Expression and Localization of Brain-Derived Neurotrophic Factor (BDNF) mRNA and Protein in Human Submandibular Gland

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Brain-derived neurotrophic factor (BDNF) promotes cell survival and differentiation in the central and peripheral nervous systems. Previously, we reported that BDNF is produced by salivary glands under acute immobilization stress in rats. However, expression of BDNF is poorly understood in humans, although salivary gland localization of BDNF in rodents has been demonstrated. In the present study, we investigated the expression and localization of BDNF in the human submandibular gland (HSG) using reverse transcription-polymerase chain reaction, western blot analysis, \textit{in situ} hybridization (ISH), immunohistochemistry (IHC), and ELISA. BDNF was consistently localized in HSG serous and ductal cells, as detected by ISH and IHC, with reactivity being stronger in serous cells. In addition, immunoreactivity for BDNF was observed in the saliva matrix of ductal cavities. Western blotting detected one significant immunoreactive 14 kDa band in the HSG and saliva. Immunoreactivities for salivary BDNF measured by ELISA in humans were 40.76±4.83 pg/mL and 52.64±8.42 pg/mL in men and women, respectively. Although salivary BDNF concentrations in females tended to be higher than in males, the concentrations were not significantly different. In conclusion, human salivary BDNF may originate from salivary glands, as the HSG appears to produce BDNF.

\textbf{Key words:} brain-derived neurotrophic factor (BDNF), human, saliva, submandibular gland

I. Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin (NT) family, which includes nerve growth factor (NGF), NT-3, -4/5, -6 and -7 [22]. BDNF is the most abundant NT in the central nervous system, and is closely involved in neural cell survival and maintenance, as well as in neural transmission [41]. In the hippocampus in particular, BDNF expression varies depending on stress [13], exercise [1], and learning [11]. Furthermore, BDNF plays an important role in facilitating the formation of neural networks. BDNF is also found in many peripheral tissues, such as the lachrymal glands [12], heart [43], and retina [44] in rodents, as well as in the testis [29], lung [35], and vascular endothelial cells [30] in human. Moreover, our previous study examined the effect of immobilization stress on BDNF expression in male rat submandibular glands [51]. Increased BDNF mRNA and protein expression were observed in duct cells as a result of immobilization stress. Furthermore, acute immobilization stress was observed to increase plasma BDNF levels, with a contribution from the submandibular glands [52].

The salivary glands consist of the major salivary glands, including the parotid, submandibular, and sublingual glands, as well as numerous minor salivary glands scattered throughout the oral cavity [15]. Their primary role is to secrete saliva, which is involved in food diges-
tion, promotion of mastication, and antimicrobial activity. However, other roles for the salivary glands may also exist, such as in stress responses, and in producing biomarkers for tumors [45, 55]. Cell growth factors, such as epidermal growth factor (EGF) and NGF, in particular, are also produced in the rat submandibular gland [6, 7]. Mouse salivary gland tissue expresses a high level of NGF [6, 7], which is released into the bloodstream in large quantities from salivary glands during fighting [3], and plasma EGF levels are reduced after damage to the major salivary glands [17]. Hence, the salivary glands may play an important role in systemic health [50].

In humans, the presence of numerous growth factors has also been observed, including EGF [8], NGF [23], and hepatocyte growth factor (HGF) [49]. Recently, Mandel et al. used immunoblotting and enzyme digestion to demonstrate that pro- and mature BDNF are present in human saliva, and that a relationship exists between salivary BDNF concentrations and the presence of the Val66Met single nucleotide polymorphism (SNP) [24, 25]. These proteins play an essential role in the protection and repair of oral and gastric soft tissue, as well as in the maintenance of gustatory tissue. Numerous animal studies have reported that sialoadenectomy (removal of the salivary glands) results in decreased wound healing [5], gastric lesions [33], epithelial keratosis, and distinct changes in taste cell structure and number [27, 32]. Furthermore, conditions of decreased saliva production in humans, such as Sjögren’s syndrome, lead to increased incidence of oral infection and frequency of taste complaints [34, 54].

