Immunohistochemical Localization of IL-17 in Induced Rat Periapical Lesions

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Abstract
Interleukin (IL)-17 is a member of a novel family of proinflammatory cytokines produced almost exclusively by a newly recognized subclass of activated T cells called “Th17” cells. From an endodontic perspective, IL-17 potently regulates cells of the innate immune system, serving as an important bridging molecule between the adaptive and innate immune systems. The purpose of this study was to investigate the immunohistochemical localization of IL-17 during the development of periapical lesions in rats. Periapical lesions developed within 28 days after mandibular first molar pulp exposure in Sprague-Dawley rats. The animals were randomly sacrificed at 0, 7, 14, 21, and 28 days after pulpal exposure. The jaws that contained the first molar were obtained and routinely prepared for histologic analysis, immunohistochemistry, and enzyme histochemistry. From day 0 to day 28, the number of IL-17–positive cells and neutrophils ascended and peaked on day 28. Osteoclast numbers substantially multiplied from day 0 to day 14 and then gradually decreased from day 14 to day 28. In addition, the osteoclast decrease contrasted with the increased number of IL-17–positive cells and neutrophils. These findings showed that IL-17 could be observed and might possibly be involved in the inflammatory response and bone resorption of periapical tissues as well as associated with periapical lesion pathogenesis. (J Endod 2009; 35:216–220)

Key Words
Interleukin-17, neutrophils, osteoclast, periapical lesions

Periapical lesions, which form as a result of a root canal infection, are characterized by a host response to continuous antigenic stimulation in the infected canals and periapical bone destruction (1, 2). The host response is complex; it involves both the recruitment of different inflammatory cells and the participation of an extensive network of immunologic mechanisms, including cytokine production. Studies have shown that a number of proinflammatory cytokines are synthesized in response to bacteria and their products, which induce and maintain an inflammatory response (3, 4). Therefore, it is clear that periapical lesion development is associated with the actions of these cytokines.

Originally cloned from a rodent T-cell hybridoma library screen, interleukin (IL)-17A (often called simply IL-17) is the founding member of a novel cytokine family of cytokines termed IL-17A through IL-17F (5, 6). IL-17 is produced almost exclusively by activated T cells and is a definitive of a new class of effector T-helper cell subsets called “Th17” that is distinct from Th1 or Th2 (7–9). Although produced primarily by T cells, IL-17 is clearly a proinflammatory cytokine with potent effects on numerous cells of the innate immune system, particularly the granulocyte lineage, and is thus considered an important bridging molecule between the adaptive and innate immune systems (6, 10). The proinflammatory functions of IL-17 have been examined in many contexts. In vitro, IL-17 has been shown to activate fibroblast, epithelial cells, endothelial cells, and osteoblasts to produce proinflammatory cytokines such as IL-6, IL-8, granulocyte colony-stimulating factor, and matrix metalloproteinases (11). IL-17 plays an essential, nonredundant role in neutrophil activation, maturation, and homeostasis (12). It could also promote bone resorption by stimulating osteoblasts to produce the receptor activator of nuclear factor-kappa B (RANKL) that affects the activity and formation of osteoclasts (13). In humans, IL-17 has been associated with the pathology of numerous autoimmune and inflammatory diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus, multiple sclerosis, psoriasis, and allograft rejection (9). Furthermore, the presence of IL-17 has also been documented in periodontitis patients, suggesting that this cytokine might mediate inflammation in periodontal diseases (14, 15).

To our knowledge, there have been few reports on IL-17 expression in periapical lesions. Therefore, we hypothesize that IL-17 might be involved in the pathogenesis of periradicular lesions, particularly the role it might play in both inflammatory response and alveolar bone destruction. The aim of this study was to examine the presence of IL-17 and discuss its relationship with inflammatory response and bone resorption at different stages in rat periapical lesions.

Materials and Methods

Induction of Periapical Lesions and Sample Preparation
Thirty-five Sprague-Dawley rats, weighing about 220 g, were purchased from the Experimental Animal Center of Wuhan University. The rats were randomly divided into five groups. All rats were anesthetized with a ketamine (0.5 mL/kg) intramuscular administration. The pulps of the lower first molars were exposed with a #1/2 round bur to a depth equal to the bur diameter so that furcal perforation would be avoided. The exposed teeth were left open to the oral environment throughout the experiment. Seven rats in each group were sacrificed at 0, 7, 14, 21, and 28 days after lesion induction. Day 0 samples served as the negative control group. Experimental procedures followed the guiding principles of the Animal Care and Use Committee in the School of Stomatology, Wuhan University.
Histologic Analysis

The jaws were removed, defleshed, and fixed in 4% paraformaldehyde for 48 hours. Then, the specimens were transferred to a 10% EDTA solution for demineralization for 4 weeks at 4°C. After that, the samples were dehydrated in an ethanol series solution and embedded in paraffin. Next, 4-μm thick frontal serial sections prepared in a mesiodistal direction were cut. One from every four sections was observed under a light microscope. The sections, which included the distal root of the first mandibular molars and exhibited a patent root canal apex representing the central portion of root canal, were selected for histologic, immunohistochemical, and enzyme histochemical analysis.

