CONTINUING EDUCATION ARTICLE

Basal nitric oxide production is enhanced by hydraulic pressure in cultured human periodontal ligament fibroblasts

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To understand orthodontic tooth movement and determine optimal orthodontic force from a biological viewpoint, nitric oxide production in cultured human periodontal ligament fibroblasts was measured at varying levels of hydraulic pressure. The fibroblasts in a culture flask were exposed to the controlled change in hydraulic pressure, and intracellular nitric oxide levels were measured in real time by a nitric oxide-binding fluorescent dye, diaminofluorescein-2. The fibroblasts produced a significantly larger amount of nitric oxide at the pressure of 75 and 100 mmHg, compared with the pressure of 0, 25, and 50 mmHg ($P < .0001$, one-way ANOVA, and $P < .05$, Tukey-Kramer test). Immunohistochemically, the cultured fibroblasts expressed brain nitric oxide synthase. The pressure level to enhance nitric oxide production was comparable to the magnitude of clinically used orthodontic force (80 g/cm^2). Nitric oxide might be a key regulator in orthodontic tooth movement. (Am J Orthod Dentofacial Orthop 2000;117:474-8)

The periodontal ligament, located between the teeth and the alveolar bone of the maxilla and mandible, plays an important role in physiologic and pathologic interactions between the 2 tissues.[1-3] Periodontal ligament fibroblasts are a specific type of cells[4] and produce many kinds of mediators in response to mechanical and biological stimuli.[5-8] We previously demonstrated that human periodontal ligament fibroblasts showed a transient rise of intracellular calcium when hydraulic pressure reached 20 to 50 mmHg.[9] Periodontal ligament fibroblasts would, therefore, play a pivotal role in the initiation of orthodontic tooth movement. Understanding the threshold for the response by these cells would lead to the determination of an optimal magnitude of force, and hence, the development of a better method in orthodontic tooth movement.

Nitric oxide is a short-lived free radical and easily diffusible across biological membranes.[10,11] Nitric oxide is generated from L-arginine by nitric oxide synthases (NOSs) in an enzymatic reaction. Its roles are so diverse as an endothelium-derived relaxing factor, a neuromodulator, and an immunologic mediator in the cardiovascular, the nervous, and the immune systems, respectively. Nitric oxide is also a regulator in homeostasis of the bone, influencing the function of osteoblasts and osteoclasts.[12,13]

In this study, we examined whether periodontal ligament fibroblasts would produce nitric oxide in response to hydraulic pressure and which level of pressure would be the threshold for this response. Until now, nitric oxide production has been estimated by the measurement of its stable end products such as nitrite and nitrate.[14] The recent development of a nitric oxide-binding fluorescent dye allowed us to monitor intracellular nitric oxide levels in real time.[15-18]

MATERIAL AND METHODS

Isolation and Culture of Human Periodontal Ligament Fibroblasts

Human periodontal ligament fibroblasts were isolated and cultured by a previously described method, with some modifications.[9,19] Briefly, tissue fragments were scaled off from normal teeth extracted for the orthodontic purpose from 2 patients (a 14-year-old girl and a 15-year-old girl), washed several times with phosphate-buffered saline (PBS) solution containing 0.02% chlorhexidine gluconate, and placed in wells of a 24-well multidish (Nunc, Naperville, Ill). To avoid the growth of epithelial cells, tissue fragments were taken mainly from the lower shaft of the teeth. Cervical parts of the roots were also discarded to avoid including epithelial rest cells. The tissues were incubated in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 100 mg/L streptomycin, and 100 mg/L ampicillin, under a humidified atmosphere of 5% carbon dioxide and 95% air at 37° C. Cells grown out of

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the tissue fragments were treated with 0.25% trypsin and 1 mmol/L EDTA for several minutes and transferred to 6-cm petri dishes (Nunc). Morphologic characteristics of the cells, such as spindle-shaped cells and overlying growth on each other, appeared the same as previously described for human periodontal ligament fibroblasts.[19]

