

Age-related changes in IGF-1 expression in submandibular glands of senescence-accelerated mice

Seiko Kobayashi[§], Yoshikazu Kamino^{†,‡}, Koichi Hiratsuka^{§,‡},
Michiko Kiyama-Kishikawa^{§,‡} and Yoshimitsu Abiko^{§,‡}

Departments of [§]Biochemistry and [†]Oral Surgery, and [‡]Research Institute of Oral Science,
Nihon University School of Dentistry at Matsudo, Matsudo, Chiba, Japan

(Received 9 December 2003 and accepted 8 April 2004)

Abstract: Saliva is known to play important roles in such functions as swallowing, mastication, speech, and taste. Furthermore, salivary glands synthesize and secrete a number of growth factors involved in cell/tissue homeostasis. It has been demonstrated that IGF-1, which is structurally analogous to insulin, has been shown to be expressed in mouse submandibular glands, and that IGF-1 stimulates DNA synthesis, amino acid uptake, protein synthesis, and glucose transport in various cells. Diminished function of the salivary glands is thought to lead to increased dental caries and periodontal diseases, which are commonly associated with aging. However, very little is known regarding the effects of age on IGF-1 expression in submandibular glands. The senescence-accelerated mouse (SAM), an experimental murine model of accelerated aging, has been extensively used to examine the mechanisms responsible for aging. In the present study, IGF-1 production and mRNA levels in the submandibular glands of SAM-P1 mice were examined. IGF-1 levels were determined by radioimmunoassay and IGF-1 mRNA levels by semi-quantitative RT-PCR. We found that IGF-1 protein levels in homogenates and IGF-1 mRNA levels decreased with age in SAMP1 mice. These findings suggest that IGF-1 synthesis in submandibular glands decreases with aging, and this may result in lower levels of cellular proliferation, regeneration and wound

healing in aged oral tissues. (*J. Oral Sci.* 46, 119-125, 2004)

Key words: senescence-accelerated mouse; aging; submandibular gland; insulin-like growth factor-1.

Introduction

The protein constituents of saliva have a number of biological functions that are intimately involved in the maintenance of oral health. These include lubrication of soft and hard tissues of the oral cavity, remineralization of enamel, initial digestion of ingested foodstuffs, antimicrobial activity for the control of infectious agents, and maintenance of integrity (1). Furthermore, biologically active proteins implicated in the maintenance of mucosal integrity and oral wound healing specificity include growth factors present in saliva (2,3), while a great number of biologically active polypeptides, have been purified or claimed to be present in the submandibular glands of mice and other species, including NGF, EGF, somatostatin (4), TGF (5), FGF (6), and IGF (7).

IGF-1 originates from local synthesis in submandibular glands (8) and is thought to play important roles in stimulation of DNA synthesis, amino acid uptake, protein synthesis, and glucose transport in a variety of tissues (9,10). Furthermore, gene expression of IGF-1 in granular convoluted tubule cells of submandibular glands has been shown using Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) methods, and expression levels are known to progressively increase in those glands during postnatal development (11).

It is common knowledge that wounds heal more slowly

Correspondence to Dr. Yoshimitsu Abiko, Department of Biochemistry and Research Institute of Oral Science, Nihon University School of Dentistry, 2-870-1 Sakaecho-nishi, Matsudo, Chiba 271-8587, Japan
Tel: +81-47-360-9328
Fax: +81-47-360-9329
E-mail: yabiko@masc.nihon-u.ac.jp

in aged tissues (12). It has also been reported that younger alveolar tissues begin progressive healing and osteoid formation whereas older tissues remain in a resting phase following tooth extraction (13). Reductions in IGF-1 mRNA levels have also been reported in several tissues, such as the liver, muscle, and brain (14). However, very little is known regarding IGF-1 expression in the submandibular glands in association with development or aging.

