

**SECRETION OF SALIVARY
CYSTATIN S BY THE
SUBMANDIBULAR GLANDS OF
RATS FOLLOWING VARIOUS
DENTAL TREATMENT, CHRONIC
TREATMENT WITH
ISOPROTERENOL AND IN
RESISTANCE AGAINST PLAQUE
FORMATION**

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This work was supported in part by a Grant-in-Aid for Scientific
Research from the Ministry of Education in Japan

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Summary

Salivary cystatin S was first discovered on the polyacrylamide gel as the large mobile (LM) protein with a relatively high electrophoretic mobility secreted by the submandibular glands of rats treated with chronic isoproterenol injections. After the LM protein was cloned and sequenced the cDNA, it was identified as a salivary cystatin S belonging to family 2 of the cystatin superfamily. Secretion of salivary cystatin S by the salivary glands was strongly associated with various dental treatment such as repeated amputation of the lower incisor teeth and orthodontic appliances such as an incisal bite plane and for the lateral expansion. In addition, induction and/or secretion of salivary cystatin S and the expression of its mRNA were dramatically observed in the submandibular glands of the plaque-resistant rats. Following various dental treatment the secretory functions of the salivary glands were significantly varied and salivary cystatin S was characteristically induced in the submandibular glands and secreted into the oral cavity. Therefore, salivary cystatin S could probably play important roles to protect oral hard and soft tissues from various invaders.

Key words : Salivary cystatin S, Plaque-resistant rat, Chronical isoproterenol injection, Orthodontic appliances, Incisor amputation

Introduction

It is well-known that the secretory functions may drastically be changed and salivary cystatin S is characteristically synthesized in the salivary glands of rats following chronic treatment with isoproterenol (IPR), repeated amputation of the lower incisor teeth, orthodontic treatment and intake of food including cysteine protease¹⁻⁹. In addition, plaque-resistant rats form virtually no plaque and do not develop gingivitis under the same dietary conditions but dramatically induce salivary cystatin S in saliva, the salivary glands and gingiva¹⁰, whereas plaque-susceptible rats with naturally occurring gingivitis, heavy plaque formation and marked periodontal pockets after being fed with commercially available powder chow^{11,12}, and have been registered as an animal model for gingivitis¹³, do not induce any salivary cystatin S in the salivary glands. Moreover, several investigators¹⁴⁻¹⁶ stated that the level of salivary cystatin S in whole saliva of periodontal patients was significantly less than that in whole saliva of healthy subjects.

The present study was thus designed to review changes of the secretory functions throughout a series of our works, especially for induction, synthesis and/or secretion of salivary cystatin S by the salivary glands subjected to various

dental treatment, chronic treatment with IPR and in resistance against plaque formation.

Materials and Methods

1. Saliva collection

Young (1 to 8 weeks of age) and adult (12-52 weeks but mainly 12 weeks of ages) male and female Sprague-Dawley (SD) rats were used in each experimental group. The plaque-resistant and plaque-susceptible rats originally generated from a Wistar-Kyoto strain were also distributed into each group. A 12-h light-dark cycle was maintained in the room by an automatic timing device with lights turned off at 18 h and the animals had ad libitum access to rat chow (Oriental Kobokogyo Co., Tokyo) and water. Ethical clearance for these studies was approved by the Asahi and Fukuoka Dental Colleges' Animal Ethics Committee.

The rats were fasted from 1700 h to 0900 h prior to the collection of saliva, then anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg body wt., i.p.), secured in the supine position with tape and tracheotomized to facilitate respiration during the saliva collection. Both submandibular ducts using thin and intramedic polyethylene tubes (PE 10, Beckton Dickinson) were intraorally cannulated by the method¹⁷. The submandibular glands did not secrete any saliva spontaneously in the absence of stimulation. With the exception of an initial drop, which was discarded, submandibular saliva was collected into microcentrifuge tubes kept in ice, and the volumes were estimated by weight, assuming the specific gravity of saliva to be 1.0. IPR (30 mg/kg), physalaemin (0.02 mg/kg), bethanechol (1 mg/kg), methoxamine (6 mg/kg) and/or phenylephrine (6 mg/kg), and oxymetazoline (5 mg/kg) and/or clonidine (10 mg/kg) dissolved in isotonic saline were employed i.p. or i.v. as secretagogues suitable for five different types of receptors¹⁸. After the saliva collection, the salivary glands were weighed to estimate salivary flow rate and to extract several compositions including nucleic acids.

