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Expression of BMP7 is associated with resistance to diabetic stress: Comparison among mouse salivary glands

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Abstract

We determined mRNA levels of bone morphogenetic protein 7 (BMP7), a growth and differentiation factor belonging to the transforming growth factor-β superfamily, in the salivary glands of mice with streptozotocin (200 mg/kg, i.p.)-induced diabetes. We also examined the effects of BMP7 on secretion of saliva and degenerative change in salivary glands in diabetic mice. In normal mice, BMP7 mRNA levels were high in the submandibular gland and low in the parotid gland, while in diabetic mice, levels were significantly decreased in the parotid gland, but not in the submandibular gland. No significant difference was observed in mRNA levels of BMP receptors between normal and diabetic mice. In diabetic mice, pilocarpine (4 mg/kg, i.p.)-stimulated salivary secretion showed a remarkable decrease in both parotid and submandibular gland, although degree of reduction was smaller in the latter. Notable degeneration with vacuolation and atrophy was also found in parotid gland, whereas degeneration of submandibular gland was slight. Administration of BMP7 (50 and 100 µg/kg, i.v.) in diabetic mice induced a significant increase in salivary secretion, with rate of recovery higher in parotid gland than in submandibular gland. In diabetic mice, BMP7 also exhibited a powerful protective effect in degenerated salivary gland, especially in parotid gland. These results suggest that BMP7 acts to prevent diabetic damage in salivary gland, and that its cytoprotective effect is closely correlated with mRNA levels in tissue.

Keywords: Bone morphogenetic protein 7 (BMP7); BMP receptor; Diabetes; Salivary gland; Real time quantitative RT-PCR
1. Introduction

Bone morphogenetic proteins (BMPs), of which more than 20 have been discovered to date, are secreted differentiation factors composing a subgroup of the transforming growth factor-β (TGF-β) superfamily. They form homo- and heterodimers and transduce intracellular signals through complexes of serine/threonine kinase type I and II receptors (Massague and Chen, 2000; Massague and Wotton, 2000; Wang et al., 2001). The type I receptors, also known as activin receptor-like kinases (ALKs), have been identified in vertebrates. ALK3 exclusively binds BMPs (Chen et al., 1998). ALK2 binds activins and TGF-βs in vitro. However, recent data demonstrate that it also has a physiological function as a type I receptor in BMP signaling (ten Dijke et al., 1994; Zhang et al., 2003). The presence of BMPs has been confirmed in a variety of organs and tissues such as bone and kidney (Li and Wozney, 2001; Simic and Vukicevic, 2005), and their function in embryonic development, regulation of cell differentiation, specification of mesoderm-derived cells, and tooth morphogenesis has been investigated (Hogan, 1996; Setoguchi et al., 2001; Plikus et al., 2005).

BMP7, also known as osteogenic protein-1, has been reported to be involved in the development of the kidney, nervous system, skeletal muscle, and eyes (Dudley et al., 1995; Luo et al., 1995; Arkell and Beggington, 1997; Furuta and Hogan, 1998; Gupta et al., 2000; Zhang et al., 2003). In the kidney, BMP7 is primarily expressed in ureteric bud epithelium and mesenchymal cells, while a decrease in BMP7 expression is found in patients with end-stage renal disease (Klahr et al., 2002). BMP7 has been reported in salivary gland and is involved in the development of embryonic branching in the submandibular gland (Jaskoll et al., 2002; Hoffman et al., 2002). The transcripts for BMP7 are mediated via its specific receptor, ALK2, and non-specific receptor, ALK3 (Maric et al., 2003).
An earlier study demonstrated that the submandibular gland was more strongly resistant to diabetic stress than the other salivary glands (Watanabe et al., 2001). These results suggested differences in sensibility, producibility, or expression of BMP7 in salivary gland. BMP7 has a cytoprotective effect in the kidney and lacrimal gland analogous to the morphogenesis of the salivary gland (Dudley and Robertson, 1997; Dudley et al., 1999; Jaskoll et al., 2002; Lund et al., 2002; Wang et al., 2003; Dichmann et al., 2003). However, the cytoprotective effects of BMP7 remain to be sufficiently demonstrated in the salivary gland.

