Eur J Med Res (2008) 13: 425-431

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Role of Msx2 as a Promoting Factor for Runx2 at the Periodontal Tension Sides elicited by Mechanical Stress*

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Abstract
Early changes of Runx2 and Msx2 expressions were examined by immunohistochemistry in mouse periodontal ligament exposed to mechanical stress. 8-week-old ddY mouse was used as experimental animal. To provide a continuous mechanical stress on periodontal ligament, rubber dam sheet was placed between upper molars of the mouse. At 20 minutes, 1 hour, 3 hours, 9 hours and 24 hours after insertion of the sheet, relevant parts of the mouse tissues were excised and fixed in 4% paraformaldehyde/0.05M phosphate buffered fixative solution. Then serial paraffin sections were prepared and histopathological evaluation as well as examination of Runx2, Msx2 and alkaline phosphatase (ALP) expressions by immunohistochemistry were performed. Control animals were not subjected to mechanical stress. In the experimental group, strong expressions of Runx2 and Msx2 were seen in periodontal fibroblasts of the tension side at 20 minutes after mechanical stress. Expressions of Runx2 and Msx2 became stronger in parallel with time, and at 24 hours after mechanical stress, the periodontal fibroblasts, cementoblasts as well as osteoblasts showed strong expression. Moreover, ALP has also demonstrated similar strong expression. On the other hand, in the control group, although expressions of Runx2, Msx2 and ALP were detected at all the experiment times, the expressions were weak. All these results strongly suggested that Runx2 promoted differentiation of osteoblasts at early stage and Msx2 worked as an activator of Runx2 function.

Key words: Mechanical stress; Periodontal ligament; Runx2; Msx2

Introduction

To establish a biological base for orthodontic treatment, researches using animal experiments have been performed in the last several decades [1]. Reitan K [2, 3] examined the tissue reactions during orthodontic tooth movement. The author pointed out the histopathological findings of bone formation and/or resorption of alveolar bone of both tension and expression sides in these experiments. However, recently periodontal fibroblast cell lines are also being used in this kind of researches. Regarding with tissue reaction occurring in orthodontic treatment, osteoblasts at surface of the tension side and osteoclasts at pressure side of the relevant periodontal space of the alveolar bone appear, and play roles in resorption and addition of bone, a well-known fact. Takano-Yamamoto et al. [4] and Cho Y [5] examined the histological and immunohistochemical and in situ hybridization findings of both tension and pressure sides of the periodontal tissues during the orthodontic tooth movement.

Recently researches related with periodontal fibroblasts have advanced and the cells have now been identified to possess characteristic features other than what fibroblasts are generally known to have. Saito et al. [6] showed expression of Runx2 in a fibroblast cell line. However, calcification does not occur in periodontium even though Runx2 expression is detected in periodontal fibroblasts. This result suggests that a mechanism, which inhibits action of Runx2, exists. In fact, Yoshizawa et al. [7] recently reported that homeobox factor Msx2 suppressed differentiation of osteoblasts by inactivating Runx2 transcription. Thus in the current study, we applied mechanical stress to periodontal tissue of the mouse and examined protein expressions of Runx2 and Msx2 by immunohistochemistry in a time period up to 24 hours.

Materials and Methods

Animals

Thirty 8-week-old male ddY mice (Japan SLC Co. Ltd., Hamamatsu, Japan) were used in the study. The mice were kept in metal cages with bedding (Paper clean, Peparlet Co. Ltd., Shizuoka, Japan) in an envi-

*This study was conducted as thesis fulfillment for the PhD degree in Dentistry of Dr Takehiro Watanabe, under the supervision of Professor Toshiyuki Kawakami, Hard Tissue Pathology Unit, Department of Hard Tissue Research, Matsumoto Dental University Graduate School of Oral Medicine.

ronment temperature of 24 ± 1 °C regulated by an air-conditioner, and were provided with water and solid food (Picolab Rodent Diet 20, Japan SLC Co. Ltd., Hamamatsu, Japan) ad libitum.

