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Original Article

# Effects of mechanical stress on the mRNA expression of S100A4 and cytoskeletal components by periodontal ligament cells

**Wagner Rodrigues Duarte[1], Yuko Mikuni-Takagaki[2], Toshio Kawase[3], Tadahiro Iimura[4], Shinichiro Oida[5], Keiichi Ohya[6], Keizo Takenaga[7], Isao Ishikawa[1], and Shohei Kasugai[6]**

*1) Department of Periodontology, Faculty of Dentistry, Tokyo Medical and Dental University; Departments of 2) Oral Biochemistry, and 3) Dental Bioengineering, Kanagawa Dental College; Departments of 4) Developmental Biology, 5) Biochemistry, and 6) Dental Pharmacology, Faculty of Dentistry, Tokyo Medical and Dental University; 7) Division of Chemotherapy, Chiba Cancer Center Research Institute*

**The periodontal ligament (PDL) functions under constant mechanical stress, and PDL cells obviously control PDL functions under such conditions. We have previously found that the mRNA expression of the $Ca^{2+}$-binding protein S100A4 and $\beta$-actin is higher in the PDL from erupted teeth than in the PDL from teeth under eruption. This suggested a role for S100A4 in the response of PDL cells to mechanical stress, possibly by coupling $Ca^{2+}$ and the cytoskeletal system. In the present study, we investigated the direct effects of cyclical stretching on the mRNA expression of S100A4 and two cytoskeletal components ($\beta$-actin and $\alpha$-tubulin) by PDL cells. In Northern blotting analysis, the expression of S100A4, $\beta$-actin, and $\alpha$-tubulin mRNAs was higher in the PDL from fully erupted and functional bovine teeth than in partially erupted ones. Similarly, when bovine PDL cells were mechanically stimulated by means of the Flexercell Strain Unit, the expression of S100A4, $\beta$-actin, and $\alpha$-tubulin mRNAs increased over the control levels. The results of our present study indicate that S100A4 is involved in the responses of PDL cells to mechanical stress possibly by coupling $Ca^{2+}$ to the cytoskeletal system in these cells.**

Corresponding Author: Wagner Rodrigues Duarte
Tokyo Medical and Dental University, 1–5–45 Yushima, Bunkyo-ku, Tokyo 113–8549, Japan.
Kanagawa Dental College, 82 Inaoka, Yokosuka 238–8580, Japan.
Chiba Cancer Center Research Institute, 6– 66–2, Nitona, Chuoh-ku, Chiba 260–8717, Japan.
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## 1. Introduction

The periodontal ligament (PDL) is located between the tooth and the alveolar bone, and its main function is to absorb and distribute the constant forces produced during mastication. Therefore, the PDL exhibits characteristics of a shock absorbing system[1], which is controlled by PDL cells. Previous studies have shown that PDL cells respond to mechanical stress by increasing their production of prostaglandin $E_2$[2,3] and interleukin 1-$\beta$[4], while decreasing the activity of alkaline phosphatase (ALP)[5]. Thus, it is likely that such inductive or inhibitory responses are essential for the physiological function of the PDL.

It has been reported that $Ca^{2+}$ and cytoskeletal components are two major participants in the cellular responses to mechanical stress[6,7]. PDL cells increase intracellular $Ca^{2+}$ levels and exhibit changes in actin filament polymerization as the most rapid responses to mechanical stress *in vitro*[8,9]. The functions of intracellular $Ca^{2+}$ are mediated by several $Ca^{2+}$-binding proteins, including members of the S100 $Ca^{2+}$-binding protein (CaBPs) family. After binding $Ca^{2+}$, the CaBPs control several cellular processes such as cell motility,

cell cycle progression, and cell metabolism[10]. In a previous study, we showed that the mRNA expression of a member of this family, known as S100A4, is higher in the PDL from erupted teeth than in that from teeth under eruption[11]. Since S100A4 is a $Ca^{2+}$-binding protein that affects cytoskeletal organization in a $Ca^{2+}$-dependent manner[12], we speculated that S100A4 mRNA expression increased as a result of PDL cells' response to mechanical stress generated by mastication and that S100A4 could couple $Ca^{2+}$ to cytoskeletal components in PDL cells.

