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Aging stimulates cyclooxygenase-2 expression and prostaglandin E_2 production in human periodontal ligament cells after the application of compressive force

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Background and Objectives: Some clinical studies show that alveolar crestal bone loss is higher in adults than in young patients during orthodontic treatment, but the causes of such a phenomenon have not been elucidated. It is known that prostaglandin E_2 (PGE_2) is a proinflammatory agent and one of the potent osteoclast-inducing factors, and is produced by human periodontal ligament cells in response to orthodontic force. The aim of this study was to investigate age-related change in the biosynthetic capacity of PGE_2 and its regulatory gene, cyclooxygenase 2 (COX-2) from periodontal ligament cells in response to mechanical stress.

Methods: Compressive force of 2 g/cm^2 was applied for 3–48 h to periodontal ligament cells obtained from human donors aged 9–50 years, and COX-2 mRNA expression in and PGE_2 production by the periodontal ligament cells in response to the compressive force were examined.

Results: Application of a compressive force of 2 g/cm^2 for 3–48 h significantly stimulated these factors in both time- and age-dependent manners. Furthermore, these increases were dramatically larger in periodontal ligament cells obtained from donors over the age of 35.

Conclusions: Periodontal ligament cells obtained from old donors have significantly greater COX-2 expression and PGE_2 production in response to compressive force than those from younger donors. The turning point of aging, where significantly larger amounts of theses factors begin production, appears to be around the age of 35. These results may be positively related to the acceleration of alveolar crestal bone loss during orthodontic treatment in adult patients.

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It is widely documented that orthodontic tooth movement enhances the loss of alveolar crestal bone height and this is greater in adults than in young patients (1). However, the causes of this phenomenon have not been elucidated. To determine these causes, it is important to investigate the effects of aging on the responses of periodontal ligament cells subjected to mechanical stress.

Prostaglandin E_2 (PGE_2) is a proinflammatory agent and one of the potent osteoclast-inducing factors (2–4) synthesized from arachidonic acid by cyclooxygenase 2 (COX-2). It is also reported that PGE_2 is produced in periodontal ligament cells in response to mechanical stress *in vivo* (5) and *in vitro* (6,7), and prostaglandins produced by periodontal ligament cells in response to mechanical stress show bone resorptive activity (8).

Some studies show that the increase in PGE_2 production in periodontal ligament cells subjected to mechanical stress from old rats (aged 60 weeks) was greater than that in cells from young rats (aged 6 weeks) (9). These studies only compared the response in cells of two different ages, and did not examine progressive changes occurring during the aging process. Furthermore, there have been no studies using human periodontal ligament cells that show age-associated changes in PGE_2 production following mechanical stress. In the present study, we investigated the effects of aging on PGE_2 production and mRNA expression for its regulatory enzyme, COX-2, in periodontal ligament cells in response to compressive force.

Materials and methods

Cell culture

Human periodontal ligament cells were prepared in accordance with the method of Somerman *et al.* (10), with minor modifications. The protocol for this experiment was reviewed and approved by the Nihon University Department of Dentistry Ethics Committee. Premolars extracted from 15 healthy patients aged 9–50 years in the course of orthodontic treatment were washed twice with 0.01 mol/l phosphate-buffered saline; tissue attached to the middle third of the root was then removed with a surgical scalpel. The coronal and apical portions of the root were not used, to avoid contamination with cells from other tissues. The tissue was minced, placed in 35-mm tissue culture dishes (Falcon, Lincoln Park, NJ, USA), and covered with sterilized glass cover slips. The medium used was α-minimal essential Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 100 U/ml penicillin-G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B (Gibco), and 10% fetal calf serum (Biofluids Division, Biosource International Rockilee, MD, USA). The cultures were maintained at 37°C in a humidified incubator containing 95% air and 5% CO_2. When the cells that grew out from the explants had reached confluence, they were detached with 0.05% trypsin (Gibco) in phosphate-buffered saline for 10 min, and subcultured in culture flasks (Falcon). Some cells still attached to the bottom of the flasks were discarded during serial passage to avoid contamination by epithelial cells, which are less easily detached than fibroblasts (11). Cells observed at confluence by phase-contrast microscopy had none of the small mats typical of epithelial cells. Cells that had been passaged five or six times were used for this experiment.

