Segregated Localization of Immunocompetent Cells and Osteoclasts in the Periodontal Ligament of the Rat Molar*

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Summary. The spatial distribution of dendritic cells, macrophages, and their respective precursor cells in the periodontal ligament of rat molars was examined by means of ACPase enzyme histochemistry and immunohistochemistry. Intense reactions for ACPase were localized in both the multinucleated-and mononucleated cells of the periodontal ligament located exclusively in the portions of physiological bone resorption due to the physiological migration of the molar teeth. Immunohistochemical staining with OX6-monomoclonal antibody that recognizes antigen-presenting cells such as dendritic cells and macrophages revealed the localization of immunopositive cells predominantly in the portions of the periodontal ligament that showed only trace reactions for ACPase. On the other hand, a large number of ED1-immunopositive cells, comprising a broad spectrum of cells of monocyte origin including dendritic cells and osteoclasts, displayed an almost even distribution throughout the periodontal ligament. Our current study is the first to show clear-cut in vivo morphological evidence that the cells of the bone-resorting, osteoclastic cell lineage and those of the non-bone resorbing, macrophagic and/or dendritic cell lineages are exclusively localized in roughly the distal and proximal regions of the periodontal ligament of rat molars, respectively. An advantage is proposed for the use of the rat molar periodontal ligament as an in vivo model system for pursuing differentiation pathways of cells of the monocyte lineage, particularly of the osteoclastic cells.

Macrophages have been known as antigen-presenting cells that have a phagocytic activity and characteristically well-developed lysosomal systems showing intense ACPase activities. In recent years the presence of dendritic cells has been well acknowledged in various tissues which have no significant ACPase activity and have an antigen-presenting capability much stronger than that of the macrophages such as sensitization of MHC-restricted T cells (VAN FURTH, 1986; STEINMAN et al., 1986). The precise origin and function of dendritic cells have been investigated (INABA et al., 1992; INABA, 1993).

Unlike in ordinary rooted teeth of limited growth, the periodontal ligament of continuously erupting rodent incisors undergoes constant remodeling, and hence is recognized as a physiological stable tissue free from infection and foreign antigens. In previous studies, we reported on the presence of a large number of dendritic cells in the periodontal ligament of rat incisors and demonstrated their region-specific localization to be different from that of macrophages and elaborated on their differentiation pathways (KAWAHARA et al., 1992; KAWAHARA and TAKANO, 1995).

The rat molar teeth are of limited growth and are known to migrate toward the distal direction constantly throughout life (VIGNERY and BARON, 1980; ROBINSON and SCHNEIDER, 1992; LASFARGUES and SAFFAR, 1993). Accordingly in the periodontal ligament, the ligament fibers and alveolar bone undergo dynamic remodeling in accordance with the distal migration of molar teeth (VIGNERY and BARON, 1980; ROBINSON and SCHNEIDER, 1992; LASFARGUES and SAFFAR, 1993). Since the biological significance of dendritic cells in the periodontal ligament of rat incisors is obscure, the present study examines the periodontal ligament of rat molars to define whether or not the dendritic cells are ubiquitous in the periodontal ligament. The results confirm the presence of dendritic cells and osteoclasts in the molar periodontal ligament, and further reveal a distinct habitat segregation between the two types of cells in the

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local environment, both of which are known to originate from monocyte lineage.

MATERIALS AND METHODS

Adult male rats of the Wistar strain weighing 260 ± 10 g were anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg body wt.) and perfused via the aorta either with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) or a mixture of 4% paraformaldehyde and 0.25% glutaraldehyde in the same buffer for 20 min. Upper jaws were dissected and processed for decalcification with neutral 5% EDTA at 4°C for 30 days. Following decalcification, the tissues were immersed in a 30% sucrose solution at 4°C overnight, and frozen in liquid nitrogen. Cross or longitudinal sections of the root of the upper molars with the surrounding alveolar bone, 20 μm in thickness, were made in a cryostat (Coldtome CM-41 Sakura corp. Japan).

Immunohistochemistry

Free floating sections were processed for immunohistochemistry by the use of ED1 monoclonal antibody (DIJKSTRA et al., 1985), and OX6 monoclonal antibody (MCMASTER and WILLIAMS, 1979), which can recognize most of the cells of monocyte-macrophage lineage and MHC-class II molecules, respectively. A solution of 0.01 M phosphate-buffered saline (PBS pH 7.4) was used to dilute the antibody and to rinse the sections. After blocking endogenous peroxidase with absolute methanol containing 0.3% H2O2, the sections were incubated for 24 h at 4°C overnight, and frozen in liquid nitrogen. Cross or longitudinal sections of the root of the upper molars with the surrounding alveolar bone, 20 μm in thickness, were made in a cryostat (Coldtome CM-41 Sakura corp. Japan).