In the present study, we examined the direct effects of stretching on the mRNA expression of S100A4 and two cytoskeletal proteins, $\beta$-actin and $\alpha$-tubulin. Our observation that the mRNA expression of S100A4, $\beta$-actin, and $\alpha$-tubulin increased in stretched PDL cells suggests that S100A4 participates in the responses of PDL cells to mechanical stress by affecting cytoskeletal organization in these cells.

## Materials and Methods

### Isolation of bovine PDL

Fresh bovine PDL was isolated as previously described[11]. Briefly,10 mandibles of approximately 1.5 year-old cattle were obtained from a local slaughter house, and the PDL from fully and partially erupted teeth was removed as follows: fully erupted permanent central incisors were extracted and their PDLs were scraped from the middle third of the root with surgical blades; contamination with surrounding tissues was carefully avoided. The PDL from partially erupted teeth was scraped as described above from the root of permanent central incisors that had not visibly reached the occlusal level when compared with adjacent teeth, and that had approximately two thirds of their roots formed. We pooled corresponding tissues and extracted and purified total RNAs as described below.

### Cell culture

Bovine PDL was removed as described above, washed twice with cold PBS, and a PDL cell culture was established as previously described[13]. Briefly, growing cells from PDL tissue explants were cultured in $\alpha$-Minimal Essential Medium ($\alpha$-MEM) (Gibco) supplemented with 10% FBS and kanamycin (60 $\mu$g/ml) at 37℃ in a humidified atmosphere of 5% $CO_2$/95% air. Cells of the 5th passage were cultured ($1 \times 10^5$ cells/well) on flexible-bottomed culture plates (Flexercell Corp., USA). For stretching the periodontal ligament cells, the bottom of the plate was deformed at 10 kPa by a computer-operated vacuum system (Flexercell Strain Unit, Flexercell Corp., McKeesport, PA). Cells were subjected to repetitive cycles of maximum 18% elongation for 2 and 24 h. Repeated sets of four stretchings for 2 s with a frequency of 1/3 Hz in 11 s followed by a relaxation period of 49 s resulted in 240 cycles of stretching in one hour.

### Total RNA extraction and Northern Blotting

Total RNA of freshly extracted PDL and PDL cell culture was extracted following the instructions supplied with the ISOGEN kit (Nippon Gene, Japan). After the RNA was measured spectrophotometrically, RNA quality was checked by electrophoresis in denaturing agarose gel. Samples containing 10 $\mu$g of total RNA were denatured by heating to 65℃ for 5 min in loading buffer containing de-ionized formamide and formaldehyde. The samples were immediately cooled on ice for 5 min and electrophoresed on a 1.2% (w/v) agarose gel containing 17% formalin, stained with ethidium bromide and photographed. Total RNAs were transferred onto nylon membranes (Zeta-Probe[R], BIO-RAD, Hercules, CA) by capillary blotting with 10 × SSC and fixed by baking at 80℃ for 30 min. The membranes were then pre-hybridized overnight at 42℃ in hybridization buffer containing 50% formamide, 0.12 M $Na_2HPO_4$, pH 4; 0.25 M NaCl; 1 mM EDTA; and 7% SDS. Hybridizations proceeded overnight at 42℃ in fresh hybridization buffer containing a radiolabeled probe. The probes used for the hybridizations were as follows: a bovine S100A4 cDNA probe[11], a *Bam*HI fragment of the 2.1 kb human $\beta$-actin cDNA clone pHF$\beta$A-1[14], and a Pst I (1.2 kb) fragment of the mouse $\alpha$-tubulin cDNA[15]. All probes were labeled with [$\alpha$-$^{32}$P] dCTP by random priming (Ready to Go Kit, Pharmacia Biotech, Uppsala, Sweden). The membranes were briefly rinsed in 2 × SSC and sequentially washed for 15 min at room temperature in 2 × SSC/0.1% SDS, and 0.5 × SSC/0.1% SDS. A final washing at 65℃ in 0.1 × SSC/0.1% SDS was performed for 15 min to remove nonspecifically bound probe. The membranes were exposed to x-ray films (X-OMAT, Kodak, Rochester, NY) at −80℃ with two intensifying screens. Autoradiographs were scanned with an imaging densitometer (BIO-RAD GS-670, BIO-RAD ), and the signal intensities were evaluated and compared with Molecular Analyst 2.0.1 Software (BIO-RAD, BIO-RAD) on a Macintosh computer. All signals were normalized to the ethidium bromide staining of the 28S

ribosomal RNA.