2. Various dental treatment

An incisal bite plane constructed from cold-cured methyl methacrylate resin was used to both upper incisor teeth with the rats. A thin metal plate was inserted into the bite plane^{4,19} as seen in Fig. 1. An orthodontic appliance for upper incisor separation was also constructed from 0.016-inch wire with an open helical loop and fixed with a cold-cured methyl methacrylate to both the left and right maxillary central incisors of rats under anesthesia⁶. Thereafter, the

orthodontic appliances were observed and modified if necessary every two days for 4 weeks.



Fig. 1 Lateral X-ray of a rat with an incisal bite plane in position. The white line between the teeth is the thin metal plate.

3. Repeated amputation of the lower incisors

The lower incisors of rats in the experimental group were amputated under ether induced anesthesia at 3- or 4-day intervals for about 28 days. At 3, 7, 14 and 28 days after the repeated amputation the animals were used to collect submandibular and parotid saliva, extracts of three major salivary glands and the nucleic acids³.

4. Chronic treatment with IPR

For chronic treatment with IPR-HCl, the drug (4 mg/kg or mainly 30 mg/kg) was administered i.p. once or twice a day for 4 or 6 days consecutively in the experimental group, while the control rats were just given sterile saline^{2,8}.

5. Saliva, extracts and sera analyses

Saliva samples were analyzed for total protein by the method²⁰ with casein as a standard and then were kept frozen at -20 C until lyophilization could be carried out. After reconstitution of the lyophilized saliva, 15 % native polyacrylamide gel electrophoresis was carried out²¹. The polyacrylamide gels were stained with Wool Fast Blue BL22. The salivary cystatin S concentrations in submandibular saliva, sera, and the extracts of the submandibular glands and

gingiva were measured with ELISA^{23, 24}. The band of salivary cystatin S was also determined by the Western blotting method²⁵, using a polyclonal antibody against purified salivary cystatin S. The submandibular glands and gingiva kept at -80°C were homogenized and then the homogenates were centrifuged at $105,000 \times g$ for 1 h at 4°C to obtain their extracts. Sera were collected from the coagulated blood by centrifugation at $10,000 \times g$ for 20 min at 4°C .

6. Antibody production to salivary cystatin S and its purification

In brief, salivary cystatin S was purified by only two steps of native polyacrylamide gel electrophoresis and Immobililine-Canal isoelectric focusing and then a polyclonal antibody against this purified sample was evoked with an equal amount of Freund's complete adjuvant⁸. This polyclonal antibody is specific to the rat salivary cystatin S-3 but can react its S-1 and S-2 with high homology separated from submandibular saliva of rats chronically treated with IPR⁸. The salivary cystatin S in submandibular saliva of plaque-resistant rats and Wistar rats subjected to chronic treatment with IPR for 4 days was also purified and antibodies against it were evoked by the method^{8,24} using two male white rabbits (2.5 kg). The IgG fraction was prepared by Affi-gel protein A column chromatography. Double immunodiffusion analysis was also performed on 1.2 % agarose gel.

7. Estimation of the nucleic acids and RNA-p

Some salivary glands of the different groups of rats were studied histologically, and others were analyzed for DNA, RNA and RNA-p contents at 3, 7, 14, 21 and 28 days after enlargement induced by a certain types of dental treatment by the methods.

8. Northern blotting method

The expression of salivary cystatin S mRNA was determined by the Northern hybridization method²⁷ with a slight modification. RNA isolated by the acid-guanidium-phenol-chloroform method was used for Northern blot hybridization. Total RNA (15 $\mu\text{g}/\text{lane}$) was subjected to electrophoresis through 1 % agarose gel containing 6.6 % formaldehyde, blotted onto positively charged nylon membranes (Amersham, UK) and cross-linked for 3 min with UV light. Digoxigenin-labelled DNA probes of salivary cystatin S were carried out by reverse transcription of mRNA with AMV reverse transcriptase XL and PCR amplification of cDNA by the instructions of the manufacturer (TaKaRa RNA PCR Kit, TaKaRa, Tokyo). The primers for amplification of salivary cystatin S cDNA with Tag DNA polymerase were commercially constructed¹⁰. With these primers, a 492-bp fragment of salivary cystatin S cDNA was amplified. The salivary cystatin S was probed with this amplified fragment and

digoxigenin-labelled 11-dUTP. The hybridization probes were detected with anti-digoxigenin-alkaline phosphatase antibody.