It is important to investigate whether BDNF and other growth factors are expressed in human submandibular gland (HSG), as the origin of salivary BDNF is not well understood and BDNF exhibits extensive function throughout the human body. In the present study, we investigated salivary BDNF in order to clarify expression patterns of BDNF in HSG. To the best of our knowledge, this is the first study that describes the expression of BDNF in HSG.

II. Materials and Methods

Tissue samples

Normal HSG tissues were obtained by neck dissections (n=12) at Kanagawa Dental College (Kanagawa, Japan). For staining with in situ hybridization (ISH) (n=4), tissue specimens were fixed in 0.01 M phosphate-buffered saline (PBS) containing 4% paraformaldehyde (pH 7.4) at room temperature for 16 hr, embedded in paraffin and serial 3-μm sections were cut. For staining with hematoxylin and eosin (HE) and immunohistochemistry (IHC) (n=8), tissues specimens were fixed in 10% buffered formalin at room temperature for 18 hr, embedded in paraffin and serial 3-μm sections were cut. Normal paraffin-embedded HSG tissues were used for staining. Normal human hippocampal tissues, ready-made paraffin-embedded or frozen tissue slides (Cosmo Bio, Tokyo, Japan), were used as positive controls for immunostaining and ISH. Total RNA from HSG tissue (n=4) was used for mRNA analysis and Ultrapure total hippocampal RNA (n=1) (Cosmo Bio) was used as a control for mRNA analysis. Total protein from the single HSG tissue was also used for western blot analysis of normal HSG. All patient materials used in this study were obtained following fully informed consent regarding the nature and the aims of the study in accordance with the Ethics Committee of the Kanagawa Dental College.

Participants and saliva collection

Fifty healthy volunteers (26 male and 24 female) from Kanagawa Dental College in Kanagawa, Japan, participated in this study. Participants had a mean age of 27±6.4 years. Participants had not consumed any food or drink, nor brushed their teeth, for 2 hr before sample collection. They were instructed not to consume alcoholic beverages for the 24 hr prior to sample collection. All participants were non-medicated non-smokers. Information about age, general and oral health was also collected. All samples were collected between 9 and 10 a.m., within a 10-min period, to minimize any possible effect of diurnal variation. All saliva samples were collected using the Salivette (Sarstedt Co. Ltd., Nümbrecht, Germany) absorbent method. The Salivette samples were collected according to the manufacturer’s instructions. Briefly, participants were instructed to chew on a cotton roll for 2 min, or until the cotton was fully saturated with saliva, and then expectorate the cotton into the Salivette tube. Participants were asked not to handle the cotton roll in order to prevent possible contamination. All saliva samples were stored on ice until handling (approximately 1 hr), at which point the tubes were centrifuged at 2000 rpm for 15 min at 4°C and the samples aliquotted. All samples were stored at −80°C until use. Upon thawing, the samples were centrifuged once more to ensure complete debris removal [46].

cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the HSG tissues (n=4) and Ultrapure total hippocampal RNA (n=1) (Cosmo Bio), stored at −80°C until use, was reverse transcribed at 50°C for 30 min, 99°C for 5 min, and 5°C for 5 min using a single-strand cDNA synthesis kit (Roche Diagnostics, Ltd., Lewes, UK) according to the manufacturer’s instructions [51]. Following the reverse transcription (RT) reaction, cDNA products were stored at −20°C until use. RT-polymerase chain reaction (RT-PCR) was performed using the RNA LA PCR™ Kit (AMV) Ver.1.1 according to the manufacturer’s instructions (TaKaRa, Tokyo, Japan). RT was primed using random 9-mers and cDNA synthesis was conducted using the following cycle conditions: 30°C for 10 min, 42°C for 20 min, 99°C for 5 min and 5°C for 5 min. Oligonucleotide primers were designed to amplify a 167-bp fragment of human BDNF. Primer sequences were 5'-CAGGGGCGAT AGACAAAG-3' (sense) and 5'-CTTCCCCCTTATA TG GTC-3' (antisense) [21]. RT products were amplified using
Tag DNA polymerase after denaturation for 10 min at 95°C, followed by 25 cycles of denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec and product extension at 72°C for 30 sec, and a final extension at 72°C for 5 min. Primers for detecting the internal control marker, a 260-bp fragment of the β-actin housekeeping gene, were 5'-CCTG TATGCCCTCTGGTGCTGA-3' (sense) and 5'-CCATCTCTT TGCTCGAAGTCT-3' (antisense) [42]. PCR products were electrophoresed on 1.5% TBE agarose gels and stained using ethidium bromide.