Application of compressive force

Periodontal ligament cells were plated onto 10-cm cell culture dishes at a density of 4.5×10^3 cells/cm^2. When the cells were subconfluent, the medium was changed to that described above, except that it contained 2%, instead of 10%, fetal calf serum. After overnight incubation, the cells were compressed continuously using the uniform compression method (7). Briefly, the confluent cell layer in the dish was covered by a thin glass plate. Cells were subjected to a 2 g/cm^2 compressive force for 3, 6, 12, 24, or 48 h according to the method of Kanzaki *et al.* (7). Cells with no compression were used as a control.

Reverse transcription–polymerase chain reaction (RT-PCR)

After the application of compressive force, total RNA was extracted from the cells using TRIZOL reagent (Gibco) and stored at −80°C. RNA (2 μg) was reverse-transcribed into cDNA, using Ready-To-Go RT-PCR Beads (Amersham Bioscience, Buckinghamshire, UK) according to the manufacturer's instructions. Aliquots of 2 μl cDNA were subjected to real-time PCR in a 25-μl solution containing 1× R-PR buffer, 1.5 mM dNTP mixture, 1× SYBR Green 1 dye, 15 mM $MgCl_2$, 0.25 U Ex-*Taq* polymerase real-time PCR version (Takara, Tokyo, Japan), and 20 mM specific primers. The primer pairs were as follows: COX-2, 5′-GGAACACAACAGAGTATGCG-3′ and 5′-AAGGGGATGGCCAGTGTATAGA-3′; glyceraldehyde-3-phoshate dehydrogenase (GAPDH), 5′-CAATGACCCCTTCATTGACC-3′ and 5′-GACAGCTTCCCGTTCTCAG-3′. These primers were designed using PRIMER 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Real-time PCR was carried out in a thermal cycler (Smart Cycler, Cepheid, Sunnyvale, CA, USA), and the data were analyzed using SMART CYCLER software (ver.1.2d). The PCR conditions were 95°C for 3 s and 68°C for 20 s for 40 cycles, and measurements were taken at the end of the annealing step at 68°C in each cycle. The level of mRNA expression was calculated and normalized to the level of GAPDH mRNA at each time-point.

Assay for PGE_2 production

The amount of PGE_2 released into the culture medium was measured by enzyme-linked immunosorbent assay using a commercially available kit (DE0100; R & D systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. The cross-reactivities of the antibody at 50% binding of maximum binding have been determined as $PGE_1 = 70\%$, $PGE_3 = 16.3\%$, $PGF_{1\alpha} = 1.4\%$, $PGF_{2\alpha} = 0.7\%$, 6-keto-$PGF_{1\alpha} = 0.6\%$, $PGA_2 = 0.1\%$, and $PGB_1 = 0.1\%$. The cross-reactivity with

13,14-dihydro-15-keto-$PGF_{2\alpha}$, 6,15-keto-13,14-dihydro-$PGF_{1\alpha}$, thromboxane B_2, and anandamide was less than 0.1%. The intra- and interassay precisions of variation for the PGE_2 assay were 8.9 and 3.0, respectively.

Immunohistochemistry for COX-2

Cells compressed for 48 h, as well as non-compressed control cells, were rinsed with phosphate-buffered saline, fixed with 2% paraformaldehyde for 10 min, and washed twice in phosphate-buffered saline. After incubation with 10% normal goat serum for 30 min at room temperature to block any non-specific reaction, the cells were incubated with the primary antibody, rabbit anti-human COX-2 (Cayman Chemical, Ann Arbor, MI, USA), at a 1000 : 1 dilution, for 15 min at room temperature. Staining was performed with avitin-biotinylated anti-rabbit immunoglobulin G as secondary antibody, conjugated with horseradish peroxidase using the catalyzed signal amplification system (DAKO Cytomation, Carpinteria, CA, USA). The number of COX-2-positive cells was counted in randomly selected areas containing 100 cells. Cell counts were determined for five different specimens. The count was expressed as number of COX-2-positive cells / total number of cells × 100.

