Increased Expression of Glutaminase in Osteoblasts Promotes Macrophage Recruitment in Periapical Lesions

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Abstract

Introduction: Recently, we have shown that tissue hypoxia stimulates the progression of periapical lesions by up-regulating glycolysis-dependent apoptosis of osteoblasts. Other facets of hypoxia-induced metabolic reprogramming in disease pathogenesis require further investigation. In this study, we examined the connection between hypoxia-augmented glutamine catabolism in osteoblasts and the development of periapical lesions. **Methods:** Primary human osteoblasts were cultured under hypoxia. The expression of glutaminase 1 (GLS1) was examined using Western blot analysis. The production of glutamate was measured by colorimetric assay. Knockdown of GLS1 was performed with small interfering RNA technology. C-C motif chemokine ligand 2 (CCL2) secretion and chemotaxis of J774 macrophages were examined by enzyme-linked immunosorbent assay and transwell migration assay, respectively. In a rat model of induced periapical lesions, the relations between disease progression and osteoblastic expression of GLS1 or macrophage recruitment were studied. **Results:** Hypoxia enhanced GLS1 expression and subsequent glutamate production in osteoblasts. Glutamate induced chemoattraction of macrophages by osteoblasts through up-regulation of CCL2 synthesis. Hypoxia promoted CCL2 secretion and macrophage recruitment through augmentation of glutaminolysis. Knockdown of GLS1 abolished hypoxia-induced effects. In rat periapical lesions, progressive bone resorption was significantly related to elevated GLS1 expression in osteoblasts and increased macrophage recruitment. **Conclusions:** In addition to the rise in glycolytic activity, the progression of periapical lesions is also associated with enhanced glutamine catabolism in osteoblasts. GLS1 may be a potential therapeutic target in the management of periapical lesions. *(J Endod 2016;■:1–7)*

Key Words

Bone resorption, C-C motif chemokine ligand 2, glutamate, glutaminolysis, hypoxia, inflammation

Significance

Increased glutaminolysis in osteoblasts is associated with the progression of periapical lesions. This results from the hypoxic state in an inflammatory environment. The hypoxia/glutaminase/glutamate/CCL2 pathway triggers macrophage chemotaxis and eventually osteoclast formation. Therefore, glutaminase is a potential target in the therapeutics of periapical lesions.

Periapical lesions are the consequence of host responses to bacterial infection originating from the root canal system (1); yet, host factors that affect the progression of periapical lesions and prognosis after treatment are not fully understood (2). Intracellular metabolism plays a pivotal role in the regulation of immune responses (3, 4); however, the influences of metabolic signals on the development of periapical lesions have seldom been addressed. In a recent study, we have shown that tissue hypoxia stimulates the progression of periapical lesions by up-regulating glycolysis-dependent apoptosis of osteoblasts (5). Other facets of hypoxia-induced metabolic reprogramming in the pathogenesis of periapical lesions deserve further investigation.

In addition to its well-known effect on the induction of glycolysis, hypoxia is involved in the regulation of glutamine metabolism (6, 7). Glutamine is the most abundant extracellular amino acid, and its use is crucial for the functions of many cell types in the body. In most cells, glutamine is metabolized to glutamate by glutaminase (8). Glutaminase is essentially a mitochondrial enzyme and exists as 2 isoforms in human, GLS1 (kidney-type glutaminase) and GLS2 (liver-type glutaminase) (9). Although GLS2 is expressed primarily in the liver, GLS1 is more ubiquitously distributed (10). Glutamine is an important nutrient for cancer cells. The link between glutaminolysis and tumor development has attracted much attention (11, 12). Glutamine metabolism also participates in the regulation of inflammatory reactions (13, 14). In brain tissue, glutamate up-regulates the expression of C-C motif chemokine ligand 2 (CCL2), a well-defined chemokine essential for the mediation of inflammation (15).

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CCL2, also known as monocyte chemoattractant protein 1, is responsible for the recruitment of osteoclast precursor cells (16). In a murine model of jaw bone inflammation, osteoblasts are the major cell type expressing CCL2 (17). In experimentally induced periapical lesions in rats, progressive bone resorption is also associated with increased CCL2 in osteoblasts (18). Hypoxic stress is known to regulate the expression of CCL2 (19, 20). However, the mechanism of hypoxia-regulated CCL2 synthesis and the relation of CCL2 and hypoxia-induced reprogramming of cellular metabolism remain to be determined.

In this study, we examined the connection between hypoxia-augmented glutaminase expression and CCL2 production in cultured human osteoblasts. Using a rat model of induced periapical lesions, the relationships between osteoblastic expression of glutaminase and disease progression or macrophage recruitment were analyzed.

