Gene expression profiles of the three major salivary glands in rats

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ABSTRACT

The protein components of saliva reflect the condition of the whole body as well as the salivary glands. The aim of this study is to characterize the gene expression profiles in each of the rat major salivary glands—the parotid, submandibular, and sublingual glands. Gene expression was analyzed using DNA microarrays, and observed differences in expression of representative genes were confirmed by quantitative, real-time polymerase chain reaction. Among the glands, the contribution to the high expression of genes encoding various proteins, specifically mucin 10, proline-rich glycoproteins, proline-rich protein 2, proline-rich proteoglycans, cystatin 10, amylase, deoxyribonuclease I, and von Ebner's gland protein, was significantly greater in the parotid gland than the other glands. The submandibular and sublingual glands had similar gene expression profiles that differed from profile of the parotid gland. For example, the genes encoding mucin 19 and ovomacroglobulin were highly expressed only in the submandibular and sublingual glands. In summary, we characterized gene expression in the rat major salivary glands and provided basic information on salivary gland marker proteins.

The parotid, submandibular, and sublingual glands are the three major salivary glands in rodents and humans, and secretions from these glands are mixed in the oral cavity and contribute 26%, 69%, and 5%, respectively, to the contents of whole saliva in humans (29). Recently, analyses of saliva components have been used to evaluate manifestation of some diseases (5, 26, 34). As the collection of saliva is safe and easy, it is useful as a diagnostic in the early detection and evaluation of diseases. For example, in the saliva from patients with Sjögren's disease, levels of pro-inflammatory cytokines, lactoferrin, and antibody against α -fodrin are higher than the levels in healthy individuals (5, 26, 34).

Additionally, salivary flow rate is an indicator of

xerostomia (31). Xerostomia arises not only from the systemic disorders, such as Sjögren's disease, but also as a response to various conditions, including adverse drug reactions and stress (9). There are differences in the innervation of the three major salivary glands (12, 25), suggesting that the effects of nerve stimulation, drugs, and stress on each salivary gland might be different. Tumor incidence also differs between the salivary glands in humans; the parotid gland has almost a 4-fold higher incidence of tumors than the other glands (16). Recently, differences in gene expression as assessed on DNA microarrays between the human parotid and submandibular glands have been reported (32).

Currently, in cases of salivary gland disease, it is difficult to identify which gland is damaged by analyzing whole saliva because the protein secretion profile of each salivary gland is not well established. Mucin is secreted from both the submandibular and sublingual glands (20), and cystatin is secreted mainly from the submandibular gland (11), but there is no evidence that these proteins are unique to the

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respective salivary glands. Therefore, further analyses are required to determine whether these proteins can be used as specific markers for each salivary gland. It is important to identify the proteins specific to each gland.

In this study, we analyzed the gene expression in rat salivary glands. Rodents are commonly used as animal models for oral biological research; therefore, a basic characterization of the protein profiles of the salivary glands in rats may be useful. We analyzed differential gene expression in the parotid, submandibular, and sublingual glands of rats using DNA microarrays and quantitative real-time polymerase chain reaction (PCR). The purpose of this study was to determine the differences and the similarities in gene expression of the rat major salivary glands, focusing on secretory and secretion-related proteins.

MATERIALS AND METHODS

RNA isolation. All procedures were conducted according to the Guidelines for the Care and Use of Laboratory Animals, The Nippon Dental University, School of Life Dentistry at Niigata. Male Wistar rats (9 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and sacrificed by cardiac puncture. Parotid, submandibular, and sublingual glands were isolated, cut into approximately 30-mg samples, washed with PBS, and soaked in 0.5 mL RNAlater (Ambion Inc., Austin, TX, USA). Total RNA was prepared using an RNeasy Plus Mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions.

Microarray analysis. Total RNA of parotid, submandibular, and sublingual glands from a single 9-week-old male rat was analyzed with the Whole Rat Genome Oligo Micro array 44K kit (Agilent Technologies Inc., Palo Alto, CA, USA). One-color microarray analysis was performed by Hokkaido System Science Co., Ltd. (Sapporo, Japan). Synthesis of cDNA and the following synthesis of the fluorescent-labeled cRNA were performed from 500-ng samples of total RNA obtained from each of the three salivary glands using the Quick Amp Labeling kit (Agilent Tech). The three fluorescent-labeled cRNA objects were applied to three different arrays on the same slide. The slides were scanned using Agilent Technologies Microarray Scanner. The data were analyzed using GeneSpring software (Agilent Tech).

