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Cervical sympathectomy causes alveolar bone loss in an experimental rat model

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Background and Objective: Periodontal disease, a pathological destructive inflammatory condition, is characterized by alveolar bone loss. Recent studies have suggested a correlation between the sympathetic nervous system and bone remodeling. To confirm the importance of the sympathetic nervous system in bone resorption, we investigated the effects of superior cervical ganglionectomy and oral challenge with *Porphyromonas gingivalis* on alveolar bone loss in rats.

Material and Methods: Rats were divided into three groups: group A underwent a sham operation as the control group; group B underwent superior cervical ganglionectomy; and group C underwent a sham operation and oral challenge with *P. gingivalis*. Horizontal alveolar bone loss was evaluated by measuring the distance between the cemento-enamel junction and the alveolar bone crest. Cytokine gene expression in the gingival tissues was assessed using reverse transcription–polymerase chain reaction analyses. The furcation areas of the mandibular molars were examined histologically.

Results: Both superior cervical ganglionectomy and oral challenge with *P. gingivalis* resulted in accelerated alveolar bone loss. Gingival tissues in the superior cervical ganglionectomy group showed increased expression of the cytokines interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6. The density of neuropeptide Y-immunoreactive fibers was decreased following superior cervical ganglionectomy. Osteoclasts were observed in the superior cervical ganglionectomy and *P. gingivalis*-challenged groups.

Conclusion: Both superior cervical ganglionectomy and oral challenge with *P. gingivalis* induced alveolar bone loss. These results provide new information on the occurrence of alveolar bone loss, in that both oral challenge with *P. gingivalis* and superior cervical ganglionectomy are important accelerating factors for alveolar bone loss. Thus, we suggest that the sympathetic nervous system is linked with the prevention of alveolar bone loss.

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Periodontal disease is a pathological destructive inflammatory condition that affects the supporting structures of teeth; it is induced by oral microorganisms which colonize the tooth surfaces and are in close contact with the gingival margin (1,2). Periodontal disease is characterized by loss of the tooth-supporting structures, connective tissue attachment and alveolar bone (3). *Porphyromonas gingivalis*, a black-pigmented gram-negative anaerobe, has been recognized as an important causative organism in adult periodontitis (4). *P. gingivalis* possesses a variety of virulence factors, including fimbriae, lectin-like adhesins, capsular polysaccharide and lipopolysaccharide, as well as numerous proteolytic enzymes. The contribution of *P. gingivalis* to alveolar bone loss

seems to be supported by the stimulation of osteoclasts, which induces bone destruction and inhibits bone formation. Psychological factors have also been suspected to increase the risk of periodontitis. The stress response is a mediating mechanism between unfavorable psychological conditions and inflammatory periodontal disease. The term 'stress' defines the psychophysiological reactions of the body to a variety of emotional or physical stimuli that threaten homeostasis. We have previously reported that the presence of restraint stress significantly enhances the progression of *P. gingivalis*-challenged periodontitis (5). Stress is one of the subject-based changing factors that were found to have an effect on the immune response and susceptibility to infection. The effect of stress on infectious diseases is thought to be mediated by products of the nervous and the neuroendocrine systems that are released during times of stress and modulate the function of neutrophils, lymphocytes and macrophages, thereby affecting the outcome of infectious diseases (6). The release of glucocorticoids and catecholamines, crucial integral hormonal mediators of the body's response to stress mounted by the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system, respectively, are speculated to play a role in the ability of stress to promote disease (7). Several studies have demonstrated that sympathetic innervation modulates bone resorption and bone cell activity. Osteoblastic activity and bone matrix formation were significantly reduced after pharmacologic and surgical sympathectomies (8). Studies on the effect of surgical sympathectomy on bone remodeling of the rat mandible found a decrease in periosteal and endosteal apposition and in the rate of mineralization, along with an increase in the number of osteoclasts and bone-resorption surfaces (9,10). In other studies, superior cervical ganglionectomy in rats resulted in a decrease in bone mineral content and in the density of the mandible (11,12). The precise mechanisms of bone remodeling and the progression of periodontitis following superior cervical ganglionectomy are still largely unknown. Recently, there is growing evidence that sympathetic nerves can directly affect cytokine production. Periapical lesions following superior cervical ganglionectomy contained significantly more interleukin-1alfa than similar lesions on the contralateral nonsuperior cervical ganglionectomy side (13). Chemical sympathetic denervation enhances the synthesis of interleukin-1beta and interleukin-6, suggesting a tonic inhibitory control of the sympathetic nerves on these inflammatory cytokines (14). Interleukin-1alfa and tumor necrosis factor-alfa are pro-inflammatory cytokines with osteoclastic activity linked to the progression of inflammatory diseases with bone destruction. Many studies of *P. gingivalis*-challenged periodontitis have been reported. However, the effects on alveolar bone loss of the sympathetic nervous system have not been investigated. Therefore, we investigated the effects of superior cervical ganglionectomy and oral challenge with *P. gingivalis* on alveolar bone loss in an experimental rat model in order to emphasize the effectiveness of the sympathetic nervous system.