Application of Hydraulic Pressure and Measurement of Intracellular Nitric Oxide

The third-to-fourth passage of human periodontal ligament fibroblasts was used in the following experiment. The cells were transferred to polystyrene tissue culture flasks with an area of 25 mm^2 (Iwaki, Funabashi, Japan) and cultured for 3 to 4 days. The cells were loaded with 20 μmol/L of diaminofluorescein-2 diacetate (DAF-2 DA: Daiichi Pure Chemicals, Tokyo, Japan) for 30 minutes at 37°C in HEPES-buffered saline solution (10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 10 mmol/L glucose, pH 7.4).[9] For a control experiment, nitric oxide synthase inhibitor, N-methyl-L-arginine (L-NMMA, Sigma, St Louis, Mo) at a concentration of 100 μmol/L was administered at the time of dye loading. Cells were washed, and the flask was then filled with HEPES-buffered saline.

Hydraulic pressure was exerted on cells in a fluid-filled closed system (Fig 1). The flask was placed on the stage of an inverted microscope, attached to an argon laser (Laser-scanning Microspectrofluorometer ACAS570: Meridian Instruments, Okemos, Mich), and hydraulic pressure was exerted on the cells.[9] The pressure inside the flask was elevated by controlled movement of the piston of a 10-mL glass syringe, connected to the flask, by means of an automatic syringe injector (Syringe Infusion Pump, Model 980324, Harvard Apparatus, South Natick, Mass), and was monitored continuously by a pressure gauge (LifeKit, Disposable Pressure Monitoring Kit: Nihon Kohden, Tokyo, Japan) that was connected through an amplifier (RMP-6004M: Nihon Kohden) to a printer (Mini Recorder SJ-3462: Atto, Tokyo, Japan).

The changes in intracellular nitric oxide were observed by an excitation wavelength of argon laser at 488 nm and an emission filter of 530 nm as the changing intensity of fluorescence every 20 seconds for 400 seconds to obtain 20 images in total. Each culture flask was used only once for the fluorescence measurement at a certain level of hydraulic pressure to avoid the effect of the preceding pressure level. Hydraulic pressure in a flask was zero at the beginning of setup, and then was elevated to either 25, 50, 75, or 100 mmHg. Four flasks were used for each pressure level. Five cells were chosen in a series of images, and the levels of fluorescence

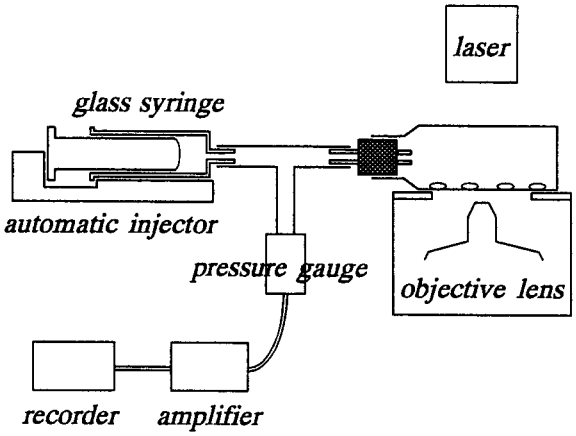

Fig 1. Schematic drawing shows fluid-filled closed system to exert hydraulic pressure on cultured cells.[9]

in each cell at each time point were quantified by integrating fluorescence with the cell area delineated by a polygon (a software attached to ACAS570). The highest level of fluorescence in one of 20 images was chosen for each cell and divided by the lowest level of fluorescence at the beginning to obtain a normalized value of fluorescence that was used for statistical analysis (20 cells for each pressure level). One-way analysis of variance (ANOVA) was to test the null hypothesis that means of fluorescence at 5 pressure levels were equal. Tukey-Kramer test for multiple comparisons was then used for differences at the alpha level of 0.05 (StatView 5.0, SAS Institute Inc, Cary, NC).

