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ORIGINAL ARTICLE

Osteoclast differentiation during experimental tooth movement by a short-term force application: An immunohistochemical study in rats

RUI XIE, ANNE MARIE KUIJPERS-JAGTMAN & JAAP C. MALTHA

Department of Orthodontics and Oral Biology, Radboud University Nijmegen Medical Centre, 309 Dentistry, Nijmegen, The Netherlands

Abstract

Objective. The origin of osteoclasts responsible for bone resorption during orthodontic tooth movement is not yet clear. Their precursors may reside within the periodontal ligament (PDL) or could be recruited from the circulation or the bone marrow. The aim of this study was to investigate the spatial and sequential distribution of osteoclast precursors during experimental tooth movement by using three differentiation markers: receptor for macrophage colony stimulating factor (c-Fms), receptor activator of nuclear factor-κB (RANK), and calcitonin receptor (CTR). ***Material and Methods.*** Six-week-old Wistar rats were used. Elastic bands were inserted between the upper 1st and 2nd molars for 1, 2, 3, and 6 days. Immunohistochemical staining for c-Fms, RANK, or CTR was performed on parasagittal sections and positive cells were counted. ***Results.*** Before force application, many c-Fms+ and a few RANK+ precursors were present in the bone marrow. No c-Fms+ osteoclast precursors were observed in the PDL. After force application, the number of RANK+ but not c-Fms+ precursors increased rapidly in the PDL. In bone marrow, the number of c-Fms+ and RANK+ precursors also increased rapidly, as did multinuclear c-Fms+, RANK+, and CTR+ cells. Subsequently, the number of c-Fms+, RANK+, and CTR+ multinuclear cells in the PDL increased. After 6 days, the expression profiles tended to return to baseline levels. ***Conclusion.*** Osteoclast precursors differentiate within the bone marrow and then migrate into the PDL during early tooth movement.

Key Words: *Calcitonin receptor, c-Fms, orthodontics, osteoclasts, RANK, tooth movement*

Introduction

Orthodontic tooth movement occurs by bone resorption at the compression side of the periodontal ligament (PDL), by bone formation at the tension side, and by remodeling of the PDL itself [1,2]. Its velocity is limited by the rate of bone resorption, and thus osteoclasts are important in its regulation. In physiological bone remodeling, osteoclast precursors reside in the periosteum close to the bone surface. They differentiate into active osteoclasts and are gradually replenished from the circulation [3]. However, the origin of osteoclasts in the PDL under orthodontic loading is not yet clear. Some studies conclude that the precursors reside within the PDL [4], while others argue that they are recruited from the circulation or the bone marrow [5].

Osteoclasts derive from the hematopoietic myeloid lineage. Pluripotent mononuclear precursors mature under the control of regulatory factors [6,7]. A close contact between stromal and bone marrow cells allows osteoclastogenesis [8], but this is also possible by adding two soluble factors, namely macrophage colony stimulating factor (M-CSF) [9] and receptor activator NFκB ligand (RANKL) [10–12]. Their respective receptors, c-Fms and RANK [13–16] are essential for *in vivo* osteoclast formation. c-Fms is considered as a marker for macrophage lineage cells and its expression is essential for the development of osteoclast precursors, since targeted disruption of the c-Fms gene results in osteopetrosis, mononuclear phagocyte deficiency and other defects [17]. During early and late stages of osteoclastogenesis *in vitro*, precursor cells sequentially express c-Fms followed by RANK, and thus c-Fms and RANK can be used to show osteoclast differentiation in tissues. This means that early-stage precursor cells are

Correspondence: Jaap C. Maltha, Radboud University Nijmegen Medical Centre, Department of Orthodontics and Oral Biology, 309 Dentistry. P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Tel: +31 24 3614005. Fax: +31 24 3540631. E-mail: orthodontics@dent.umcn.nl

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c-Fms+RANK-, whereas late-stage precursor cells are c-Fms+RANK+ [18].

Committed osteoclast precursors fuse to multinuclear cells, attach to the bone surface, and develop ruffled borders [6]. Other markers of osteoclasts are tartrate-resistant acid phosphatase (TRAP), vitronectin receptor (VNR), and calcitonin receptor (CTR). TRAP and VNR are easy to visualize, but they are also expressed in activated macrophages, while CTR is exclusively expressed on active osteoclasts [19–21]. Therefore the latter was chosen as marker for active osteoclasts.