**SDS-PAGE and western blot analysis**

Total proteins (40 μg) from the single HSG tissue and whole saliva (n=5) were boiled for 3 min with Laemmli sample buffer (Wako, Tokyo, Japan) and allowed to cool to room temperature. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels with 2.5 ng of recombinant BDNF (rBDNF) (R&D Systems, Minneapolis, MN, USA) used as a positive control. The Precision Plus All Blue Standard (Bio-Rad, Tokyo, Japan) molecular weight standard was used to monitor protein migration during electrophoresis. Proteins were transferred onto PVDF membranes (Millipore Corp., Bedford, MA, USA) in transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH) for 1 hr at 100 V and 4°C, then blocked for 1 hr at room temperature in blocking buffer (PBS, 0.1% Tween-20, 1% NP-40) with 5% non-fat dry milk (NFDM; w/v). For immunodetection, PVDF membranes were incubated overnight at 4°C with anti-BDNF rabbit polyclonal antibody (1:1000; molecular weight 14 kDa; sc-546, Santa Cruz Biochemistry, Santa Cruz, CA, USA) in blocking buffer with 5% NFDM. After washing in Tween-phosphate-buffered saline (PBST, 0.1% Tween 20, 50 mM Tris, pH 7.6, 150 mM NaCl), membranes were incubated in anti-rabbit IgG conjugated with HRP (Dako Cytomation, Glostrup, Denmark), diluted 1:2000 in blocking buffer with 5% NFDM, for 1 hr at room temperature. Membranes were washed again and the ECL Plus Chemiluminescence system (Amersham Biotech, Piscataway, NJ, USA) was used for detection. For negative control experiments, non-immunized rabbit or mouse IgG was used instead of the primary antibody. To determine the binding specificity, a competitive assay was also conducted using rBDNF (R&D Systems) [20, 39].

**BDNF ELISA analysis**

Human BDNF was detected by sandwich ELISA according to the manufacturer’s instructions (CYT306; Millipore Corp.). All assays were performed in F-bottom 96-well plates (Nunc, Wiesbaden, Germany). Tertiary antibodies were conjugated to horseradish peroxidase (HRP) and color was developed with tetramethylbenzidine (TMB) and measured at 450/570 nm. BDNF content was quantified against a standard curve with a detection limit was <4 pg/mL that was calibrated with known amounts of BDNF. All samples were tested twice, and the mean was calculated. Cross-reactivity to related neurotrophins (NGF, NT-3, and NT-4) was less than 3%. Intra- and inter-assay coefficients of variation were 3.7% and 8.5%, respectively. Concentrations were expressed as pg/mL [14].

**Statistical analysis**

Statistical analyses were carried out using the SPSS (Version 17.0; SPSS Inc., Chicago, IL, USA) statistics program. Student’s t-test was used to assess differences in BDNF concentrations between men and women (expressed as mean±SD). A probability level of 0.05 or less was accepted as significant.

**III. Results**

**RT-PCR measurement of BDNF gene expression in HSG**

Amplified products corresponding to human BDNF transcripts were detected in RT-PCR samples derived from human hippocampus and HSG (lanes 1 and 2, respectively;
Human β-actin transcripts were also detected in RT-PCR samples derived from human hippocampus and HSG (lanes 3 and 4, respectively; Fig. 1). The sizes of the amplified fragments for BDNF and β-actin were 167 and 260 bp, respectively. In addition, BDNF gene expression was observed in all cases.

**Western blot analysis of BDNF in saliva and HSG**

Immunoblotting of rBDNF, as a positive control, with antiserum against human BDNF revealed the expected specific mature full-length 14 kDa band (Fig. 2). In HSG and whole saliva, specific immunoreactive bands were also detected at 14 kDa (Fig. 2). Full-length BDNF protein expression was observed in all cases.