9. Standard RT-PCR and competitive RT-PCR of mRNA for salivary cystatin S

The RT-PCR and competitive RT-PCR were carried out to estimate the expression of mRNA for salivary cystatin S with the constructed primers of salivary cystatin S and a competitor²⁸.

10. Statistical analysis

All data were analyzed by the unpaired t-test and factorial analysis of variance. When factorial analysis of variance gave a significant F value, Duncan's New Multiple-Range Test was carried out on the adjusted means.

Results

1. Enlargement with hypertrophy and hyperplasia of the salivary glands following a certain types of dental treatment and chronic IPR administration

The submandibular and sublingual, but not parotid, glands were significantly enlarged at 7 to 28 days after repeated amputation of the lower incisors and a certain types of dental treatment with orthodontic appliances. The weights of submandibular and sublingual glands were about less twice their normal glands. In contrast to dental treatment, the submandibular and parotid, but not sublingual, glands after chronic injection with IPR were significantly enlarged by 36 h and reached to the maximum levels by 7 to 10 days after treatment. The weight of the parotid glands was approximately 4 to 5 times the weight of the control glands, whereas the submandibular glands were about

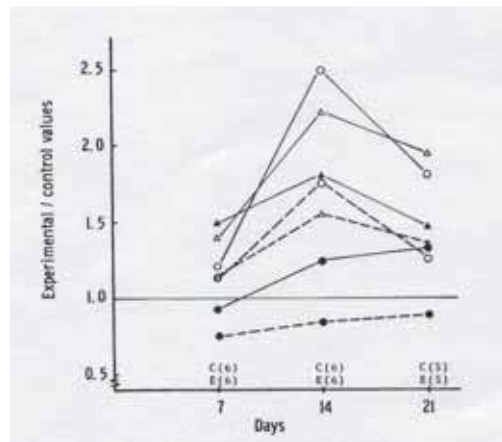


Fig. 2
 Changes of the DNA, RNA and RNA-p contents of the submandibular glands of rats subjected to repeated amputation of the lower incisor teeth for 21 days.
 ●—□ : DNA/gland,
 ●...● : DNA/mg gland,
 □—□ : RNA/gland,
 □...□ : RNA/mg gland
 —○ : RNA-p/gland,
 ○...○ : RNA-p/mg gland,
 ▲—▲ : RNA/DNA

twice their normal glands. Together with the results of the histological studies, the DNA and RNA contents had significantly increased in the salivary glands of rats which suggested that both hypertrophy and hyperplasia had occurred in these three major salivary glands after dental treatment and chronic treatment with IPR29 (Fig. 2).

Enlargement was completely abolished with atropine for only the sublingual glands but with phenoxybenzamine for both the submandibular and sublingual glands (Fig. 3).

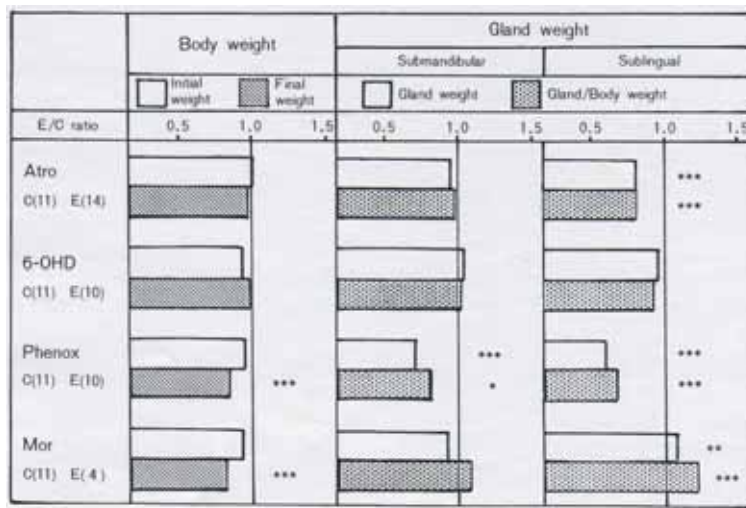


Fig. 3 The effects of orthodontic treatment with different types of autonomic antagonists and morphine on body weight and the weights of the submandibular and sublingual glands for 1 week.

