The Expression of Macrophage and Neutrophil Elastases in Rat Periradicular Lesions

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Abstract
Macrophage elastase and neutrophil elastase are involved in tissue destruction in periradicular lesions. The purpose of this study was to examine these elastases immunohistochemically during development of periradicular lesions induced in rat mandibular first molar by pulpal exposure for 7, 14, 21, 28, and 42 days. Histologically, periradicular inflammation developed from 7 to 21 days and then subsided after 28 days. The area of these lesions gradually increased from 7 to 28 days and subsequently decreased at 42 days. Macrophage elastase was first detected at 7 days and predominantly shown from 14 to 28 days, whereas neutrophil elastase gradually increased from 14 to 28 days. Macrophage elastase was significantly greater than neutrophil elastase from 7 to 21 days. These results suggest that macrophage elastase was enhanced from an early stage during the development of these lesions and that neutrophil elastase was related to the expansion of periradicular tissue destruction including bone resorption.

Key Words
Elastase, macrophage, neutrophil, periradicular lesion, rat

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A periradicular lesion evoked by bacterial infection consists of periapical inflammation with destruction of the periodontal ligament and resorption of the alveolar bone and root (1). The periodontal ligament is a dense connective tissue localized between the cementum and the alveolar bone and supports the tooth. The destruction of the apical periodontal ligament is initiated by degradation of the extracellular matrix (ECM). ECM is composed mainly of collagen fibers, and elastic-system fibers including oxytalan fibers and elastin-containing fibers (ie, elastic and elaunin fibers) are ubiquitous components of this ligament (2). Elastic-system fibers have been investigated in the rat molar periodontal ligament including its apical portion (3). The enzymes of ECM degradation are both serine proteinases and matrix metalloproteinases (MMPs).

MMPs are a family of zinc-dependent endopeptidases that possess the ability to degrade all components of the ECMs and play important roles in many physiologic and pathological processes (4, 5). Especially, MMP-2 (geratinase A), -7 (matrilysin), -9 (geratinase B), and -12 (macrophage metalloelastase) have elastinolytic properties (6). MMP-12 was discovered in thioglycolate-stimulated mouse peritoneal macrophages (7). Murine MMP-12 complementary DNA was cloned (8), and, subsequently, human and rat homologues of MMP-12 complementary DNA were reported (9–11). MMP-12 has the ability to degrade ECM components by attacking elastin (12) and other basement membrane components such as fibronectin or collagen type V but not gelatins (13, 14). Collagen type IV or laminin is also degraded by murine or human MMP-12 (13, 14) but not by rat MMP-12 (11). Macrophages with those proteolytic activities are able to penetrate basement membranes and invade several tissues (14). Various roles of MMP-12 in disease models have been revealed to date, such as the development of lung emphysema (15), prevention of tumor growth through angiostatin generation (16, 17), and degeneration of the aortic medium in abdominal aortic aneurysm (18). By using the complementary DNA microarray analysis, MMP-12 in rat periradicular lesion was upregulated compared with healthy control (19). But, to date, few reports have been published with respect to the role of MMP-12 in periodontal and periradicular tissues.

Neutrophil elastase, which is involved in the degradation of elastin, is one of the serine proteinases and it has strongly degradative action toward several proteins. Secreted cathepsin G and proteinase 3 also have an elastinolytic property in serine proteinase (20). Polymorphonuclear leukocytes (or neutrophils) contain an abundance of lysosomal granules filled with proteolytic enzymes and other components. Neutrophil granules can be differentiated into specific and azurophilic (or primary) granules, the latter containing the serine proteinases such as elastase and cathepsin G as well as other enzymes (21). Neutrophil elastase secreted by activated neutrophils can bind to and degrade ECM. This protease has wide substrate specificity and has strong competency in degrading a variety of the proteins including elastin (22). There is a possibility that it evokes the severe tissue destruction seen in pulpitis (23) and periradicular lesions (24). In human periapical disease, neutrophil elastase levels of periapical exudates associated with clinical symptoms (25) and there may be a possibility that it serves as a diagnostic marker (26).

