compression area at 72 h in some samples (data not shown).

**Immunohistochemistry**

Figure 2B,C illustrates the staining of MMP9 and cathepsin K, respectively. Both antibodies stained the same population of multinuclear cells. The immunohistochemical staining patterns of ED1 and MMP9 are shown in Fig. 2D,E. An example of negative-control staining, in which MMP9 or ED1 antibodies were omitted, is shown in Fig. 2F. In both MMP9 and ED1 staining, the same secondary antibody (biotinylated donkey–anti-mouse IgG) was used. Therefore, only one negative control is shown.

No statistically significant time effect was found in any of the observed areas at the control sides. Mononuclear ED1⁺ or MMP9⁺ cells were mainly present within the PDL, and the ED1⁺ cells were present at statistically significant higher numbers than the MMP9⁺ cells, both at the mesial side and at the distal side (Fig. 3A). ED1⁺ and MMP9⁺ multinuclear cells were almost exclusively present on the distal bone surfaces, where they were present in equal numbers (Fig. 3B).

**Compression areas**

In the compression areas, the number of ED1⁺ mononuclear cells at the bone surfaces was low. However, they showed a statistically significant increase in number at 96 and 120 h, and the number of MMP9⁺ mononuclear cells showed a statistically significant increase from 48 to 96 h (Fig. 3C). The vast majority of ED1⁺ or MMP9⁺ mononuclear cells appeared within the PDL. An initial decrease in the number of ED1⁺ mononuclear cells was found to occur from 12 to 24 h. Thereafter, their numbers returned to control levels and higher. The number of MMP9⁺ cells in the PDL showed a statistically
significant increase from 48 to 96 h (Fig. 3D). Adding up all cell counts from the compression areas showed the same tendencies and statistical significances as the data from the PDL (data not shown).

The vast majority of ED1+ and MMP9+ multinuclear cells were found along the bone surface. The number of ED1+ and MMP9+ multinuclear cells on the bone surface increased gradually from 36 h onwards (Fig. 3E). There was a tendency for the numbers of ED1+ and MMP9+ multinuclear cells in the PDL and on the root surface to increase from 48 h onwards and from 72 h onwards, respectively (Fig. 3F,G). However, as a result of the low number and large variance in the PDL and on the root surface, these tendencies were not statistically significant.

Tension areas
In tension areas, the only statistical significances were found at the bone surface, where the number of ED1+ and MMP9+ mononuclear cells showed a tendency to decrease from 48 h onwards; however, this was only statistically significant at 72 and 96 h for ED1+ cells and at 48 h for MMP9+ cells (Fig. 3H). In the PDL, there were some increases and decreases, but these were unrelated to time (Fig. 3I). If the overall numbers of ED1+ and MMP9+ mononuclear cells at the tension areas were considered, no time dependency was found (data not shown).

In tension areas, ED1+ and MMP9+ multinuclear cells were almost exclusively found at the bone surfaces. The number of ED1+ and MMP9+ multinuclear cells decreased after force application. After 12 h, the total number of ED1+ multinuclear cells already showed a statistically significant decrease. This decrease continued over time, and from 36 h onwards almost all ED1+ multinuclear cells had disappeared. The same was found for MMP9+ cells, but here the decrease was only statistically significant from 24 h onwards. No statistically significant difference between the number of ED1+ and MMP9+ cells was found at any time-point (Fig. 3J).

Bone marrow
The number of ED1+ mononuclear cells tended to be higher in the experimental sides than in the control sides between 12 and 48 h, but this was not statistically significant (Fig. 3K). The number of ED1+ and MMP9+ multinuclear cells showed a statistically significant temporary increase at the experimental sides from 24 to 48 h (Fig. 3L).

Discussion
Osteoclast recruitment during orthodontic tooth movement has been studied in rats using different force systems at different time-points (22–24). In our study, we used a controlled and a well-standardized force of 10 cN on three molars (27). Even with such a small force, hyalinization was observed after force application in all samples of 24 h and later.

While the appearance of osteoclasts at compression sides has been described in several studies (18–24), we focused on the origin of the osteoclasts, which is still largely unknown. As osteoclast differentiation and recruitment after force application are rather quick processes (23), we limited the experimental period to the first 120 h.