Statistical methods

Values were calculated as the mean ± SD. Data were subjected to one-way and two-way analyses of variance (ANOVA) as indicated below. Student's *t*-test was used for analysis of the difference between control and compression groups.

Results

The time–course of the effects of compressive force on COX-2 mRNA expression in periodontal ligament cells from individual donors is shown in Fig. 1(A–C). In most periodontal ligament cells examined, a statistically significant increase in COX-2 mRNA expression was observed 12–48 h after application of the compressive force. COX-2 mRNA expression in response to compressive force was significantly

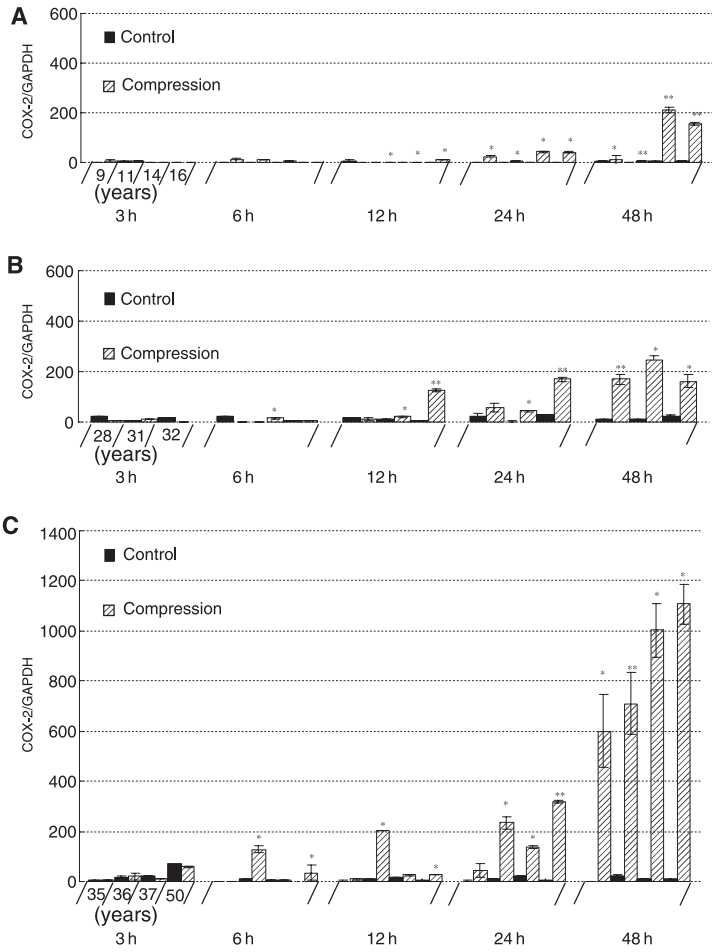

Fig. 1. Time–course of the effects of compressive force and aging on COX-2 mRNA expressions in human periodontal ligament cells. (A) 9–16 years old; (B) 28–32 years old; (C) 35–50 years old. Values are mean ± SD of three separate measurements in 11 individual cells. COX-2 expression was increased in a time- and age-dependent manner ($P < 0.001$, two-way ANOVA). Increases were significantly different from corresponding control cells (*$P < 0.01$, **$P < 0.001$).

increased in a time-dependent and age-dependent manner, as shown by two-way ANOVA ($P < 0.001$). Because COX-2 expression was highest 48 h after the application of compressive force, these values of 15 individual donors were extracted and are shown in Fig. 2. COX-2 expression increased in an age-dependent manner (one-way ANOVA, $P < 0.001$) but its increase was not constant. The increase was fivefold in cells from 9- to 11-year-olds, 15-fold in those from 14- to 31-year-olds, dramatically increased in the cells from 36-year-olds, and was further increased to 100-fold in cells from 50-year-olds.

PGE_2 production at 48 h after application of the compressive force was analyzed in periodontal ligament cells from different-aged subjects. A significant increase in PGE_2 production in response to compressive force was found in all cells from donors except the 11-year-old. This increase was also age-dependent by one-way ANOVA ($P < 0.001$; Fig. 3).