## Results

First, we conducted a Northern Blot analysis to examine the expression of S100A4, $\alpha$-tubulin and $\beta$-actin mRNAs in the PDL from fully and partially erupted teeth. As shown in Fig. 1, the expression of all mRNAs was higher in the PDL from fully erupted teeth than in the PDL from partially erupted ones. Similar results were obtained from two sets of separate experiments. The relative values for signal intensity of S100A4 mRNA, after normalization to the ethidium bromide staining of the 28S ribosomal RNA, are shown in Fig. 2.

To test the direct effects of mechanical stress on the mRNA expression of S100A4, $\alpha$-tubulin, and $\beta$-actin, PDL cells were cultured under mechanical stress and Northern Blotting was performed. Hybridization with the S100A4 cDNA probe showed a high baseline expression level of the S100A4 mRNA by bovine PDL cells. When the cells were cultured under mechanical stress, however, the S100A4 mRNA expression level was upregulated (Fig. 3), and it was 2.5-fold higher after 24 h (Fig. 4). Similarly, the expression of $\alpha$-tubulin and $\beta$-actin mRNAs increased in the same manner (Fig. 3), reaching expression levels 2.8-fold ($\alpha$-tubulin) and 2.4-fold ($\beta$-actin) higher than the control level after 24 h of stretching (Fig. 4). Similar results were obtained from two sets of separate experiments. The

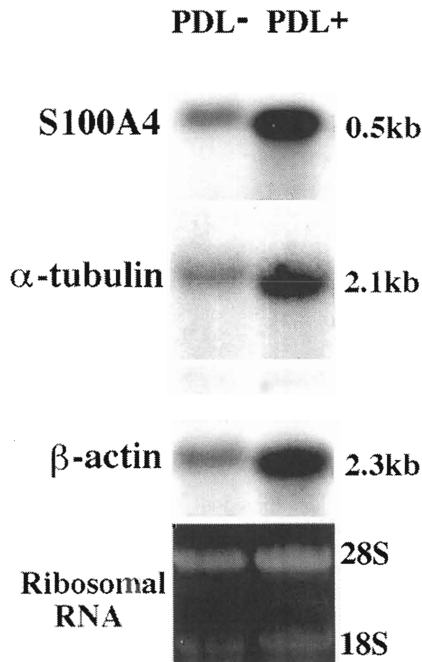


**Fig 1.** Northern blotting analysis of the expression levels of S100A4, $\alpha$-tubulin, and $\beta$-actin mRNAs in the PDL from fully and partially erupted teeth. Total RNA was extracted as described in Materials and Methods; samples containing 10 $\mu$g of total RNA were fractionated on 1.2% agarose gels, transferred to nylon membranes, and hybridized with cDNA probes for bovine S100A4, mouse $\alpha$-tubulin, and human $\beta$-actin. The result of the ethidium-bromide staining of the 28S / 18S ribosomal RNAs is shown in the lower panel. PDL-, PDL from partially erupted teeth; PDL+, PDL from fully erupted teeth.