The initial response in the compression areas was a decrease in the number of ED1+ and MMP9+ multinuclear cells, which started at 12 h and lasted until 24 h. This may be related to hyalinization of the PDL, when all cellular components, including macrophages, gradually disappear (28). After 24 h, ED1+ macrophages re-appeared, probably through recruitment from the surroundings of the hyalinized area. The number of ED1+ macrophages gradually returned to normal values and tended to become higher than in the controls. The number of MMP9+ mononuclear cells showed a pattern comparable to that of the ED1+ mononuclear cells, suggesting...
Fig. 3. (A, B) Box-plots of mononuclear and multinuclear cells at the control sides. ED1\(^+\) mononuclear cells were present in statistically significantly higher numbers than MMP9\(^+\) cells. ED1\(^+\) and MMP9\(^+\) multinuclear cells were present almost exclusively at the distal bone surface. No statistically significant differences were found between the numbers of ED1\(^+\) and MMP9\(^+\) mononuclear or multinuclear cells at any time-point. (C–L) Differences observed in the numbers of positive cells between the experimental and control compartments during the experimental period. Means and standard errors (SE) are given. (C, D) Mononuclear cells at the compression areas, respectively, on the bone surface and in the PDL. The number of ED1\(^+\) cells on the bone surface showed a statistically significant increase at 96 and 120 h, and the number of MMP9\(^+\) mononuclear cells showed a statistically significant increase from 48 to 96 h. The number of ED1\(^+\) mononuclear cells in the PDL showed a statistically significant decrease at 12 and 24 h, and thereafter returned to control levels or higher. The number of MMP9\(^+\) cells showed a statistically significant increase from 48 to 96 h. (E) Multinuclear cells on the bone surface of the compression areas. A comparable, statistically significant increase in the number of ED1\(^+\) and MMP9\(^+\) cells was found from 36 h onwards. (F, G) Multinuclear cells in compression areas, respectively in the PDL and on the root surface. There were tendencies that the number of ED1\(^+\) and MMP9\(^+\) multinuclear cells in the PDL and on the root surface increased, respectively, from 48 to 72 h onwards. However, these tendencies were statistically not significant. (H, I) Multinuclear cells in the tension areas, respectively on the bone surface and in the PDL. The number of ED1\(^+\) and MMP9\(^+\) cells on the bone surface were very low and they showed a small decrease in the experimental compartments. However, no overall time dependency was found. The number of ED1\(^+\) cells in the PDL was rather variable. The only statistical significance was found at 96 h. The number of MMP9\(^+\) cells showed no changes until 36 h. It tended to be lower thereafter, but this was only statistically significant at 48 h. (J) Multinuclear cells in the tension areas at the bone surface. The number of ED1\(^+\) cells decreased from 12 h onwards and were almost absent from 36 h onwards. The number of MMP9\(^+\) cells showed a comparable decrease, which was statistically significant from 24 h onwards. (K) Mononuclear cells in the bone marrow at the experimental sides. The number of ED1\(^+\) mononuclear cells tended to be higher in the experimental sides than in the control sides between 12 and 48 h. However, this was statistically not significant. (L) Multinuclear cells in the bone marrow at the experimental sides. The number of ED1\(^+\) and MMP9\(^+\) cells showed a statistically significant increase from 24 to 48 h. Thereafter, they returned to control levels. MMP9, matrix metalloproteinase-9; PDL, periodontal ligament.
that the newly recruited ED1⁺ mononuclear cells in the compression areas are also MMP9⁺ and therefore can be considered as migrating macrophages involved in the removal of the hyalinized tissue (29–31).

The most prominent change after force application at the compression areas was the gradual increase of multinuclear cells at the bone surface. This was statistically significant from 36 h to the end of the experimental period. Such an increase was also found in the PDL and at the root surface, but the effect was small and it started later than on the bone surface, at 48 and 72 h, respectively. These data suggest that the osteoclasts or their multinuclear precursors migrate from the bone marrow to the bone surface and subsequently to the PDL and the root surface. The bone lining osteoclasts start direct bone resorption, the multinuclear cells within the PDL may play a role in the removal of hyalinized tissue, and the root-related osteoclasts eventually start root resorption (18, 19, 32).

The increase in the number of multinuclear cells at the bone surface at the compression areas was preceded by reactions within the bone marrow, which might be evoked by chemotactic effects of the hyalinization process (33–35) or through mechanosensitivity of osteocytes (36). Both mononuclear and multinuclear cells in the bone marrow increased temporarily from 24 to 48 h, although this was only statistically significant for the multinuclear cells, as a result of the wide variation in the number of mononuclear cells. After 48 h, the numbers of both cell types again decreased to control levels.

The data suggest a rapid differentiation of macrophage-lineage cells in the bone marrow in the first 24 h of orthodontic force application. They probably fuse rapidly within the bone marrow and start to produce MMP9. After attachment to the bone, these multinuclear osteoclasts start undermining resorption. After removal of the alveolar bone they probably migrate to the periodontal bone surface, where they may contribute to the removal of hyalinized tissue and start direct bone resorption (19, 23).

The number of ED1⁺/MMP9⁺ multinuclear cells at the tension areas was low in the PDL and at the root surface throughout the experimental period. At the bone surface, however, the number of pre-existing osteoclasts

![Fig. 3. (continued)](image-url)
showed a rapid decrease over time, which is probably caused by apoptosis (37). From 36 h onwards, osteoclasts are almost completely absent at the tension areas. This reflects the change from a distal drift to a mesial orthodontic tooth movement.

The data support the hypothesis that orthodontic force application will initially lead to an increased number of ED1 macrophages within the bone marrow, which subsequently fuse and start to express MMP9. Then, they further differentiate into osteoclasts that start undermining resorption. The number of osteoclasts within the bone marrow, however, subsequently decreases again rapidly to reach normal levels. In the meantime, a transient decrease in macrophage-lineage cells within the PDL is reversed to an increase. Concomitantly, an increase in the number of osteoclasts within the PDL is taking place. This strongly suggests that osteoclasts responsible for tooth movement originate from the bone marrow in the case of hyalinization and from recruited precursors in the PDL in the case of direct bone resorption.

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