Material and methods

Animal study

A total of 18, 3-wk-old male Sprague-Dawley rats were used in the experiments; they were obtained from a commercial farm (Nihon SLC, Shizuoka, Japan). The rats were housed in cages throughout the experimental period to facilitate successful isolation. They were fed a standardized diet of hard briquettes and water, and were maintained under a 12-h light/dark cycle (lights on from 08:00 to 20:00 h) at a temperature of 22°C and relative humidity of 50%. The rats were given sulfamethoxazole (1 mg/mL) and trimethoprim (200 μg/mL) in their drinking water, which was available *ad libitum*, for 4 d to reduce the original oral flora; this was followed by a 4-d antibiotic-free period before surgical sympathectomy and oral challenge with *P. gingivalis*. Each cage contained six rats that belonged to the same group. The rats were divided into the following three groups (Fig. 1): group A underwent a sham operation (control group); group B underwent superior cervical ganglionectomy; and group C underwent a sham operation and oral challenge with *P. gingivalis* (*P. gingivalis*-challenged group). All rats recovered without signs of distress during the experimental period. At the end of the experimental period (30 d), all rats were killed (by decapitation) under anesthesia, which was induced by an intramuscular injection of Nembutal®(50 mg/mL of pentobarbital sodium, at 0.5 mL/kg body weight; Dainippon Sumitomo Pharma, Osaka, Japan). The rats were killed in the morning, between 08:00 and 10:00 h. The experimental procedures of this study were reviewed and approved by the Committee of Ethics on Animal

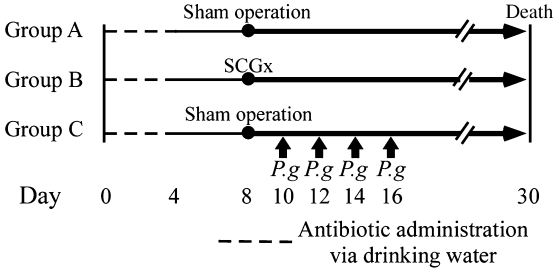

Fig. 1. Experimental procedure. Rats were divided into three experimental groups ($n = 6$). Group A underwent a sham operation (control group); group B underwent superior cervical ganglionectomy (superior cervical ganglionectomy group); and group C underwent a sham operation and oral challenge with *Porphyromonas gingivalis* (*P. gingivalis*-challenged group). Superior cervical ganglionectomy and the sham operation were performed on day 8. Rats were orally challenged with *P. gingivalis* ATCC 33277 by oral gavage on days 10, 12, 14 and 16. On day 30, all rats were killed. *P.g*, *Porphyromonas gingivalis*; SCGx, superior cervical ganglionectomy.

Experiments of Kanagawa Dental College and were performed under the guidelines for animal experimentation of Kanagawa Dental College.

Superior cervical ganglionectomy

Surgical sympathectomy was performed by right superior cervical ganglionectomy on day 8 of the study. The rats were anesthetized by an intraperitoneal injection of Nembutal® (50 mg/mL of pentobarbital sodium, at 0.5 mL/kg body weight). A ventral midline incision was made aseptically in the neck; the right sternocleidomastoid muscle was exposed and deflected; and the right carotid artery was displaced laterally to expose the superior cervical ganglion. The right superior cervical ganglion was excised by transecting the cervical preganglionic sympathetic nerve and the internal and external carotid nerves. The wound was closed with a 4-0 silk suture (15–17). In the sham operation, the rats were anesthetized and incised, and the superior cervical ganglion was localized in the same way as it was localized for superior cervical ganglionectomy. However, the right superior cervical ganglion was not excised.