Experimental Protocol

The experimental method in the current study was performed according to our previous publication [8]. First of all, mice were anesthetized with intraabdominal injection of pentobarbital sodium solution (Nembutal, Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan), and were fixed to handmade experimental table. The mouse was placed on the table in an open-mouth condition by pulling the upper anterior teeth in superior direction with a kite string and by pulling the lower anterior teeth in inferior direction with a rubber string (Fig. 1a). In this condition, continuous mechanical stress was applied by inserting an angle cut, and 2 layers of 2 x 2 mm rubber dam sheet (Ivory, Premium Rubber Dam Pure Latex: Heraeus Kulzer GmbH & Co. KG, Hanau, Germany) was bent and placed between upper right first molar and second molar as described previously [8] (Fig. 1b). Fig. 1 Diagram shows sagittal surface of the mouse upper molar in the current experiment. From mesial side, 3 teeth are arranged as first molar (M1), second molar (M2) and third molar (M3), and rubber dam sheet (green oval) was inserted between M1 and M2. Histopathological horizontal serial sections (red line) of the relevant periodontal tissue were prepared. As shown in Fig. 1H, M1, M2 and M3 were observed as periodontal mass. The experimental group was divided into 5 subgroups according to mechanical stimulus time (20 minutes, 1 hour, 3 hours, 9 hours and 24 hours). Table 1 shows number of the animals in each subgroup. Upon completion of each time period, the anesthetized animals were killed by ether inhalation, and the relevant tissues were extracted as one block. As control, same tissue part of the mouse opposite left side was used. The current experiment was performed according to the Guidelines for Animal Experiments of Matsumoto Dental University.

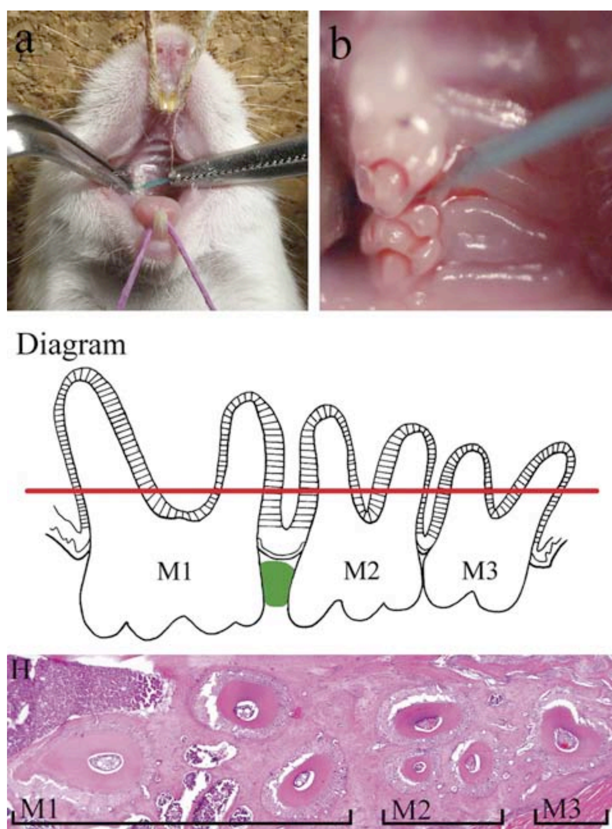

Fig. 1. Photograph of rubber dam sheet insertion between mouse molars (a) and its high magnification image (b). Insertion part of rubber dam sheet (green oval image) and diagram of horizontal section (red line) as well as histopathological image are seen (H x16).

Histology

The materials ware immediately fixed in 4% paraformaldehyde/0.05M phosphate-buffered solution and decalcified in 10% ethylenediamine tetraacetic acid. The materials were then dehydrated by passage through a series of ethanols and embedded in paraffin. Samples were cut at 5µm serial sections. Serial sections were then collected onto silane-coated slide and examined by histopathological (hematoxylin and eosin) and immunohistochemical (IHC) techniques.

Immunohistochemistry

For IHC, deparaffinized sections were prepared after pre-treating with 0.13% pepsin for 30min at 37 °C. Examination was carried out using a Dako Envision + Kit-K4006 (Dako, Glostrup, Denmark) and three primary antibodies: anti-rabbit Runx2 (M-70, Runx2: 1/1000), anti-chick Msx2 (4GI, Msx2: 1/1000) and anti-human alkaline phosphatase (B4-78, ALP: 1/50). The Runx2 antibody was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. The Msx2 antibody was developed by Jessell and Brenner-Morton [9]. The ALP antibody was developed by Katzumann [10]. Both antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development of the NIH and maintained by The University of Iowa, Department of Biological Science, Iowa City, IA, USA. Immunohistochemical reaction products were visualized by diaminobendizine. Samples were then counterstained with hematoxylin. For negative control, sections were incubated with omission of the primary antibody under the same protocol.