Immunohistochemistry showed that cultured cells from both young donors (14 years; young cells) and old donors (50 years; old cells) were densely packed and had spindle-like or polygonal morphology. There was no significant difference in size and shape between young and old cells. The COX-2 protein level was similar in both young and old cells. However, compressive force increased COX-2 protein, which stained brown in the

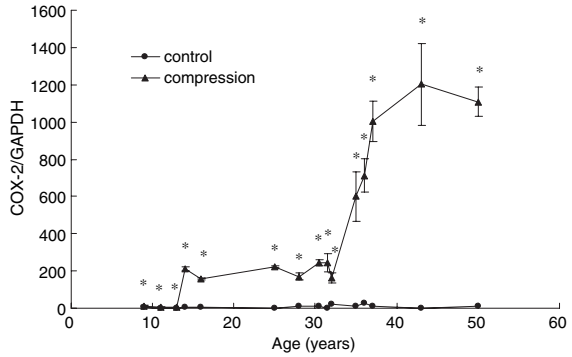

Fig. 2. COX-2 mRNA expression at 48 h after application of compressive force. COX-2 expression was increased in an age-dependent manner ($P < 0.001$, one-way ANOVA), and increases were significantly different from corresponding control cells ($P < 0.01$). Values are mean ± SD of three separate measurements in 15 individual cells.

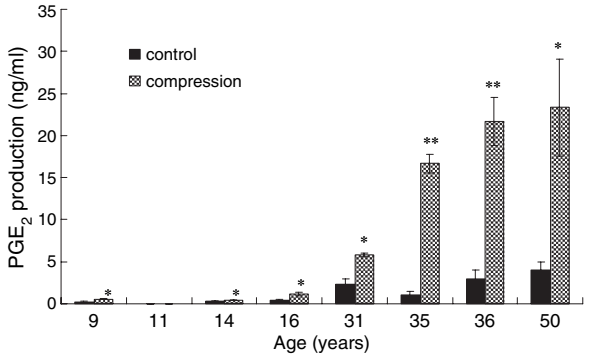

Fig. 3. Effects of compressive force and aging on PGE_2 production after 48 h. PGE_2 production was increased in an age-dependent manner ($P < 0.001$, one-way ANOVA). Increases were significantly different from corresponding controls ($^*P < 0.05$, $^{**}P < 0.01$). Values are mean ± SD of three separate measurements in eight individual cells.

cytoplasm surrounding the unstained nucleus. Staining intensity and the number of stained cells were much higher in old cells than in young cells following application of compressive force, but the staining intensity varied from cell to cell in both types of cell (Fig. 4A). The number of COX-2-positive cells was significantly increased from 3 to 19% in young cells and from 5 to 41% in old cells 48 h after application of compressive force ($P < 0.05$). There was no significant difference between young and old control cells, but compressive force significantly increased the number of COX-2-positive old cells compared with young cells ($P < 0.05$; Fig. 4B).

Discussion

We investigated the effect of aging on the initial pattern of COX-2 gene expression in human periodontal ligament cells after application of a compressive force. COX-2 expression and PGE_2 production were both significantly increased in response to this compressive force in an age-dependent manner.

In this study, we used a compressive force of 2 g/cm^2, according to previous studies (7,12,13). Kanzaki *et al.* (7) showed that periodontal ligament cells induce factors such as PGE_2, receptor activator of nuclear factor-κB ligand and osteoprotegerin in response to 2 g/cm^2 of compressive force. These previous studies suggested that cells were only stimulated once in each experiment, and static compressive force is thought to mimic the *in vivo* system applied in orthodontic treatment. Therefore, we used this system to simplify the experimental model.