The purpose of the present study was to examine the involvement of macrophage elastase and neutrophil elastase in the development of periradicular lesions in rats.
Material and Methods
The Induction of Periradicular Lesions

Twenty-four male Wistar rats, weighing about 250 g, were used in the present study. Periradicular lesions were induced as described previously (27, 28). In brief, all animals were anesthetized, and the pulpal tissues in the left and right mandibular first molars were exposed so that perforation of the furcation would be avoided. The exposed areas were left open to the oral environment until the animals were killed.

Tissue Preparation and Immunohistochemistry

Animals were sacrificed at 7, 14, 21, 28, and 42 days (each period, \( n = 4 \)) after the pulpal exposure. Animals without the exposure were used as the control (day 0, \( n = 4 \)). At the time of sacrifice, the mandibles of all animals were removed and fixed in periodate-lysine-paraformaldehyde solution for 24 hours at 4°C. The mandibles were decalcified by being soaked in 5% EDTA solution until complete decalcification (approximately 60 days) at 4°C. Then, these were embedded in OCT compound (Miles Scientific, Naperville, IL) and sectioned serially at 5 \( \mu \)m in the mesiodistal plane.

The presence of macrophage elastase and neutrophil elastase in the periapical tissue were examined immunohistochemically. The primary polyclonal antibodies reactive with macrophage elastase (sc-8839) and neutrophil elastase (sc-9521) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Normal goat serum was used as the negative control. The sections were stained by use of ABC Staining System (sc-2018, Santa Cruz Biotechnologies). The slides were exposed to hydrogen peroxide in deionized phosphate-buffered saline (PBS) for 10 minutes to quench the endogenous peroxidase activity and subsequently reacted with 1.5% goat serum diluted in PBS for an hour to block nonspecific antibody binding. These slides were incubated with each primary antibody for 12 hours, followed by biotinylated antibody for 30 minutes. Then, they were incubated with avidin-biotinylated peroxidase complex for 30 minutes. For the final chromogenic reaction, the slides were exposed to the substrate solution consisting of diaminobenzidine-tetrahydrochloride and hydrogen peroxide and were counterstained with hematoxylin. The periapical tissue of the mesial root of the mandibular first molar was examined for both antigen-positive cells.

Histometry

Quantitative analysis was performed on 4 serial sections from each molar. The area of the periradicular lesion was measured histometrically as described previously (26, 27). The area of the lesion was the

Figure 1. Immunohistochemical staining for macrophage elastase. (A) In the mandibular first molar at 7 days after the exposure, some elastase-expressing cells detect in the residual pulpal tissue and around the root apex. B is a high-magnification view of cropped area in A. These cells gradually increase at (C) 14 and (D) 21 days. (E) By 28 days, these cells increase in number and are observed in and around the abscess. F is a high-magnification view of cropped area in E. G is a cropped area in F. The arrows indicate macrophage elastase-positive cells. These cells are round-shaped monocytes in high magnification. By 42 days, these cells decrease in number, but some still remain in the abscess (H). A, C, D, E, and H scale bars = 1.0 mm. B and F scale bars = 0.25 mm. G scale bar = 0.025 mm.
periodontal ligament between the root apex and alveolar bone in the mesial root. The cells reactive with antimacrophage and antineutrophil elastases were counted in an area 0.6 mm square of the periapical portion surrounding the root apex of the mesial root, and the number of antigen-positive cells per unit area (cells/mm²) was calculated (28).

**Statistical Analysis and Animal Protocol**

The results of all measurements were presented as mean values ± standard deviations. The average values were determined for each animal, and the average values were calculated for each group. The statistical significance of differences was determined by the Mann-Whitney U test. The principles of laboratory animal care according to the animal protocol institutionally approved by the ethics committee of School of the Dentistry of Aichi-Gakuin University were followed.

