Original Article

Changes of Caspase-1 after the Application of Orthodontic Forces in the Periodontal Tissues of Rats

Xiulin Yan[a]; Jiang Chen[b]; Yuquan Hao[c]; Yan Wang[d]; Li Zhu[e]

ABSTRACT

Objective: To investigate the changes of caspase-1 in orthodontic tooth movement and to determine whether the changes are phase-specific.

Materials and Methods: Eighty Wistar rats were included in this study. Sentalloy closed-coil springs were placed to induce a mesial traction force on the lower right first molar. The animals were killed after 1, 3, 7, and 14 days (n = 20 at each time point). The mandibles of 10 rats were sampled for histomorphometric analysis and immunohistochemical assay, and the periodontal tissues of 10 other rats were sampled for detecting caspase-1 mRNA and protein by real-time RT-PCR and by Western blotting, respectively.

Results: The inflammatory reaction was evident in paraffin sections with hematoxylin-eosin staining. The immunohistochemical assay showed that orthodontic forces significantly increased the number of caspase-1-positive cells in the periodontal ligament (PDL). Mechanical force triggered an increase of caspase-1 mRNA in periodontal tissues. The expression of caspase-1 mRNA increased from day 1, reached the peak on day 3, and then decreased. The results of Western blotting indicated that the levels of both procaspase-1 and P20 subunit significantly increased after the application of orthodontic forces, compared with those in controls ($P < .05$).

Conclusion: Caspase-1 level increases during orthodontic tooth movement and changes with different phases, which might play a significant role in orthodontic tooth movement. (*Angle Orthod.* 2009;79:1126–1132.)

KEY WORDS: Orthodontics; Rats; Caspase-1; Western blotting; Messenger RNA

INTRODUCTION

Orthodontic tooth movement is based on force-induced periodontal ligament (PDL) and alveolar bone remodeling. The process is described as the necrosis of PDL on the pressure side with formation of a cell-free hyaline zone followed by osteoclastic resorption of the neighboring alveolar bone and bone apposition by osteoblasts on the tension side.[1] During these events, the local reaction is an inflammatory reaction. It is necessary that the body eliminate the hyalinized tissue during the inflammatory reaction.[2] It has been reported in experimental studies that stress produced by orthodontic forces would cause a marked increase in the staining intensity of interleukin (IL)-1α and IL-1β in all cell types of PDL.[3,4] Even TNF-α,[3,4] PGE2,[5] COX-2,[6] and γIFN[7] were observed in PDL in experimental tooth movement. Meanwhile, alveolar bone remodeling depends not only on activation, differentiation, and maturation of osteoblasts and osteoclasts, but also on cell apoptosis and necrosis. So the inflammation process and the apoptosis process go on simultaneously during orthodontic tooth movement.

Caspases, a family of 14 known cysteine proteases, participate in one of two distinct signaling pathways: activation of proinflammatory cytokines and promotion of apoptotic cell death. Caspase-1 seems to be an ex-

[a] Lecturer, Department of Orthodontics, School of Stomatology, China Medical University, Liaoning, China.

[b] Lecturer, Editorial Office of Journal of China Medical University, China Medical University, Liaoning, China.

[c] Lecturer, Department of Prosthodontics, School of Stomatology, China Medical University, Liaoning, China.

[d] Lecturer, Department of Endodontics, School of Stomatology, China Medical University, Liaoning, China.

[e] Research Assistant, Department of Developmental Biology, Key Laboratory of Cell Biology, China Medical University, Ministry of Public Health of China, Liaoning, China.

Corresponding author: Ms Xiulin Yan, Lecturer, Department of Orthodontics, School of Stomatology, China Medical University, 117# Nanjing North Street, Heping District, Shenyang, Liaoning, 110002 China
(e-mail: chbyxl@163.com)

Accepted: February 2009. Submitted: October 2008.

 DOI: 10.2319/100508-519.1

ception among the caspase families, as it is involved in both the production of active cytokines, including IL-1, IL-18 (interferon γ-inducing factor), and IL-33, and the apoptosis. Inflammation and apoptosis, including the activation of caspase-1 and its subsequent cytokines, are common features in ischemic events in the brain[8] and heart.[9] The root, PDL, and alveolar bone are, to some extent, suffering from chronic ischemic events after application of orthodontic force. But the role of caspase-1 in inflammatory response and apoptosis is unclear in orthodontic tooth movement.