The senescence-accelerated mouse (SAM) was developed through selective inbreeding of the AKR/J strain (15), and there are currently 15 strains; 10 that are accelerated senescence-prone, short-lived (SAMP) and 5 that are accelerated senescence-resistant, long-lived (SAMR) strains. The degree of senescence as well as histopathological observations, in addition to growth patterns of body weight and age when reproductive capability is attained, all suggest that a characteristic aging feature common to all SAMP strains is the rapid advancement of senescence. Furthermore, all SAM strains represent a unique model to study the aging process in higher organisms (16).

The recent advent of microarray technology allows thorough analysis of gene expression patterns under different environmental conditions (17). In this approach, individual DNA probes are arrayed on a small glass surface, and labeled first-strand cDNA from specific tissue or cell sources is then hybridized onto the array. The amount of fluorescence at each DNA probe spot is correlated to the abundance of specific mRNA transcripts in the cells. DNA microarray technology has recently been utilized for research in the field of gerontology and is considered to be useful (18,19).

We previously analyzed gene expression changes in the submandibular glands of 1- and 12-month-old SAMP1 mice using a cDNA microarray, and found that IGF-1 gene expression at 12 months was lower than that at 1 month (20). In the present study, we analyzed changes in mRNA levels of IGF-related genes based on cDNA microarray analysis results. Furthermore, IGF-1 mRNA levels in submandibular glands from neonatal, adult, and aged SAMP1 mice were determined using real-time PCR, and phenotypic expression changes in IGF-1 levels were also examined.

Materials and Methods

Animals

SAMP1 mice were kindly provided by the Council for SAM Research, Institute for Frontier Medical Sciences, Kyoto University, and were bred under conventional conditions with free access to food and water. For cDNA

microarray analysis, 1- and 12-month-old male mice were used. For real-time PCR and ELISA experiments, 1-, 5- and 9-month-old male mice were used as neonatal, adult and aged models, respectively.

Preparation of submandibular glands

Mice were killed under ether anesthesia, and the submandibular glands were removed and homogenized with 0.025 M sucrose buffer. The homogenate was centrifuged at $30,000 \times g$ for 30 min at 4°C. Supernatant fractions of cell-free extracts were used for radioimmunoassay (RIA) examination of IGF-1. For RNA extraction, glands were homogenized in guanidine thiocyanate-phenol-chloroform solution, as previously reported (21).

Measurement of IGF-1

Amounts of IGF-1 in submandibular glands from SAMP1 mice were determined by radioimmunoassay, using a commercially available kit containing ^{125}I -labeled IGF-1 as a tracer (Amersham, Arlington Heights, IL). Radioactivity levels were determined with a γ -well counter (Aloka, Tokyo, Japan).

Gene expression monitoring by cDNA microarray

cDNA microarray hybridization was used to identify temporal transcription changes in the submandibular glands and mRNA from the glands was used to synthesize cDNA labeled with Cy3-deoxyuridine triphosphate (dUTP) or Cy5-dUTP. These labeled-cDNA probes were mixed and simultaneously hybridized to a UniGEM V human cDNA microarray bearing 7,276 different genes (Incyte Genomics, St. Louis, MO), where they competitively reacted with the arrayed cDNA copies.

Endpoint RT-PCR

RNA was isolated using an acid guanidinium thiocyanate-phenol-chloroform extraction method (21). cDNA synthesis was carried out using a Superscript II RnaseH (-) reverse transcriptase system (Invitrogen) with oligo d(T)₁₂₋₁₈ primer at 42°C for 1 hour.

PCR was carried out using a Gene Amp PCR reagent kit (Applied Biosystems, USA) as instructed by the manufacturer under the following conditions; 34 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds. DNA primers for the nucleotide sequences of mouse IGF-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were evaluated using Primer 3 design software (http://www.frod.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The PCR primers used were as follows; $^5\text{-ATCACCATCTTCCAGGAG-}^3$ (forward primer for GAPDH); $^5\text{-ATGGACTGTGGTCATGAG-}^3$

(reverse primer for GAPDH); expected size, 318 bp, 5'-CTGGACCAGAGACCCTTTGC-³ (forward primer for IGF-1); 5'-AGAGCGGGCTGCTTTTGTAG-³ (reverse primer for IGF-1), expected size, 218 bp. For each primer set, PCR thermal-cycle conditions were optimized to achieve a single-band PCR product on 1.5% agarose gel electrophoresis with ethidium bromide staining.