In this study, we investigated the mRNA expression of BMP7 and receptors ALK2 and ALK3 in salivary gland using real time quantitative RT-PCR. We also investigated the cytoprotective effects of BMP7 on the secretion of saliva and degenerative change in salivary gland in mice with streptozotocin-induced diabetes. To the authors’ knowledge, this is the first report to provide evidence that BMP7 is associated with resistance of salivary gland tissues to diabetic stress.

2. Materials and methods

2.1. Chemicals

Streptozotocin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Pilocarpine hydrochloride and recombinant human BMP7 were purchased from Wako Pure Chemical Industries (Osaka, Japan) and R&D Systems Inc. (Minneapolis, MN, USA), respectively. All other reagents used were of analytical grade.

2.2. Animals

Male ddY mice weighing 20 to 23 g were obtained from Japan SLC, Inc. (Hamamatsu, Japan). All animals were housed in an air-conditioned room (temperature: 21 ± 2°C; humidity: 55 ±
10%) under a 12-h light/dark cycle (lights on between 6:00 am and 6:00 pm) and maintained on commercial laboratory chow and water for at least 3 days before being used. All animals were treated within the Guidelines for the Treatment of Experimental Animals approved by The Japanese Pharmacological Society and Tokyo Dental College.

2.3. Induction of diabetes mellitus

A single intraperitoneal injection of streptozotocin at 200 mg/kg in a volume of 0.2 ml per 10 g body weight was administered in each mouse. The streptozotocin was dissolved in physiological saline. Control mice were given an equal volume of physiological saline. Two and five days after administration of streptozotocin, urinary glucose levels were determined using test strips of Uropaper II (Eiken, Tokyo, Japan). Mice that tested negative were excluded from the experiment. Fourteen days after administration of streptozotocin, blood glucose levels were further measured with the Glucose CII-test Wako (Wako Pure Chemical Industries, Osaka, Japan). Mice with blood glucose levels of >700 mg/dl were used in the experiment.

2.4. Administration of BMP7

The diabetic mice were given an intravenous injection of BMP7 at 50 and 100 µg/kg in a volume of 0.1 ml per 10 g body weight on days 7, 9, 11, and 13 after administration of streptozotocin. BMP7 was dissolved in physiological saline. Control mice were given an equal volume of physiological saline. Collection of saliva and excision of the salivary gland for histological examination were performed 24 h after the last administration of BMP7.

2.5. Extraction of Total RNA and real time quantitative RT-PCR

The parotid, submandibular, and sublingual glands of mice anesthetized with pentobarbital (50 mg/kg, i.p) were rapidly excised, and total cellular RNA was isolated with the RNagents Total RNA Isolation System (Promega, Madison, WI, USA). Total RNA was reverse-transcribed
using a Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The primers used were as follows: (5’-GAAAACACGACGACGTGACCA-3’, 5’-GGTGGCGTTCATGTAGGAGT-3’) for BMP7; (5’-TTCGGATTGTGCTGTCCATA-3’, 5’-GTTTCCCACATCAAGCTGGT-3’) for ALK2; (5’-CTTCTCCAGCTGCTTTTGT-3’, 5’-ATAGCGGCCTTTACCAACCT-3’) for ALK3; (5’-AACCCTTGGCATTGTGGAAG-3’, 5’-ACACATTGGGGTGAAGAACA-3’) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. cDNA was amplified by real time quantitative RT-PCR using the DyNAmo SYBR green qPCR kit (Finnzymes, Espoo, Finland) on the DNA Engine Opticon 2 System (MJ Research, MA, USA). All samples were quantified by the comparative cycle threshold (Ct) method for relative quantification of gene expression. PCR was conducted with 35 cycles according to the following protocol: 2-min predenaturation 96°C, 30 s denaturation at 94°C, 30 s at 59°C, followed by a 30-s extension at 79°C. Data were expressed as the ratio of BMP7, ALK2, or ALK3 mRNA and GAPDH mRNA. A portion of each PCR product was separated on 2% agarose gel and visualized by staining with 1% ethidium bromide. A 100-bp DNA ladder (Invitrogen) was used as the molecular weight standard.