PGE_2 is one of the potent osteoclast-inducing factors (2–4) and a proinflammatory agent that can cause pain or hyperplasia (14). It is synthesized from arachidonic acid by COX, which is a prostaglandin endoperoxidase (15). There are two isozymes, COX-1 and COX-2. COX-2 is induced in the response to growth factors, various proinflammatory agents such as cytokines, mitogens and lipopolysaccharides (16,17), and mechanical stress (6). The early phase of tooth movement involves an acute inflammatory response related to PGE_2 produced by periodontal ligament cells in response to mechanical stress (6). PGE_2 has also been shown to be a potent bone-resorptive factor during tooth movement (5). Therefore, PGE_2 produced by periodontal ligament cells may be highly associated with the resorption of alveolar crestal bone. Many orthodontists may have experienced that gingival recession following alveolar crestal bone resorption during orthodontic treatment was higher in adults than in young patients. Some studies have evaluated alveolar bone loss in adult orthodontic patients (1,18,19). Nelson (18) reported that multiple linear regression analysis revealed a positive relationship between age and alveolar crestal bone loss, and a negative relationship between initial bone level and subsequent bone loss in adult orthodontic patients. However the etiology of alveolar bone loss in adult orthodontic patients has not been yet clarified. Our previous study (9) showed that PGE_2 production was enhanced by mechanical stress in periodontal ligament cells from young (aged 6 weeks) and old (aged 60 weeks) rats, and the production of PGE_2 in the cells from old rats was significantly higher (2.2-fold) than in those from young rats.

We also reported using an *in vitro* aging system (different population doubling levels), described by Hayflick (20), which showed that mechanical tension stimulates IL-1β and plasminogen activator production in human periodontal ligament cells. Stimulation was significantly higher in old cells (18–20 passages) compared with young cells (5–6 passages) (21,22). IL-1β is a potent stimulator of PGE_2 production in periodontal ligament cells. Although these studies support our present results, they examined only two differ-