Real-time PCR analysis

Two-step quantitative detection of IGF-1 mRNA levels was carried out using an OPTICON™ DNA Engine (MJ Research INC., MA). Twenty micrograms of total RNA was reverse-transcribed using Superscript II (Gibco BRL, Gaithersburg, MD). PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen, Chatsworth, CA), with amplification reactions performed in a final volume of 20 µl containing 10 µl of 2 × QuantiTect SYBR Green PCR Master Mix, 0.25 µM of IGF-1-specific primers, and 5 µl of 500-fold diluted cDNA solution. To reduce variability between replicates, PCR premixes, which contained all reagents except cDNA, were prepared and aliquoted into 0.2-ml thin-wall strip tubes (MJ Research., MA). DNA primers and thermal cycling conditions were the same as used for endpoint RT-PCR. Experiments were performed in quadruplicate for each data point.

Results

Changes in the gene expression of IGF-related genes in SAMP1 submandibular glands from 1- and 12-month-old mice were determined by cDNA microarray. Of the 11 IGF-related genes spotted on the microarray, only mouse IGF-1 mRNA showed a more than 2-fold change (Fig. 1, Table

1). Furthermore, mRNA of the IGF-1 gene in 12-month-old mice exhibited lower expression than that in 1-month-old mice.

Next, submandibular glands isolated from 1-, 5-, and 9-month-old SAMP1 mice and IGF-1 gene expression levels were compared using endpoint RT-PCR. As shown in Fig. 2, a 218-bp expected band for the IGF-1 gene was observed, and the DNA band intensity at 1 month was highest, while

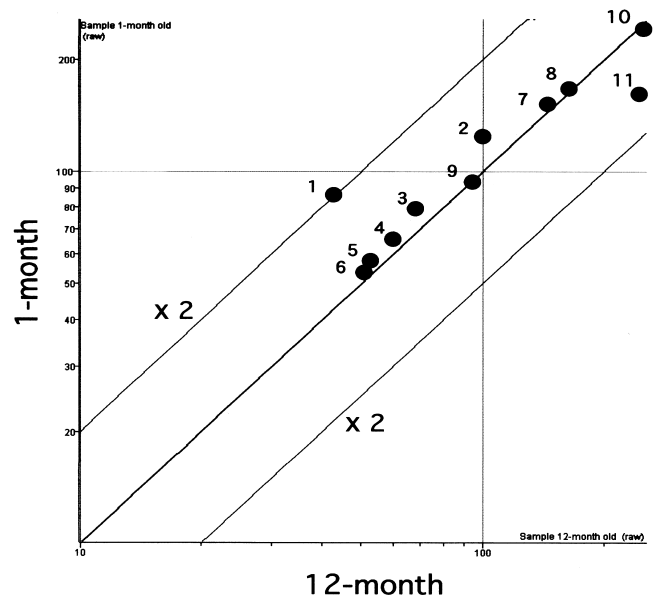


Fig. 1 Scatter plot of IGF-related gene expression in SAMP1 submandibular glands. For each gene, mRNA levels in 1- and 12-month-old SAMP1 are given on the x-axis and expression levels for the same gene in elderly mice are plotted on the y-axis. Two-fold change in expression level.

Table 1 Microarray analysis of IGF related genes

Gene ID	Ratio (1M/12M)	Gene description
1 W10072	2.00	IGF-1 (mouse)
2 W89253	1.25	IGF binding protein 5 (mouse)
3 AA198560	1.15	IGF binding protein 3 (human)
4 W83086	1.12	IGF binding protein (mouse)
5 AA277831	1.10	IGF-1A (human)
6 AA423061	1.06	IGF binding protein complex acid labile (human)
7 AA241784	1.04	IGF binding protein 5 (mouse)
8 AI322387	1.00	IGF-2 (human)
9 AA049649	0.98	IGF binding protein complex acid labile (human)
10 AA387288	0.98	IGF-1A (human)
11 AA028475	0.65	IGF-2 (mouse)

The mRNA level changes are expressed as ratio of intensity from 1-month / 12-month old mice, Ratio (1M/12M), by cDNA microarray analysis. Each gene ID number is shown as closed dot in Fig. 1.

that at 9 months was lowest. In contrast, the 318-bp band for the housekeeping gene GAPDH had a similar intensity in 1-, 5- and 9-month-old SAMP1 mice.