Atro; atropine, 6-OHD; 6-hydroxydopamine, Phenox; phenoxybenzamine, Mor; morphine, C; 1 week after application, E; 1 week after application with different types of antagonists.

*, p < 0.05, **, p < 0.02; ***, p < 0.01 by unpaired t-test. Values in the parenthesis denote No. of rats.

However, no enlargement was seen in any salivary gland of plaque-susceptible and plaque-resistant rats at any weeks of age.

2. Induction and/or secretion of salivary cystatin S by the submandibular glands of rats after various dental treatment, chronic treatment with IPR and in resistance of plaque formation

Induction of salivary cystatin S by the submandibular glands of rats following dental treatment, chronic treatment with IPR and in the

plaque-resistance were clearly observed (Fig. 4).

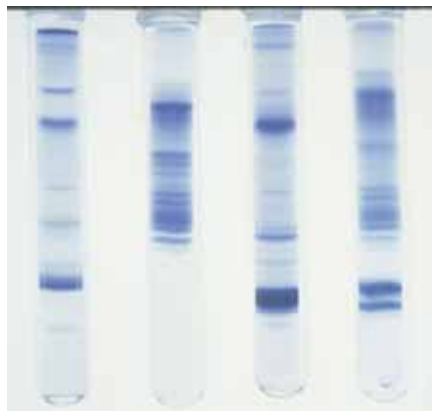


Fig.4 Electrophoretograms on 10 % native polyacrylamide gels including large mobile (LM) proteins. The columns from left to right denote isoproterenol (the β -type protein), methoxamine (the α -type protein) stimuli in normal rats, and isoproterenol and methoxamine stimuli in rats subjected to chronic treatment of isoproterenol.

In rats subjected to repeated amputation of the lower incisor teeth and a certain types of orthodontic treatment, salivary cystatin S in the submandibular glands were induced at later stage than in rats subjected to chronic treatment with IPR and secreted into saliva by 28 days after dental treatment judged immunologically, electrophoretically and immunohistologically²⁹. By the chemical sympathectomy with 6-hydroxydopamine, the induction and/or secretion of salivary cystatin S was dramatically enhanced by 7 days after a certain types of dental treatment³⁰. In contrast, salivary cystatin S in the submandibular glands of rats subjected to chronic treatment with IPR was dramatically induced by a single IPR injection by 24 h and the amount increased dramatically to 400,000-fold by 7 days although in control rats salivary cystatin S was detectable from only two sources, submandibular saliva elicited by IPR and submandibular gland extracts, but could not be detectable in any other samples. The salivary cystatin S levels in submandibular saliva dramatically increased by 8 days and reached 30 % of the total salivary protein after chronic treatment with IPR 24. Salivary cystatin S was also present in the parotid and sublingual glands, and esophagus, small intestine, stomach, kidney and gingiva as well as sera of rats, probably translocated from submandibular saliva into, subjected to chronic treatment with IPR^{24,31}.

In contrast, the concentrations of salivary cystatin S in submandibular saliva, extracts of the submandibular glands and gingiva of plaque-resistant groups were significantly higher but after both groups had been subjected to chronic treatment with IPR they were not significantly different from those in plaque-susceptible groups. The concentration of salivary cystatin S in submandibular saliva of plaque-resistant, but not plaque-susceptible, groups increased significantly with increasing age (Table 1).

Week Group	4		8		12	
	RES	SUS	RES	SUS	RES	SUS
Salivary cystatin S concentration	2157.6**	57.3	12348.5**	144.8	43916.4**	12.5
	±	±	±	±	±	±
	400.0	16.2	1400.0	14.2	6200.0	2.7
No. of rats	8	8	8	10	9	9

RES and SUS; ng/mg protein

Table1. Comparison of salivary cystatin S concentrations in submandibular saliva between male RES and SUS rats in response to isoproterenol during development (means \pm S.E.)