**Results**

**Histologic Findings**

In the mandibular first molar with no exposure, the periapical tissue did not change. At 7 days after the pulp exposure, a few inflammatory cells were noted in the periapical tissue. At 14 days, slight inflammation and bone resorption were observed. By 21 days, a small abscess was found around the root apex. Severe inflammation and bone resorption were investigated. By 28 days, the abscess had extended. Resorption of the apical root and alveolar bone was evident at this time. The abscess and inflammation in periapical tissue had decreased by day 42.

**Macrophage Elastase**

No macrophage elastase-expressing cells were present in the periapical tissue at day 0. At 7 days after the exposure, however, a few elastase-expressing cells were detected around the root apex (Fig. 1A and B). By 14 days, these cells had increased in number (Fig. 1C). At 21 days, such cells were observed in and around the apical abscess (Fig. 1D). By 28 days, many cells were seen there (Fig. 1E, F, and G). By 42 days, these cells decreased in number, but some still remained (Fig. 1H).

**Neutrophil Elastase**

No neutrophil elastase-expressing cells were observed in the periapical tissue at day 0 or 7 days (Fig. 2A). At 14 days, a few elastase-expressing cells were detected around the root apex (Fig. 2B and C). By 21 days, such cells were increased in number (Fig. 2D). At 28 days, these cells decreased in number, but some still remained (Fig. 2E and F). By 42 days, these cells decreased in number, but some still remained (Fig. 2G and H).

**Figure 2.** Immunohistochemical staining for neutrophil elastase. (A) At 7 days, a few neutrophil elastase-expressing cells are observed in the residual pulpal tissue, whereas no cells are present in periapical tissue. (B) By 14 days, antigen-positive cells detect in apical periodontal ligament. C is a high-magnification view of cropped area in A. These cells increase in number from (D) 21 to (E) 28 days. At 28 days, these scatter in apical periodontal ligament. F is a high-magnification view of cropped area in E. G is the cropped area in F. Arrows indicate the macrophage elastase-positive cells. Arrows point to the neutrophil elastase-positive cells. (G) These cells are polymorphonuclear-shaped and polynuclear in high magnification. (H) By 42 days, their number decrease, but some remain in the apical abscess. A, B, E, and H scale bars = 1.0 mm. C and F scale bars = 0.25 mm. G scale bar = 0.025 mm.
TABLE 1. Changes in Mean and SD of the Area of the Periradicular Lesion and the Number of the Macrophage and Neutrophil Elastase-expressing Cells per Unit Area

<table>
<thead>
<tr>
<th>Experimental Period</th>
<th>Area of Periradicular Lesion (mm²)</th>
<th>Macrophage Elastase (cells/mm²)</th>
<th>Neutrophil Elastase (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>0.229 ± 0.110</td>
<td>0.88 ± 0.85</td>
<td>1.20 ± 0.62</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.264 ± 0.089</td>
<td>13.70 ± 2.91</td>
<td>2.21 ± 13.70</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.437 ± 0.067</td>
<td>24.60 ± 3.78</td>
<td>4.30 ± 3.60</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.776 ± 0.141</td>
<td>62.00 ± 10.20</td>
<td>42.90 ± 6.62</td>
</tr>
<tr>
<td>Day 28</td>
<td>1.038 ± 0.116</td>
<td>84.20 ± 16.40</td>
<td>73.20 ± 19.60</td>
</tr>
<tr>
<td>Day 42</td>
<td>0.832 ± 0.096</td>
<td>49.30 ± 13.90</td>
<td>53.10 ± 17.40</td>
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</table>

21 days, these cells had increased in number and were seen in the apical abscess (Fig. 1D). By 28 days, these cells were still observed there (Fig. 2E, F, and G). At 42 days, these cells were fewer in number, but some still remained (Fig. 2H).

Histometrical Result

The area of periradicular lesion and the numbers of macrophage and neutrophil elastase-expressing cells are presented in Table 1. The area increased gradually from 0 to 28 days after pulpal exposure, whereas it decreased by 42 days. There was no significant difference in area between the control rats without exposure and those rats with pulpal exposure for 7 days. This area from 14 to 42 days was significantly larger than the control one (p < 0.05). Especially, this area showed the greatest enlargement from 14 to 21 days.