Macrophage lineage cells and osteoclasts have been shown in the PDL [22,23]. However, still little is known about the origin of the newly recruited osteoclasts. We hypothesize that, as in physiological bone remodeling, osteoclast precursors reside within the PDL close to the alveolar wall. After force application, the residing osteoclast precursors differentiate into active osteoclasts, and the precursors are replenished from the circulation. Therefore, in this study, the localization of early and late osteoclast precursors, and mature osteoclasts, was investigated during the initial phase of experimental tooth movement in rats using c-Fms, RANK, and CTR as markers.

Material and methods

Animal preparation

Eight male Wistar rats, aged 6 weeks, were used in this study. The animals were acclimatized for at least 1 week before the start of the experiment and were housed under normal laboratory conditions, with powdered laboratory chow (Sniff, Soest, The Netherlands) and water *ad libitum*. Ethical permission for the study was obtained in accordance with the guidelines of the Board for animal experiments of Radboud University Nijmegen.

Orthodontic forces were applied at all maxillary left and right sides, except four, which served as baseline (day = 0). Forces were applied according to the method of Waldo & Rothblatt [24]. Under inhalation anesthesia (5% isoflurane for induction and 2–3% for maintenance), orthodontic elastic bands (59-700-14, GAC, Bohemia, NY, USA) were inserted between the upper 1st and 2nd molars. Rats were killed at different time-points after inhalation anesthesia (5% isoflurane for induction and 2–3% for maintenance) and perfused with 4% freshly made paraformaldehyde in phosphate buffer (PBS) in order to obtain samples at baseline (day = 0) and after 1, 2, 3, or 6 days of force application. The maxillae were dissected and fixed in 4% paraformaldehyde for 24 h, then decalcified with 10% EDTA and embedded in paraffin.

Immunohistochemistry

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 hematoxylin and eosin for general tissue survey. For immunohistochemical evaluation, sections were selected that contained the radicular pulp of the mesial and distal roots of the maxillary 2nd molar.

Staining techniques

c-Fms. After deparaffinization and rehydration through a graded series of ethanol solutions, the slides were first treated with citrate buffer (pH 6.0) in a microwave for 10 min, followed by 1% trypsin (Difco Laboratories, Detroit, Mich, USA,) at 37°C for 5 min. The sections were then treated with H_2O_2 in methanol to inhibit endogenous peroxidase activity. Non-specific binding of the secondary antibody was blocked with 20% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, Pa., USA). The sections were exposed sequentially to: (1) a rabbit polyclonal antibody to c-Fms (1:100) (sc-13949; Santa Cruz Biotechnology, Santa Cruz, Calif., USA); (2) the biotinylated goat-anti-rabbit IgG (Chemicon, Temecula, Calif, USA); (3) Vectastain ABC-Elite kit (Vector Laboratories, Burlingame, Calif., USA), and (4) the staining solution of 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, Mo., USA). After immunostaining, the slides were treated with 5% copper sulphate for 5 min and faintly counterstained with hematoxylin.

RANK and CTR. The general treatments were the same as for c-Fms, only the first antibody was replaced by anti-RANK 1:400 (sc-9072; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) or anti-CTR 1:200 (kind gift from Maria Morfis, Neurobiology Unit, St. Vincent Institute of Medical Research, Victoria, Australia). RAW 264.7 cells were used as positive controls according to the datasheet. Negative controls were performed by omitting the first antibodies.

Measurements

The 2nd maxillary molars were tipped to the distal by separation elastics and therefore the cervical regions of the distal sides of the roots of the 2nd molar are pressure areas. These sides were used to evaluate the differentiation and recruitment of osteoclasts and their precursors. Comparable areas were used for baseline (day = 0) measurements. Control molars moved slightly to the distal by physiological drift. The measurements were

restricted to a 300 × 700 μm rectangle comprising the pressure areas of the PDL and the inter-radicular bone marrow. Per side, at least three sections, about 25 sections apart, were evaluated by counting positive mononuclear and multinuclear cells in the PDL and the bone marrow.