Oral challenge with *P. gingivalis*

The bacterial strain *P. gingivalis* ATCC 33277 was used successfully in this study to induce experimental alveolar bone loss in rats. *P. gingivalis* cells were grown at 37°C for 18 h in a brain–heart infusion broth (Difco, Detroit, MI, USA) supplemented with 5 mg/mL of yeast extract, 5 μg/mL of hemin and 0.2 μg/mL of vitamin K_1, in an anaerobic chamber with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. Rats were orally challenged with *P. gingivalis* ATCC 33277, which was suspended in 5% carboxymethylcellulose, and each rat received 0.5 ml (5×10^9 cells/mL) of the suspension by oral gavage on days 10, 12, 14 and 16 of the study. The recovery of orally challenged *P. gingivalis* was assessed using the polymerase chain reaction (PCR) method. *P. gingivalis* was recovered from rats in the *P. gingivalis*-challenged group at the end of the experimental period.

Body, thymus and spleen weights

The body weight of the rats was recorded in the morning, every 2 d throughout the experimental period. After killing the rats, the thymus and spleen were removed, washed in saline and weighed.

Measurement of alveolar bone loss in rats

The right and left sides of the upper jaws were used as dry specimens for measuring horizontal alveolar bone loss. The upper jaws were defleshed after 10 min in an autoclave at 15 pounds/inch^2 (p.s.i.), and were then immersed in 3% hydrogen peroxide, rinsed and air dried. Horizontal alveolar bone loss from around the maxillary molars was evaluated morphometrically. The distance between the cemento-enamel junction and the alveolar bone crest was measured at seven palatal sites in each rat (4,5). Measurements were made under a dissecting microscope (×40) fitted with a digital high-definition system (Digital HD microscope VH-7000; Keyence, Osaka, Japan) that was standardized to provide measurements in millimeters.

Reverse transcription–PCR analysis of cytokines

Gingival tissues were gently dissected from the right and left sides of the upper jaws in order to conduct reverse transcription-PCR (RT-PCR) analysis of the cytokines present in these tissues. Dissected gingival tissues were minced and extracted, and the extracts were purified and concentrated. Total RNA was isolated from the gingival tissues using an RNeasy® Mini Kit, according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). cDNA was reverse transcribed and amplified using the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen Corporation, San Diego, CA, USA). The following primer pairs (including the PCR product size in parentheses) were synthesized by Invitrogen (18–20): interleukin-1alfa, 5′-CTA AGA ACT ACT TCA CAT CCG CAG C-3′ and 5′-CTG GAA TAA AAC CCA CTG AGG TAG G-3′ (623 bp); tumor necrosis factor-alfa, 5′-CAC GCT CTT CTG TCT ACT GA-3′ and 5′-GGA CTC CGT GAT GTC TAA GT-3′ (616 bp); interleukin-6, 5′-CAA GAG ACT TCC AGC CAG TTG C-3′ and 5′-GGA GAA AGC TTC CCA ACT TTT G-3′ (335 bp); and beta-actin, 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′ and 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′ (759 bp). Cycling conditions for denaturation, annealing and extension were as follows. Interleukin-1alfa: 35 cycles at 94°C for 60 s, 58°C for 45 s and 72°C for 45 s; tumor necrosis factor-alfa: 35 cycles at 94°C for 60 s, 58°C for 45 s and 72°C for 45 s; interleukin-6: 45 cycles at 94°C for 60 s, 58°C for 45 s and 72°C for 45 s; and beta-actin: 35 cycles at 94°C for 60 s, 58°C for 45 s and 72°C for 45 s. PCR was performed using an iCycler™ (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. An image analyzer (AE-6905 H Image Saver HR; Atto, Tokyo, Japan) was used to detect signal intensity. Band sizes were confirmed with reference to molecular size markers (One STEP Ladder 50®; Nippon Gene, Tokyo, Japan). Values for each cytokine mRNA were normalized against the amount of beta-actin mRNA, which was used as a housekeeping gene for each experimental condition.

Histological findings

Tissue blocks containing all three mandibular molars, alveolar bone and surrounding soft tissues were dissected from the right side of the lower jaws separately and used as histological specimens. All tissue blocks were fixed in 10% buffered formaldehyde (pH 7.4) overnight, rinsed in 0.1 M phosphate buffer, decalcified in 10% EDTA for approximately 6 wk, rinsed again in 0.1 M phosphate buffer, soaked in 30% sucrose overnight and stored frozen at −80°C. The tissue blocks were randomly divided for immunohistochemistry and hematoxylin and eosin staining. Tissue blocks for immunohistochemistry were embedded