At 12 weeks of age, the concentration of salivary cystatin S in submandibular saliva of the plaque-resistant groups was over 3,500 times than in saliva of plaque-susceptible rats¹⁰. The concentration of salivary cystatin S in the extract of the submandibular glands of male and female plaque-resistant groups was dramatically higher by 4,500-6,300 times than that of male and female plaque-susceptible groups. The salivary cystatin S in the extract of gingiva in the male plaque-resistant group was detectable by the ELISA method, but was not in the male plaque-susceptible group¹⁰. By the Western blotting method¹⁰, positive bands were detected only in submandibular saliva obtained from male and female plaque-resistant groups, but not from plaque-susceptible groups.

3. Agonist-dependent changes of secretory functions of the submandibular glands enlarged following various dental treatment, chronic treatment with IPR and in resistance of plaque formation

By the enlarged submandibular glands of rats subjected to repeated amputation of the lower incisor teeth and a certain types of orthodontic treatment, the secretory function did not greatly change except that the protein secretion were significantly reduced due to malnutrition devoid of the normal occlusion, following the excessive reduction of body weight. In contrast, the enlarged glands characteristically induced salivary cystatin S and secreted it into saliva after various dental treatment. This enlargement of the submandibular glands of rats was dramatically followed with hypertrophy and hyperplasia of the acinar cells, but not the granular convoluted tubule (GCT) cells. Therefore, the components such as proteases, epidermal growth factor and nerve growth factor, and α -adrenoceptors, which were responsible for the α -type proteinsecretion in response to α -adrenoceptor agonists, present in the GCT cells, were

dramatically reduced following these dental treatment as well as chronic treatment with IPR. Therefore, a number of GCT cells were relatively reduced following such treatment, but their secretory functions were still conserved persistently for the α -type protein secretion following a certain types of dental treatment except that following repeated amputation of the lower incisors at 28 days after treatment, the α -type protein secretion was almost completely disappeared in the submandibular glands (Fig. 5).

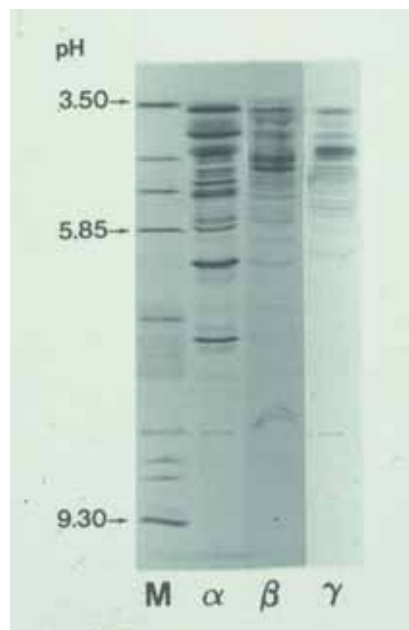


Fig. 5. The IEF electrophoretograms of three types (α -, β - and γ) of protein in rat submandibular saliva on gradient pH 3.5-9.0 gels. M denotes standard substances for pH.

In both groups of plaque-resistant and susceptible rats at all ages the proteins secreted by the submandibular glands in response to IPR, physalaemin and bethanechol were of the β -type as seen in the normal SD rats in contrast that the proteins were of the α -type in response to phenylephrine and oxymetazoline in both groups. These observation meant that the GCT cells were almost completely intact and could secrete their components different from those mentioned above. However, a little modifications were seen in response to

oxymetazoline in plaque-resistant rats which usually secreted the γ -type proteins in the normal submandibular glands of SD rats□

The agonist-dependent changes of secretory functions of the submandibular glands of plaque-resistant and plaque-susceptible rats were observed. For submandibular saliva elicited by IPR, physalaemin and bethanechol, the flow rates were significantly less in the plaque-resistant rats. In contrast, the concentration of protein in submandibular saliva in response to IPR, physalaemin and phenylephrine in plaque-resistant groups was significantly higher.