Statistics

The median values and 50% confidence intervals of the measurements were calculated. As most of the data were not normally distributed, non-parametric tests were used for the analysis over time and differences between groups. For the analysis, the data from the control sides were considered as experimental day 0. The Kruskal-Wallis test was used to evaluate time differences in the number of positive mono- or multinuclear cells for each marker and the Mann-Whitney test to compare the medians at each time-point. Differences were assumed to be significant if $p < 0.05$.

Results

Figure 1 illustrates the area that was observed and the staining for c-Fms, RANK, and CTR, respectively. A micrograph of the tension side is given as a control in Figure 1E. The median values of the number of positive cells are given in Figure 2.

Bone marrow

Mononuclear cells (Figure 2A). No mononuclear CTR+ cells were found in the marrow. The distribution of c-Fms+ and RANK+ cells was almost the same in day 0 and day 1 samples, the number of c-Fms+ cells being about three times as high as the number of RANK+ cells. At day 2, the number of c-Fms+ cells and RANK+ cells both increased. On day 3 the number of c-Fms+ cells had returned to control level, but the number of RANK+ cells had further increased and now differed significantly from day 0 ($p = 0.012$) and was about four times as high as the number of c-Fms+ cells. At day 6, the number of RANK+ and c-Fms+ cells had decreased again, but the number of RANK+ cells was still significantly higher than at day 0 ($p = 0.010$).

Multinuclear cells (Figure 2B). The number of multinuclear cells in the bone marrow was far lower than the number of mononuclear cells, and the pattern of their markers was quite different. At baseline (day = 0), only a few RANK+ and CTR+ cells were present; no c-Fms+ cells were found. Already at day 1 the number of cells in the marrow had increased, now showing about equal numbers of c-Fms+, RANK+, and CTR+ cells. At day 2 the numbers of all three markers showed a tendency to increase further. At day 3 they had all decreased in number again and at day 6 the pattern of their markers was the same as at baseline.

PDL at pressure sides

Mononuclear cells (Figure 2C). The only marker found in mononuclear PDL cells was RANK. At baseline, they were present at relatively low numbers. On days 1, 2, and 3, their number was elevated, but this was not significant. At day 6 their number had decreased to baseline levels again.

Multinuclear cells (Figure 2D). At baseline (day = 0), almost no multinuclear c-Fms+, RANK+, or CTR+ cells were found, but they were present in small and equal numbers at day 1. Their numbers increased further at days 2 and 3, and at day 3 were significant higher compared to day 0 ($p = 0.001$, $p = 0.001$, $p = 0.014$, respectively). At day 6 the number of all three categories of positive cells had decreased considerably. However, this decrease was only significant for CTR+ cells ($p = 0.023$). For all three categories, the numbers of positive cells were higher at day 6 than at day 0 ($p < 0.05$) and they appeared in equal numbers.

Discussion

This study reports on the spatial and temporal changes in three osteoclast markers during experimental tooth movement: c-Fms, RANK, and CTR. These markers also permit estimations of the level of osteoclast differentiation. The main conclusion is that, contrary to our hypothesis, in which osteoclast differentiation is assumed to be initiated within the PDL, osteoclasts first differentiate within the bone marrow and then migrate into the PDL during tooth movement. Although this is not a new observation, this study serves as confirmation of previous work using a different experimental approach than has been employed previously.

In this study, a tipping molar movement was induced with the Waldo-Rothblatt method [24]. This model has its limitations when force levels and amount of tooth movement need to be quantified, while insertion of the elastic band also causes gingival inflammation. However, in the present study, the method was only used to evaluate the differentiation and recruitment of osteoclasts; tooth movement parameters were not considered to be important for our purposes.