The number of neutrophils that infiltrated the inflammatory tissues was also analyzed. The counting area definition was centered at a fixed distance from the distal root apical foramina throughout the selected section of each different specimen. For morphometric analysis, the number of neutrophils was counted by using specimens stained with hematoxylin and eosin. On average, we counted the number of neutrophils was counted by using specimens stained with hematoxylin and eosin. On average, we counted the number of neutrophils at 10 different sites under a high power field (hpf) at a magnification of ×400 in each specimen. Within these designated areas, the dark-stained cells with a multilobed, horseshoe-shaped nucleus were deemed neutrophils (16, 17). Observations were verified by an oral pathologist. The number of neutrophils was then calculated.

Enzyme Histochemistry (Tartrate-resistant Acid Phosphatase Assay)

Tartrate-resistant acid phosphatase (TRAP) is usually a histochemical marker specifically for osteoclasts. TRAP-positive cells ranged in color from dark red to purple. A TRAP kit (Sigma, St Louis, MO) was used to detect TRAP activity and identify osteoclasts. Sections that contained the periapical region were selected and subjected to TRAP activity examination. Procedures were performed as previously described (18, 19). Briefly, after the sections had been rehydrated and washed, they were incubated in a Naphthol AS-Bl phosphoric acid solution (Sigma, St Louis, MO) and Fast Garnet GBC for 1 hour at 37°C. After incubation, the sections were then stained with hematoxylin. Some sections were incubated in a substrate-free medium to serve as TRAP activity controls. Quantitative analysis of TRAP-positive osteoclast numbers was determined by counting multinucleated TRAP-positive cells under a microscopic hpf at a magnification of ×400 in five randomly fields that had direct contact with alveolar bone in the periapical regions. The technician who performed this task was not aware of the original specimen.

Immunohistochemistry

Immunohistochemistry was performed by the streptavidin-biotin complex method. Rabbit polyclonal antibodies against human origin IL-17 (Santa Cruz Biotechnology, CA, sc 7927) at a 1:100 dilution were used as the primary antibody. The sections were washed and stained using the streptavidin-biotin complex kit (Boshiide, Wuhan, China) according to the manufacturer’s manual. The procedures were as follows: after the sections had been rehydrated, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes at room temperature. After being washed in distilled water, the sections were first incubated with 5% BSA for 20 minutes at room temperature and then with the primary antibody for 24 hours at 4°C. Next, the sections were washed with phosphate-buffered saline and then incubated with streptavidin-biotin complex for 20 minutes. Finally, the sections were developed with 3, 3’-diaminobenzidine (Boshiide, Wuhan, China) and confirmed under a microscope. Counter staining was performed by hematoxylin. Regarding the negative controls, the nonimmune goat serum was used instead of the primary antibody. Next, the cells reactive with the anti–IL-17 in the periapical portion were counted. The vicinity was defined as the area centered at a fixed distance from the distal root apical foramina throughout the selected section of each different specimen. The areas that were subjected to cell counting were strictly defined by the research design. Under a microscopic hpf at a magnification of ×400, a technician randomly picked up five visual fields in the area and counted cells according to the single blind principle. The number of antibody-reactive cells per hpf (cells/hpf) was then calculated.

Statistical Analysis

All measurement results were presented as mean values ± standard deviation. Average values were determined for each specimen and then were calculated for each group. Data were analyzed with SPSS13.0 software (SPSS Inc, Chicago, IL). Statistical differences in each group were subjected to a one-way analysis of variance test (α = 0.05). Pair-wise multiple comparisons were performed in cases in which the analysis of variance test showed significant differences.

Results

Histologic Analysis

The periapical region was intact, and neither inflammation nor bone resorption could be observed in the negative controls on day 0. On day 7, a slight infiltration of inflammation cells could be observed. Then small areas of periapical alveolar bone resorption occurred. On day 14, lots of inflammation cells in the periapical tissues and alveolar bone resorption were observed. On day 21, a small abscess was seen around the root apex with inflammatory cell infiltration, and alveolar bone resorption was found. On day 28, the apical abscess enlarged, and alveolar bone resorption was still found.