The purpose of this study was to determine whether the expression of caspase-1 is changed during orthodontic tooth movement in rats, and whether the changes in expressions of caspase-1 protein and mRNA are phase specific. For this purpose, we detected the expressions of caspase-1 protein and mRNA after different durations of the application of orthodontic forces.

MATERIALS AND METHODS

Eighty 2-month-old male Wistar rats with an average weight of 206 g (±11 g) were used. Ethical permission was obtained from the Department of Experimental Animals, China Medical University, China. All rats were anesthetized with 10% chloral hydrate (3 mL/kg body weight) to receive orthodontic devices. The method modified from that described by Kobayashi et al[10] was used to induce a mesial traction force on the lower right first molar. The ligature-wire loops around the cervical aspect of the teeth were used to attach Sentalloy closed-coil springs (Grikin, Beijing, China), and each spring delivered approximately 50 g of force (Figure 1). The lower left first molars of the same rats were used as the controls.

During the study, the rats were kept in plastic cages with a standard 12-hour light/dark cycle, and were fed a soft diet and water *ad libitum*. The body weights of the rats were recorded daily. The rats were killed by dislocation of the cervical vertebra on days 1, 3, 7, and 14, respectively (n = 20 each time). The mandibles from 10 rats were removed and fixed in 10% neutral-buffered formalin for histologic analysis. Approximately 5 mm^3 volumes of bone and root tissues dissected from the mesial aspect of molars of five rats were used for instant extraction of total mRNA, and the tissues sampled in the same area from five other rats were stored at −70°C for later extraction of total protein.

Immunohistochemical Assay

The mandibles were fixed in 10% formalin for 72 hours followed by complete decalcification in 10% EDTA for 4 to 8 weeks (confirmed radiographically). The tissues were embedded in paraffin for preparation of 4 μm sagittal sections. Some sections were stained with hematoxylin and eosin (HE) for light microscopy. Other sections were selected for immunohistochemical assay. As the primary antibody, the goat polyclonal antibody against caspase-1 (Santa Cruz Biotechnology, Santa Cruz, Calif) was used at 1:100 dilution. An SP kit (Zhongshan Biotechnology Company, Beijing, China) was adopted to localize the secondary antibody. Staining was visualized with DAB. Simultaneously, negative control was performed without the primary antibody.

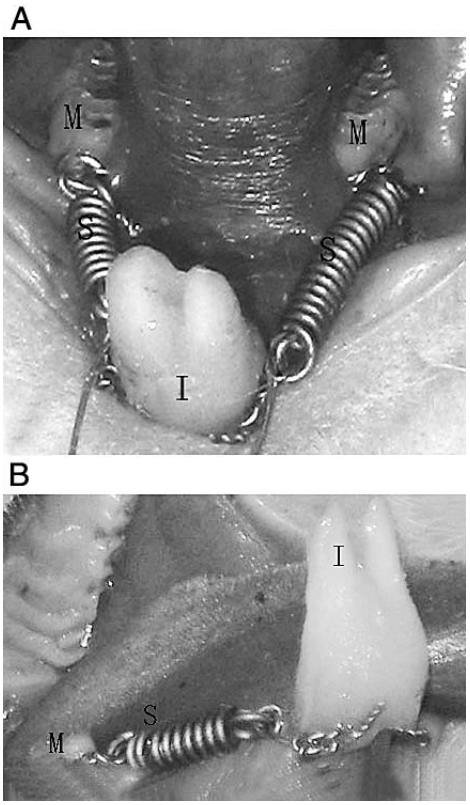

Figure 1. Orthodontic device *in situ* applied to rat molar to induce a mesial traction force. Sentalloy closed-coil spring (S) is attached to molar (M) and incisor (I), as described in text.

Extraction of Total mRNA

The tissues were washed three times with Dulbecco's phosphate-buffered saline (D-PBS) containing 0.1 mM EDTA without calcium and magnesium, and then the bone and root tissues were homogenized under liquid nitrogen with a mortar and pestle. The resulting tissue suspensions in liquid nitrogen were evaporated into disposable plastic Petri dishes before TRIZOL reagent (Invitrogen, Carlsbad, Calif) was added. Total RNA was isolated according to the protocol. RNA yield and purity were determined by UV-spectrophotometry and agarose gel electrophoresis.