2.6. Collection of saliva from parotid and submandibular/sublingual glands

Saliva was collected as described previously (Watanabe et al., 2001). Briefly, the mice, anesthetized with pentobarbital sodium (50 mg/kg i.p), were secured in a supine position, and a tracheal tube was inserted to support respiration. The tapered end of each capillary cannula (PE-50; Clay-Acams, Becton Dickinson, MD, USA) was inserted into the parotid and submandibular ducts, and the other end was placed inside a 0.5-ml microtube to collect secreted saliva. Saliva from the glands was collected every 30 min for 2 h by stimulation with pilocarpine, which was dissolved in physiological saline and intraperitoneally administered at 4
mg/kg in a volume of 0.1 ml per 10 g body weight. Quantification of the amount of saliva secreted was calculated by converting the weight (mg) into volume (µl), assuming the same gravity as water.

2.7. Histological examination

The parotid, submandibular, and sublingual glands of normal and diabetic mice anesthetized with pentobarbital (50 mg/kg, i.p) were removed and fixed immediately with a 10% formalin solution containing phosphate buffer. The tissues were embedded in paraffin for making sections. Tissue sections (5 µm in thickness) were stained with hematoxylin/eosin for histological examination. To detect lipid droplets, the tissues were stained with Oil red-O.

2.8. Statistical analysis

The data are expressed as the mean ± S.E.M. Significant differences between two groups were determined with Student’s t-test and the Aspin-Welch t-test in the case of equal and unequal variance, respectively. In the experiment on functional recovery achieved with BMP7, significant differences were analyzed with an ANOVA and a t-test. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Expression of BMP7, ALK2, and ALK3 mRNA in mouse salivary gland

BMP7 mRNA expression in mouse salivary gland was determined by real time quantitative RT-PCR. Tenfold serial dilutions of cDNA were used to construct a standard curve from $10^6$ to $10^1$ copies. The standard curve was generated from threshold cycle values, and linear regression analysis provided a straight-line plot, which indicated that our system was able to accurately quantify BMP7 cDNA. Melting curve analysis also revealed that the BMP7-specific
primer pair amplified a single predominant product at a melting temperature of 83.5°C (data not shown). Similar results were obtained with the specific primers for the BMP7 receptors ALK2 (BMP7 specific) and ALK3 (BMP7 non-specific), and GAPDH as the housekeeping gene (data not shown). These results confirmed that real time quantitative RT-PCR yielded specific products solely derived from target mRNAs.

3.2. Expression of BMP7, ALK2, and ALK3 mRNA in salivary gland of diabetic mice

In normal mice, we found that expression of BMP7 mRNA in the submandibular gland was approximately 10-fold the level of that in the parotid and sublingual glands (Fig. 1). In diabetic mice, a notable reduction in BMP7 mRNA expression was observed in the parotid gland (Fig. 1). Values in this gland decreased to about 4% of the normal level. The mRNA levels in the submandibular and sublingual glands also showed a tendency to decrease, although this was not significant.

In normal mice, ALK2 and ALK3 mRNA levels were also nearly the same in the three salivary glands examined (Fig. 2). No marked change was observed in their levels in diabetic mice.

3.3. RT-PCR product of BMP7, ALK2 and ALK3 in salivary gland

The PCR of salivary gland cDNA with the primer for BMP7 produced a single product of the expected size, 159bp (Fig. 3A). In diabetic mice, electrophoresis revealed a clear decrease in the intensity of this protein band in parotid gland compared with that in submandibular and sublingual gland (Fig. 3B). Single bands for ALK2 and ALK3 corresponding to the predicted length based on the primer used, 153 and 229bp, respectively, were also detected in the three salivary glands, and these showed no change in diabetic mice (data not shown).

3.4. Protective effect of BMP7 on salivary secretion in diabetic mice

In diabetic mice, pilocarpine-stimulated secretion of saliva was significantly reduced, with
secretion from the parotid and submandibular glands decreasing to 12% and 30% of normal levels, respectively (Fig. 4). However, secretion recovered dose-dependently on administration of BMP7 (50 and 100 µg/kg, i.p.). Recovery rate was higher in the parotid gland than in the submandibular gland. BMP7 had no effect on salivary secretion from normal mouse gland.

3.5. Protective effect of BMP7 on morphological changes in salivary gland in diabetic mice

We further examined the effect of BMP7 on histological changes in salivary gland in diabetic mice (Fig 5). Parotid gland in diabetic mice exhibited notable degeneration compared with normal tissue, showing vacuolation and atrophy. Tissue samples were Oil red-O negative (data not shown). Vacuolation was also found in submandibular gland in diabetic mice, but to a far lesser degree than that in parotid gland. On the other hand, in BMP7-treated mice, both parotid and submandibular gland showed little degenerative damage and a reduction in vacuolation.