The neutrophils in the negative controls were seldom observed on day 0. After the rats were induced with lesions, neutrophils could be readily seen in the inflamed periapical regions on day 7 and subsequently increased on day 14 (Fig. 1A). On day 21, the number of neutrophils ascended, especially in the abscess tissue. Climax was reached on day 28 (Fig. 1B). From 1 week to 4 weeks after pulpal exposure, there was a significant difference in the number of neutrophils in each group compared with the other time group (Table 1).

Enzyme Histochemical Observation

No multinucleated osteoclasts were observed in the normal un-inflamed periapical areas. On day 7, a small amount of osteoclasts could be seen in the periapical tissues. From day 7 to day 14, the number of osteoclasts increased and climax on day 14 (Fig. 1C). Then, on day 21, osteoclast numbers decreased dramatically. Few osteoclasts could be seen on day 28 (Fig. 1D). The cell counts and their corresponding standard deviation values from day 7 to day 28 are depicted in Table 1.

Immunohistochemical Finding

It was observed that IL-17–positive cells were present in all phases of the induced rat periapical lesions, and the number of IL-17–positive cells increased during the lesion expansion periods. The lymphocyte was the predominant IL-17–positive cell type. Some IL-17–positive cells were found 7 days after pulpal exposure. Subsequently, a significant increase in cell number was detected at 14 days (Fig. 1E). Then, on day 21, the number of IL-17–positive cells was still rising and then peaked at 28 days (Fig. 1F). The cell counts and their corresponding standard deviation values from day 7 to day 28 are also depicted in Table 1.
Discussion

As we have known, periapical lesions are thought to be a periapical tissue reaction to bacterial infection and consist of both periapical inflammation and alveolar bone destruction. These lesions are characterized by the persistent migration and infiltration of inflammatory cells such as neutrophils, lymphocytes, plasma cells, macrophages, and mast cells (20). Previous investigations have suggested that CD4+ T lymphocytes are the predominant inflammatory cells that infiltrate the pathogenesis of periapical lesions and play a key role in the disease (21, 22). IL-17A is a proinflammatory cytokine that is primarily secreted from T lymphocytes, mediators of adaptive immunity. Recently, IL-17 was shown to be the defining cytokine of a new CD4+ T-helper subset termed “Th17” and has been linked to a number of T-cell–driven inflammatory conditions (7–9). The IL-17 is particularly fascinating because of its many novel properties. Structurally, IL-17 bears little resemblance to other well-described cytokine families. In culture systems, IL-17 behaves much like classic inflammatory cytokines such as TNF-α and IL-1β, triggering upregulation of the same genes and using similar signaling pathways. However, unlike TNF-α and IL-1β, IL-17 is produced by a novel CD4+ T-cell subset (Th17 cells). IL-17 promotes the expansion and recruitment of innate immune cells such as neutrophils and also cooperates with TLR ligands, IL-1β, and TNF-α to enhance inflammatory reactions. Therefore, IL-17 appears to be a signal from the adaptive immune system that alerts the innate immune system to amplify inflammation and mobilize neutrophils (6, 10). Because of the role that IL-17 appears to play in immune system regulation and its possible contribution to clinical disorders, the identification and characterization of related molecules have been of particular interest. Soon after its discovery, IL-17 was linked to the bone destructive pathology seen in RA. IL-17 overexpression either by an adenovirus or direct intra-articular IL-17 injection resulted in joint erosion (13, 23). Recently, numerous studies have also indicated a potential role for IL-17–mediated

Figure 1. Representative views of histologic, enzyme histochemical, and immunohistochemical observation of induced rat periapical lesions. (A) Neutrophils could be observed in capillaries and inflamed periapical regions on day 14; (B) a large amount of inflamed cells that infiltrated periapical areas were observed on day 28 (many of them were neutrophils); (C) a great number of multinucleated osteoclasts were found in 14-day specimens; (D) fewer multinucleated osteoclasts were observed in 28-day specimens; (E) some IL-17–positive cells were observed on day 14; and (F) intense immunoreaction for IL-17 occurred in 28-day specimens (×400).
inflammation in both the initiation and progression of periodontal destruction (24, 25). Based on these considerations, it is possible to speculate that this cytokine is involved in periapical inflammatory response development and alveolar bone resorption.