**BDNF in situ hybridization**

In normal HSG tissue, hybridization signals were predominantly identified in serous cells rather than mucous cells (Fig. 3A). Ductal cells were weakly positive to antisense probes for BDNF mRNA (Fig. 3A) and sense probes failed to produce hybridization signals (Fig. 3B). Hybridization signals for BDNF mRNA were predominantly observed in serous cells, and weakly expressed in ductal cells in all cases. These results corresponded well with the immunohistochemical distribution profiles.

**BDNF immunohistochemistry**

In normal HSG tissue, BDNF was mainly localized in serous cells (Fig. 4A), whereas most mucous cells were immunonegative for BDNF (Fig. 4A). Furthermore, distribution of BDNF protein was more intense in serous cells than in mucous cells (Fig. 4B). Ductal epithelia, including intercalated, striated (Fig. 4C) and interlobular, intralobular (Fig. 4D) and excretory ducts, were weakly immunopositive. Saliva matrix secreted into ductal cavities was also BDNF immunopositive (Fig. 4D). However, BDNF protein was not observed in myoepithelial cells. In absorption control sections of submandibular glands (Fig. 4E), no expression of BDNF was detected with the pre-absorbed antibody. In accordance with the expression of BDNF mRNA, distribution of BDNF protein was predominantly detected in serous cells and weakly detected in ductal cells in all cases.
Salivary BDNF concentrations

A comparison of salivary BDNF concentrations in males and females, as determined by ELISA, is presented in Table 1. Although salivary BDNF concentrations in females tended to be higher than in males, the concentrations were not significantly different.

IV. Discussion

We previously reported that BDNF expression in the rat submandibular gland is up-regulated by chronic stress.

Table 1. ELISA determination of salivary BDNF concentrations in adult humans

|          | n  | BDNF (pg/ml) |
|----------|----|--------------|
| Men      | 26 | 40.76±4.83   |
| Women    | 24 | 52.64±8.42   |
| Total    | 50 | 46.58±4.80   |

Concentrations of salivary BDNF are presented as geometric means±SD. There were no significant differences between men and women, p>0.05, Student’s t-test.
levels in human saliva [31, 37]. In a previous study, De et al. demonstrated [51]. While previous studies indicate that the salivary glands are a major source of NGF, and high concentrations of NGF in the submandibular gland have been reported in animals [26, 53], there are currently very few reference values for measuring the expression and localization of NGF in human salivary glands [10]. However, there are many reference values for NGF levels in human saliva [31, 37]. In a previous study, De Vincente et al. examined the expression and localization of neurotrophin proteins in 14 human (4 parotid, 6 submandibular, and 4 sublingual glands) and 5 mouse salivary glands using IHC [10], and found that neurotrophins were not detected in human salivary glands. In the mouse, NGF was the only neurotrophin found in salivary glands (submandibular gland) [10]. The aim of the present study was to clarify the expression and localization of BDNF protein and mRNA in the HSG. We demonstrated that BDNF mRNA and protein were primarily localized in two cell types in the HSG, serous and ductal cells, with the former exhibiting higher expression levels. In addition, BDNF expression and distribution patterns were highly consistent when observed with IHC and ISH. These results provide the first evidence that BDNF is produced in HSG serous and ductal cells. However, fewer positive cells were detected using ISH as compared to IHC. Since we previously reported that expression of BDNF increases in salivary glands under stress conditions [39, 51], we interpret the present results as an indication that individual responses are specific to the stress conditions utilized. Furthermore, we were not able to use the specimens in this study to examine the effects of differences in tissue fixation conditions.

In previous studies, we examined the expression of the BDNF-receptor tyrosine receptor kinase B (TrkB) in rat salivary glands under immobilization stress conditions [51, 52]. Since expression of TrkB was not observed in rat salivary glands, we surmised that BDNF originating from salivary glands does not function in an autocrine fashion. Additionally, since TrkB expression was not observed in the surrounding oral tissues of the rat, including oral mucosa and esophageal mucosa, we concluded that BDNF of salivary gland origin would function in a paracrine manner on other remote organs. However, as we did not examine the expression and localization of TrkB in human salivary glands in the present study, we do not know whether BDNF would function in an autocrine or paracrine manner.

