High-mobility Group Box 1 Is Associated with the Inflammatory Infiltration and Alveolar Bone Destruction in Rats Experimental Periapical Lesions

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Abstract

Introduction: This study was conducted to observe the immunohistochemical localization of high-mobility group box 1 (HMGB1) and its receptor, Toll-like receptor 4 (TRL4), in the development of periapical lesions induced in rats. The possible role of these molecules in the pathogenesis of periapical lesions was also explored. **Methods:** Periapical lesions developed within 35 days after mandibular first molar pulp exposure in Wistar rats. The animals were randomly killed at 0, 7, 14, 21, 28, and 35 days after pulp exposure. The jaws that contained the first molar were obtained and prepared for histologic analysis, enzyme histochemistry, immunohistochemistry, and double immunofluorescence staining. **Results:** From day 0 to 35, the areas of periapical bone loss increased and appeared to be stabilized on day 35. A few HMGB1-positive, TLR4-positive cells and osteoclasts could be observed on day 7. From day 7 to 28, the HMGB1 and TLR4 protein expression increased and subsequently remained stable. The number of osteoclasts multiplied from day 0 to 14 and then gradually decreased from day 14 to 35. Double immunofluorescence staining results showed HMGB1-positive, TLR4-positive cells around periapical lesions surrounding the apical foramen. **Conclusions:** Thus, HMGB1 and TLR4 may be associated with the pathogenesis of the periapical lesions. *(J Endod 2016;■:1–6)*

Key Words

High-mobility group box 1, osteoclast, periapical lesions, toll-like receptor 4

Significance

The interaction between HMGB1 and TLR4 plays an important role at different stages of rat periapical lesion development and provides a number of potential therapeutic targets for attenuating pathological bone resorption in such inflammatory diseases.

High-mobility group box 1 (HMGB1), also called amphoterin or HMG1, is a 30-kd abundant nonhistone nuclear protein expressed in all eukaryotic cells that can be released extracellularly by monocytes and macrophages stimulated by gram-negative bacterial lipopolysaccharides (LPSs), tumor necrosis factor alpha, or interleukin 1 beta (1). Plasma and tissue levels of HMGB1 have been shown to participate in inflammatory processes, such as rheumatic disease, sepsis, and stroke (2–4). HMGB1 serves as a danger signal that evokes an inflammatory response by activating various immune-related cells when released extracellularly (5). HMGB1-induced inflammatory response occurs in association with the recruitment of macrophages and certain T cells (6). One study has reported that the ability of HMGB1 to induce cellular activation and generate inflammatory responses is similar to those initiated by LPSs (7). HMGB1 can induce Kupffer cells to secrete several inflammatory cytokines, including tumor necrosis factor alpha and interleukin 1 beta, as well as upregulate the activities of p38 mitogen-activated protein kinase, c-Jun-N-terminal kinase, and nuclear factor kappa B after burn injury (8). Extracellular HMGB1 is a physiologically/pathologically relevant osteoclastogenic cytokine that promotes osteoclast differentiation, and extracellular HMGB1 stimulates the differentiation of osteoclast precursors in the presence of receptor activator of nuclear factor kB ligand (RANKL) *in vitro* and *in vivo* (9).

Extracellular HMGB1, which functions as a proinflammatory cytokine, can bind to cell-surface receptors, such as the receptor for advanced glycation end-products and Toll-like receptors 2 and 4 (TLR2 and TLR4), to adjust cellular responses to infection, injury, and inflammation (10). HMGB1 signaling through TLR2 and TLR4 but not the receptor for advanced glycation end-products contributes to LPS-induced inflammation (7). The TLR4 loss-of-function mutation partially protects against alveolar bone loss and improves glucose homeostasis in mice with periodontitis that were fed a high-fat diet (11). TLR4 is a principal signaling receptor for LPS and is indispensable for LPS-induced bone resorption *in vivo* (12). The TLR signaling pathway induces the expression of RANKL in fibroblastlike synoviocytes, and the expression of RANKL consequently promotes the differentiation of osteoclasts. Therefore, targeting specific TLRs

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may be a useful approach to prevent inflammatory bone destruction in the pathogenesis of rheumatoid arthritis.

A periapical lesion denotes the inflammation and destruction of periradicular tissues caused by etiologic agents of endodontic origin. The invasion of bacteria or bacterial toxins into the periapical region from an infected root canal system initially leads to infiltration of inflammatory cells and the production of proinflammatory cytokine reactions (13, 14). In addition to the direct toxic effects of microbial products, an immune response has also been suggested to play an important role in the pathological progress of periapical lesion formation (15). Although HMGB1 has been shown to activate TLR4 in immune cells and TLR4 is associated with alveolar bone loss in rats with periodontitis (7, 11), a potential link between HMGB1 and TLR4 signaling in periapical lesions in rats remains largely unexplored. Therefore, we hypothesized that HMGB1 and TLR4 may be involved in the pathogenesis of periapical lesions.