in Tissue-Tek OCT compound (Sakura, Zoeterwoude, the Netherlands), and serial sagittal sections of 30-μm thickness were made using a freezing slide microtome in a −20°C cryostat. All sections were mounted on gelatin-coated slides and air dried. For concrete immunohistochemical procedures, sections were rinsed several times in phosphate-buffered saline and treated for 15 min with methanol containing 3% hydrogen peroxide to inhibit endogenous peroxidase activity. Sections were incubated in polyclonal neuropeptide Y antibody (1:4000 dilution) (Yanaihara Institute, Shizuoka, Japan), raised in rabbits, for 72 h at 4°C. Following several rinses in phosphate-buffered saline, antigen–antibody complexes were localized by staining the sections for 30 min in Histofine Simple Stain Rat MAX-PO (Nichirei, Tokyo, Japan). For final visualization, the sections were stained for 15 min in Simple Stain DAB (Nichirei) solution containing 0.003% hydrogen peroxide. Tissue blocks for hematoxylin and eosin staining were carefully oriented and embedded in paraffin with the axis of the teeth parallel to the cutting direction. Tissue blocks were serially cut into 5-μm sections in the mesial-distal direction. The most central section of each tooth was selected for analysis, stained with hematoxylin and eosin, and mounted.

Statistical analysis

Differences among experimental groups were analyzed using Fisher's protected least significant difference by one-way analysis of variance. A significance level of 5% was selected for rejecting the hypotheses. Computations were performed using a statistical software program (STATVIEW version 5.0; Abacus Concepts, Berkeley, CA, USA).

Results

Body, thymus and spleen weights

All the rats had similar body weights (approximately 90 g) at the beginning of the experimental period. Mean body weights exhibited a similar rate of increase during the course of the experiment in all the groups (data not shown). The total gain was approximately 167% in all groups from the beginning (approximately 90 g) to the end (approximately 240 g) of the experimental period (Table 1) ($p < 0.05$). The thymus and spleen weights were similar in all the groups at the end of the experimental period ($p < 0.05$).

Alveolar bone loss

To determine the effects of superior cervical ganglionectomy and oral challenge with *P. gingivalis* on alveolar bone loss, the bone level differences of the rats were determined between the experimental groups. Figure 2A shows the mean [± standard error of the mean (SEM)] cemento-enamel junction : alveolar bone crest (i.e. the distance between the cemento-enamel junction and the alveolar bone crest) value at each of the seven measurement sites. The alveolar bone loss was measured as described previously (4). As alveolar bone decreased, the cemento-enamel junction : alveolar bone crest increased. In the right side of the upper jaws (Fig. 2A,B), both the superior cervical ganglionectomy group and the *P. gingivalis*-challenged group showed a distance between the cemento-enamel junction and the alveolar bone crest that was greater than that of the control group ($p < 0.05$), indicating alveolar bone loss caused by superior cervical ganglionectomy and oral challenge with *P. gingivalis*. In the left side (Fig. 2A,B), the cemento-enamel junction : alveolar bone crest was greater in the *P. gingivalis*-challenged group than in the control group ($p < 0.05$), as a result of the effect of oral challenge with *P. gingivalis*. However, because the left side was not exposed to superior cervical ganglionectomy, the increasing effect of superior cervical ganglionectomy on alveolar bone loss was not shown entirely in the superior cervical ganglionectomy group ($p < 0.05$).

RT-PCR analysis of cytokine gene expression in gingival tissues

To substantiate the functional changes in the gingival tissues of rats as a result of superior cervical ganglionectomy and oral challenge with *P. gingivalis*, the gene-expression profiles of interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6 were compared using RT-PCR analyses. The expression of interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6 was much stronger in the right side of the superior cervical ganglionectomy group compared with the control and *P. gingivalis*-challenged groups (Fig. 2C), indicating that superior cervical ganglionectomy resulted in increased expression of those cytokines.

Histopathological findings

Sections were placed under a Nikon microscope (Nikon, Tokyo, Japan). Sections from experimental sites were magnified 20× in order to investigate alveolar bone resorption. Osteoclasts were found in the furcation area of the mandibular molars in the *P. gingivalis*-challenged group (Fig. 3C). Moreover, osteoclasts were also found in the superior cervical ganglionectomy group (Fig. 3B). However, the control group exhibited no notable

Table 1. A comparison of the body, thymus, and spleen weights at the end of the experimental period

	Weight		
	Body (g)	Thymus (g)	Spleen (g)
Group A	240.22 ± 6.43	0.51 ± 0.41	0.53 ± 0.02
Group B	242.86 ± 6.06	0.54 ± 0.02	0.57 ± 0.02
Group C	240.00 ± 7.36	0.51 ± 0.01	0.53 ± 0.02

Group A, sham operation (control group); group B, superior cervical ganglionectomy group; group C, sham operation and oral challenge with *Porphyromonas gingivalis* (*P. gingivalis*-challenged group). The results are expressed as means ± standard error ($p < 0.05$).