4. Discussion

BMP, a secreted differentiation factor, is a member of the transforming growth factor-β superfamily (Hogan, 1999). BMP7, also known as osteogenic protein-1, has been identified as a key epithelial signaling molecule in tooth mesenchymal and mandibular arch patterning (Wang et al., 1999; Piscione et al., 2001). Interestingly, BMP7 gene knockout mice showed an abnormal phenotype, with a disorganized mesenchyme and a decrease in epithelial branching (Jaskoll et al., 2002).

An earlier study found a great difference in susceptibility to diabetic stress between types of salivary gland in streptozotocin-injected rats (Watanabe et al., 2001). The submandibular gland exhibited greater resistance to diabetic stress than the parotid gland, both functionally and histologically. It has been reported that the positive regulatory action of BMP7 in the
development of kidney, guts, lung, and prostate involves branching morphogenesis (Setoguchi et al., 2001; Lund et al., 2002; Wang et al., 2003; Maric et al., 2003). The morphological similarity between salivary gland and organs such as kidney, and the potential protective effect of BMP7 on diabetic damage in salivary gland were, therefore, taken into account in carrying out the present study.

In this study, we first examined the mRNA expression of BMP7 and its receptors, specific ALK2 and non-specific ALK3, in mouse salivary gland. We found transcripts for BMP7 and its receptors in all three salivary glands examined. In normal mice, the BMP7 gene was highly expressed in the submandibular gland, and less so in the parotid and sublingual glands (Fig. 1). However, in diabetic mice, expression showed a significant decrease in parotid gland, but not in submandibular or sublingual gland (Fig. 1). The PCR products of BMP7 in diabetic mice approximately reflected the levels of BMP7 mRNA in the glands (Fig. 3). On the other hand, no significant difference was observed in expression levels of ALK2 or ALK3 between normal and diabetic mice (Fig. 2). This result led us to hypothesize that BMP7 administered in diabetic mice transduces its signal and modulates cell reactions, as BMP7 acts via receptors ALK2 and ALK3, in which no change was found in diabetic mice. It has been reported that submandibular gland with high levels of BMP7 gene expression exhibited resistance to stress induced by diabetic dysfunction in streptozotocin-injected rats and post-radiotherapy in humans, and exerted a positive effect on cell survival in mice (Dudley et al., 1999; Watanabe et al., 2001; Maes et al., 2002). BMP7 has also been proposed to be involved in promoting cell differentiation and preventing apoptosis (Dudley et al., 1999; Piscione et al., 2001; Setoguchi et al., 2001; Hoffman et al., 2002). Furthermore, BMP7 appears to be antifibrogenic, suppressing some of the profibrogenic effects of TGF-β (Wang et al., 2001 and 2003).
present results and those of earlier reports suggest that BMP7 is involved in preventing loss of salivary gland function and enhancing its recovery.

To elucidate the functional effect of BMP7, we further examined the effect of BMP7 on secretion of saliva and morphological change in salivary gland in diabetic mice. The results strongly supported our hypothesis that BMP7 was capable of reversing diabetic dysfunction. In diabetic mice, salivary secretion showed a significant decrease compared to that in normal mice (Fig. 4). Notably, secretion in the parotid gland showed a reduction to 12% of normal levels. Secretion in the submandibular gland also decreased, but to a lesser degree than that in parotid gland. Our results are consistent with those of other studies on streptozotocin-induced diabetes (Hand and Weiss, 1984; Anderson, 1987; Kimura et al., 1996; Watanabe et al., 2001). Differences in rate of reduction between salivary glands may be associated with amounts of BMP7 mRNA in tissues. Furthermore, one cause of decrease in salivary secretion with diabetes may be change in susceptibility of receptors in salivary gland membrane. Watanabe et al. (2001) reported that, in addition to being induced by water loss, decrease in salivary secretion in diabetic rats was also closely associated with lowered susceptibility of muscarinic receptors in salivary gland membrane.