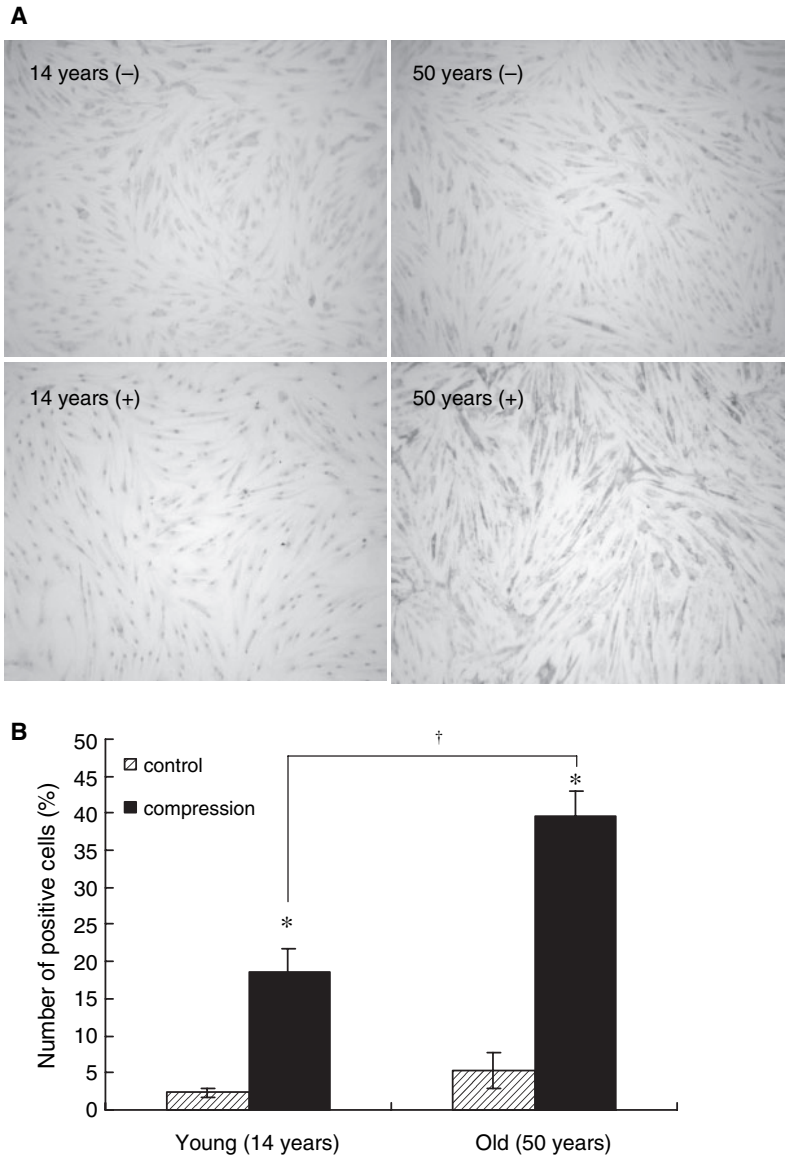

Fig. 4. (A) Immunohistochemistry of COX-2 protein expression in human periodontal ligament cells from young (14 years) and old (50 years) donors with (+) or without (–) application of compressive force for 48 h. Cells expressing COX-2 were few in both young and old control (–) cells. After the application of compressive force for 48 h, staining intensity and number of stained cells increased in both cell ages, but the increase was much higher in the old cells. Staining intensity varied among cells in both young and old cells. (B) Number of COX-2-positive stained cells. *Significantly different from corresponding control cells ($P < 0.05$). †Significantly different from corresponding young cells ($P < 0.05$).

ent ages of young and old cells, and did not study the consecutive changes induced by aging of periodontal ligament cells. Also, it is possible that artifacts are mixed during *in vitro* aging.

Furthermore, it is clinically important to establish the turning point of acute changes during aging. In our present study, which examined periodontal ligament cells from donors of several different ages, we found not only age-related increases in COX-2 and PGE_2 expression in human periodontal ligament cells in response to compressive force, but also the turning point of aging at which the production of significantly large amounts of bone resorptive factors begins. COX-2 gene expression and PGE_2 production at 48 h after the application of compressive force were rapidly increased in cells from donors over the age of 35. These increases were clearly different from those in younger cells. The results are consistent with those of Streckfus *et al.* (23). There was a significant multiple linear regression model relationship between oral bone loss and aging, the amount of bone lost rapidly increased from 30 to 50 years of age. Moreover, oral hygiene was a factor but contributed only slightly to the overall model. It is therefore most likely that the sensitivity of periodontal ligament cells to mechanical stress, such as occlusal and/or orthodontic force, increases in an age-dependent manner. Periodontal ligament cells from older patients, especially those over the age of 35, produced significantly higher amounts of bone resorptive factors, and consequently, resorption of alveolar crestal bone may occur in these older patients more readily than in young patients.

In the present study, the production of bone resorptive factors was similarly small in both young and old cells in the absence of stimulation. These phenomena are supported by the results of Abdellatif and Burt (24), who found that oral hygiene is of greater relative importance than age as a determinant of periodontitis. In other words, it may be difficult for alveolar crestal bone resorption to take place if occlusal and/or orthodontic forces are not applied to the teeth. Therefore, it may be necessary to avoid exposure to excessive force in older patients to maintain healthy periodontal tissues. These results have led us to ensure the use of light force, less tooth movement, and shorter treatment periods, so as not to induce resorption of alveolar crestal bone during orthodontic treatment.

Some studies have reported periodontal ligaments composed of heterogeneous cell populations (25,26) with cells at different stages of differentiation and displaying different phenotypes, such as osteoblasts, cementoblasts, or fibroblasts. In this immunohistochemical study, old periodontal ligament cells expressed COX-2

protein more strongly than young cells, with a significant increase in the number of stained cells in response to compressive force. This suggests that the sensitivity of periodontal ligament cells to compressive force clearly increased, and the number of periodontal ligament cells responsive to the force may also have increased.

The reason why old cells express higher levels of COX-2 and PGE_2 than young cells is still unclear. Although phospholipase A_2 is a key enzyme in eicosanoid production, mammalian cells contain structurally diverse forms of phospholipase A_2. Since cytosolic phospholipase $A_{2\alpha}$ from these isoforms plays a key role in PGE production by osteoblasts and in osteoclastic bone resorption in response to IL-1 (27), a study into the effects of ageing and compressive force in periodontal ligament cells on cytosolic phospholipase $A_{2\alpha}$ expression may provide a solution to this question.

In conclusion, this study demonstrates that periodontal ligament cells obtained from older donors have significantly greater COX-2 gene expression and PGE_2 production in response to compressive force than those obtained from younger donors. The turning point of aging, where significantly larger amounts of these factors begin production, appears to be around the age of 35. These factors have potent bone resorptive activity; therefore, alveolar crestal bone loss during orthodontic treatment in adult patients may be related to the high production of these factors in periodontal ligament cells that have increased their sensitivity to mechanical forces during aging.

Acknowledgements

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