Wet weights (mg/BW) of the submandibular glands, the volumes of saliva secreted and flow rates of plaque-resistant groups after chronic treatment with IPR were significantly less than those of plaque-susceptible groups¹⁰. In contrast, the concentration of protein in the plaque-resistant groups was significantly higher than that of plaque-susceptible groups subjected to chronic treatment of IPR.

4. The gene expression of salivary cystatin S

The expression of mRNA of salivary cystatin S was also determined by the Northern blotting method (Fig. 6), RT-PCR and competitive RT-PCR. The gene expression of salivary cystatin S was observed in the submandibular glands of rats after 7 days subjected to a certain types of orthodontic treatment (Fig. 7) and in resistance against plaque formation, and the submandibular glands of Wistar rats subjected to chronic treatment with IPR for 4 days, but not in plaque-susceptible groups, or in Wistar and SD rats without chronic treatment with IPR and any orthodontic appliance (Fig. 6).

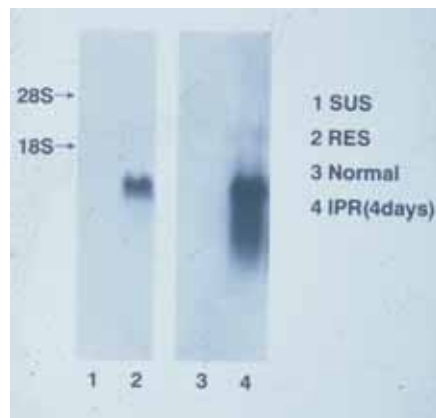


Fig. 6 Northern blotting of salivary cystatin S in the submandibular glands of the plaque-resistant, plaque-susceptible and Wistar rats with and without isoproterenol-treatment for 4 days. The gene expression of salivary cystatin S is seen in the plaque-resistant and isoproterenol-treated rats, but not in the plaque-susceptible and normal Wistar rats. 1 indicates plaque-susceptible (SUS), 2 plaque-resistant (RES), 3 normal Wistar and 4 isoproterenol-treated submandibular glands of Wistar rats.

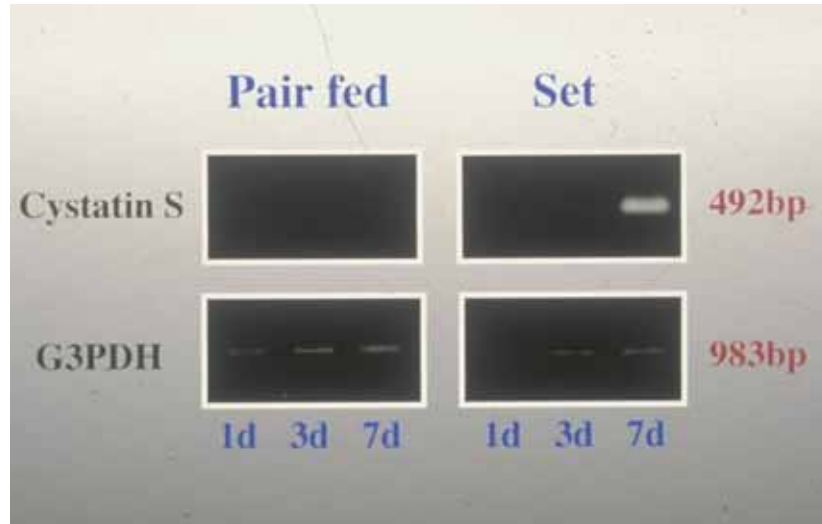


Fig. 7. The gene expression of salivary cystatin S in the submandibular glands of rats subjected to orthodontic treatment for the lateral expansion was observed after 7 days (7 D) by RT-PCR. Pair fed denotes control rats with paired food and Set the experimental groups. G3PDH denotes glyceraldehyde 3-phosphate dehydrogenase.

The gene expression of salivary cystatin S in the submandibular glands of SD rats during development was predominant at 7 weeks of age which coincided developmentally with the regression of terminal tubule cells and the differentiation of the GCT cells, respectively²⁸.