Materials and Methods

Animal Experiment and Sample Preparation

A total of 42 male Wistar rats weighing 200–250 g were purchased from the Experimental Animal Center of Hubei Province, Wuhan, China. The rats were randomly divided into 6 groups. All rats were anesthetized with ketamine (90 mg/kg intraperitoneally). The mandibular first molars of rats were used. The pulps of the lower first molars were exposed with a 1/4 round bur to a depth equal to the bur diameter so that furcal perforation would be avoided. The pulps were then left open to the oral environment. Six rats were killed on days 0, 7, 14, 21, 28, and 35. Day 0 samples served as the negative control group. The mandibles were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) (pH = 7.4) at 4°C for 48 hours. After rinsing with 0.1 mol/L PBS (pH = 7.4), the specimens were decalcified with 10% EDTA acid at 4°C for 8 weeks. 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. The experimental procedures followed the guiding principles of the Animal Care and Use Committee in the School of Stomatology at Wuhan University, Wuhan, China.

Morphometric Analysis of Periapical Lesions

Paraffin frontal serial sections that included the distal root of the first mandibular molars and exhibited an obvious root canal apex representing the central portion of the pulp and root canal were selected for hematoxylin-eosin staining. After deparaffinization and rehydration, the sections were stained with hematoxylin-eosin; then, the sections were dehydrated through increasing concentrations of ethanol and xylene. One from every 4 sections was subjected to observation under a light microscope and a fluorescence microscope.

Enzyme Histochemistry

Tartrate-resistant acid phosphatase (TRAP) is usually a histochemical marker specifically for osteoclasts. A TRAP kit (Sigma-Aldrich, St Louis, MO) was used to detect TRAP activity and identify osteoclasts. After the sections had been rehydrated and washed, they were incubated in a solution of Naphthol AS-BI phosphoric acid (Sigma-Aldrich) and Fast Garnet GBC (Sigma-Aldrich) for 1 hour at 37°C. After incubation, those sections were washed with water and then stained with hematoxylin. Serving as controls for TRAP activity, some sections were incubated in a substrate-free medium. TRAP-positive cells ranged in color from dark red to purple. TRAP-positive cells containing 2 or more nuclei were counted as osteoclasts.

Immunohistochemistry

After deparaffinization and rehydration, antigen was retrieved with pepsin (Zhongshan, Beijing, China) for 20 minutes at 37°C. Then, we treated the antigen with 3% hydrogen peroxide for 15 minutes to block the activity of endogenous tissue peroxidase. Rabbit polyclonal antibodies against human HMGB1 (Abcam, Cambridge, UK) and goat polyclonal antibodies against human TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:100 and 1:100, respectively, were used as the primary antibody for 16 hours at 4°C. The sections were washed and stained using the SP kit (Maixin, Fuzhou, China) and PV kit (Zhongshan, Beijing, China) according to the manufacturers' manual. The sections were then developed with 3, 3′-diaminobenzidine (Boshide, Wuhan, China), and the slides were completely rinsed with fresh water and counterstained with hematoxylin. Negative controls were obtained by the omission of primary antibodies, which were substituted with PBS.

Double Immunofluorescence Labeling

Double immunofluorescence labeling experiments were used to analyze the spatial distribution and localization of HMGB1 and TLR4 in the apical area. Briefly, the sections were rehydrated and then treated with PBS 0.3% Triton X-100 (Sigma-Aldrich) for 5 minutes at room temperature. After that, 1% bovine serum albumin was used to prevent unspecific staining for 2 hours. The sections were then incubated at 4°C overnight with HMGB1 rabbit polyclonal antibody (1:50). After washing in PBS, the sections were incubated with tetramethylrhodamine isothiocyanate–labeled donkey antirabbit immunoglobulin G (1:200 diluted in PBS [Invitrogen, Carlsbad, CA]) for 1 hour at 37°C. The process was repeated for the goat polyclonal antibodies against human TLR4 (1:50) for 2 hours at 37°C, and then secondary donkey antigoat CY3 antibodies (1:200 diluted in PBS [Beyotime Institute of Biotechnology, Jiangsu, China]) were added for 1 hour at room temperature; 4′,6-diamidino-2-phenylindole was counterstained to reveal the nuclei. Figures were then obtained using a fluorescent microscope with a camera (Leica, Wetzlar, Germany).

Cell Counting and Statistics Analysis

In each specimen, multinuclear TRAP-positive cells and HMGB1- and TLR4-positive cells in the periapical tissues were counted under high-power magnification (400×). Counting was performed by a blinded observer randomly picking up 5 visual fields in the areas. The average number per high-power field in each group was then subjected to statistical analysis with 1-way analysis of variance and Pearson correlation at $\alpha = 0.05$ using SPSS 13.0 (SPSS Inc, Chicago, IL).

Results

Histologic Observation

Sections with the complete root canal, including the apical foramen, were selected for measurement to ensure that each section represents the largest periapical lesion area. The extent of lesion areas significantly increased from day 0 to day 28 and stabilized thereafter (Fig. 1*A–L*). On day 0, the periapical region was intact, and no bone resorption was observed. On day 14, numerous inflammation cells appeared in the periapical tissues, and alveolar bone resorption became more prominent. On day 35, lesion expansion seemed to be stabilized. Statistically significant differences are listed in Table 1.

Enzyme Histochemical Observation

TRAP-positive cells were dark red to purple in color around the bone edge. On day 0, no osteoclast was found in the periapical area (Fig. 2*G*). On day 7, a small number of osteoclasts were found in the periapical tissues, and then the number of osteoclasts peaked on day