Immunohistochemistry

Cultured cells in a 6-cm petri dish were fixed for 30 minutes with 4% paraformaldehyde in PBS and incubated for 30 minutes with 0.3% hydrogen peroxide in methanol to inactivate endogenous peroxidase. Cells were incubated with normal goat serum for 20 minutes to block nonspecific binding, and then with anti-bNOS or anti-eNOS rabbit antibody (antinitric oxide synthase, brain or endothelial, IgG fraction, ×500 dilution, Sigma) for 30 minutes at room temperature. After being washed 3 times each for 5 minutes with PBS containing 0.05% Tween-20 (Bio-Rad Laboratories, Hercules, Calif), cells were incubated with biotinylated mouse antibody against rabbit IgG for 30 minutes, and washed again. Cells were incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (ABC: Vector Stain Elite ABC Kit, Vector Laboratories, Burlingame, Calif) for 30 minutes, and washed. The color was developed by incubation with 3,3'-diaminobenzidine (Sigma Fast DAB Peroxidase

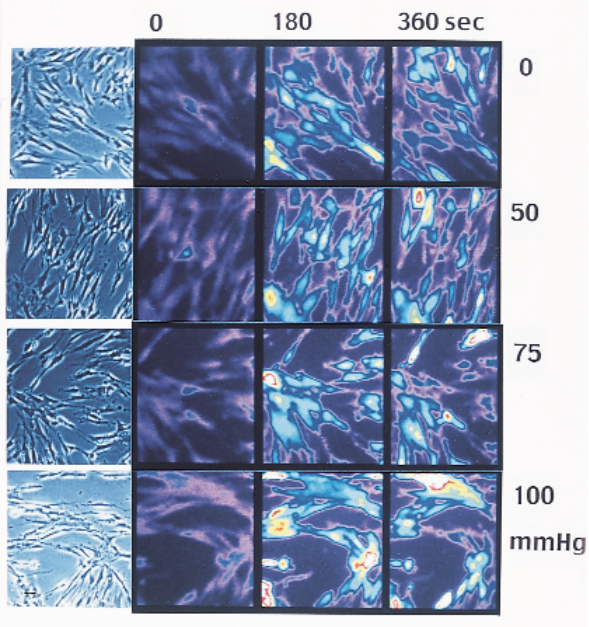

Fig 2. Spontaneous changes in intracellular nitric oxide levels of human periodontal ligament fibroblasts at hydraulic pressure of 0, 50, 75, and 100 mmHg (top to bottom). Nitric oxide levels are higher at the pressure of 75 and 100 mmHg, compared with 0 and 50 mmHg. Each frame is obtained at 0, 180, and 360 seconds (left to right). Changing intensity of fluorescence, indicative of nitric oxide levels, is shown in pseudocolor. Bar = 20 μm.

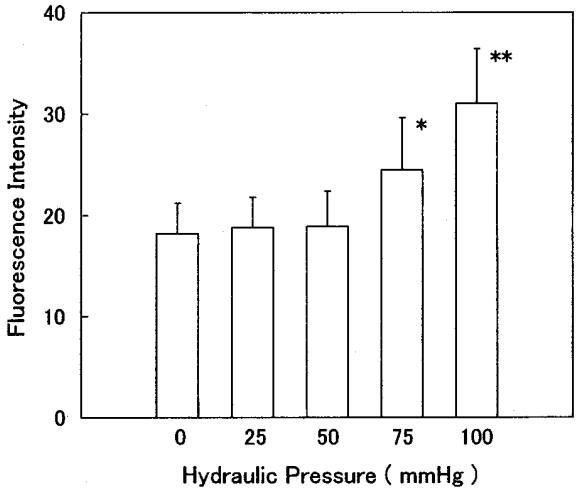

Fig 3. Peak levels of intracellular nitric oxide in human periodontal ligament fibroblasts at different levels of hydraulic pressure. Nitric oxide levels are significantly higher at the pressure of 75 and 100 mmHg, compared with those at the pressure of 0, 25, or 50 mmHg (P < .0001, one-way ANOVA; * and **, P < .05, Tukey-Kramer test, n = 20 for each level of hydraulic pressure). Fluorescence intensity is normalized by dividing a peak level by a lowest level at the beginning of observation. (T bars represent standard deviation.)

Substrate Tablet Set, Sigma) for 30 minutes at room temperature. Normal rabbit serum in place of the primary antibodies served as negative controls. Six dishes were used each for staining of either brain or endothelial nitric oxide synthase or for control.