cDNA Synthesis and Real-Time RT-PCR

Reverse-transcription polymerase chain reaction (RT-PCR) was performed on two steps with the SYBR PrimeScript RT-PCR Kit (Perfect Real Time) (TaKaRa Bio Inc, Shiga, Japan).The total high-quality RNA (1 μg) was reverse-transcribed in a 20 μL reaction on the PCR system (Bio-Rad Lab, Hercules, Calif). Then, 2 μL of the complete RT-sample was used as the template for real-time PCR in a volume of 25 μL on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, Calif) according to the manufacturer's protocol. The primer sequences were as follows: Caspase-1 forward: 5′- CCAGAGCACAAGACTTCTGAC; reverse: 5′- TGGTGTTGAAGAGCAGAAAGC (339 bp, GenBank Accession No. NM_012762). A constitutive gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to normalize the data by using the same amount of cDNA. Real Time RT-PCR Primer (Rn006) (TaKaRa Bio Inc) was used as GAPDH primer. The amplification conditions were as follows: 95°C for 10 seconds, 40 cycles of 95°C for 5 seconds, and 60°C for 34 seconds. The data were output and analyzed simultaneously by Relative Quantitation Experiment Workflow.

Protein Preparation and Western Blotting

The tissues were homogenized following the procedure of extraction of total mRNA. The homogenized tissues were prepared with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate, protease inhibitors) for 30 minutes on ice. Then the lysates were centrifuged twice for 10 minutes at 10,000 × *g* at 4°C. Protein concentrations were determined using the Bradford method. Protein extracts (20 g per sample) and a biotinylated molecular weight marker (Cell Signaling Technology, Danvers, Mass) were denaturated in Laemmli sample loading buffer at 95°C. Equal amounts of proteins were loaded and separated by SDS-PAGE, and then were transferred to the PVDF membrane (Millipore, Bedford, Mass) using Semi-dry transfer cell (Bio-Rad Lab). The PVDF membrane was blocked with 5% nonfat milk and incubated with goat polyclonal anti-caspase-1 (Santa Cruz Biotechnology) overnight at 4°C. The blots were incubated with an HRP-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature, performed with the ECL Kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). Immunoreactive bands were visualized by the Las-3000 Mini (FUJIFILM, Tokyo, Japan). Quantification of band density was done using Alpha Ease Fc software (Alpha Innotech, San Leandro, Calif).

Statistical Analysis

The data for plotting are the means of all animals in one group. To analyze statistically significant differences between different sets of data, a two-tailed Student's *t*-test was performed, and the *P* value was obtained.

RESULTS

Physiologic Parameters

Two rats died of anesthetic accident and were discarded from this study. Two rats were also discarded since their appliances were damaged. The other 76 rats tolerated the orthodontic appliances well and were able to eat and drink without problems. They gained weight steadily after 4 to 5 days.

Light-Microscopic Observations

Examination of paraffin sections stained with HE showed resorption lacunae associated with an inflammatory reaction on the mesial root surfaces of teeth subjected to orthodontic forces in 32 of 40 rats. Some multinucleated cells were located in both lacunae and PDL (Figure 2). Other sections stained for immunohistochemical assay showed that orthodontic forces significantly increased the number of caspase-1-positive cells in PDL (Figure 3), compared with controls.

Caspase-1 mRNA Expression

Mechanical force triggered an increase of caspase-1 mRNA in PDL. This effect was evident after 1 day, was greatest after 3 days, and decreased from day 7 to day 14. However, compared with controls, the caspase-1 mRNA level increased by 88% after 1 day, 280% after 3 days, 310% after 7 days, and 226% after 14 days (all $P < .05$) (Figure 4).

Caspase-1 Protein Expression

Both 45 kDa (procaspase-1) and 20 kDa bands (active P20 subunit of caspase-1) were found after 1, 3, 7, and 14 days, and were blackest after 3 days in teeth subjected to orthodontic forces and control teeth. The results of Western blotting showed that levels of procaspase-1 increased by 75% on day 1, 110% on day 3, 72% on day 7, and 119% on day 14, and P20 subunit levels increased by 151% on day 1, 147% on day 3, 36% on day 7, and 81% on day 14, compared with controls (all $P < .05$) (Figure 5).