Double staining of OX6 immunohistochemistry and ACPase enzyme histochemistry by Azo dye method

First, free floating sections were processed for immunohistochemistry by use of OX6 as described above except that the DAB treatment was omitted. The sections immunostained for OX6 were then subjected for ACPase activity reactions by the Azo dye method (BURSTONE, 1961; MAEDA et al., 1993). Briefly, the sections were incubated for 10 min in a medium consisting of 0.7% fast red violet LB salt (Sigma, St. Louis, USA), 0.8% naphthol AS-MX phosphate (Sigma, St. Louis, USA), and 0.1 M acetate buffer (pH 5.3), at room temperature. An incubation with the medium containing 10 mM NaF or with a substrate-free medium completely abolished the ACPase reaction in both experiments. After completion of the histochemical staining for ACPase activity, the OX6 immunoreaction site was visualized by incubation in the DAB medium.

Distribution density of immunopositive cells in the periodontal ligament

Differences in the distribution density of the OX6-immunopositive cells in the periodontal ligament between the ACPase reactive area and non-reactive area were examined in cross sections of the central 1/3 of the upper second molar roots. On the photomicrographs, the periodontal space of each root was divided evenly into 36 units by drawing straight lines extending radially from the center of the dental pulp as shown in Figure 5. The mean cell density per unit area of the periodontal ligament was calculated independently in the intensely ACPase positive portion and ACPase weak portion of the ligament, and values compared between the two. Since the unit surface area of the examined periodontal ligament differed among the specimens, a paired T-test between the immuno-positive cell density from the ACPase positive- and ACPase weak portions in the respective roots was executed. The ACPase positive- and ACPase weak portions of the periodontal ligament in each examined root were distinguished precisely by staining the adjacent cryosections for ACPase by the Azo dye method. The roots observed were randomly selected from those of the upper second molars, and no specific type of root was chosen. Twenty-eight cut surfaces of the roots were selected for counting OX6-immunopositive cells, and thirteen cut surfaces for ED1-immunopositive cells.

RESULTS

ACPase activity in the periodontal ligament

Intense reactions for ACPase were localized in cells of the periodontal ligament located more or less in the distal portions of the periodontal space toward which a physiological migration of the molar is known to take place (Fig. 1). Very intense ACPase reactions appeared in the osteoclasts on the bone surface. Among the cut surfaces of the roots of upper second molars, the portions of the alveolar bone surface, enriched with intensely ACPase reactive osteoclasts and undergoing bone resorption, were not necessarily restricted to the distal side and differed among the individual roots. A number of resorption cavities were occasionally seen on the distal root
Fig. 1. ACPase reactions (azo dye method) in the periodontal ligament of the upper second molar (cross section). a. Note a region-specific localization of ACPase reactive cells in the ligament of each root (*). A alveolar bone, p periodontal liga-
ment, d dentin, pu dental pulp, v blood vessels. ×24. b. Enlarged view of the disto-
buccal root, showing intense ACPase reactions of the osteoclasts (single arrows) and mononuclear cells around the root (arrow heads). Double arrows indicate resorption cavities on the root surface. ×56

All the pictures are aligned so that the right hand side indicates the distal direction, and the top of the pictures, the buccal direction.
surface extending through the cementum to the dentin matrix (Fig. 1b).

In the ACPase reactive areas of the ligament, there were some small, round cells showing intense ACPase reactions equal to that of the osteoclasts (Fig. 1b). Moreover, fibroblasts in this area also showed some ACPase activity, whereas the ACPase reaction was almost negative in the rest of the periodontal ligament where the osteoclasts were lacking (Fig. 1b).

**OX6-immunopositive cells and ACPase reaction**

The presence of a large population of OX6-immunopositive cells in the periodontal ligament of rat molars was confirmed (Fig. 2). In longitudinal sections of molar roots, OX6-immunopositive cells showed a fairly even distribution from the cervical to the apical portion of the periodontal ligament (Fig. 2a). Examination of cross sections of the molar roots, however, revealed an apparent heterogeneity in the distribution of OX6-immunopositive cells in the periodontal ligament (Fig. 2b). In cross sections, the OX6-immunopositive cells were shown to be much smaller in number in the areas where a large number of multinucleated osteoclasts, which were immunonegative to OX6, were located. Many of the OX6-immunopositive cells had large cytoplasmic projections and displayed
Fig. 3. A double staining for ACPase histochemical reaction (red) and OX6 immuno-reactivity (black). a. A habitat segregation between OX6-immunopositive cells and intensely ACPase reactive cells in the periodontal ligament is displayed. A alveolar bone, d dentin, pu pulp, * ACPase reactive area. ×20. b. Higher magnification of the disto-lingual root. ×48
Fig. 4. Legend on the opposite page.
dendritic profiles, while some showed spindle or ovoid contours.