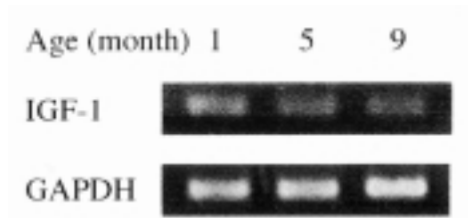


Fig. 2 Measurement of IGF-1 mRNA levels by endpoint RT-PCR. The ethidium bromide staining pattern of amplified PCR products on agarose gel electrophoresis is shown. The RT-PCR method and DNA primers used are described in Materials and Methods. The GAPDH gene was used as a control.

Because it is difficult to quantitatively analyze mRNA levels using endpoint RT-PCR when copy number is low, further experiments to determine mRNA levels of IGF-1 were performed using real-time PCR. Figure 3 shows fluorescence intensity curve of real-time PCR analysis of RNA standards and the IGF-1 mRNA sample (Fig. 3A). The calibration curve for fluorescence signal against the cycle is shown in Fig. 3B. The melting curve profiles of PCR end products of IGF-1 are shown in Fig. 3C; all samples showed a single peak with the same characteristic melting curves. The melting curve profiles of GAPDH mRNA also appeared as a single peak (data not shown). These results suggest that fluorescence acquisition in each cycle of the amplification program eliminated nonspecific fluorescence signals and ensured an accurate quantification of the desired gene product. Furthermore, standard curves for IGF-1 and GAPDH mRNA levels were constructed by