DISCUSSION

The results of this study show that the levels of caspase-1 mRNA and procaspase-1 and active P20 sub-

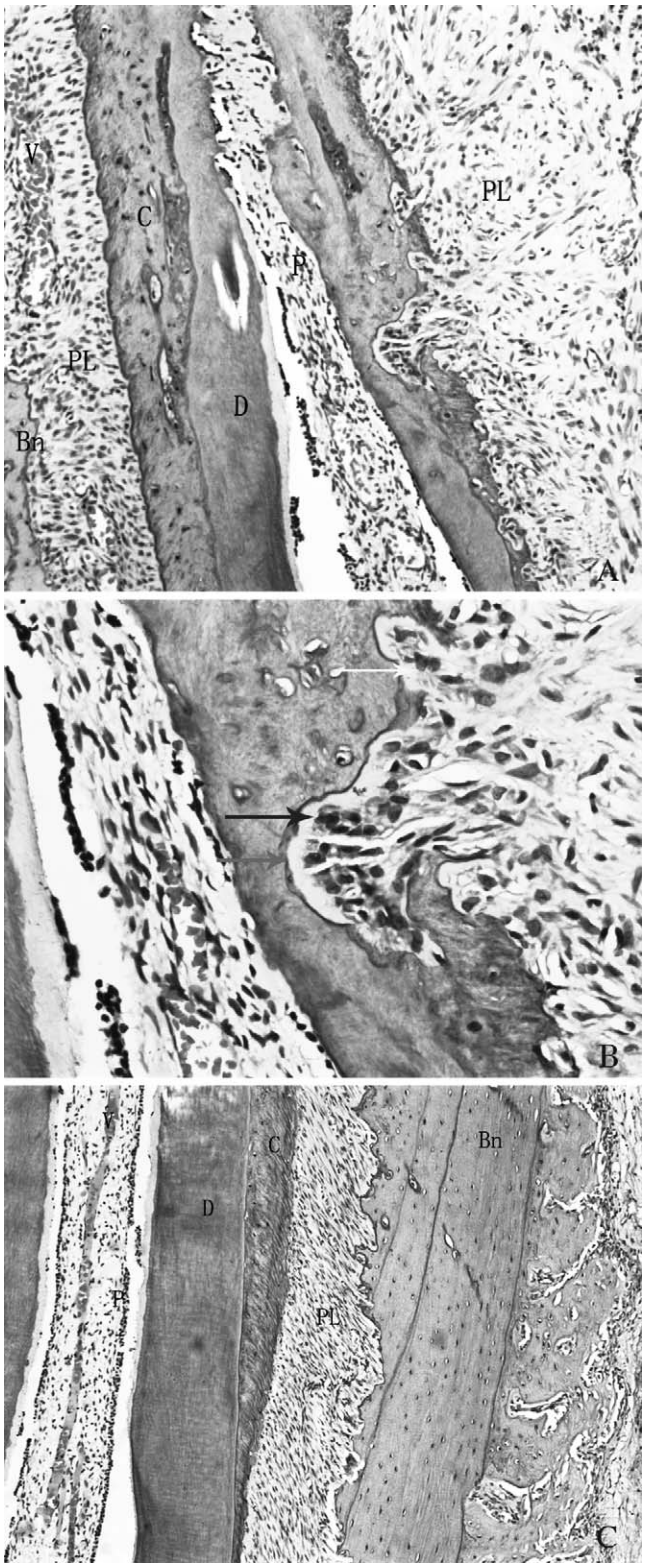

Figure 2. Paraffin sections of mesial tooth surfaces (HE). (A) Tooth subjected to orthodontic force for 14 days (×100). (B) A part of picture A (×400). (C) Control tooth (×100). In teeth subjected to orthodontic force, resorption lacunae were noted, often eroding through cementum into dentin (*grey arrows*). Inflammatory infiltrate and multinucleated giant cells (*black arrow*) also exist, although occasionally not within lacunae (*white arrows*).