Real-time quantitative PCR. First-strand cDNA was synthesized from 5 µg of total RNA template using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) and oligo dT primers according to the manufacturer's instructions. LightCycler (Roche) amplification using a TaqMan probe selected from the Universal ProbeLibrary (Roche) was used for the quantitative real-time PCR assay according to the manufacturer's instruction. Selection of the TaqMan probe and design of the primers listed in Table 1 were performed at the Universal ProbeLibrary Assay Design Center Website. A partial GAPDH cDNA was used as a standard. The concentration of the GAPDH cDNA was determined by measuring the absorbance at 260 nm. The crossing points of unknown samples were determined from the regression line, and the corresponding concentrations were calculated. The transcript copy number was then calculated. The analysis was performed in triplicate using RNA samples from three separate rats.

RESULTS

Differential gene expression in the three major salivary glands by DNA microarray analysis

To characterize the genes expressed in the three major salivary glands, we performed a DNA microarray analysis. The total gene expression profile of these salivary glands is shown in Fig. 1. All pre-normalized intensity signals from each probe were collated. There were 6211, 7250, and 7505 genes with expression values over 500, for the parotid, submandibular, and sublingual glands, respectively, and these were classified into seven groups as shown in the Venn diagram (Fig. 1a). Group D (genes expressed in the submandibular and sublingual glands) contained a large number of genes (1325 genes), whereas groups B (genes expressed in the parotid and submandibular glands) and F (genes expressed in the parotid and sublingual glands) contained fewer genes, 53 and 96 genes, respectively.

Gene ontology (GO) analyses were performed to characterize the representation of GO terms of cellular component in groups A, B, C, D, E, F, and G (Fig. 1b). The result showed that all the groups (A-F), except group G, contained the extracellular region GO terms, including the genes related to secretory proteins. All groups that included the parotid gland (groups A, B, and F), except group G, showed a higher ratio of extracellular region GO terms than the other groups.

Table 1 Primers used for real-time PCR

Gene symbol	GenBank accession	Primer	sequence (5' → 3')	Amplicon size
<i>Amy1</i>	NM_001010970	Forward	AGAGGAAACAAAGGCTTCATTG	74
		Reverse	GGAAGACCAGTCTGTAGAGTTGTTG	
<i>Cst10</i>	ENSRNOT00000007336	Forward	CCTTAGCAGGCCAATACGAG	61
		Reverse	AAAGTTCTTCCCAGACACAACC	
<i>Gpx1</i>	NM_030826	Forward	CGACATCGAACCCGATATAGA	60
		Reverse	ATGCCTTAGGGGTTGCTAGG	
<i>Muc19</i>	XM_235593	Forward	GATATCCTGGTACCACAGTCACAA	69
		Reverse	TCCCATCGGAGCCACTATTA	
<i>Psp</i>	NM_052808	Forward	TGCCCCTGGTTTTTGAAAT	70
		Reverse	AAGGAAACAGCAACGTCGAG	
<i>Tf</i>	NM_001013110	Forward	TCCAGAAGGTACCACATATGAAGA	107
		Reverse	GGAAAGTGCAGGCTTCTAGG	
<i>Ca6</i>	ENSRNOT00000051309	Forward	AGGATTCCGCCACCATCT	61
		Reverse	TGATCCATTACCGCATTCTCT	
<i>Pip</i>	NM_022708	Forward	CAATGAAGGCCAGGATAATGA	75
		Reverse	TCGTCAGGTGTACTTGGAACA	
<i>GAPDH</i>	NM_017008	Forward	TGGGAAGCTGGTCATCAAC	78
		Reverse	GCATCACCCCATTTGATGTT	

a. Venn diagram

b. GO analysis: Cellular component

Fig. 1 Gene expression profiles of rat parotid, submandibular, and sublingual glands by DNA microarray. The signals flagging absent and missing values were excluded, and the genes with the expression values over 500 after correction by GAPDH expression were included. A Venn diagram of the genes expressed in the three salivary glands is shown in **a**. Group A includes 354 genes; group B includes 53 genes; group C includes 164 genes; group D includes 1325 genes; group E includes 376 genes; group F includes 96 genes; group G includes 5708 genes. Cellular component GO analysis on these seven groups classified in **a** is shown in **b**. The ratio of the number of the GO term in the category to the number of the total GO terms in each group is shown as a pie chart. subM: submandibular, subL: sublingual.