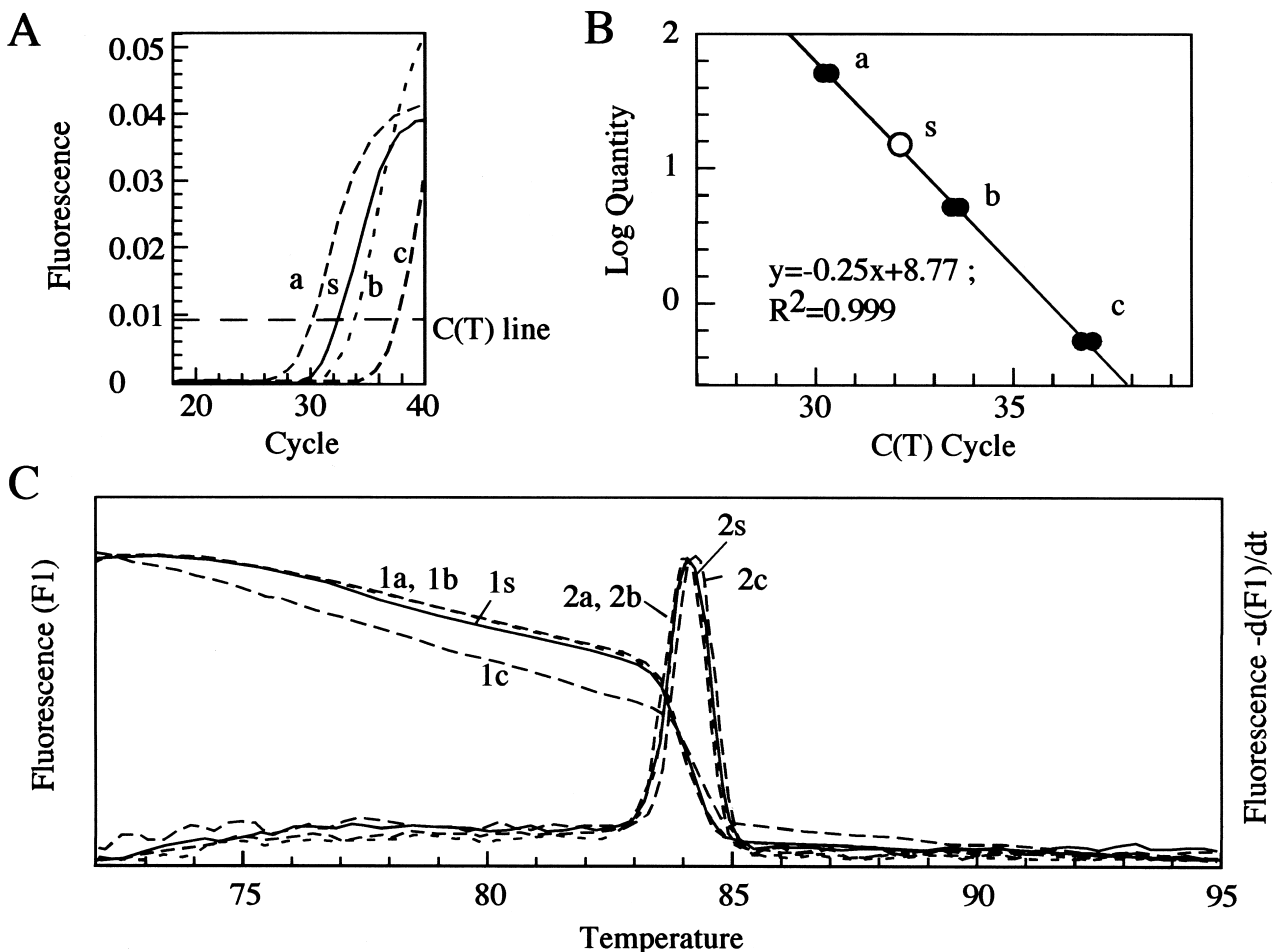


Fig. 3 Measurement of IGF-1 mRNA levels by real-time PCR. (A), Plot of fluorescence against cycle number. (B) Standard curve. Template solution was serially diluted and cDNA equivalent to 50 ng (a), 5 ng (b), and 0.5 ng (c) of total RNA was used as the template for quantification; S indicates the sample RNA. The threshold limit was set above the noise signal. (C) Melting curve profiles of PCR end product. External copy number standards and RNA sample from submandibular gland were used in the same plates. All PCR end products showed a single peak with same melting temperature, and no background products are observed.

plotting the crossover point against the number of standard copies. The number of target copies in each sample was then calculated automatically with reference to this curve.

The normalized IGF-1 values against GAPDH expression level are shown as ratios in Fig. 4. IGF-1 mRNA levels at 1 month were highest and those at 9 months were lowest, and the differences between the results after 1, 5 and 9 months were significant. The ratio of IGF-1 mRNA levels relative to those of GAPDH at 1, 5 and 9 months were 100, 68.1 and 45.6%, respectively. These results clearly demonstrate that IGF-1 gene expression levels in the developmental stage were high, and that they reduced with age.

The phenotypic expression of IGF-1 in submandibular glands at 1, 5 and 9 months was also examined. As shown in Fig. 5, the reduction of IGF-1 protein levels confirmed a significant effect of development and aging. The amount of IGF-1 in gland homogenates was highest in 1-month-old SAMP1 mice and this amount decreased significantly with age (lower by 52.9% and 42.1% at 5 and 9 months, respectively, $P < 0.01$).

Discussion

The SAM strain of mice was developed by the Department of Senescence Biology, Kyoto University by selective inbreeding of the AKR/J strain of mice, based on a graded score for senescence, life span, and pathologic phenotype. There are 10 strains of SAM mice, including SAMP1 (15). In the course of development, it was shown that SAMP strains manifest various pathologic phenotypes that are sufficiently characteristic to differentiate them from other SAM strains (16). For example, SAMP1 shows accelerated senescence coupled with a short lifespan and general aging characteristics as a genetic trait, and has been widely used in gerontological research.