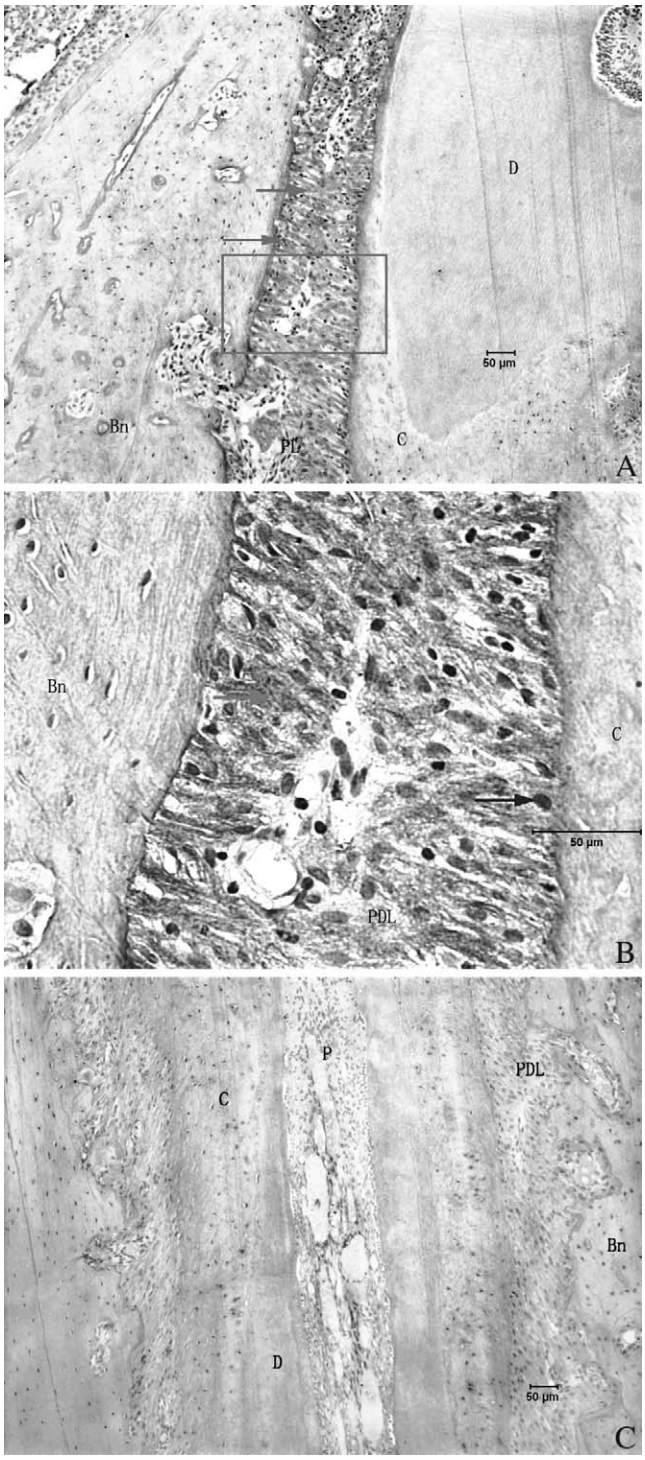

Figure 3. (A) Caspase-1 is expressed in PDL of tooth subjected to orthodontic force for 3 days (×100; the caspase-1-positive cells are marked by grey arrows). (B) A part of picture A (×400; fibroblast-like cells are marked by grey arrows; mononucleated macrophage-like cells are marked by black arrow). (C) Control tooth (×100).