Discussion

In saliva secreted by the enlarged submandibular glands of rats subjected to repeated amputation of the lower incisor teeth, the concentrations of inorganic (K, Na, Ca and inorganic P) and organic substances (urea and sialic acid), and amylase activity did not greatly change during the experimental period for about 1 month with some exceptions, although the enlarged submandibular glands were significantly reduced their secretory functions for the protein secretion from 14 to 28 days after treatment²⁹ as likely seen in the submandibular glands after orthodontic treatment with an incisal bite plane⁴. Incidentally, the parotid gland, atrophied following these dental treatment, did not change their secretory functions during 1 month²⁹. However, in the sublingual glands at only 3 days, but not 7 days with an exception, subjected to orthodontic treatment, salivary flow rate, the concentrations of sialic acid and Ca, and the amounts of protein secreted

in saliva were significantly reduced¹⁹. From these results it was suggested that such variations of the salivary components were caused by the daily malnutrition following inadequate intake of foods due to irregular occlusion.

The mechanism for enlargement of the sublingual and submandibular glands of rats subjected to various dental treatment was relatively different from that of rats subjected to chronic treatment with IPR by which caused enlargement of the parotid and submandibular glands of rats. The contents of DNA, RNA and RNA-p in the submandibular and sublingual glands, but not parotid glands, of rats subjected to various dental treatment were significantly higher at 7 and 14 days after treatment than those of control rats. Atropine completely inhibited enlargement of sublingual glands in contrast to that phenoxybenzamine (adrenergic α -blocker) also inhibited enlargement of both submandibular and sublingual glands³⁰. Furthermore, in both submandibular and sublingual glands subjected to a certain types of orthodontic appliances, the contents of substance P significantly increased at both 3 and 7 days, but not at 14 and 28 days after treatment⁶. Therefore, both parasympathetic and sympathetic nerves as well as sensory nerves could probably associate with such events in three major salivary glands after various dental treatment.

In contrast, the contents of both DNA and RNA in the submandibular and parotid glands subjected to chronic treatment with IPR were significantly higher than those of control rats at the relatively early stages. No enlargement was occurred in the sublingual glands of rats subjected to chronic treatment with IPR. The acinar cells were greatly enlarged with hypertrophy and hyperplasia in the submandibular and parotid glands of rats. Only enlarged submandibular gland subjected to a certain types of dental treatment and chronic treatment with IPR could drastically induce and secrete salivary cystatin S. It has not yet been elucidated the reasons why salivary cystatin S was drastically induced and secreted into the oral cavity by the submandibular glands of rats subjected to such treatment³¹. Further studies are necessary to elucidate genuine roles of salivary cystatin S secreted into the oral cavity, although some investigators elucidate that salivary cystatin S probably plays a role to protect the oral membrane and cavity from protease attacks derived from several bacteria³².

It would be very useful to identify salivary biomarkers which distinguish patients with dental caries, gingivitis or periodontitis from healthy persons. No clear compositional differences have been discovered except some reports on salivary cystatin C or S in human whole and parotid saliva¹⁴⁻¹⁶, although many biomarkers, namely antibacterial factors, metabolic products, bacterial enzymes, cytokines and enzymes of host cells have been elucidated in saliva and gingival crevicular fluids obtained from patients with dental caries or periodontitis³³. The levels of salivary cystatin S in submandibular saliva, extracts of the submandibular glands and gingiva in plaque-resistant rats increased

substantially with increasing age and were significantly higher than those in plaque-susceptible rats¹⁰ in consistent with a report¹⁶.