We selected the SAMP1 strain as an experimental model to examine the effects of aging on IGF-1 expression in submandibular glands. We determined senescence grading scores during breeding in our animal house and categorized the SAMP1 neonatal, adult and aged stages at 1, 5 and 9 months of age, respectively. Previously, we analyzed changes in the expression of 6,500 genes in SAMP1 submandibular glands that occurred between 2 and 15 months of age using Affymetrix gene chip analysis (22). However, because IGF-1 mRNA levels were low in 15-month-old SAMP1 mice, the results of that gene chip analysis showed IGF-1 gene expression as absent due to low copy number. The number and kind of gene probes incorporated in the Affymetrix gene chip and Incyte DNA microarray (7,300 genes) analyses differ (for example, the Incyte DNA microarray contains more IGF-related

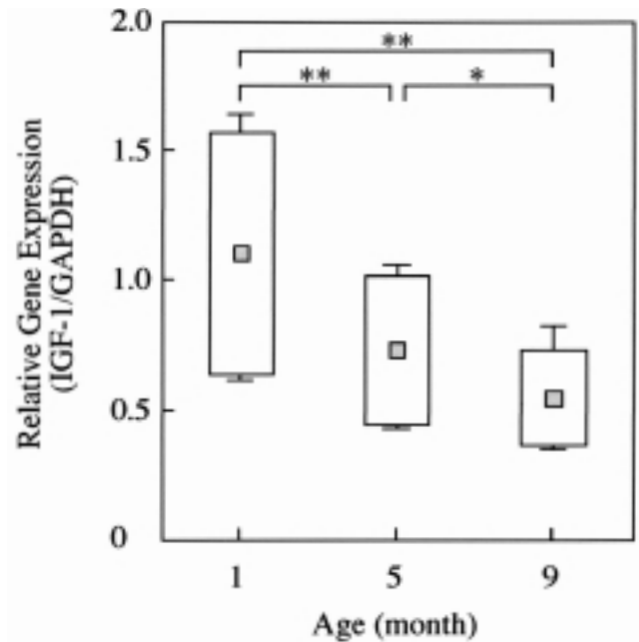


Fig. 4 Comparison of IGF-1 mRNA levels in SAMP1 submandibular glands. Total RNA was isolated from submandibular glands of SAMP1 mice of different ages. IGF-1 mRNA levels were calculated as the ratio of IGF-1 mRNA levels to GAPDH mRNA levels from 3 different experiments. Results represent the means (open square) \pm SD (box) of relative expression; * $P < 0.01$, ** $P < 0.05$.

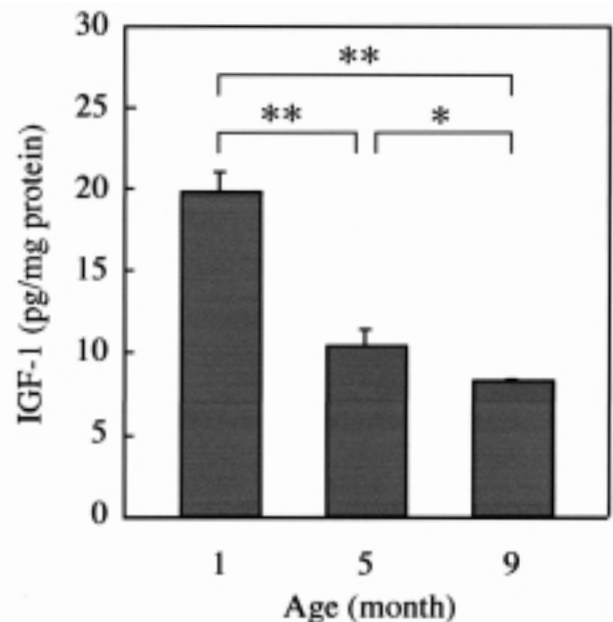


Fig. 5 Comparison of IGF-1 amounts in SAMP1 submandibular glands. Cell-free extract was isolated from submandibular gland homogenates of SAMP1 mice of different ages. Amount of IGF-1 was measured as described in Materials and Methods. Values (means \pm SE) were determined from 3 different SAMP1 submandibular glands; * $P < 0.01$, ** $P < 0.05$.

genes than the Affymetrix gene chip), and thus we analyzed the changes in gene expression using the Incyte Mouse DNA microarray in this study.