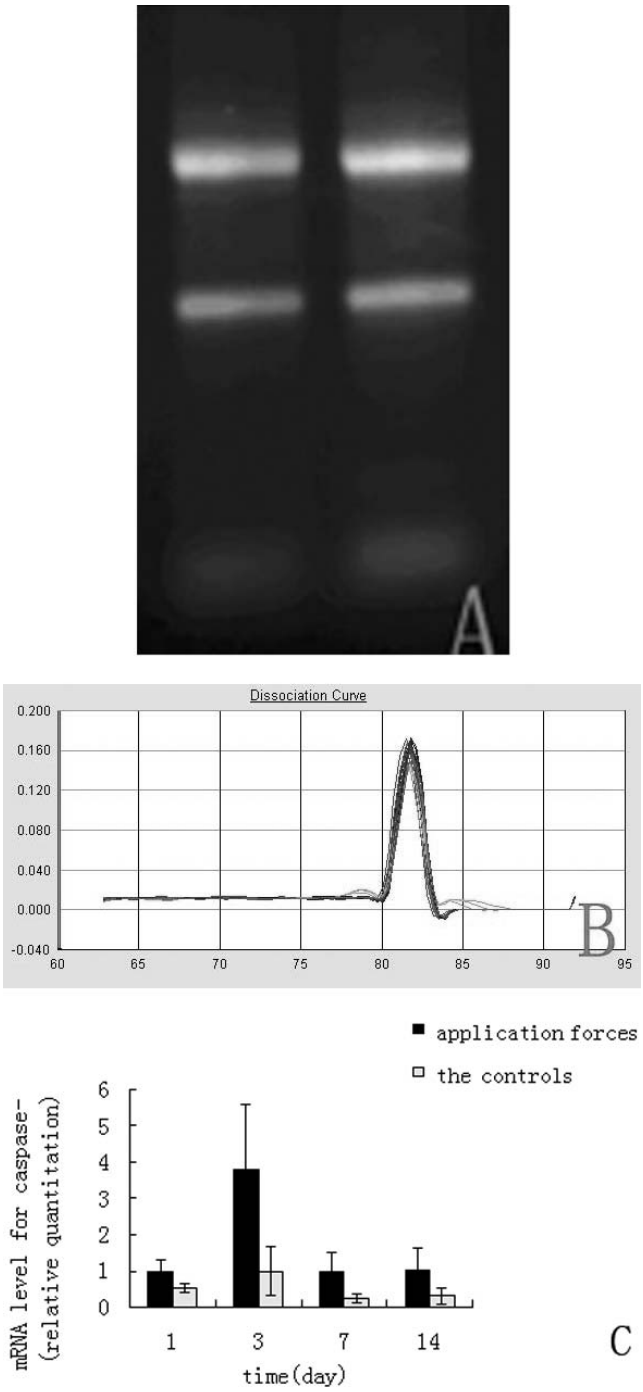

Figure 4. (A) RNA electrophoresis. (B) The dissociation curve for caspase-1. (C) Real-time RT-PCR for caspase-1.

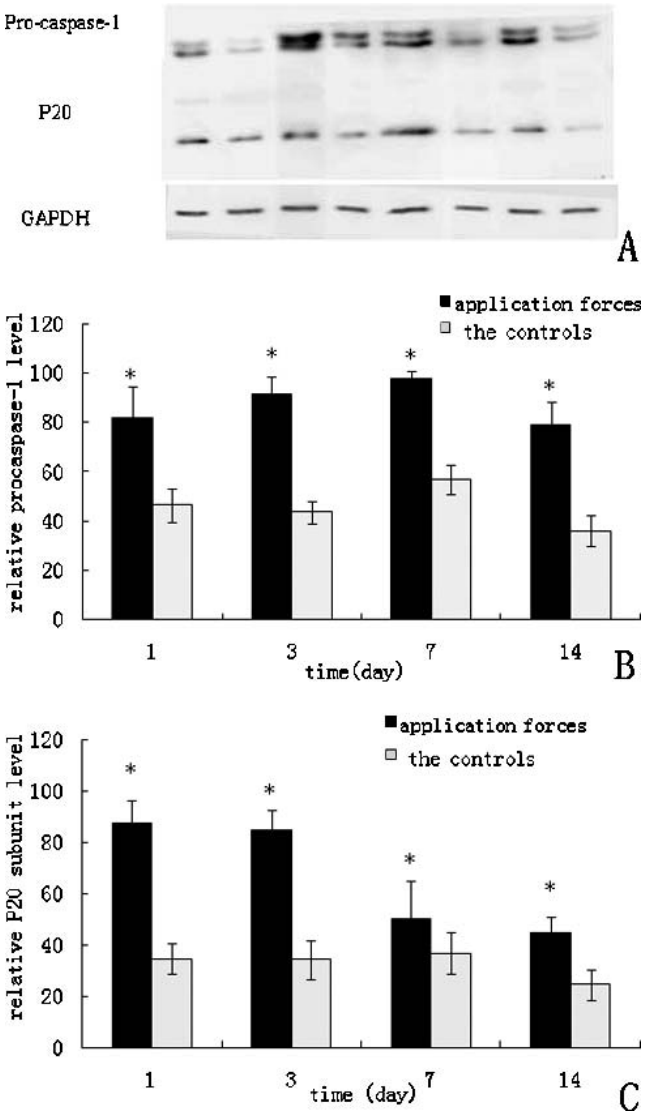

Figure 5. (A) Representative Western blotting for caspase-1. (B) The relative protein levels of procaspase-1. (C) The relative protein levels of P20 subunit.

unit increase after the application of orthodontic forces and change with different durations of orthodontic forces. Evidence that the level of caspase-1 increases and is phase-specific in orthodontic tooth movement supports a causal link between caspase-1 activity and the application of orthodontic forces. Caspase-1 lies in the first line of the innate immune response and host defense, so the innate immune response may play an important role in orthodontic tooth movement.