We also demonstrated here that a 14 kDa protein, corresponding to BDNF, was detected as a single band in western blot analysis of HSG tissue. Similarly, BDNF was detected in saliva samples as a single band of the expected size. At the mRNA level, RT-PCR analysis of these samples yielded a specific band corresponding to BDNF. Based on these results, mature BDNF protein is present in HSG and whole saliva. Although BDNF mRNA and protein expression has been observed in rodent submandibular glands [48, 51], we did not investigate the presence of BDNF in rodent saliva. In addition, a recent proteomic analysis of human saliva failed to identify growth factors such as NGF and BDNF [9]. Recently, Mandel et al. performed deglycosylation and plasmin treatment of saliva, and observed that saliva samples contained many bands corresponding to BDNF (24, 32 and 34 kDa) [24]. There was considerable variation in the expression and relative concentrations of each form of the protein, and not all participants expressed every form. In their study, the specificity of the anti-BDNF antibody was confirmed by peptide neutralization, as well as by a lack of cross reactivity with the other neurotrophins, thereby verifying that the antibody specifically binds BDNF. The existence of multiple higher molecular weight forms of BDNF (24, 32 and 34 kDa) has also been reported in experiments using cultured neuronal and non-neuronal cells [28, 47]. It has been suggested that the various bands represent differentially glycosylated and glycosulphated forms of pro- and mature BDNF, and that salivary pro-BDNF is cleaved to mature BDNF by plasmin. To our knowledge, this is the first report of BDNF in salivary secretions, whether in humans or other species [24]. However, the observation of single bands in this study is consistent with the fact that we did not perform deglycosylation or plasmin treatment of saliva and HSG tissues.

In the present study, we used ELISA to measure salivary BDNF concentrations in a total of 50 adult men and women. Our results indicate that although salivary BDNF concentrations tended to be higher in females than in males, there were no significant differences between men and women. It is important to note that all samples were collected between 9 and 10 a.m. in this study. In contrast to the previous observations, Mandel et al. reported that women had significantly higher levels of salivary BDNF than men [25], with all samples being collected between 12 and 1 p.m. There are numerous factors known to affect salivary protein levels, including circadian rhythm, salivary flow-rate, stress, and infection [36]. Furthermore, since we have found that salivary BDNF concentrations exhibit diurnal variation, the observation of significant gender differences in saliva BDNF concentrations may be subject to diurnal variation (data not shown). In the future, we plan to further investigate the effect of various parameters on salivary BDNF concentrations, including diurnal variation, gender, daily variation, salivary flow-rate, stress, and infection.

With regard to the physiological roles of the various growth factors present in saliva, it has been reported that wound licking accelerates wound healing in rodents [4, 16]. We also propose that BDNF may interact with and complement other salivary growth factors in maintaining the balance between proliferation, survival, and death of cells. We measured the concentration of HGF, using an ELISA system, in saliva and blood before and after an operation for...
salivary gland tumor and found that HGF levels were significantly increased after surgery [49]. Thus, it is highly possible that the presence of growth factors in saliva serves to accelerate wound healing. Conversely, blood NGF concentrations decrease in mice that have undergone submandibular gland resection [2]. Although the details of the route taken by the growth factors produced in the salivary glands to the blood are unknown, reabsorption from the sublingual area is considered a likely explanation. Indeed, nitroglycerin tablets are administered at the sublingual area because this location has a thin mucous membrane and an abundance of blood vessels. The openings of the submandibular and sublingual glands are located in this area and it is reasonable to hypothesize that growth factors in saliva can be reabsorbed from the sublingual area. Although NGF is not detected in human salivary glands, other unknown neurotrophic factors could be produced in the salivary glands and reabsorbed from the sublingual area, thereby affecting the central nervous system. We propose that saliva could be assessed as a useful indicator of growth factor production, assuming that growth factors are beneficial to the living body.

In summary, the present study represents the first description of the expression of BDNF in serous and ductal cells of the HSG, indicating that salivary BDNF originates in the submandibular gland. In subsequent studies, we are planning to focus on further analysis of BDNF function in saliva.

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VI. References

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