Table 1. Experimental Periods and Number of Animals.

Periods	Cont	20min	1hr	3hr	9hr	24hr	Total
Number	5	5	5	5	5	5	30

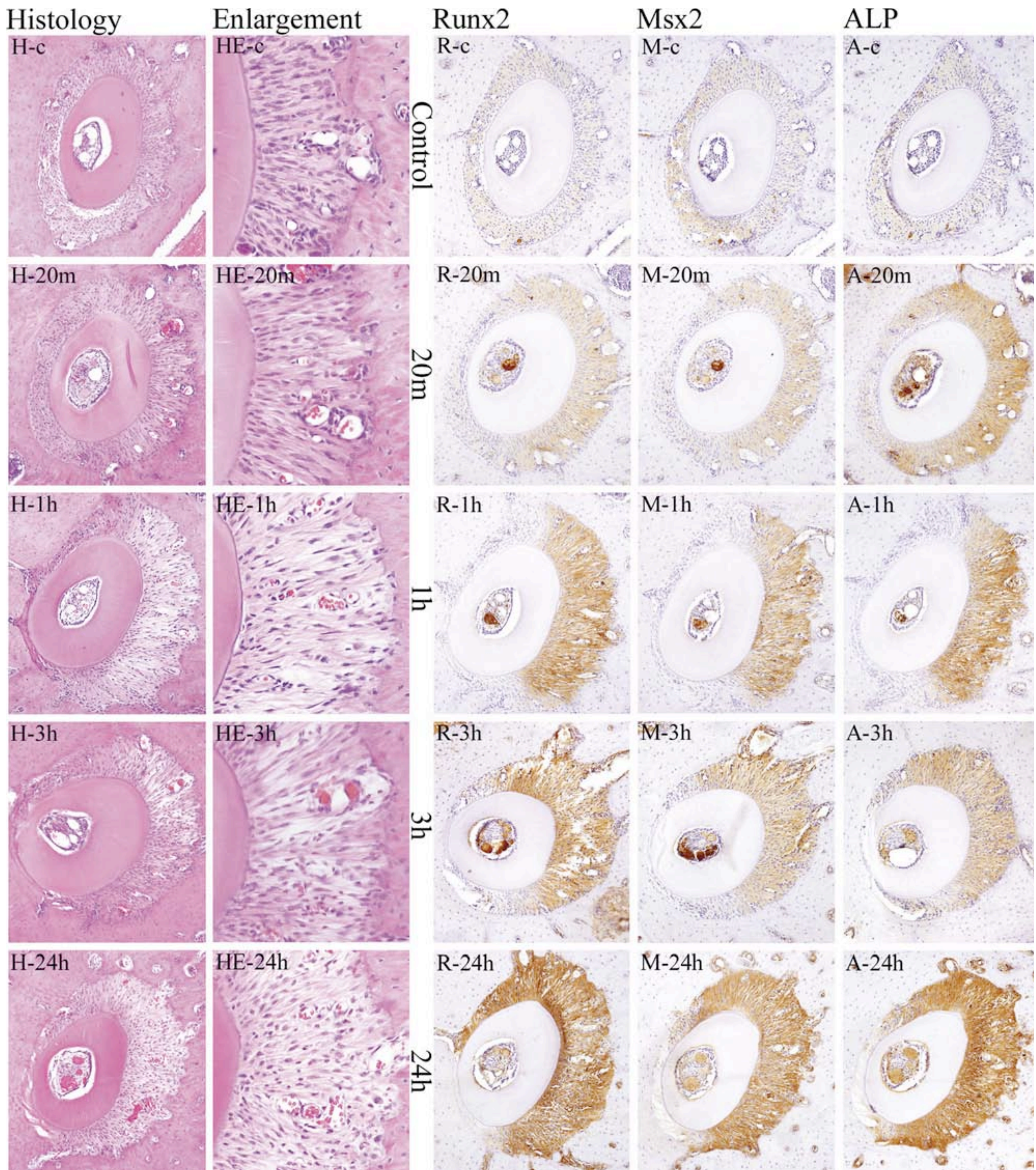

Fig. 2. Histopathological as well as immunohistochemical results. Left column shows histopathological image of the alveolar bone socket morphology (x 60) and right column next to it is high magnification image of tension side (x 160). Right 3 columns reveal results of Runx2, Msx2 and ALP immunohistochemical stainings (x 60). Most upper row is the control group and the next four rows display groups of 20 minutes, 1 hour, 3 hours and 24 hours.