RESULTS

During the observation period of 400 seconds, the basal level of intracellular nitric oxide at hydraulic pressure of 0 mmHg showed a spontaneous increase followed by a decrease with its peak at different time points varying from cell to cell (Fig 2). This fluctuation of nitric oxide was completely abolished by the addition of L-NMMA, inhibitor of nitric oxide synthase, indicating that the increase of nitric oxide was attributable to the enzymatic reaction.

The level of hydraulic pressure was then elevated to 25, 50, 75, and 100 mmHg. At the pressure of 25 and 50 mmHg, neither the latency to a peak nor peak levels of the spontaneous increase of nitric oxide showed any difference from those at the pressure of 0 mmHg. When hydraulic pressure was maintained at 75 and 100 mmHg, peak levels of the spontaneous increase of intracellular nitric oxide were significantly higher than those at 0, 25, and 50 mmHg (P < .0001, one-way ANOVA, and P < .05, multiple comparisons by Tukey-Kramer test, Fig 2 and Fig 3). The preceding incubation with L-NMMA resulted in no increase of nitric oxide levels at any pressure.

Immunohistochemically, human periodontal ligament fibroblasts in culture were positive for brain nitric oxide synthase, but negative for endothelial nitric oxide synthase (Fig 4).

DISCUSSION

In this in vitro study, we clearly demonstrated that basal production of nitric oxide in cultured periodontal ligament fibroblasts was enhanced at pressure levels of 75 and 100 mmHg, compared with levels of 0, 25, and 50 mmHg. There was no significant change in nitric oxide production between the pressure of 0 mmHg and 50 mmHg. These facts indicate that a threshold for a significant increase of nitric oxide production in cultured periodontal ligament fibroblasts lies between the pressure of 50 mmHg and 75 mmHg. A closed fluid-filled system used in this study consisted of a culture flask and tubes connecting a syringe and a pressure gauge. Hydraulic pressure inside this closed system

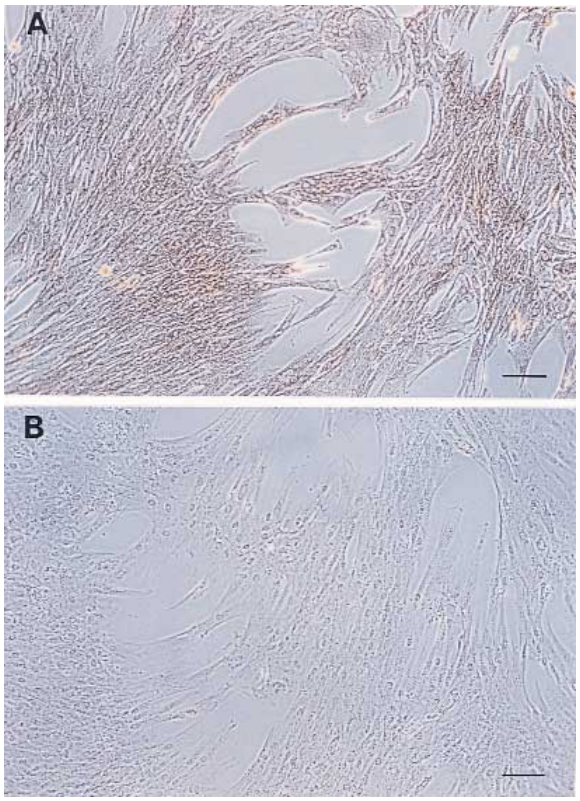

Fig 4. Immunohistochemistry of nitric oxide synthases on human periodontal ligament fibroblasts in culture. The fibroblasts are positive for brain nitric oxide synthase (**A**), while negative for endothelial nitric oxide synthase (**B**). Bar = 100 μm.

was directly monitored by the pressure gauge. The wall of a polystyrene flask could be stretched by fluid-filling in addition to the elevation of hydraulic pressure. Cultured cells in the flask would, therefore, receive a small magnitude of stretching in addition to compression by hydraulic pressure.