Elastic band insertion results in a gap between the 1st and 2nd molars, which can be as wide as 0.5 mm after 3 days [25]. The force decays to zero if the gap opens more than 0.3 mm [26]. This means that the elastic band delivers a rapidly decreasing stimulus that fades away completely within 3 days. This might

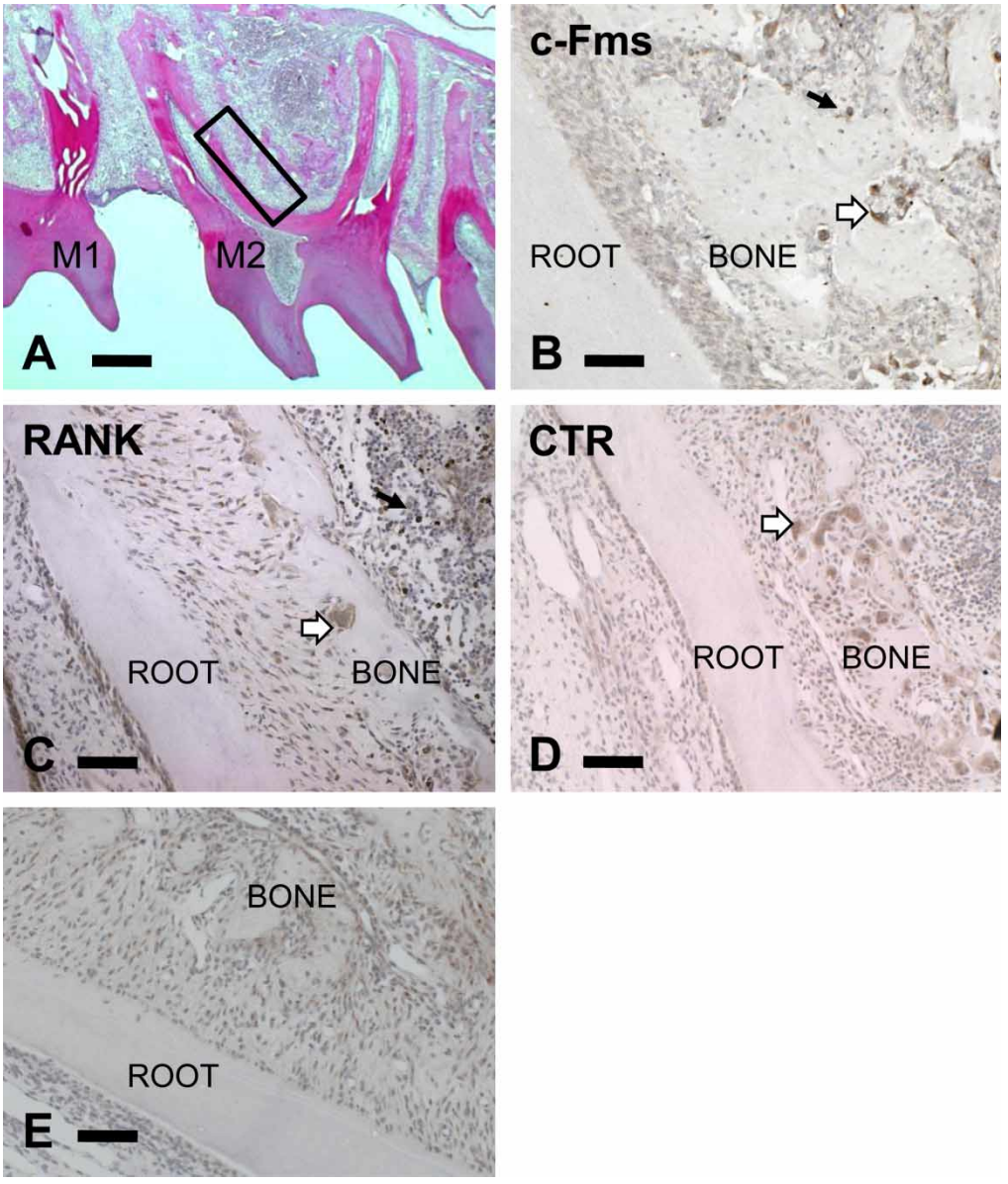

Figure 1. A. HE staining showing the area observed. M1: upper 1st molar. M2: upper 2nd molar. Bar = 500 μm. B. Staining for c-Fms after 2 days of elastic band insertion. c-Fms+ mononuclear cells (black arrow) and c-Fms+ multinuclear cells (white arrow). Bar = 100 μm. C. Staining for RANK after 3 days of elastic band insertion. RANK+ mononuclear (black arrow) and multinuclear cells (white arrow). Bar = 100 μm. D. Staining for CTR after 3 days of elastic band insertion. Only multinuclear CTR+ cells are present. Bar = 100 μm. E. Tension area showing that osteoclast formation occurs primarily only on the pressure area. RANK staining. Bar = 100 μm.