The number of macrophage elastase-expressing cells in the periapical tissue increased gradually from 7 to 28 days after pulpal exposure, whereas it decreased at 42 days. Their numbers from 7 to 42 days were significantly larger than the control one (p < 0.05). Few neutrophil elastase-expressing cells were detected at 7 and 14 days after the exposure. The number of these cells increased gradually from 21 to 28 days, whereas it decreased at 42 days. There was not a significant difference in the number between the rats without exposure (control) and those exposed for 7 or 14 days, but the numbers for the animals with pulpal exposure for 21 to 42 days were significantly larger (p < 0.05). The number of macrophage elastase-expressing cells was larger than that of neutrophil elastase-expressing ones from 7 to 21 days (p < 0.05). However, there was no significant difference between the 2 cell types at 28 or 42 days.

Discussion

The presence of macrophage elastase and neutrophil elastase was evaluated during the process of periradicular lesion formation. The macrophage elastase after 7 days and neutrophil elastase after 14 days were shown in the periapical tissue after pulpal exposure. Both elastases increased during the development of this lesion with alveolar bone destruction. At 42 days, the periradicular lesion contained fewer neutrophil and macrophage elastase-expressing cells than those at 28 days, and the lesion did not show further expansion. Various serine proteases and MMPs, which degrade ECM in periapical tissue, are involved in the development of periradicular lesions. Both elastases have elastolytic properties and may play an important role in the lesion formation.

Macrophage elastase is one of the MMP family members produced specifically by macrophages and destroys ECM to degrade elastin with other MMPs. In other MMPs, collagenase (MMP-2 and -9) and matrilysin (MMP-7) also have elastolytic properties. MMP-12 degrades not only elastin but also collagen type IV, fibronection, and other ECM components. Human macrophage elastase degrades α1-antitrypsin, the primary physiologic inhibitor of human leukocyte elastase (13). In the present study, macrophage elastase first appeared in the periradicular lesion at 7 days and predominated at 14, 21, and 28 days. The most evident increase in OX-6-positive cells (macrophages) in this lesion was observed at an early stage postoperatively (29). When macrophages are activated, they may participate in triggering the development in this lesion (30). Dendritic/Langerhans cells, which are positive for ED1 (a general marker of mononuclear phagocytes), can release macrophage elastase (31). Because macrophages are activated in the initial stage of periradicular lesion, macrophage elastase may contribute to the early destruction of ECM in periapical tissue.

Neutrophil migration and degranulation in the affected regions of inflamed pulp require the actual presence of bacteria. After the bacterial infection has spread beyond the apical foramen, activated neutrophils assemble in the periapical tissue and phagocytize the bacteria. Neutrophil has the defense mechanism involved in the killing of invading bacteria by the liberation of reactive oxygen species (32) and lysosomal enzymes (20). In these serine proteinases, elastase, cathepsin G, and proteinase 3 have elastolytic activity. Especially, this elastase that is a strong enzyme can kill bacteria (25). It may secrete neutrophil elastase in the periapical lesion as result of their degranulation with other serine proteinases. A positive relationship exists between pulpitis and the increase of neutrophil elastase activity in humans (23). Polymorphonuclear leukocyte elastase was observed in the periapical tissue of patients clinically diagnosed as having irreversible pulpitis (24). Neutrophil elastase is detected in the periodontal ligament of patients with periodontitis (33). Although the neutrophil elastase was already known to be increased in the inflammation of other tissues, the increase was investigated in the periapical tissue with inflammation. The macrophage elastase was first detected at 7 days, whereas the neutrophil elastase was observed in and around the apical abscess after 14 days. Neutrophil appeared later compared with macrophage elastase. These results suggest that the expansion of this lesion with alveolar bone destruction may follow the increase of neutrophil elastase.

In conclusion, these investigations have indicated that macrophage elastase activity enhanced from an early stage during the development of periradicular lesions and that neutrophil elastase activity was related to the expansion of periapical tissue destruction including bone resorption.

References