In our experiment, a rat periapical lesion model was created to investigate the pathogenesis of apical periodontitis. Through histologic analysis, it was found that a rapid period of lesion expansion and bone resorption occurred between day 0 and day 14 (active phase) after pulp exposure, with enlargement slowly increasing thereafter (chronic phase), which agrees with Wang and Yang’s studies (18, 19). In the immunohistologic study, it was observed that IL-17-positive cells were present in all induced rat periapical lesion phases. In addition, the number of IL-17-positive cells increased during the lesion expansion periods. The lymphocyte was the predominant IL-17-positive cell type, which agrees with previous findings that T lymphocytes were the predominant inflammatory cells infiltrating periapical lesions (21) and that IL-17 is produced almost exclusively by activated T cells (9). Our results were also in accordance with Golic et al. (26) who found that mononuclear cells, isolated from periapical lesions, produce significant amounts of IL-17 upon additional stimulation, and the IL-17 concentration correlated with the proportion of CD3+ and CD4+ cells. These results indicate that IL-17 might be an important factor in both the initiation and development of periapical lesions. However, using other more sophisticated techniques, such as in situ hybridization and Western blot, may yield better results for being both more sensitive and more specific.

Like many inflammatory cytokines, IL-17 is a double-edged sword; it plays a particularly significant role in regulating neutrophil recruitment, granulopoiesis, and activation. In some settings such as RA, IL-17 is a pathological factor that promotes bone resorption. However, in the context of some infectious diseases, IL-17 plays an essential role in protecting the host by recruiting neutrophils to infected tissues (10). Recently, numerous studies have shown that neutrophils play a protective role in the control of infection-stimulated alveolar bone resorption in experimental periapical lesions and periodontitis (17, 27). Such findings could be used to explain the current study’s results; the osteoclast number rapidly rose on day 7 and then gradually decreased on day 21 and day 28, and the decrease in osteoclast numbers contrasted with the increased number of IL-17-positive cells and neutrophils. It is well known that the neutrophils usually infiltrate in an early inflammation stage and act as the first line of defense against invading bacterium. But according to our results, which agree with the work of De Rossi et al. (17), periapical lesions might occur as an inflammatory response to bacterial infection and possibly consist of inflammatory cell infiltration. Neutrophils are involved in all phases of lesion development and are understood as a long-term persistent infection caused by root canals left open to the oral environment. The findings also showed the concept that an accumulation of inflammatory cells such as lymphocytes and neutrophils might represent a protective host response aimed at eradicating the invading microorganisms and prevents the host from direct bacterial bone invasion surrounding the tooth apex (28).

### Table 1. The Number of IL-17-positive Cells, Osteoclasts, and Neutrophils per High-power Field (hpf × 400) in Rat Periapical Lesions

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>IL-17-Positive Cells/hpf</th>
<th>Osteoclasts/ hpf</th>
<th>Neutrophils/hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0d</td>
<td>13</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7d</td>
<td>11</td>
<td>4.63 ± 1.36</td>
<td>3.00 ± 0.89</td>
<td>8.36 ± 1.43</td>
</tr>
<tr>
<td>14d</td>
<td>12</td>
<td>13.17 ± 2.86</td>
<td>7.42 ± 1.08</td>
<td>22.67 ± 3.82</td>
</tr>
<tr>
<td>21d</td>
<td>11</td>
<td>32.27 ± 5.97</td>
<td>5.18 ± 1.25</td>
<td>40.55 ± 7.05</td>
</tr>
<tr>
<td>28d</td>
<td>11</td>
<td>52.27 ± 10.71</td>
<td>3.09 ± 0.83</td>
<td>70.91 ± 12.03</td>
</tr>
</tbody>
</table>

The greatest number of IL-17-positive cells and neutrophils per high power field both occurred on day 28. In addition, there were significant differences in the counts at all time points. Regarding osteoclasts, the amount climax on day 14. No significant difference was found between day 7 values and day 28 values. Significant differences were found in the counts at all other time points.

Based on previous findings and present research, it could be speculated that IL-17 might exert both bone protective and bone destructive effects in the initiation and development of periapical lesions. On one hand, IL-17 involved in periapical lesion pathogenesis might be one of the factors responsible for stimulating the expression of inflammatory effectors, most of which have been shown to have an impact on bone metabolism and promote osteoclastogenesis that indirectly favor alveolar bone resorption. On the other hand, IL-17 is important for neutrophil recruitment and activation, which might lead to the result that despite its apparent pathologic role in promote inflammatory response, IL-17 might play a protective role by preventing against infection-stimulated alveolar bone loss in periapical disease.

In conclusion, the results of our histologic, enzyme histochemical, and immunohistochemical studies showed that IL-17 is expressed in experimental rat periapical lesions. It might be involved in both the inflammatory response and bone resorption of periapical tissues and associated with the periapical lesion pathogenesis. However, the role of IL-17 in periapical lesions is still equivocal; it is uncertain whether the overall effect of IL-17 is to combat ongoing infection or contribute to bone destruction by promoting osteoclastogenesis. Further studies should elucidate the role of IL-17 and the intrinsic mechanism implicated in periapical lesion development and bone destruction so as to maximize host protective effects and minimize host deleterious effects.

### References