In the present study, mRNA levels of IGF-1 related genes in 1- and 12-month-old SAMP1 submandibular gland were examined using the Incyte Mouse cDNA microarray. Among these genes, only IGF-1 mRNA showed significant changes; IGF-1 expression was 2-fold higher at 1 month when compared to that at 12 months. We therefore focused on the IGF-1 gene to study and quantify its mRNA levels in submandibular glands at the neonatal, adult, and aged stages using endpoint RT-PCR and real time PCR assays. Furthermore, IGF-1 phenotypic expression in SAMP1 submandibular glands was also determined at these different stages. Our results provide clear evidence that IGF-1 protein and mRNA levels were highest at the neonatal stage and then significantly declined with age.

Previous studies of other rodent strains in addition to non-human primates and humans have found that the amplitude of growth hormone pulses decreases with age, resulting in a decline in plasma levels of IGF-1 (23); however, IGF-1 infusion improved nitrogen balance in dietary-restricted, catabolic humans (24). The therapeutic potential of recombinant human IGF-I treatment is being investigated in various growth hormone-resistant and insulin-resistant disorders and IGF-I may be substituted for growth factor in order to promote linear growth in children with growth hormone insensitivity (25). In addition, IGF-1 administration in aged rats was shown to increase cardiac heavy chain myosin and restore aortic elastin levels, thus suggesting that it may affect specific proteins that decline with age (23). On the other hand, Amano et al. (26) demonstrated that IGF-1 levels increased proportionally with the postnatal development of GCT cells in rat submandibular glands and that IGF-1 mRNA was also expressed at much lower levels throughout the acinar and duct systems, irrespective of age.

In the present study, IGF-1 mRNA levels in SAMP1 submandibular glands were shown to decrease with age using a DNA microarray, endpoint RT-PCR and real-time PCR analysis. Furthermore, IGF-1 phenotypic expression was also reduced in submandibular gland homogenates of SAMP1. These findings suggest that IGF-1 gene expression and production in submandibular glands in SAMP1 decrease with age.

Further studies are needed to evaluate IGF-1 expression in different strains of mice, as well as in other animals and humans. In recent years, gene therapy and tissue engineering have captured the imagination of the general public, and both offer the potential for treating clinical conditions that are now considered impossible or extremely

difficult to manage by conventional therapeutic measures. Applications of gene therapy and tissue engineering to salivary glands have been developed with a focus on the repair of irreversible gland damage in experimental models (27). Furthermore, salivary glands have been presented as an interesting and suitable target organ for gene therapeutic applications (28). Effective and safe methods of IGF-1 gene transfer to aged submandibular glands may help to recover physiologic roles in the oral cavity.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (B2-13470395) from the Japan Society for the Promotion of Science, and a Grant from the Ministry of Education, Culture, Sports, Science, and Technology to promote multidisciplinary research projects.