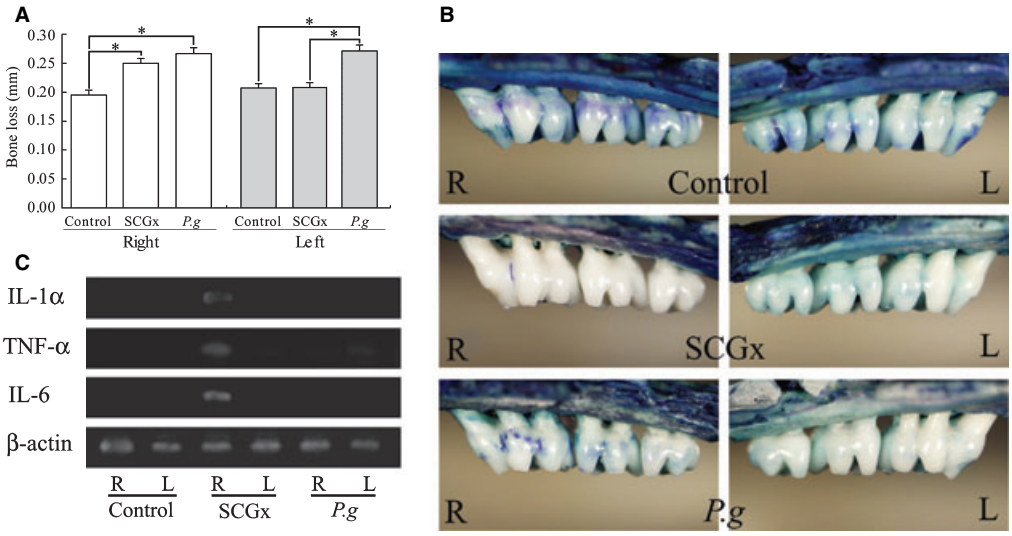

Fig. 2. The effects of superior cervical ganglionectomy and oral challenge with *Porphyromonas gingivalis* on alveolar bone loss. (A) Horizontal alveolar bone loss from around the maxillary molars was evaluated morphometrically on the right and left sides of the upper jaws. Columns represent the means of the data obtained from six rats (expressed as mean ± standard error) (*$p < 0.05$). Bone loss (mm) represents the distance between the cemento-enamel junction and the alveolar bone crest. (B) Morphometric bone levels. In the right side, alveolar bone loss was greater in the superior cervical ganglionectomy and *P. gingivalis*-challenged groups compared with the control group. In the left side, alveolar bone loss was similar in the superior cervical ganglionectomy group and greater in the *P. gingivalis*-challenged group compared to the control group. (C) Comparison of cytokine (interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6) expression in gingival tissues, using reverse transcription–polymerase chain reaction analyses. The expressions of the cytokines interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6 were strongly represented in the right side of the superior cervical ganglionectomy group when compared with the control and *P. gingivalis*-challenged groups. The experiment was repeated twice with similar results obtained on each occasion. Beta-actin was used as a loading control. Control, control group (group A); SCGx, superior cervical ganglionectomy group (group B); *P.g*, *P. gingivalis*-challenged group (group C). IL-6, interleukin-6; IL-1α, interleukin-1alfa; L, left; R, right; TNF-α, tumor necrosis factor-alfa.

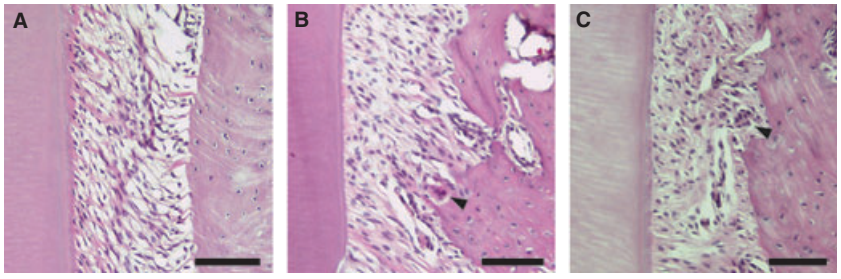

Fig. 3. Periodontal tissues in the right side of the lower jaws of the control group (A), in the superior cervical ganglionectomy group (B) and in the *Porphyromonas gingivalis*-challenged group (C). Osteoclasts (arrow) were found in the furcation area of the mandibular molars in the superior cervical ganglionectomy and *P. gingivalis*-challenged groups. However, the control group exhibited no notable microscopic changes in the periodontal tissue. Original magnification: 20×. Scale bar = 100 μm.

microscopic changes in the periodontal tissue (Fig. 3A).