partly explain the decrease in osteoclast numbers after 3 days.

c-Fms expression in early osteoclast precursors has been shown *in vitro* [27,28], while, *in vivo*, c-Fms mRNA and protein expression was only found in late precursors and mature osteoclasts [29,30]. In tooth-supporting tissues, RANK expression was found in mature osteoclasts [31]. Since in *in vitro* osteoclastogenesis RANK expression followed the expression of c-Fms [18], c-Fms+RANK- mononuclear cells were considered as early-stage precursor cells, whereas c-Fms+RANK+ mononuclear cells were considered as late-stage precursor cells. CTR is assumed to be a marker for mature osteoclasts, since it is not expressed in macrophage polykaryons [32,33].

At baseline, cells in all stages of osteoclast differentiation resided in the bone marrow. In the pressure areas of the PDL, only very few multinuclear CTR+ cells were found; these were probably responsible for the distal drift. These findings indicate that under normal physiological conditions the differentiation of mature osteoclasts takes place within the bone marrow and that only very few mature osteoclasts migrate to the PDL at the pressure area.

After 1 day of elastic band insertion, the results suggest fusion of mononuclear precursors into multinuclear cells and further differentiation within the

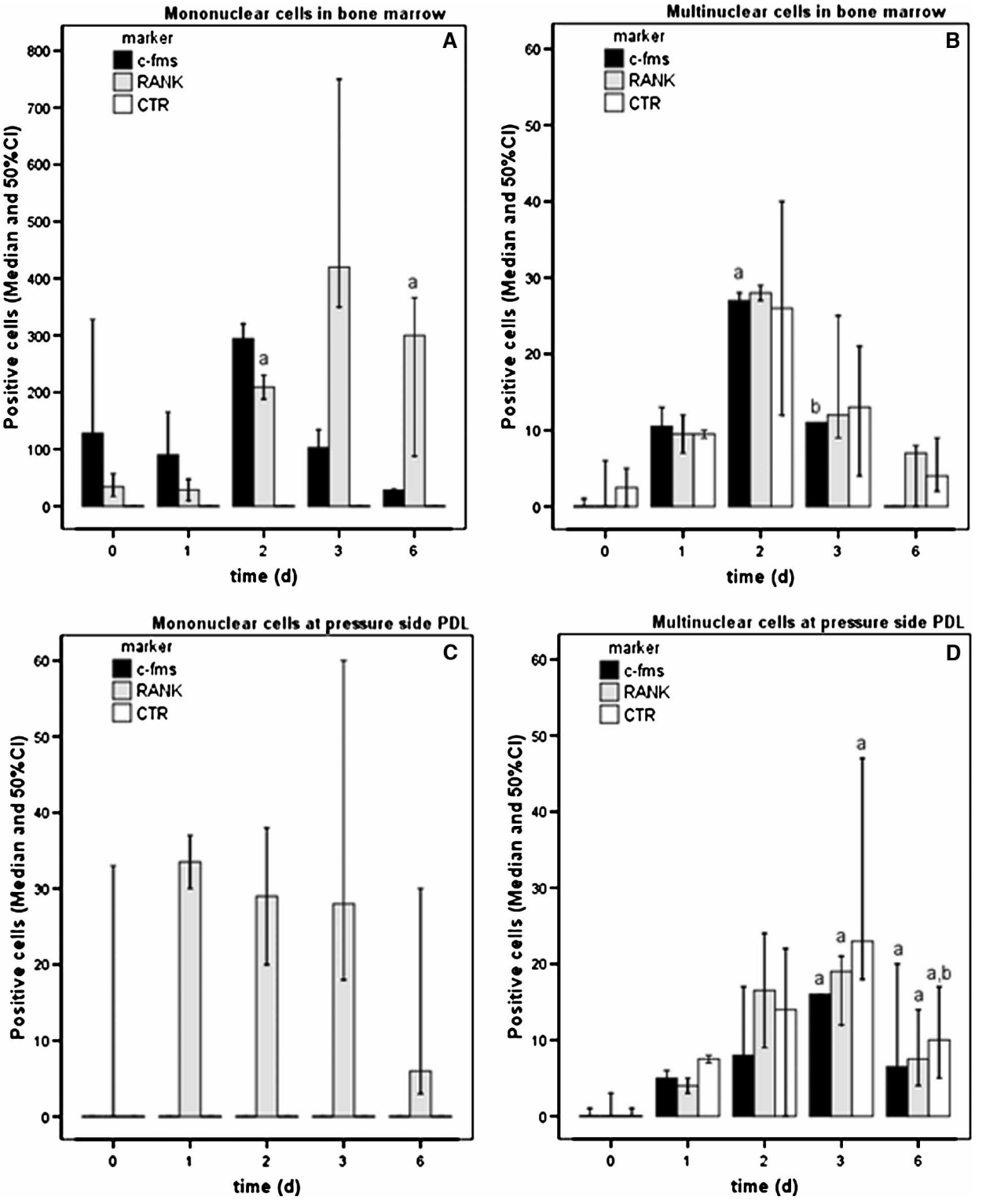

Figure 2. Number of positive mono- and multinuclear cells for each marker in bone marrow and in the pressure areas of the PDL. a: increase is significant; b: decrease is significant**.** A. Mononuclear cells in the bone marrow. The number of c-Fms+ mononuclear cells reached a maximum at day 2, while RANK+ mononuclear cells reached a maximum at day 3. No CTR+ mononuclear cells were observed. B. Multinuclear cells in the bone marrow. The number of c-Fms+, RANK+, and CTR+ multinuclear cells reached a maximum at day 2. From day 3 on, their numbers decreased again. C. Mononuclear cells in the pressure areas of the PDL. Neither c-Fms+ nor CTR+ mononuclear cells were found in the PDL throughout the entire experimental period. RANK+ mononuclear cells were present in the pressure areas of PDL and their number increased after force application. This increase remained until day 3. On day 6 the number of RANK+ mononuclear cells had decreased again. D. Multinuclear cells in pressure areas of PDL. Multinuclear cells increased after force application and reached a maximum at day 3; their numbers decreased thereafter.