Comparison of gene expression of salivary protein groups in the three major salivary glands

The contribution of the individual salivary glands to the composite salivary protein profile has not been established, although there have been a number of studies on salivary proteins in the whole saliva and in each salivary gland. Therefore, we compared expression among the salivary glands of genes encoding proteins that either might be secreted into saliva or important for secretion.

The expression levels of genes encoding growth factors and mucins, both of which are abundant in human and animal saliva (20, 36), are shown in Fig. 2a. It has been reported that the granular duct cells in the mouse submandibular and parotid glands are rich in nerve growth factors (19). In the present study, *Ngfg* encoding nerve growth factor (NGF) gamma was the most prominently expressed growth factor in the three salivary glands, and its expression level was similar among the three glands. The submandibular and sublingual glands expressed similar, high levels of *Muc19*, as reported previously (37). The expression of *Muc10*, which encodes mucin 10—also called submandibular gland mucin (1)—was higher in the parotid gland than in the submandibular and sublingual glands.

The expression of the genes encoding the proline-rich protein (PRP) family is shown in Fig. 2b. The genes encoding PRPs were predominantly expressed in the parotid gland, as reported previously (4), although some genes coding PRPs (*e.g.*, *PRP-2*, *Prp3*, and *XM_344035*, which encodes basic PRP) were expressed in all the three salivary glands at very similar levels.

The expression of genes encoding protease inhibitors, which include the cystatin family, is shown in Fig. 2c. Salivary SD-type cystatins are expressed in serous acini and demilunes in the human salivary glands (11), but only cystatin S is known to be a saliva-specific cystatin in rats (7). The expression of *CysS*, which encodes cystatin S and has been reported to be augmented by β -adrenergic stimulation in the rat (11), was lower than the other cystatins in all the three glands. Cystatin C, encoded by *Cst3*, is a typical cystatin that is distributed to the salivary glands and widely throughout all the organs, as well as cystatin B and cystatin 6. Expression of *Cst3*, *Cstb*, and *Cst6*, which encode ubiquitous cystatins, were similar among the three salivary glands in the rat. The highest expression of *Cst10*, which encodes cystatin 10 (also called carminerin) and contributes to chondrocyte calcification in the mouse (17), was observed predominantly in the parotid gland.

Various proteins showing enzyme activities have been identified in human and animal saliva (10). Genes encoding the salivary enzymes were highly expressed in all salivary glands (Fig. 2d). The genes encoding well-known salivary enzymes, such as amylase, deoxyribonuclease (DNase), carbonic anhydrase VI, lactoperoxidase, and ribonuclease, were predominantly expressed in the parotid gland. Genes encoding the enzymes that are not specific to saliva, such as ribonuclease P and 6, arginase, glutathione peroxidase I, and kallikrein, were expressed at similar levels in all three salivary glands.

The expression of the other secretion-related proteins is shown in Fig. 2e. Salivary proteins, including parotid secretory protein (PSP) (15); cysteine-rich secretory protein (encoded by *Crisp1* and *Crisp2*) (18); and glycoprotein 340 (encoded by *DMBT1*) (24), were expressed at higher levels in the parotid gland than in the other two glands. Albumin (encoded by *Alb*), α -2 macroglobulin (encoded by *A2m*), β -2 microglobulin (encoded by *B2m*), and glutathione-S-transferase (encoded by *Gsp1*) are produced mainly in the liver and usually detected in the blood plasma (30), and these genes showed the similar expression levels in the three salivary glands. The submandibular and sublingual glands expressed genes encoding murinoglobulins (*Mug1* and *Mug2*) and ovomacroglobulin at higher levels than the parotid gland. The genes encoding calgranulins (*S100a8* and *S100a9*), prolactin-induced protein (*Pip*), and von Ebner's gland proteins (*Vegp1* and *Vegp2*), which are all detected in human saliva (14), were mainly expressed in the parotid gland.