Results

Histopathological Examination

Control group

When distal buccal root of the upper first molar was horizontally examined, the alveolar bone socket was in oval shape with a long diameter of 320 μm and a short diameter of 250 μm though a small variation of size between the samples existed (Fig. 2 H-c). Although localization of the root in the alveolar bone socket showed a slight deviation to the mesial direction, the root with periodontal tissue was mainly localized in the center with a diameter of 50 μm. Periodontium was basically arranged between the alveolar bone and cemental surface of the root with radiated as well as bundle shaped fibers. However, in some part direction of the arrangement was slightly different in the mesial side. In these parts, fibroblasts between the roots lo-

calized in transverse direction possibly to be able to keep continuity of the roots after branching. On the other hand in the distal side of the root, vertical or oblique images were observed. Capillaries between bundles of the periodontium were seen. The fibroblasts with long elliptical nucleus within spindle shaped cytoplasm were detected in the periodontium through running direction of the periodontium (Fig. 2 HE-c). On the other hand, cementoblasts were localized at the cemental surface as one layer with an elliptical nucleus and scanty cytoplasm. Osteoblasts with nucleus in oval shape and scanty cytoplasm showed slight flatness, and were arranged in surface of the alveolar bone. And remodeling line representing resorption and addition of the bone was seen. In some part of the alveolar bone, a resorption-like tissue with osteoclasts was observed. Osteoclasts were also detected in surface of the cementum.

Experimental group

Regarding with findings of the experimental group, differences from the control group will be emphasized.

At 20 minutes group, the root clearly moved about 20 μm to the mesial direction representing the pressure side. The root was enlarged about 70μm in the distal side (Fig. 2 H-20m). Thus at the tension side, radiated arrangement of the periodontium became clear, and the periodontal fibroblasts showed an appearance of spindle shape cytoplasm and long oval or flat-like nucleus (Fig. 2 HE-20m). On the other hand, disorder in arrangement of the periodontal fibroblasts was recognized at the pressure side.

At 1 hour group, relative location of the root in the alveolar bone further moved mesially, and width of the periodontium of the same part was narrow with a diameter of about 10 μm. On the other hand, periodontal space of the opposite distal tension side enlarged to a width of 90 μm (Fig. 2 H-1h). Periodontium of the same part was considerably stretched and interfibrillar area became loose. Cytoplasm and nucleus of the periodontal fibroblasts were in long spindle shape. On the other hand, at the pressure side, various degenerative changes in the cells, which formed the periodontium, were recognized. Many osteoblasts and cementoblasts distributed at the alveolar bone surface were confirmed as elliptical shape cells (Fig. 2 HE-1h).

At 3 hours group, width of the periodontium at the distal tension side enlarged to a size of 100 μm, resulting in relative mobility of the root and stretching of the periodontal fibers with formation of interfiber space. The root at pressure side approached further to the alveolar bone (Fig. 2 H-3h). At the tension side, cytoplasms of the periodontal fibroblasts were in long spindle shape and their nuclei showed a shape from long oval to long spindle. Many osteoblasts at the alveolar bone surface with a shape of oval or cylinder were observed (Fig. 2 HE-3h).

At 9 hours group, the periodontal space at the distal tension side was about 100 μm due to relative movement of the root in the alveolar bone. At the mesial pressure side, the root extremely approached to the alveolar bone, resulting in degeneration and atrophy of the tissue. Thus the cells were almost not recognized at this side. At the tension side, space of the periodontium decreased. Cytoplasms of the periodontal fibroblasts were in short spindle shape and their nuclei were in long elliptical shape. On the other hand, cementoblasts on the root surface were also in elliptical or cylindrical shape. Osteoblasts coming in contact with the alveolar bone revealed elliptical or cylindrical shape, and increase of their number was recognized.