Materials and Methods

Cell Culture

National Taiwan University's Institutional Review Board approved the study protocols, and written informed consent was obtained from the patients. Primary cultures of osteoblasts were established from alveolar bone explants obtained during odontectomy of third molars as previously described (21). The osteoblasts were grown in induction medium under normoxic (21% oxygen) or hypoxic (2% oxygen) conditions using an Invivo2 Hypoxia Workstation (Ruskinn Technology Limited, Bridgend, UK). Murine macrophage cell line J774 cells (American Type Culture Collection, Manassas, VA) were grown in Roswell Park Memorial Institute 1640 medium (GE Healthcare Life Sciences, Logan, Utah) (18).

Western Blot Analysis

Western blot analysis was performed as described earlier (18). The membranes were incubated with antibodies against GLS1 (1:1000; Epitomics, Burlingame, CA) or mitochondrial transcription factor A (1:2000; Abcam, Cambridge, MA) at concentrations suggested by the manufacturers. Primary antibodies were visualized with horseradish peroxidase–conjugated secondary antibodies and an enhanced chemiluminescence detection system (Amersham, Little Chalfont, Buckinghamshire, UK).

Knockdown of GLS1 Gene

Osteoblasts were transfected with 30 pmol GLS1 small interfering RNA (Genepharma, Shanghai, China) by Lipofectamine RNAiMAX (Thermo Fisher, Waltham, MA) according to the manufacturer's instructions. After transfection, osteoblasts were cultured for an additional 24 hours with fresh medium and then prepared for the indicated experiments.

Glutamate Assay

The levels of glutamate in cultured cells and the medium were determined using the Amplex Red Glutamic Acid Assay Kit (Thermo Fisher) according to the manufacturer's instructions. After treatment, culture supernatants and cells were collected separately. Cells (6×10^5) were resuspended in lysis buffer (0.5 mol/L Tris-HCl, pH = 7.5) and sonicated for 30 seconds. Cell lysates and conditioned media were analyzed for glutamate level. The enzymatic assay recognized glutamate as a specific substrate and produced hydrogen peroxide. The Amplex Red reagent (10-actel-3,7-dihydroxyphenoxazine) then reacted with hydrogen peroxide to develop proportional fluorescence. The amount of glutamate was quantified using a fluorescence microplate reader (excitation at λ = 530 nm, emission at λ = 590 nm [M5; Molecular Devices, Sunnyvale, CA]). The experiments were performed in triplicate for each condition.

Measurement of CCL2

Supernatants were evaluated for CCL2 levels using a human CCL2 enzyme-linked immunosorbent assay kit (Bender MedSystems, Vienna, Austria) as previously described (18). The absorbance was measured with 450 nm as the primary wavelength and 620 nm as reference. For each condition, 3 independent experiments were performed.

Cell Migration Assay

The migration of J774 macrophages was assessed with a transwell chamber system (3.0-μm pore size, Thermo Fisher) as previously described (18). Briefly, 2.5×10^4 cells were seeded into the upper chambers. Conditioned media collected from various experiments were added to the lower chambers. J774 cells were allowed to migrate from the upper chambers toward the lower chambers for 24 hours at 37°C. Cells that had migrated to the lower chamber were harvested and counted microscopically at 100×. All experiments were performed in triplicate.

Animal Model of Periapical Lesions

The experimental protocol was approved by the Center of Laboratory Animals, College of Medicine, National Taiwan University, Taipei, Taiwan, and the animals were maintained following the Guide to Management and Use of Experimental Animals (Ministry of Science and Technology, Taiwan). Periapical lesions were induced in 20 seven to eight-week-old Sprague Dawley rats as previously described (18). Pulp exposures were performed at the distal fossa of the right mandibular first molars and left open for 10 or 30 days (n = 10 for each time point) to allow the establishment of periapical lesions. Afterward, the animals were euthanized, and the mandibles were obtained for radiographic and immunohistochemical analyses.

Radiography and Image Analysis

The jaws were radiographed and analyzed using the Digora image analysis system (Soredex, Helsinki, Finland) as previously described (18). The radiographic border of each periapical lesion at the distal root apex of the right mandibular first molar was determined, and the area of the lesion was quantified in pixels. Data were then converted to square millimeters using the following equation: 1 mm^2 = 256 pixels.

Immunohistochemistry and Cell Counting

After imaging, the mandibles were subjected to immunohistochemistry as previously described (18). Sections containing the periapical lesions were incubated with antibodies against GLS1 or CD68 (a pan-macrophage marker [Serotec, Oxford, UK]). Bonded antibodies were detected by sequential incubation with biotinylated secondary antibody, streptavidin-peroxidase conjugate, and diaminobenzidine. The sections were then counterstained with hematoxylin and observed under a light microscope. Digital images were loaded to Image J software (National Institutes of Health, Bethesda, MD). Osteoblasts expressing GLS1 were quantified in 5 representatives and consecutive high-power fields (400×) for each specimen. Areas of bone surface with the adjacent lining tissues were encircled, and the number of osteoblasts that stained positive for GLS1 was counted. For quantification of cells expressing CD68, immunostained cells in 5 consecutive microscopic fields of 200× magnification were counted.