Administration of BMP7 (50 and 100 µg/kg, i.v.) produced a dose-dependent increase in secretion from both parotid and submandibular gland in diabetic mice (Fig. 4). A remarkable increase was observed in the parotid gland, where level of BMP7 gene expression is normally lower than that in submandibular gland. These results are consistent with reports that BMP7 was effective against diabetic damage in kidney (Vukicevic et al., 1996; Wang et al., 2001; Klahr et al., 2002), and also suggest that BMP7 strongly acts as a protective factor against diabetic damage to salivary gland, as well as to kidney.
Histological examination of diabetic mice revealed remarkable degeneration, with tissue showing vacuolation in parotid gland, but only minor change in submandibular gland (Fig. 5). In BMP7-treated mice, both parotid and submandibular gland tissue revealed a clear reduction in damage, with a decrease in vacuolation, despite diabetic conditions (Fig. 5). This result reflected the effect of BMP7 on secretion of saliva in diabetic mice, and indicated that BMP7 plays a key role in cytoprotection in salivary gland in diabetic mice.

BMP7 is a critical morphogen expressed in mouse salivary gland. The protective effect of BMP7 in salivary gland may be related to a recapitulation of its developmental actions, as BMP7 stimulates epithelial cell differentiation and provides a survival signal in mesenchymal cells during morphogenesis (Luo et al., 1995; Dudley et al., 1995; Vukicevic et al., 1996). The precise molecular mechanism of BMP7’s action remains to be clarified. Further investigation is necessary to elucidate its mechanism in more detail.

In conclusion, the present results demonstrate that administration of BMP7 in diabetic mice protects against decrease in salivary secretion and induction of cell degeneration in salivary gland, and that the cytoprotective effect of BMP7 may be closely correlated with amount of BMP7 mRNA in tissue.

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Figure legends

Figure 1
BMP7 mRNA levels in salivary gland of mice with streptozotocin-induced diabetes. Levels were measured 14 days after administration of streptozotocin (200 mg/kg, i.p.). BMP7 mRNA levels were normalized against GAPDH mRNA levels. Open and filled columns show values for normal and diabetic mice, respectively. Data represent mean ± S.E.M. of 5-6 samples. *P<0.05, significantly different from corresponding value.

Figure 2
Levels of ALK2 (A) and ALK3 (B) mRNA in salivary gland of mice with streptozotocin-induced diabetes. Levels were measured 14 days after administration of streptozotocin (200 mg/kg, i.p.). Levels of ALK2 and ALK3 mRNA were normalized against levels of GAPDH mRNA. Open and filled columns show values for normal and diabetic mice, respectively. Data represent mean ± S.E.M. of 5-6 samples.

Figure 3
Analysis of BMP7 mRNA expression in mouse salivary gland. A: BMP7 in normal mice, B and C: BMP7 and GAPDH in mice treated with streptozotocin (200 mg/kg, i.p.) for 14 days, respectively. Numerical value on right indicates product size.

Figure 4
Effect of BMP7 on salivary secretion in mice with streptozotocin-induced diabetes. Fourteen days after administration of streptozotocin (200 mg/kg, i.p.) or streptozotocin plus BMP7 (50 and 100 µg/kg, i.v.), pilocarpine (4 mg/kg, i.p.) was administered and secreted saliva was collected for 2 h. Open and filled columns show amount of saliva collected from parotid and submandibular glands, respectively. Data represent mean ± S.E.M. of 5-6 mice. *P<0.05,
significantly different from corresponding value.

Figure 5

Pathological analysis of salivary gland in BMP7-treated mice. Mice were dissected 14 days after administration of streptozotocin (200 mg/kg, i.p.) or streptozotocin plus BMP7 (100 µg/kg, i.v.), and parotid and submandibular glands were removed and stained with hematoxylin/eosin. (A), normal mice; (B), streptozotocin-treated mice; (C), streptozotocin plus BMP7-treated mice. (a), (b), and (c) show magnifications of areas of (A), (B), and (C), respectively. Arrowheads in (b) and (c) show typical vacuolation.
Figure 2 (Izumi et al.)

(A) ALK2 mRNA / GAPDH mRNA

(B) ALK3 mRNA / GAPDH mRNA
Figure 3 (Izumi et al.)

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<th>Parotid gland</th>
<th>Submandibular gland</th>
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Figure 4 (Izumi et al.)

The graph shows the saliva volume (μL) across different conditions: Normal, Streptozotocin, Streptozotocin + BMP7 (50), and Streptozotocin + BMP7 (100). The data indicates significant differences marked with asterisks (*) between the groups.
Figure 5a

parotid gland

A

B

C

a

b

c

30 μm

5 μm

5 μm
Figure 5b

submandibular gland