At 24 hours group, at the pressure side the periodontal tissue approached further to the alveolar bone and cell components were almost not recognized (Fig. 2 H-24h). On the other hand, at the tension side, cytoplasms of the periodontal fibroblasts were in spindle shape, and their nuclei were in slight thick elliptical shape. Osteoblasts on the alveolar bone surface were in mostly cylindrical or short elliptical shape. Similar appearance was also detected in the cementoblasts (Fig. 2 HE-24h).

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Control group

Weak expression of Runx2 peptide was observed at periodontium of distal buccal side of the first molar root during all mechanical stress time intervals. This expression was slightly stronger at the mesial side. When observed in detail, this expression was attributed to mainly cytoplasms of the fibroblasts (Fig. 2 R-c). Msx2 expression was also similar to expression pattern of Runx2. However, Msx2 expression was stronger at the mesial side as compared to Runx2 expression (Fig. 2 M-c). Again weak expression of ALP was also detected at all the mechanical stimulus time similar to Runx2 expression (Fig. 2 A-c).

Experimental group

At 20 minutes group, regarding with Runx2 expression, strong reaction in cytoplasms of the periodontal fibroblasts at the tension side was detected. On the other hand, at the pressure side, slightly weak staining as compared to the control group was observed (Fig. 2 R-20m). Although positive staining of ALP expression similar to Runx2 pattern was observed at the tension side, density was stronger as compared to Runx2 expression (Fig. 2 A-20m).

At 1 hour group, strongly stretched cytoplasms of the periodontal fibroblasts and the cementoblasts showed Runx2 protein and this expression was stronger as compared to the 20 minutes group. And Runx2 expression almost disappeared at all areas of the pressure side (Fig. 2 R-1h). Expression and distribution of Msx2 protein were identical to Runx2 protein expression (Fig. 2 M-1h). And ALP expression state was similar to Runx2 expression pattern. However, ALP expression was much stronger at the tension side of the periodontal fibroblasts as compared to Runx2 expression (Fig. 2 A-1h).

At 3 hours group, Runx2 demonstrated positive expression in cytoplasms of the pretty stretched and enlarged periodontal fibroblasts and cementoblasts. Positive staining area extended to the pressure side as compared with 1 hour group. This expression state was also detected in cytoplasms of the osteoblasts (Fig. 2 R-3h). Positive Msx2 expression at the periodontal fi-

broblasts and cementoblasts was slightly weaker than Runx2 expression (Fig. 2 M-3h). ALP expression findings were also similar to Msx2 expression (Fig. 2 A-3h).

At 9 hours group, Runx2 revealed positive reaction in cytoplasms of the strongly stretched periodontal fibroblasts, cementoblasts and osteoblasts. This immunoreaction further extended to the pressure side as compared with the 3 hours group. At this period, Msx2 expression result was similar to Runx2 expression. And ALP expression was also confirmed to be similar with Rux2 expression findings.

At 24 hours group, Runx2 revealed strong positive reaction at all areas of the root periodontal fibroblasts, cementoblasts and osteoblasts except the areas of the root closely attached to the alveolar bone at the pressure side. The reaction was strongest at this period (Fig. 2 R-24h). Msx2 result was also similar to Runx2 findings (Fig. 2 M-24h). Furthermore ALP findings were also similar to staining of the others. However, strong expression of ALP was also shown in the osteoclasts located at the alveolar bone surface (Fig. 2 A-24h).

Discussion

To understand the biological mechanism in orthodontic treatment, researches using animal experiments have been performed in the last several decades. Thereafter efforts have focused on all aspects of tissue change upon mechanical stress. Especially researches focused on bone resorption and addition at the tension side after mechanical stimulus by showing existence of osteoclasts and osteoblasts, respectively. Because tissue changes at early period have recently been interested in, examination of tissue type shifted from hard to soft one, i.e. periodontal tissue. Recently increasing number of researches investigating cell differentiation and morphogenesis, which are regulated by mediators such as cytokines and transcription factors, have been reported. Osteoblasts and osteoclasts have a major role for cell differentiation in orthodontic dental treatment, and many researches using these cells have also been performed [11-13]. Thus we planned the current research based on recent success of Saito et al. [6] and Yoshizawa et al. [7] as mentioned in the introduction section.