Differential gene expression by real-time PCR

To confirm the microarray results, the genes that encode secretory proteins and had distinct expression patterns in the microarray analysis were selected for real-time PCR analysis. The eight genes analyzed by real-time PCR were *Muc19*, which encodes mucin 19 and was expressed at the high levels both in the submandibular and sublingual glands; *Amy1* (encoding amylase) and *Psp* encoding parotid secretory protein, which are observed in abundance in human and rat saliva; *Gpx1* (encoding glutathione peroxidase 1), the level of which may reflect oxidative stress due to diabetes or drug addiction (2); *Tf*, which encodes transferrin and is secreted from the rat parotid acinar cells (22); *Ca6* (encoding carbonic anhydrase VI); *Pip* (14); and *Cst10*. *GAPDH* expression was used as an internal standard, and the expression of each gene was represented relative to *GAPDH* expression. The results from the real-time

Fig. 2 Comparison of the gene expression in the parotid, submandibular, and sublingual glands on growth factors and mucins (a), proline-rich proteins (b), protease inhibitors (c), enzymes (d), and the other secretion-related proteins (e). Data were indicated as log₂ value of the intensity after correction with *GAPDH* expression. The corrected average of *GAPDH* was 12.0. Boolean flags “IsPosAndSignif”, which indicate the mark if the mean signal of a feature is greater than the corresponding background and if this difference is significant, were used to pick up genes for the analysis. White columns represent the sublingual gland, striped columns represent the submandibular gland, and black columns represent the parotid gland.

C

Protease inhibitors

PCR were compared with the results from the DNA microarray analysis of one rat (Fig. 3). Relative expression of *Cst10*, *Amy1*, *Psp*, *Tf*, *Ca6*, and *Pip* was higher in the parotid gland than in the submandibular and sublingual glands, whereas *Muc19* was specifically expressed in the submandibular and sublingual glands, and the expression of *Gpx1* was apparently higher in the submandibular glands than in the other glands. The results from the real-time PCR were, in the aggregate, consistent with the results from the DNA microarray analysis, although the relative expression levels were not the same between the results from the two methods.

DISCUSSION

There are a number of studies on the expression and distribution of proteins in the parotid, submandibular, and sublingual glands (4, 10, 11, 14, 20, 24). However, there are few reports that simultaneously compare gene expression in the three major salivary glands, although the gene expression of human parotid and submandibular glands has been compared for tumor incidence in a previous study (32). In this investigation, we analyzed gene expression in the rat salivary glands using DNA microarrays and assessed the microarray results using real-time PCR. The results on eight genes from the real-time PCR were largely consistent with the results from the microarray analysis (Fig. 3); therefore, the results on the large number of genes in the DNA microarray analysis may be useful in comparing gene expression in the three salivary glands.

It has been reported that the submandibular gland is a mixed gland with serous and mucous acini, the sublingual gland contains mainly mucous acini, and the parotid gland is composed of only serous acini (13). Our results suggested that the gene expression profiles of the submandibular and sublingual glands were very similar and that the profile of the parotid gland was considerably different from the profiles of the submandibular and sublingual glands (Fig. 2).

Salivary mucins are the component that make saliva viscous and are generally known to be secreted from the submandibular and sublingual glands. Our investigation revealed that large numbers of mucin genes were also highly expressed in the parotid gland, although only *Muc19* (encoding mucin 19), which showed the highest expression among the mucins investigated, was much more highly expressed in both the submandibular and sublingual glands than in the parotid gland. Mucins encoded by *MUC2*, *MUC5AC*, *MUC5B*, *MUC6*, and *MUC7* are

known as the secreted mucin in human saliva. The genes of mucin 5ac and 5b (*Muc5ac* and *Muc5b*), as well as *Muc6*, were expressed in all three rat salivary glands, but mainly in the parotid gland. These results indicated that a number of mucins may be expressed not only in the mucous cells but also in the serous cells. *Muc10* was expressed in the rat parotid gland, whereas the protein mucin 10 has not been detected in human saliva as well as mucin 19 (1). In the rodent salivary glands, *Muc10* may be expressed specifically in the parotid gland, and *Muc19* specifically in the submandibular and sublingual glands.

It has been reported that saliva contains various growth factors (36) and that the submandibular gland secretes NGF and transforming growth factor (TGF) β (28). In our results, all three salivary glands expressed the genes encoding TGF, epidermal growth factor (EGF), and NGF, and the expression of these genes was highest in the parotid gland. The submandibular gland is the largest salivary gland in rodents and humans; therefore, it may produce and secrete the largest amount of growth factors.