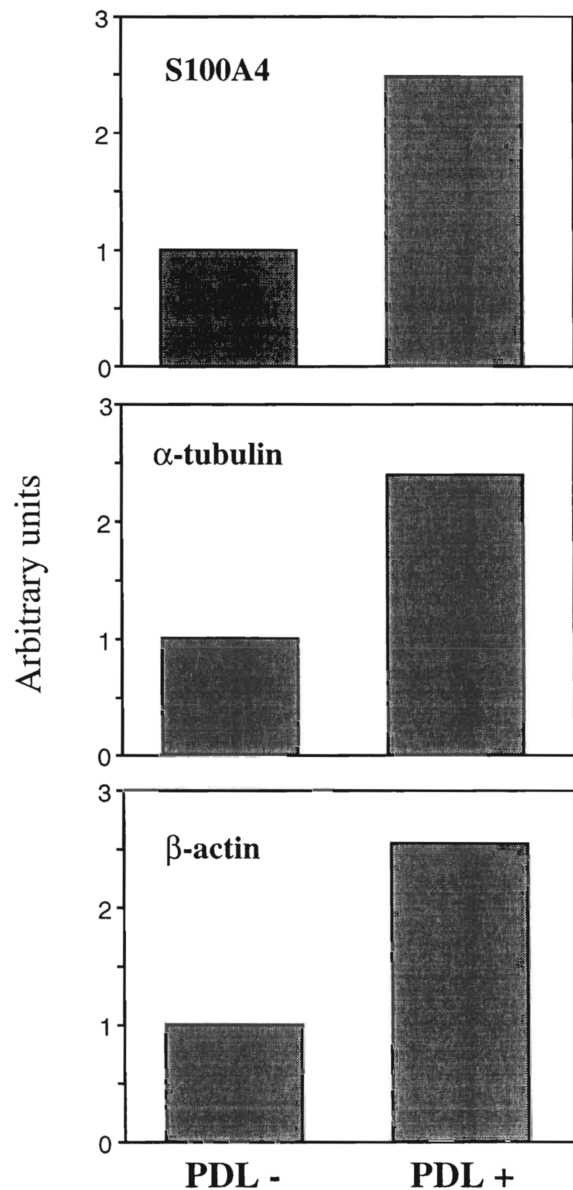


**Fig 2.** Relative values for signal intensity of S100A4, $\alpha$-tubulin, and $\beta$-actin mRNAs after normalization to the ethidium bromide staining of the 28S ribosomal RNA. PDL-, PDL from partially erupted teeth; PDL+, PDL from fully erupted teeth.

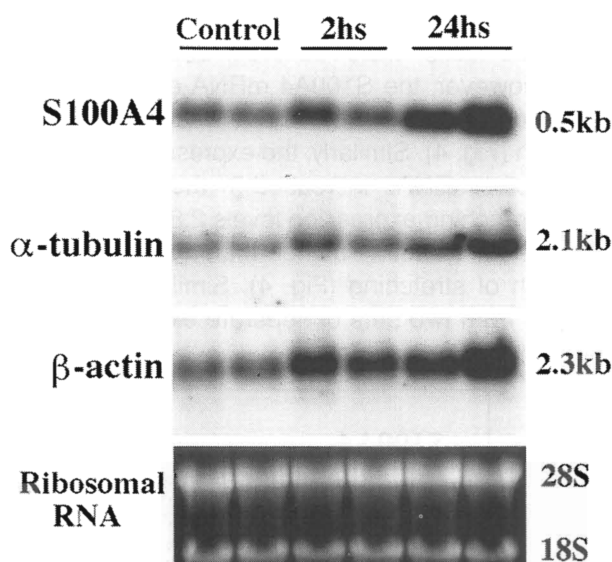


**Fig 3.** Northern blotting analysis of the expression levels of S100A4, α-tubulin, and β-actin mRNAs in PDL cells cultured under mechanical stress. PDL cells were cultured under mechanical stress as described in Materials and Methods for 2 and 24 h. Total RNA was as extracted; samples containing 10 μg of total RNA were fractionated on 1.2% agarose gels, transferred to nylon membranes, and hybridized with cDNA probes for bovine S100A4, mouse α-tubulin, and human β-actin. The results of the ethidium-bromide staining of the 28S / 18S ribosomal RNAs is shown in the lower panel.