Neuropeptide Y-immunoreactive fibers

The right sides of the control and superior cervical ganglionectomy groups were examined using immunohistochemistry to confirm the success of superior cervical ganglionectomy. In the control group, an increase in the density and staining of the neuropeptide Y-immunoreactive fibers was found in the alveolar bone around the furcation area of the mandibular molars (Fig. 4C). However, neuropeptide Y-immunoreactive fibers in the superior cervical ganglionectomy group were not as dense and stained more weakly than those of the control group (Fig. 4D).

Discussion

In this study, we investigated the effects of surgical sympathectomy and oral challenge with *P. gingivalis* on alveolar bone loss in an experimental rat model. We found that superior cervical ganglionectomy and oral challenge with *P. gingivalis* accelerated alveolar bone loss and osteoclastic activity when compared with the control group. The

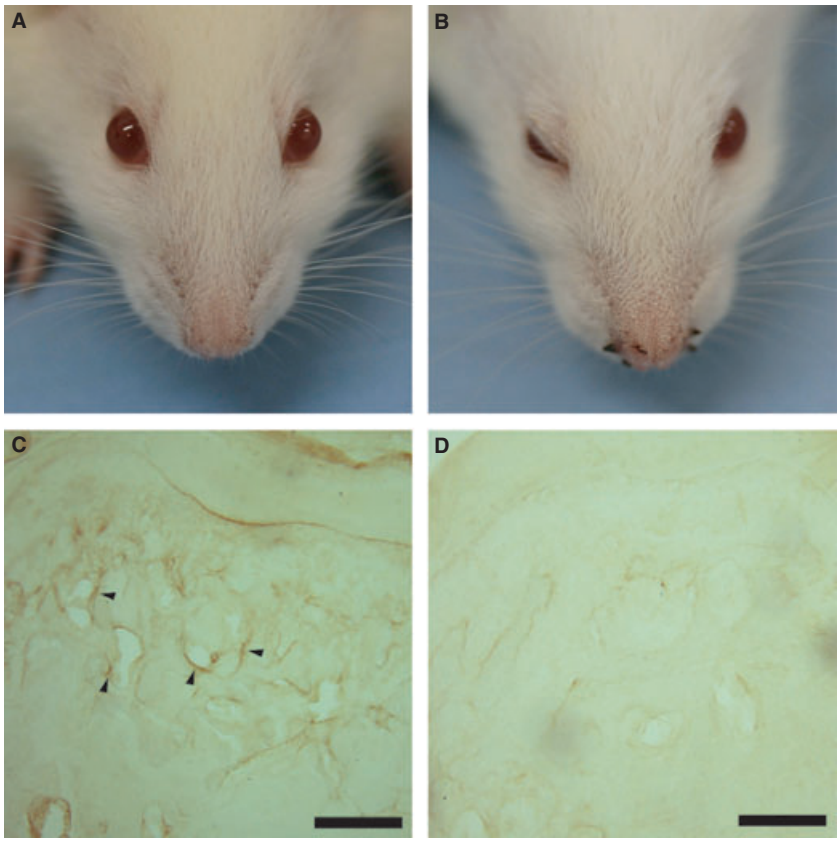

Fig. 4. The presence of ptosis in the control group (A) and in the superior cervical ganglionectomy group (B). Successful superior cervical ganglionectomy was confirmed by the rapid onset of ptosis (B), a clinical sign of successful sympathetic denervation, on the right side of the rats. The rats of the control group had normal eyes (A). Light microscopic distribution of neuropeptide Y-immunoreactive fibers (arrows) in the right sides of the control group (C) and the superior cervical ganglionectomy group (D) were examined using immunohistochemistry to confirm the success of superior cervical ganglionectomy. An increase in the density and staining of neuropeptide Y-immunoreactive fibers was found in alveolar bone around the furcation area of the mandibular molars in the control group. However, neuropeptide Y-immunoreactive fibers in the superior cervical ganglionectomy group appeared thinner and stained less intensely than those in the control group. Decreased neuropeptide Y-immunoreactive fibers can also indicate successful superior cervical ganglionectomy. Original magnification: 20×. Scale bar = 100 μm.