Before discussing results of the current study, experimental system will be verified. Based on the method established by Waldo [1], rubber dam sheet was placed between the mouse molars, and degeneration of the periodontal tissue was observed. Insertion of rubber dam sheet between the mouse molars provides easy and simple application of the method. Thus mastering this technique will let examination of the many animals be performed in a short time. However, one shortcoming of this technique is the possibility that the tooth can move by time and the power enforced to rubber dam sheet cannot be fixed. Another method described by Andrade et al. [14] used coil string between anterior teeth and first molars for mechanical stress. Although a fixed power can be applied in this method, its application is extremely difficult and it is impossible to use many experimental animals in a short time. This time we examined expression and distribution of the factors related with bone formation in periodontium by immunohistochemistry. To increase reliability of the current study, use of many samples was needed. Therefore we used Waldo method [1] in this study. Regarding with experiment time, we limited the time up to 24 hours based on our recent report, confirming the changes of Runx2 and Msx2 proteins within 24 hours in mechanically stimulated periodontium [8]. Reitan[3] reported that acute cellular remodeling reaction in mechanically stimulated bone is the start of bone formation within 1-2 days. In the current research, changes of osteoblast differentiation-related factors such as Runx2 and Msx2 expressions within 24 hours were considered to reflect this kind of reaction. And Kawarizadeh et al. [15] applied mechanical stress to the rat molar for 15 minutes and observed increased expression of Runx2 in the relevant periodontal fibroblasts. In our study, we used a long range of time intervals from 20 minutes to 24 hours.

Mesial side of second molar root was observed in our previous study [8]. However, the root was relatively thin and its rotation occurred due to insertion of rubber dam sheet, and fixation at the tension as well as pressure sides was not properly done. Due to these reasons, time-dependent observation was quite difficult. To overcome these difficulties, mesial distal side of the first molar root was observed in the current study.

In the results of histopathological observation in the current study, early morphological changes of the periodontal fibroblasts were observed at 20 minutes group. The periodontium showed enlargement at the tension side and constriction at the pressure side, and the periodontal space enlargement was in highest state at the 3 hours group. The results demonstrated prominent alteration of the periodontal fibroblasts. The nucleus was in spindle shape, and the cytoplasm was strongly pulled out. Proliferation of the osteoblasts distributed at the tension side surface of the alveolar bone was confirmed in the groups from 3 to 9 hours. On the other hand, the cementoblasts localized at cementum surface did not show a major change. At 24 hours group, width of the periodontal space was enlarged in maximum state. The cells with thick elliptical nuclei and rich cytoplasm increased. Reitan [2] stated that during bone formation of the alveolar bone, precursor cells of the osteoblasts at the periodontium differentiated into osteoblasts and appeared at surface of the alveolar bone, followed by active bone formation. In results of the current research, within 24 hours the cells from periodontium were considered to proliferate and differentiate into osteoblasts.

Before discussing results of the immunohistochemical staining, several facts about Runx2 and Msx2 will be mentioned. Komori [16] clearly showed that Runx2 promoted differentiation process of osteoblasts at early stage but oppositely suppressed the differentiation at later stage. That is, although Runx2 induces differentiation of mesenchymal stem cell into osteoblast at early stage, it keeps the osteoblast in immature differentiated state by suppressing the process at later stages. Runx2 was mentioned to inhibit differentiation of osteoblasts into osteocytes. On the other hand, Msx2, a transcrip-