Caspase-1 is an essential regulator of inflammatory responses and apoptosis through its capacity to process and activate proIL-1β (the most potent factor of bone resorption), proIL-18, and proIL-33. Caspase-1 exists as an inactive precursor in the plasma membrane. It is activated within the inflammasome, a macromolecular complex assembled by members of the NOD-LRR family of proteins in response to ''danger signals.'' These signals, which are most commonly bacterial products or alterations in the intracellular ionic milieu, appear to act in a specific manner, inducing the assembly of specialized inflammasomes. The activation can be induced by a variety of ''danger sig-

nals,'' including ischemia[11] and increased cAMP,[12] and by proinflammatory cytokines like TNF-α and γIFN.[13,14]

The loss or gain of caspase-1 activity/expression forms the basis of a number of diseases caused by deregulation of inflammatory and apoptotic pathways. Deformation of the periodontal tissues, which may be a ''danger signal'' in the body, appears after the application of orthodontic force. As a result, ischemia[15] and increased cAMP[16] exist, and the levels of many inflammatory cytokines, such as IL-1,[3,4] IL-6,[17] IL-8,[18] IL-10,[19] TNF-α,[3,4] PGE2,[5] γIFN,[7] and COX-2,[6] change in the periodontal tissues during this process. If the activity/expression of caspase-1 increases, the local inflammation response might be intensified under an orthodontic force of the same magnitude, which could induce certain side effects, such as irreversible root resorption. In this study, we found that the levels of caspase-1 mRNA were highest after 3 days, but the levels of activated caspase-1 marked by active P20 subunit reached the peak on day 1. What down-regulated the translation process of caspase-1 mRNA on day 3 has not been elucidated. It can be supposed that there may be a negative feedback mechanism in the local area, which inhibits the translation process of caspase-1 mRNA and avoids the side effects such as irreversible root resorption, that results from the local excessively serious inflammatory response.

In this study, we also found that caspase-1 was expressed on fibroblast-like, mononucleated macrophage-like cells, and multinucleated giant cells in untreated periodontal tissues and was hyperexpressed in treated periodontal tissues in immunohistochemical assay. The most visible inflammatory response (Figure 3) was found in the periodontal tissue on day 3 and was consistent with the expressions of caspase-1 mRNA and proteins. It is interesting to note that the visible resorption lacunae were found in the periodontal tissues stained by HE (Figure 2) in most but not all rats. This result indicated that the tolerances of different rats to the same orthodontic forces were different. The same forces applied to some animals could result in serious root resorption, but in other animals, they may not cause any side effect.

If some disorders characterized by increased activated caspase-1 belong to one kind of disease, it can be supposed that patients with these diseases such as rheumatoid arthritis[20] and inflammatory bowel disease,[21] in which the expression of caspase-1 increases, may be the high-risk group for root resorption in orthodontic tooth movement. In turn, the patients with serious root resorption in orthodontic tooth movement may be the high-risk group for these autoinflammatory and autoimmune disorders.

If caspase-1 plays a significant role in orthodontic tooth movement, the inhibitors of caspase-1 activity such as Pralnacasan[22] and VX-765[23] may be useful for avoiding serious root resorption due to the local excessively severe inflammatory response. It can be a new method for improving the safety of orthodontic treatment.

How caspase-1 is activated in orthodontic tooth movement and which cytokine substrates are responsible for active caspase-1 have not yet been determined. More detailed studies need to be done to identify the exact mechanism of force-induced caspase-1 activation.

CONCLUSION

- The caspase-1 level increases during orthodontic tooth movement and changes with different phases, which might play a significant role in orthodontic tooth movement.

ACKNOWLEDGMENTS

We thank the Education Department of Liaoning Province for funding this project, and Dr. Y. H. Chen and Dr W. D. Zhao of the Department of Developmental Biology, Key Laboratory of Cell Biology, Ministry of Public Health of China, China Medical University, for generously granting us time and helpful advice.

REFERENCES

1. Rygh P, Bowling K, Hovlandsdal L, Williams S. Activation of the vascular system: a main mediator of periodontal fiber remodeling in orthodontic tooth movement. *Am J Orthod.* 1986;89:453–468.
2. Brudvik P, Rygh P. Root resorption beneath the main hyalinized zone. *Eur J Orthod.* 1994;16:249–263.
3. Jager A, Zhang D, Kawarizadeh A, Tolba R, Braumann B, Lossdorfer S, Gotz W. Soluble cytokine receptor treatment in experimental orthodontic tooth movement in the rat. *Eur J Orthod.* 2005;27:1–11.
4. Tzannetou S, Efstratiadis S, Nicolay O, Grbic J, Lamster I. Comparison of levels of inflammatory mediators IL-1beta and betaG in gingival crevicular fluid from molars, premolars, and incisors during rapid palatal expansion. *Am J Orthod Dentofacial Orthop.* 2008;133:699–707.
5. Seifi M, Eslami B, Saffar AS. The effect of prostaglandin E2 and calcium gluconate on orthodontic tooth movement and root resorption in rats. *Eur J Orthod.* 2003;25:199–204.
6. Jerome J, Brunson T, Takeoka G, Foster C, Moon HB, Grageda E, Zeichner-David M. Celebrex offers a small protection from root resorption associated with orthodontic movement. *J Calif Dent Assoc.* 2005;33:951–959.
7. Najat A, Lars F, Pongsri B, Moiz B. Orthodontic movement induces high numbers of cells expressing IFN-gamma at mRNA and protein levels. *Journal of Interferon & Cytokine Research.* 2000;20:7–12.
8. Felderhoff-Mueser U, Sifringer M, Polley O, Dzietko M, Leineweber B, Mahler L, Baier M, Bittigau P, Obladen M, Ikonomidou C, Buhrer C. Caspase-1-processed interleukins in hyperoxia-induced cell death in the developing brain. *Ann Neurol.* 2005;57:50–59.
9. Westphal E, Rohrbach S, Buerke M, Behr H, Darmer D, Silber RE, Werdan K, Loppnow H. Altered interleukin-1 receptor antagonist and interleukin-18 mRNA expression in