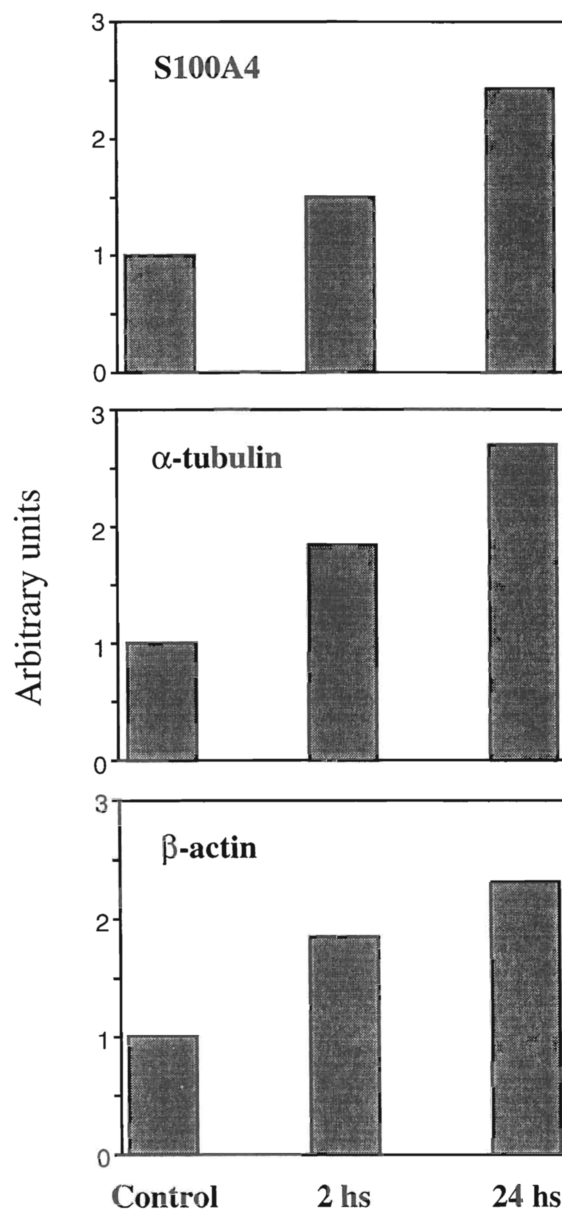


**Fig 4.** Mean relative values for signal intensity of S100A4, α-tubulin, and β-actin mRNAs after normalization to the ethidium bromide staining of the 28S ribosomal RNA.

mean relative values for the signal intensity of all mRNAs after normalization to the 28S ribosomal RNA stained with ethidium bromide are shown in Fig. 4.

## Discussion

In a previous study, we showed that the PDL from erupted and fully functional teeth expressed higher levels of S100A4 and β-actin mRNAs than did the PDL from teeth under eruption[11]. The results of the present study confirmed our previous findings and further demonstrated that the expression of α-tubulin mRNA is also higher in the PDL from fully functional teeth. This difference could reflect the response of PDL cells to the mechanical stress produced by mastication. Indeed, mechanical stimuli elicit several responses in the PDL which are controlled by PDL cells[16]. Therefore, the direct effects of mechanical stress on PDL cells were analyzed at the cellular level. Similarly to the results of the tissue study, PDL cells responded to mechanical stress *in vitro* by increasing the mRNA expression of S100A4 mRNA, β-actin, and α-tubulin.

The mRNA expression of β-actin has been frequently used as a control for Northern Blotting. Several studies have shown, however, that its expression is inconstant and depends on the stage of cell differentiation, stage of tissue development, and origin of the tissues[17,18,19,20]. In this study, we showed that the mRNA expression of β-actin increased in the same manner as that of S100A4 and α-tubulin. Taken together, these results indicate that mechanical stress upregulates the expression of all these mRNAs by PDL cells and that these molecules play certain roles in the

response of PDL cells to mechanical stress.

It has been shown that PDL cells increase their intracellular $Ca^{2+}$ concentration and exhibit changes in actin filament polymerization among the earliest responses to mechanical stress *in vitro*[8,9]. Interestingly, S100A4 binds to F-actin in the presence of $Ca^{2+}$ [21] and associates with other cytoskeletal proteins, such as non-muscle myosin[22] and non-muscle tropomyosin[23] in a $Ca^{2+}$-dependent manner. Furthermore, the interaction of S100A4 with these cytoskeletal components affects cytoskeletal organization and dynamics[21,24]. Therefore, as the expression of S100A4 increases after the application of mechanical stress, it is assumed that the increase in intracellular $Ca^{2+}$ concentration is coupled to the changes in cytoskeletal organization by S100A4.

Several reports have shown that PDL cells respond to mechanical stress by increasing their production of prostaglandin $E_2$[3] and interleukin 1-$\beta$[4], while decreasing the activity of alkaline phosphatase (ALP) with decreased expression of ALP mRNA[5]. Interestingly, the increased production of bone resorption stimulators (prostaglandin $E_2$ and interleukin 1-$\beta$) together with the decreased activity of a marker of bone formation (ALP) indicate that mechanical stress induces responses important for the functional maintenance of the PDL space. Our recent study indicates that S100A4 is an inhibitor of the mineralization process which is secreted by PDL cells[25]. Although it is not conclusive whether S100A4 secretion by PDL cells increases under mechanical stress, the increased expression of S100A4 mRNA could also be connected to the inhibitory effects of mechanical stress on the expression of osteoblastic phenotypic markers by PDL cells, which is essential for maintenance of the PDL space.

Since the PDL is constantly exposed to mechanical stress, it is conceivable that PDL cells have specialized mechanisms for adapting to such conditions. The results of the present study suggest that S100A4 is a component of this adaptive mechanism and that S100A4 is an important molecule for the physiological function of the PDL.

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