tion regulatory factor with homeodomain, was identified as mammalian homolog of drosophila cell cycle related gene, Msh [17,18]. Msx was identified to have two variants in human (Msx1 and Msx 2) and 3 variants in mouse (additional Msx3). High expression of Msx2 was shown in bone and joint. Homeobox gene product is known to activate or inactivate transcription of other genes, and it is considered as one of the genes with inhibitory function for morphogenesis. Satokata et al. [19] reported that Msx2 knockout mouse is born alive but abnormality of multiple ectodermal tissues is seen. Similar to human diseases, incomplete formation of calvaria, abnormal tooth formation and cartilage hypoplasia were detected. Thus Msx2 was considered to play an important role in early differentiation process including activation as well as inactivation states. Ichida et al.[20] investigated role of Msx2 in differentiation of pluripotent mesenchymal stem cells of C3H10T1/2 and C2C12 as well as mouse first generation cultured osteoblasts, and showed ALP increase in C3H10T1/2 and C2C12 cells and promotion of calcification in mouse first generation cultured osteoblasts by Msx2. That result strongly suggests that Msx2 plays a crucial role as a transcription factor for osteoblast differentiation. Cheng et al. [21] similarly showed that Msx2 functioned as an inhibitory factor for lipocyte differentiation and an acceleration factor for osteoblast differentiation. This result shows that Msx2 in addition to its dedifferentiation induction function induces osteoblasts in priority depending on change of its amount. And Ishii et al. [22] reported that Msx2 and Twist by cooperating each other are involved in differentiation and proliferation of osteoblasts. Yanagisawa et al. [23] examined expression of transcription factors in osteoblasts by western blotting and confirmed increased expression of Runx2 and Msx2, suggesting that they play important roles in osteoblast differentiation.

As mentioned in the introduction section, regarding with characteristics of the periodontal fibroblasts, Yoshizawa et al. [7] examined mRNAs of the periodontal cells PDL-L2 and the osteoblast cells MC3T3-E1 by microarray method for comparison of gene expressions. Their results showed that Msx2 expressed in high amount in PDL-L2 cells. When change of Msx2 expression was examined by time, although Msx2 expression did not display any alteration in PDL-L2 cells, its expression in MC3T3 cells decreased by differentiation of the cells and finally disappeared with formation of evident calcification. When Msx2 in PDL-L2 cells was deleted, the cells showed calcification. Oppositely, when Msx2 was forced to express in MC3T3 cells, calcification cannot occur, and stimulation with BMP2 results in only a small amount of calcification. Thus Msx2 is the gene responsible for inhibition of calcification, and calcification ability of the cells can be regulated in vitro by adjusting expression of Msx2. In another study, Liu et al. [24] suggested that Msx2 suppressed differentiation of osteoblast precursor cells as well as immature osteoblasts, and promoted proliferation of osteocytes. Moreover Shirakabe et al. [25] reported that Msx2 functioned as an inhibitory factor for Runx2 activity during morphological formation of human cranial bones.

In results of the current study, Runx2 showed weak activity in periodontal fibroblasts of the control group at all the time intervals. This result was compatible with report of Saito et al.[6], which mentioned low transcriptional activity of Runx2 expression in PDL-L2 cells. In our results, Msx2 revealed also weak expression in the periodontium space during all the experiment time intervals similar to Runx2 expression pattern. Our data was in vivo confirmation of the in vitro results of Yoshizawa et al. [7], in which Msx2 expression was reported to block differentiation of osteoblasts.

After application of mechanical stress, Runx2 expression disappeared at the pressure side at 1 hour. However, at the tension side, Runx2 protein expression appeared in the periodontal fibroblasts as early as 20 minutes after mechanical stress, and became stronger in parallel with the time. This result suggests that this process is even faster than its appearance in osteoblasts in alveolar bone at the tension side. Our results supported the opinion of Komori [16], who reported that Runx2 promoted differentiation of osteoblasts at early stage.

And Msx2 protein reaction similar to Runx2 appeared in the periodontal fibroblasts as early as 20 minutes and became stronger with time. Expression of an osteoblast marker, ALP, also showed strong expression with parallel to Msx2 expression. Thus our results suggest that Msx2 activates Runx2 function as similarly reported by Ichida et al. [19] but opposite to results of Yoshizawa et al. [7], which mentioned Msx2 as an inhibitory factor for osteoblast differentiation.

In conclusion, application of mechanical stress on the periodontal fibroblasts resulted in strong expressions of ALP, Runx2 and Msx2 at the tension side with time, which were found to be in low level in the control group. Our results suggested that Runx2 induced differentiation of osteoblasts, and Msx2 functioned as a promoting factor for Runx2 activity at the tension side in the cells exposed to mechanical stress.

Acknowledgment: This research was partially supported by a Grant-in-Aid for Scientific Research (C) (#19592374 and #20592419) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received: May 21, 2008 / Accepted: June 30, 2008

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