References

1. Fox PC (1989) Saliva composition and its importance in dental health. *Compend Contin Educ Dent* 13, S456-S460
2. Herrera JL, Lyons II MF, Johnson LF (1988) Saliva: Its role in health and disease. *J Clin Gastroenterol* 10, 569-578
3. Zelles T, Purushotham KR, Macauley SP, Oxford G E, Humphreys-Beher, MG (1995) Saliva and growth factors: the fountain of youth resides in us all. *J Dent Res* 74, 1826-1832
4. Barka T (1980) Biologically active polypeptides in the submandibular gland. *J Histochem Cytochem* 28, 836-859
5. Yeh YC, Guh JY, Yeh HW (1989) Transforming growth factor type alpha in normal human adult saliva. *Mol Cell Endocrinol*, 67, 247-255
6. Hiramatsu Y, Kagami H, Kosaki K, Shigetomi T, Veda M, Kobayashi S, Sakanaka M (1994) The localization of basic fibroblast growth factor (FGF-2) in rat submandibular glands. *Nagoya J Med Sci* 57, 143-152
7. Costigan DC, Guyda HJ, Posner B (1988) Free insulin-like growth factor I (IGF-1) and IGF-II in human saliva. *J Clin Endocrinol* 66, 1014-1018
8. Ryan J, Mantle T, McQuaid S, Costigan DC (1992) Salivary insulin-like growth factor I originates from local synthesis. *J Endocrinol* 135, 85-90
9. Melvin HA (1993) Age Factor in Human Alveolar Repair. *J Oral Implant* 140, 138-142
10. Zapf J, Froesch E, Humbel R (1981) The insulin-like growth factors (IGF) of human serum: chemical and biological characterization and aspects of their physiological role. *Curr Top Cell Regul* 19, 257-309

11. Amano O, Iseki S (1993) Expression, localization and developmental regulation insulin-like growth factor I mRNA in rat submandibular gland. *Arch Oral Biol* 38, 671-677
12. Goodson WH, Hunt TK (1979) Wound healing and aging. *J Invest Dermatol* 73, 88-91
13. Melvin HA (1993) Age Factor in Human Alveolar Repair. *J Oral Implant*, 140, 138-142
14. Ferber AC, Chang C, Sell C, Ptasznik A, Cristofalo VJ, Hubbard K, Ozer HL, Adamo M, Roberts CT, LeRoith D, Dumenil G, Baserga, R (1993) Failure of senescent human fibroblasts to express the insulin-like growth factor-I gene. *J Biol Chem* 268, 17883-17888
15. Takeda T, Hosokawa M, Takeshita S, Irino M, Higuchi K, Matsushita T, Tomita Y, Yasuhira K, Hamamoto H, Shimizu K, Ishii M, Yamamuro T (1981) A new murine model of accelerated senescence. *Mech Ageing Dev* 17, 183-194
16. Takeda T, Hosokawa M, Higuchi K (1997) Senescence-accelerated mouse (SAM): a novel murine model of senescence. *Exp Gerontol.* 32, 105-109
17. Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, 270, 467-470
18. Helmborg A (2001) DNA-microarrays: novel techniques to study aging and guide gerontologic medicine. *Exp Gerontol* 36, 1189-1198
19. Kirschner M, Pujol G, Radu A (2002) Oligonucleotide microarray data mining: search for age-dependent gene expression. *Biochem Biophys Res Commun* 298, 772-778
20. Hayakawa M, Kamino R, Hiratsuka K, Kobayashi S, Abiko Y (2003) DNA microarray analysis of growth factor gene expressions in mouse submandibular gland by ageing. *Med Biol* 145, 93-96
21. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1162, 156-159
22. Hiratsuka K, Kamino Y, Nagata T, Takahashi Y, Asai S, Ishikawa K, Abiko Y (2002) Microarray analysis of gene expression changes in aging in mouse submandibular gland. *J Dent Res* 81, 679-682
23. Sonntag WE, Lehan JE, Ingram RL (1992): Effects of Aging and dietary restriction on tissue protein synthesis: relationship to plasma insulin-like growth factor-1, *J Gerontology* 47, B159-B163
24. Clemmons DR, Underwood LE (1994) Uses of human insulin-like growth factor-I in clinical conditions. *J Clin Endocrinol Metab* 79, 4-7
25. Bondy CA (1994) Clinical uses of insulin-like growth factor-I. *Ann Int Med* 120, 593-601
26. Amano O, Iseki S (1993) Expression, localization and developmental regulation insulin-like growth factor I mRNA in rat submandibular gland, *Arch Oral Biol* 38, 671-677
27. Baum BJ (2000) Prospects for re-engineering salivary glands. *Adv Dent Res* 14, 84-88
28. Hoque AT, Yamano S, Baccaglioni L, Baum BJ (2001) Using salivary glands as a tissue target for gene therapeutics. *J Drug Target* 9, 485-494