experimental rat model used in this study was a relatively simple *in vivo* model in which *P. gingivalis* was used to infect the animal, leading to destructive periodontitis. Therefore, we used a *P. gingivalis*-challenged animal model in order to emphasize the effectiveness of the sympathetic nervous system in protecting against alveolar bone loss. The sympathetic nervous system, being part of the autonomic nervous system, is one of the major pathways connecting the brain to the periphery and is responsible for up-regulating and down-regulating many homeostatic mechanisms in living organisms (14). Autonomic nerve fibers are found in the periosteum, endosteum and cortical bone and, in many cases, the free-running fibers are associated with blood vessels that enter the bone through Volkmann's canals (11,12). Many studies have suggested that stress may increase the susceptibility to periodontitis (21–25). Under stress, the sympathetic nervous system is activated (13).

Surgical sympathectomy was performed by right superior cervical ganglionectomy to investigate the effects on alveolar bone loss of the sympathetic nervous system (15–17). The efficiency of superior cervical ganglionectomy was confirmed by the rapid onset of ptosis, a clinical sign of successful sympathetic denervation, on the right side of the rats (Fig. 4B) (9,10,13,16,26). It persisted throughout the experimental period. The body, thymus and spleen weights in all the groups of rats were similar at the end of the experimental period (Table 1). In fact, although slight increases in the body, thymus and spleen weights were found in the superior cervical ganglionectomy group, the differences were not statistically significant ($p < 0.05$). These results indicated that the body, thymus and spleen weights showed normal growth during the entire experiment, regardless of whether superior cervical ganglionectomy or oral challenge with *P. gingivalis* was employed. In the right side of the upper jaws, both superior cervical ganglionectomy and oral challenge with *P. gingivalis* resulted in accelerated alveolar bone loss (Fig. 2A,B). In an experimental orthodontic tooth-movement model, root resorption was

shown to be increased after superior cervical ganglionectomy (27). In the pulp-exposure model, large periapical lesions and an increased number of osteoclasts were found following superior cervical ganglionectomy (28,29). In the present study, alveolar bone loss on the left side of the upper jaws (Fig. 2A,B) was greater in the *P. gingivalis*-challenged group than in the control group, as shown on the right side of the figure panels. The effect of superior cervical ganglionectomy on alveolar bone loss was not demonstrated entirely on the left side of the upper jaws of the superior cervical ganglionectomy group because superior cervical ganglionectomy was not performed on this side. Because superior cervical ganglionectomy, which is indicated to result in increased alveolar bone loss, was performed only on the right side of the upper jaws, the differences in the rates of alveolar bone loss between the right and left sides of the superior cervical ganglionectomy group were shown. Histopathologically, after hematoxylin and eosin staining, osteoclasts were found in the furcation area of the mandibular molars in the superior cervical ganglionectomy and *P. gingivalis*-challenged groups, but not in the control group (Fig. 3). We suggest that superior cervical ganglionectomy stimulates osteoclastic activity and induces alveolar bone loss, similarly to oral challenge with *P. gingivalis*.