In brief, in saliva secreted by the submandibular glands of SD rats three types of protein were discovered and designated by us as the α -, β - and γ - types^{10, 18, 20-22, 34-40} (Fig. 5). In saliva in response to β 2- adrenoceptor, cholinergic, peptidergic and β 1-adrenoceptor, at relatively low doses, agonists, the β - type protein, probably derived from the acinar cells, were dominantly secreted. In contrast, in saliva in response to β 1-adrenoceptor agonists such as tyrosine metabolites (catecholamines), at relatively high doses, the α -type protein, probably derived from the GCT cells, was characteristically secreted. The third type, which we have designated as the γ - type protein, could probably be present in human saliva and were secreted in response to 2-imidazoline derivatives such as clonidine, oxymetazoline, naphazoline and tetrahydrozoline, but not α -aminoclonidine which could secrete the α -type protein. The origins of the γ - type protein have not been yet clear. Therefore, further studies are necessary to elucidate the secretion route for the γ - type protein by the submandibular glands of rats. The γ - type protein contains significantly higher albumin and sIgA than the α - and β -types of proteins^{34,41}. By the submandibular glands of rats subjected to various dental treatment and chronic treatment with IPR, the α -type protein was relatively, but not completely, disappeared due to hypertrophy and hyperplasia of the acinar cells and devoid in part of the GCT cells of the salivary glands. In both plaque-resistant and plaque-susceptible rats the proteins in submandibular saliva were of the β -type in all samples in response to IPR, physalaemin and bethanechol and in rats subjected to chronic treatment with IPR, as seen in normal SD rats¹⁸. These results suggest that the acinar cells of the submandibular glands could still work normally in both types of rats. In contrast to the β -type of protein, the α -type of protein was secreted in both groups of rats in response to oxymetazoline, which used to secrete the γ -type protein by SD rats^{18,34}. Significantly higher levels of catecholamines in the salivary glands of plaque-resistant and plaque-susceptible groups than those of normal SD rats may be accountable for this discrepancy⁴². The plaque-susceptible groups responded to chronic treatment with IPR more strongly than plaque-resistant groups. It has already been suggested that a small amount of neurotransmitter release from very few impulses in postganglionic sympathetic secretomotor nerves is sufficient to activate acinar cells, whereas the GCT cells require a much higher local concentration of neurotransmitter and this greater release of neurotransmitter can only be achieved by high frequency impulse formation⁴². From data on the catecholamine content in the submandibular and adrenal glands, it may be suggested that plaque-resistant rats, as seen in a strain of the rat with hypertension, may have a much augmented adrenal-sympathetic system^{43,44}.

The isoelectric point (pI 4.5), molecular weight (14,500), electrophoretic profile on pH 3.5-5 or 3.5-9.0 isoelectric focusing gels and sequence of the 26 N-terminal amino acids of the salivary cystatin S purified from submandibular saliva of plaque-resistant groups were identical to those of the rat salivary cystatin S-3 purified from submandibular saliva of a different strain, SD rats subjected to chronic treatment with IPR^{8,10}. The gene expression of salivary cystatin S in the submandibular glands of rats subjected to repeated amputation of the lower incisor teeth and an application of an incisal bite plane has never been checked so far but similar results, as seen in the rats with an orthodontic appliance for the lateral expansion, could probably be obtained. The expression of the mRNA of salivary cystatin S in the submandibular glands was clearly observed in both male and female plaque-resistant, but not in plaque-susceptible or Wistar rats unless these were subjected to chronic treatment with IPR for 4 days. However, it has been elucidated in detail by quantitative competitive RT-PCR that the salivary cystatin S gene was already expressed at 7 days of age and that its concentration increased gradually. Then it reached a maximum at 28 days of age and decreased to constant levels after 26 weeks of age²⁸. These expression patterns of salivary cystatin S gene were coincided with the regression of terminal tubule cells and the differentiation of the GCT cells. Incidentally, in the gene expression of the five adrenoceptor subtypes, unexpected results were obtained in the α 1a-adrenoceptor subtype, which was most abundant and over 70 % within total five adrenoceptor subtypes (α 1a, α 1b, α 2A, β 1 and β 2) in the submandibular glands of both plaque-resistant and plaque-susceptible rats. The gene expression of β 1-adrenoceptor subtypes, which probably play a role to synthesize salivary cystatin S in the acinar cells of the salivary glands, was unexpectedly low in both groups⁴⁵. Therefore, further studies are necessary to elucidate the role of the gene expressed salivary cystatin S in the salivary glands.

It can be concluded that salivary cystatin S induced following various dental treatment and chronic treatment with IPR could probably be a good biomarker to elucidate the roles of the salivary cystatin S in the oral cavity and also to discriminate two groups of plaque-resistant and plaque-susceptible rats. However, further studies in detail are necessary to elucidate the preventive actions of salivary cystatin S in the oral cavity.

This paper was presented at the II Congress of BaSS - Belgrade

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