myocardial tissues of patients with dilated cardiomyopathy. *Mol Med.* 2008;14:55–63.
10. Kobayashi Y, Hashimoto F, Miyamoto H, et al. Force-induced osteoclast apoptosis in vivo is accompanied by elevation in transforming growth factor beta and osteoprotegerin expression. *J Bone Miner Res.* 2000;15:1924–1934.
11. Liu X, Drogmitz O, Neeff H, Benz S, Hopt UT. Apoptosis is caused by prolonged organ preservation and blocked by apoptosis inhibitor in experimental rat pancreatic grafts. *Transplant Proc.* 2004;36:1209–1210.
12. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature.* 2006;440:237–241.
13. Jain N, Sudhakar CH, Swarup G. Tumor necrosis factor-alpha–induced caspase-1 gene expression. *FEBS J.* 2007; 274:4396–4407.
14. Gu Y, Kuida K, Tsutsui H, et al. Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. *Science.* 1997;275:206–209.
15. Brodin P, Linge L, Aars H. Instant assessment of pulpal blood flow after orthodontic force application. *J Orofac Orthop.* 1996;57:306–309.
16. Santos De Araujo RM, Oba Y, Moriyama K. Role of regulator of G-protein signaling 2 (RGS2) in periodontal ligament cells under mechanical stress. *Cell Biochem Funct.* 2007; 25:753–758.
17. Yamaguchi M, Ozawa Y, Mishima H, Aihara N, Kojima T, Kasai K. Substance P increases production of proinflammatory cytokines and formation of osteoclasts in dental pulp fibroblasts in patients with severe orthodontic root resorption. *Am J Orthod Dentofacial Orthop.* 2008;133:690–698.
18. Maeda A, Soejima K, Bandow K, Kuroe K, Kakimoto K, Miyawaki S, Okamoto A, Matsuguchi T. Force-induced IL-8 from periodontal ligament cells requires IL-1ß. *Dent Res.* 2007;86:629–634.
19. Garlet TP, Coelho U, Silva JS, Garlet GP. Cytokine expression pattern in compression and tension sides of the periodontal ligament during orthodontic tooth movement in humans. *Eur J Oral Sci.* 2007;115:355–362.
20. Ku G, Ford P, Raybuck SA, Harding MW, Randle JCR. Selective interleukin-1b converting enzyme (ICE/Caspase-1) inhibition with Pralnacasan (HMR 3480/VX-740) reduces inflammation and joint destruction in murine type II collagen-induced arthritis (CIA). *Arthritis Rheum.* 2001;44:166–276.
21. Loher F, Bauer C, Landauer N, et al. The interleukin-1{beta}–converting enzyme inhibitor Pralnacasan reduces dextran sulfate sodium-induced murine colitis and T helper 1 T-cell activation. *J Pharmacol Exp Ther.* 2004;308:583–590.
22. Rudolphi K, Gerwin N, Verzijl N, Van Der Kraan P, Van Den Berg W. Pralnacasan, an inhibitor of interleukin-1[beta] converting enzyme, reduces joint damage in two murine models of osteoarthritis. *Osteoarthritis Cartilage.* 2003;11:738–746.
23. Stack J, Beaumont K, Larsen PD, Straley KS, Henkel GW, Randle JCR, Hoffman HM. IL-converting enzyme/caspase-1 inhibitor VX-765 blocks the hypersensitive response to an inflammatory stimulus in monocytes from familial cold autoinflammatory syndrome patients. *J Immunol.* 2005;175: 2630–2634.