The range of 50 to 75 mmHg corresponds to 68 to 102 g/cm^2, as 1 mmHg is equal to 1.35951 g/cm^2. Currently, the magnitude of orthodontic force used clinically is about 80 g/cm^2, which happens to fall within the range of 50 to 75 mmHg as demonstrated in this in vitro study. The periodontal fibroblasts in a culture flask are different from an in vivo situation in the periodontal ligaments. For example, periodontal ligament fibroblasts in vivo are surrounded by extracellular matrix, such as collagens and proteoglycans, which provide matrix-cellular signals. There is a limitation to apply the in vitro results to an in vivo situation of the periodontal ligament.

The enhanced production of nitric oxide in response to flow-induced shear force, mechanical strain, stretching, or compression has been shown in other types of cells such as vascular endothelial cells,[20-29] bone cells (osteoblasts),[30-32] and chondrocytes.[33,34] Periodontal ligament fibroblasts can be added to the list of cells that produce nitric oxide in response to mechanical stimuli. It should be noted that the threshold for nitric oxide production was also determined in our experimental system, because the system could exert a controlled level of hydraulic pressure on cultured cells.

Spontaneous fluctuation of intracellular nitric oxide levels in each cell was observed even at the pressure of 0 mmHg in the present experiments. This increase of nitric oxide might be attributable to fluid shear force in a culture flask that was generated by continuous vibration of the stage of the microscope to scan images during the observation period.[25-27] Laser damage to cells as a possible cause for the nitric oxide increase could be denied by the fact that a spontaneous increase of intracellular calcium was not observed at the pressure of 0 mmHg in the same experimental system.[9]

A previous study demonstrated that the activity of NADPH diaphorase, indicative of nitric oxide synthase activity,[35,36] was found histochemically in the dental pulp, periodontal ligament, alveolar bone marrow crypts, and gingival epithelium.[37] Immunohistochemically, the subtype of nitric oxide synthases, expressed in cultured human periodontal ligament fibroblasts, was brain type (bNOS), but not endothelial type (eNOS). Nitric oxide produced in response to hydraulic pressure was thus derived from the activity of bNOS.

Brain and endothelial nitric oxide synthases are known to be activated by calcium/calmodulin.[10,11] In our previous study, human periodontal ligament fibroblasts were demonstrated to show a transient rise of intracellular calcium in the same experimental system when hydraulic pressure reached 20 to 50 mmHg.[9] The enhanced production of nitric oxide at higher levels of hydraulic pressure in human periodontal ligament fibroblasts might be attributed in part to activation of brain nitric oxide synthase by calcium/calmodulin. Nitric oxide then activates guanylyl cyclase in periodontal ligament fibroblasts, leading to an increased level of cyclic GMP.[10,11] Furthermore, prostaglandins are synthesized through direct activation of cyclooxygenase by nitric oxide.[38] Nitric oxide may also diffuse to the dental pulp and reach the vascular endothelial cells thereof, resulting in a change of the vascular tone.[39]

Nitric oxide may diffuse to the alveolar bone and influence the function of osteoclasts and osteoblasts. Nitric oxide has been shown to enhance osteoblastic differentiation[40] and inhibit osteoclastic activity.[41-44] Nitric oxide is also demonstrated to activate metalloproteinases.[45] These facts are not necessarily consis-

tent with the remodeling process of the alveolar bone on the compression side in orthodontic tooth movement. Nitric oxide might rather play a protective role for the teeth and alveolar bone during tooth movement. How nitric oxide production could be related to orthodontic tooth movement has to wait further studies.

CONCLUSION

Human periodontal ligament fibroblasts in culture produced nitric oxide in response to hydraulic pressure. Brain nitric oxide synthase was shown to be expressed in cultured periodontal ligament fibroblasts. The threshold of pressure for the enhanced production of nitric oxide in the fibroblasts, lying between 50 and 75 mmHg, is comparable to the magnitude of force used clinically in orthodontic tooth movement.[46,47] Understanding the role of nitric oxide in initiation of orthodontic tooth movement might lead to a better strategy of orthodontic tooth movement in the future.

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