A double staining of sections for OX6-immunohistochemistry and ACPase enzyme histochemistry clearly indicated that a predominant population of OX6-immunopositive cells was located in the ACPase weak or negative portion of the periodontal ligament. There was only a small number of immunoreactive cells in the intensively ACPase reactive area of the ligament where bone resorption by the osteoclasts appeared to be in progress (Fig. 3a, b.).

The eccentric distribution pattern of the OX6-immunopositive cells in the periodontal ligament of the doubly stained specimens was similar to that in the specimens simply stained for OX6 alone and, hence, should not be considered as an artifact (compare Fig. 2b and Fig. 3a).

**ED1-immunopositive cells**

A large number of ED1-immunopositive cells, being recognized as a broad spectrum of cells of monocyte origin, appeared and showed almost equal distributions in the periodontal ligament (Fig. 4a). Large sized osteoclasts were easily recognized due to particularly intense ED1-immunoreactions. ED1-immunopositive mononuclear cells in the osteoclast-rich area mostly had rich cytoplasm and hardly showed typical dendritic profiles (Fig. 4c), whereas those in the osteoclast-free areas showed relatively slim cell bodies and displayed rather weaker and somewhat granular immunoreactions in the cytoplasm (Fig. 4b).

**OX6- and ED1-immunopositive cell densities in the osteoclast-rich area and osteoclast-free area**

On the cross sections of the molar roots, OX6- and ED1-immunopositive cell densities in both the osteoclast-rich area and osteoclast-free area of the periodontal ligament were respectively computed and statistical analyses of the differences were made. As shown in Table 1 and Figure 6, there was a significant difference between the values from the ACPase reactive area and ACPase weak area of the periodontal ligament on both the OX6- and ED1-immunopositive cell densities. OX6-immunopositive cells showed a distinct difference in cell density between the two areas, being enriched in the ACPase weak area. ED1-immunopositive cells that optically appeared to have equal distribution in the periodontal ligament also showed a slight but significant difference in cell density between the two areas. In the case of ED1-immunopositive cells, the density was higher in the ACPase reactive, osteoclast-rich area.

**DISCUSSION**

**Differences in distribution of antigen presenting cells in incisor- and molar periodontal ligament**

In previous studies we demonstrated the presence of a large number of immunocompetent cells, the dendritic cells in particular, in the lingual periodontal ligament of rat incisors and compared their spatial distribution and morphological as well as immunohistochemical features with those of the macrophages known to have an antigen presenting capability as the dendritic cells (Van Furth, 1986; Steinman et al., 1986). In the lingual aspect of the periodontal ligament of rat incisors we examined, the distribution pattern and morphological profiles of the cells, immunoreactive respectively to OX6- and ED1-monoconal antibodies, were basically similar. Accordingly, most, if not all, of the cells immunopositive to either of the two types of the antibodies appeared to belong to the same group of antigen presenting cells, though a double immunostaining with OX6- and ED1-antibodies was not successfully attempted (Van Furth, 1986; Steinman et al., 1986).

In the present study, an abundance of dendritic cells was also confirmed in the periodontal ligament of rat molars, indicating that the OX6-immunopositive dendritic cells were the cellular constituent common to the intact periodontal ligament. However, when viewed in the cross cut sections of the molar roots, the spatial distribution of the dendritic cells in the periodontal ligament was eccentric and
different from that of the ED1-immunopositive cells that showed an almost even distribution throughout the ligament. As shown in Figure 4c, the ED1 monoclonal antibody recognizes osteoclasts and its precursor cells. It is therefore possible to conclude that the difference between the distribution patterns of the OX6- and ED1-immunopositive cells in the molar periodontal ligament is due in part to the exclusive localization of the cells of the osteoclastic lineage in the distal aspects of the periodontal ligament that show immunoreactivity to ED1 but not to OX6, and also to the scarceness of the other types of OX6-immunopositive cells in such areas.

Since the rat molar teeth are known to undergo physiological distal migration throughout life, it can be expressed that the OX6-immunopositive dendritic cells and immunopositive macrophages are preferentially localized in the proximal, bone forming aspects of the periodontal ligament, whereas they are sparse in the distal, bone resorbing aspects of the periodontal ligament in rat molar. Thus our current observation is the first to provide morphological evidence showing a clear-cut habitat segregation between the bone-resorbing osteoclastic cell lineage and non-bone resorbing macrophagic and/or dendritic cell lineage occurring in vivo.