bone marrow. Possibly, these multinuclear cells subsequently migrate to the PDL, while some of them lose their c-Fms expression. Apart from that, the appearance of RANK+/c-Fms- mononuclear cells in the PDL suggests that mononuclear precursors also migrate to the pressure area of the PDL and lose their c-Fms expression.

After 2 days, the bone marrow showed a rapid increase of mononuclear and multinuclear cells. The numbers of c-Fms+, RANK+, and CTR+ multinuclear cells were about equal. This indicates that differentiation is activated within the bone marrow. Also the number of multinuclear cells in the PDL increased, probably as a result of undermining bone resorption, destruction of the alveolar cortex, and migration of marrow multinuclear cells. Also, migration of RANK+ mononuclear cells from the bone marrow into the PDL occurred.

After 3 days the force had dissipated. Although RANK+ mononuclear cells in bone marrow still increased in number, c-Fms+ mononuclear cells decreased, suggesting a halt to the early differentiation of marrow cells. A continuous migration of multinuclear cells from the bone marrow to the PDL seems to occur, as they decreased in the marrow and increased in the pressure areas of the PDL.

After 6 days, all positive cells tended to return to baseline levels, although not completely. This is probably related to the vanishing force. As a consequence, bone resorption stops by cessation of osteoclast differentiation and/or apoptosis [34].

c-Fms+ mononuclear cells were never found in the PDL, indicating that early osteoclast precursors only exist in the bone marrow, whereas RANK+ mononuclear cells were found in the PDL, indicating the presence of later osteoclast precursors. This points in the same direction as the findings of Rody et al. [5], although we used a different experimental approach.

In conclusion, the normal rat PDL is devoid of early mononuclear osteoclast precursors; only very few active osteoclasts are present and these are probably responsible for distal drift. After elastic band insertion, early mononuclear precursors in the bone marrow differentiate into committed mononuclear precursors, fuse, and migrate into the PDL at pressure areas where they differentiate into active multinuclear osteoclasts. Alternatively, multinuclear osteoclasts are activated within the bone marrow and migrate into the PDL through undermining resorption and continue with direct bone resorption.

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