It has been reported that PRPs are secreted from the parotid gland (4). However, our results revealed that the submandibular and sublingual glands also expressed the basic PRP gene and several other types of PRP genes, indicating that PRPs are not specific to the parotid gland. Proline-rich proteoglycans (6) were much more highly expressed in the parotid gland than the other glands, and their expression may be specific to the parotid gland.

The ubiquitous cystatins, *Cstb*, *Cst3*, and *Cst6* (encoding cystatin E/M), were expressed in all the three glands at very similar levels, consistent with previous results (11). *Cst10* which has been reported in developing cartilage of the mouse but not in humans (17) was expressed at the high levels mainly in the rat parotid gland. This is the first report showing *Cst10* expression in the soft tissues, but the expression and the role of carminerin encoded by *Cst10* in the parotid gland is unknown. The gene *Cyss*, which encodes cysteine S and has been reported to be produced in response to β -adrenergic stimulation (7), was expressed at low levels in all three glands. Further examination is required to determine in which gland(s) *Cyss* is up-regulated in response to β -adrenergic stimulation.

The parotid gland contributed to the expression of almost all the genes encoding salivary-specific enzymes that were investigated. Specifically, the genes encoding amylase, DNase, and carbonic anhydrase

Fig. 3 Comparison of gene expression of secretory and secretion-related proteins among the parotid (black columns), submandibular (striped columns), and sublingual glands (white columns) of rats by real-time PCR analysis (right) and DNA microarray (left). The bar graphs represent expression relative to the GAPDH standard. Statistical analyses of the differences between the relative expressions in the salivary glands were evaluated by ANOVA. ($n = 3$, for the real-time PCR; * $P < 0.05$; ** $P < 0.01$).

VI were expressed in the parotid gland, while the genes encoding lactoperoxidase, glutathione peroxidase, kallikrein, and lysozyme (none of which are salivary specific) were expressed at similar levels in all the three glands, including the parotid gland. It is well-known that amylase and carbonic anhydrase VI are mainly secreted from the serous cells of the parotid glands, but the origins of the other enzymes have not been established. Further investigation of the expression of these enzymes is required.

Genes encoding proteins that could not be classified into any of the categories described above were also investigated. The gene encoding ovomacroglobulin, which is an $\alpha 2$ -macroglobulin-like protease inhibitor, was expressed in the submandibular and sublingual glands. *Crisp1* encoding a member of the

cysteine-rich secretory proteins (CRISPs), which may play a role in the innate immune system (18), and *PSP*, which encodes a bacteria-agglutinating peptide (15), were mainly expressed in the parotid gland. The genes encoding some blood proteins were expressed at similar levels in all the salivary glands. Specifically, the genes encoding $\beta 2$ -microglobulin, which is reported to occur at higher density in saliva of patients with Sjögren's syndrome (21); $\alpha 2$ -macroglobulin, which is a protease inhibitor (3); and transferrin, which is an iron-binding protein, were highly expressed in all three glands. These proteins may not originate from blood but rather produced locally in the salivary glands. Further investigation for examination of this possibility is required. *Lfn* coding lactoferrin was expressed only at low levels

in rat salivary glands, which is interesting given that it has an antibacterial effect in the oral cavity of human (33).

Our investigation revealed that a large number of genes encoding secretory proteins were expressed mainly in the parotid gland and that some secretory proteins were highly expressed in both the submandibular and sublingual glands with low parotid gland expression. Our results suggest that the analysis of salivary proteins may be useful in distinguishing the disorders of the parotid gland from those of the submandibular and sublingual glands. In contrast, analysis of salivary proteins may not be useful in distinguishing the disorders of the submandibular gland from those of the sublingual glands.

The investigation of proteins in human saliva (14) demonstrates that many proteins encoded by the genes analyzed in our investigation are also detected in human saliva; therefore, these proteins may be useful as markers for the different salivary glands in humans. However, there are some genes which are highly expressed but are known not to be translated into proteins. For example, the expression of mucin 19 has not been confirmed in human saliva, whereas the encoding gene *Muc19* has been detected in the human submandibular gland (8). Detection of genes in saliva may be useful in assigning specific transcripts to each salivary gland rather than specific proteins. Further examination will be required to determine whether the gene expression profiles of the human salivary glands correspond to the appearance of proteins in human saliva.

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