**Fig. 5.** Schematic diagram showing the areas for the statistical analysis of immunopositive cells. A cross-cut sectional image of a rat molar root.

**Fig. 6.** A diagrammatic representation of OX6- or ED1-immunopositive cell densities in the ACPase-reactive and ACPase-weak or non-reactive regions of rat molar periodontal ligament. The values in the ACPase-reactive regions represent the density of immunopositive cells in regions undergoing bone resorption, whereas those in ACPase weak or non-reactive regions, those undergoing bone formation. See text for details.
Differentiation pathways of dendritic cells and osteoclasts

It is generally accepted that both the OX6- and ED1-immunopositive cells originate from the hematopoietic stem cells in the bone marrow. Some of the marrow cells have been shown to leave the marrow early, moving to the peripheral connective tissue via the blood stream where they proliferate and differentiate to become resident macrophages such as Kupffer cells in the liver or other types of antigen presenting cells under the influence of local factors, while most of the macrophages and dendritic cells derive from further differentiated monocytes arriving via the blood stream (NAITO, 1993).

As regards the origin of the osteoclasts, a possibility remains that they differentiate secondarily in the peripheral tissues from monocytes as is the case for the macrophages. However, a large body of experimental evidence has suggested that the differentiation pathway of the cells of the osteoclast lineage is determined prior to the differentiation of monocytes (SCOTT, 1967a, b; RIFKIN et al., 1980; HORTON et al., 1984; EJIRI and OZAWA, 1984; SCHEVEN, 1986; MARKS and POPFF, 1988; PETER and GROOTH, 1992; SUDA et al., 1992). Nevertheless, most of the above data are of in vitro experiments and, hence, a clear-cut distinction between the macrophagic and osteoclastic precursor cells in the peripheral connective tissue can not be made before the latter type of cells further differentiate and acquire a phenotypical expression typical of bone resorbing cells: large numbers of mitochondria and relatively small amounts of rough-surfaced endoplasmic reticulum, and cytoplasmic vesicles showing intense ACPase activity (EJIRI, 1983; EJIRI and OZAWA, 1984).

A morphological marker allowing a discrimination between the two types of the precursor cells either of the bone resorbing, osteoclastic cell lineage and of the non-bone resorbing, macrophagic and/or dendritic cell lineage in vivo has thus been awaited.

Molar periodontal ligament as a model system

As already mentioned, our current study is the first to show in vivo morphological evidence that the cells of the bone-resorptive, osteoclastic lineage and those of the non-bone resorptive, macrophagic and/or dendritic cell lineages are respectively localized in roughly the distal and proximal regions of the periodontal ligament of rat molars. To our knowledge, the periodontal ligament of rat molars is so far the only tissue where an apparent habitat segregation between the bone resorptive-and non-bone resorptive cells of monocyte origin has been identified.

Although no conclusive explanation has yet been given as to why the dendritic cell population is so small in the portion of the periodontal ligament where cells showing intense ACPase activities are prevalent, it is important to note that such a condition is maintained and lasts throughout life in the molar periodontal ligament due to the physiological distal migration of molar teeth (VIGNERY and BARON, 1980; ROBINSON and SCHNEIDER, 1992; LASFARGUES and SAFFAR, 1993). It is speculated that if an orthodontic force would be applied to rat molars so that the direction of tooth movement changed from the physiological one to the opposite direction, a drastic shift of the sites of bone resorption and formation would be induced rather rapidly. In this context, the periodontal ligament of rat molars is expected to become an exquisite model tissue whereby the origin and differentiation pathways of the bone resorptive- and non-bone resorptive cells of monocyte origin can be examined further in vivo.

Our preliminary ultracytochemical observations have in fact shown undifferentiated mononuclear wandering cells displaying osteoclast-like intensely...
ACPase reactive tubulovesicular structures in the cytoplasm to be localized exclusively in the distal aspects of the periodontal ligament, and their absence in the proximal ligament of rat molars (unpublished data). Histocytochemical and immunohistochemical observations of the molar periodontal ligament under an orthodontic force are currently in progress in our laboratory by means of light-and electron microscopy.

In conclusion, a clear-cut habitat segregation between the cells of the bone resorative cell lineage and non-bone resorative, macrophagic and/or dendritic cell lineage has been confirmed in the rat molar periodontal ligament by immunohistochemistry and enzyme histochemistry. An advantage of the use of the rat molar periodontal ligament as a model system for pursuing differentiation pathways of cells of the monocyte lineage is proposed.

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REFERENCES


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