Hormonal factors (estrogens, parathormone, vitamin D), growth factors (insulin-like growth factor-1, transforming growth factor-beta, bone morphogenetic proteins), cytokines (interleukins, tumor necrosis factor-alfa, osteoprotegerin) and membrane receptors (low-density lipoprotein receptor-related protein-5) are the most influential regulators of bone cell activity (30). Although the mechanisms of bone remodeling following superior cervical ganglionectomy appear complex and are not completely known, it has been considered that the sympathetic nervous system affects the production of cytokines. Oral microorganisms, including *P. gingivalis*, can elicit the release of pro-inflammatory cytokines (interleukin-1, tumor necrosis factor-alfa and interleukin-6) (31,32). In this study, we investigated three cytokines (interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6) that are closely linked to bone resorption. Interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6 were expressed more strongly on the right side of the upper jaws of the superior cervical ganglionectomy group compared with same side of the control group (Fig. 2C), indicating that superior cervical ganglionectomy resulted in increased expression of these cytokines. However, interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6 were expressed only weakly in the *P. gingivalis*-challenged; this is contrary to the effects of oral challenge with *P. gingivalis*, which induces the release of pro-inflammatory cytokines. It was thought that because interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6 were released only during the early days after oral challenge with *P. gingivalis*, they would be present at only low levels in the *P. gingivalis*-challenged group at the end of the experimental period. The differences ininterleukin-6 expression in blood serum were not observed in all the groups (data not shown). Sympathetic nervous system denervation enhanced the synthesis and production of interleukin-6 (14). Following superior cervical ganglionectomy, periapical lesions in rats contained significantly more interleukin-1alfa when compared with similar lesions on the contralateral nonsuperior cervical ganglionectomy side (13). The sympathetic nervous system, along with catecholamines, can alter the type 1/type 2 T-helper cell balance, shifting it from a pro-inflammatory (T helper 1) response to an anti-inflammatory (T helper 2) response (33). Locally released noradrenaline, neuropeptide Y, or circulating catecholamines affect lymphocyte traffic, circulation and proliferation; they also modulate cytokine production and the functional activity of different lymphoid cells. The binding of noradrenaline released from sympathetic nerve terminals to specific adrenergic receptors on immune cells importantly modulates a number of immune and inflammatory responses (34). Sympathetic nerves and neurotransmitters generally exert inhibitory effects on immune mechanisms and inflammation (14). Neuropeptide Y, a co-transmitter with norepinephrine in peripheral sympathetic nerve fibers, has by far the highest concentrations among neuropeptides identified in bone tissue. Haug *et al.* reported that surgical sympathectomy resulted in an almost complete loss of neuropeptide Y-immunoreactive fibers in the right superior cervical ganglionectomy jaws (28). Moreover, chemical and surgical sympathectomies caused the disappearance of pulpal neuropeptide Y-immunoreactive fibers (35). Our immunohistochemistry results also revealed a decrease in the density and staining of neuropeptide Y-immunoreactive fibers in the alveolar bone around the furcation area of the mandibular molars in the superior cervical ganglionectomy group. Thus, after superior cervical ganglionectomy, cytokine expression was increased in gingival tissues because the sympathetic nerves have an inhibitory control over bone-resorptive cytokines (interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6). This effect presumably depended on the deprivation of adrenergic neurotransmitter input at a local level. A decreased number of neuropeptide Y-immunoreactive fibers resulted in increased alveolar bone loss and osteoclastic activity; they can also indicate successful superior cervical ganglionectomy. Sympathetic nerves have a vasoconstrictor function. Neuropeptide Y potentiates the vasoconstrictive action of noradrenaline and is present in noradrenergic sympathetic nerves. Neuropeptide Y-immunoreactive and catecholaminergic fibers were sparse in the periosteum except as associated with blood vessels, suggesting that a major role of these substances in the periosteum may be in regulating the blood flow to bone (36). Neuropeptide Y-immunoreactive fibers in the alveolar bone exist in the bone marrow, principally surrounding blood vessels. In the periodontal ligament, the few neuropeptide Y-immunoreactive fibers present are located mainly in the apical third (29). Thus, we suggest that bone resorption after sympathec-

tomy seems to be caused by transient vascular changes.

The results of the present study clearly showed that both superior cervical ganglionectomy and oral challenge with *P. gingivalis* accelerate alveolar bone loss and osteoclastic activity. Cytokine expression was also increased in the superior cervical ganglionectomy group. Alveolar bone loss induced by superior cervical ganglionectomy is dependent on the change in expression of bone-resorptive cytokines (i.e. interleukin-1alfa, tumor necrosis factor-alfa and interleukin-6). These cytokines are regulated by changes in adrenergic neurotransmitters. However, further study of these observations is needed. Both sensory (calcitonin gene-related peptide-immunoreactive and substance P-immunoreactive) and sympathetic (neuropeptide Y-immunoreactive) nerve fibers are widely distributed in the periosteum of membranous bones and long bones (36). The sprouting of sensory nerve fibers occurred 4 d after dental injury and increased in number until 28 d, with a decrease thereafter (37,38). Thus, comparisons of sensory and sympathetic nerve fibers will be needed to establish the precise mechanism of the sympathetic nervous system on alveolar bone loss. In addition, knockout animal studies with cytokine ablation and/or systemic chemical sympathectomy by drugs will be investigated.

In conclusion, we confirmed that superior cervical ganglionectomy induced alveolar bone loss, similarly to oral challenge with *P. gingivalis*. These results gave new information on the occurrence of an increase in alveolar bone loss. Both oral challenge with *P. gingivalis* and surgical sympathectomy were important factors accelerating alveolar bone loss. Thus, we suggest that the sympathetic nervous system is linked with the prevention of alveolar bone loss.

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