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Molecular expression of adiponectin in human saliva

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ABSTRACT

Adiponectin (APN) is an adipocyte-specific secretory protein that is highly and specifically expressed in adipose tissue. Serum APN consists of trimers, hexamers, and larger high-molecular-weight (HMW) multimers, and these HMW multimers appear to be of more bioactive forms. Evidence indicates that APN is produced by salivary gland epithelial cells, might be implicated in the regulation of local immune responses.

Salivary APN was investigated in 52 healthy individuals. Western blotting under non-reducing conditions revealed that salivary APN consisted predominantly of a super HMW (SHMW) form of APN. In Western blotting, no significant differences were observed in SHMW APN levels in saliva samples with or without occult blood contamination, but non-SHMW APN levels were elevated in the samples with occult blood contamination. In the saliva samples without occult blood contamination, APN levels were significantly elevated in females than in males, in agreement with the results of previous reports for serum samples.

In summary, salivary SHMW APN is suggesting a possible promising oral biomarker.

1. Introduction

Recent studies have revealed that adipose tissue produces and secretes bioactive molecules called adipocytokines, including leptin, adiponectin (APN), tumor necrosis factor-α, and plasminogen activator inhibitor type-1 [1–4]. Furthermore, adipocytokine dysregulation is linked to obesity-associated metabolic diseases [5–9]. We identified APN as an adipocytokine from the human adipose tissue cDNA library [10]. APN is an adipocyte-specific secretory protein and is a member of the soluble defense collagen family that includes a collagen-like domain. In this domain, three APN peptides form one stable trimer, and the trimers further multimerize to form bouquet forms [11,12]. Plasma APN levels are low in obesity and type 2 diabetes [13,14]. The biological functions of APN include improvement of glucose and lipid metabolism [7,8] and prevention of inflammation and atherosclerosis [15–20].

Saliva is a body fluid that can be easily collected in a non-invasive manner. APN is produced by salivary gland epithelial cells [21]. Several studies have found very low APN levels in human saliva relative to plasma [22–24]. APN receptors (AdipoR1 and AdipoR2) were expressed in human gingival fibroblasts and periodontal ligament cells, which exert anti-inflammatory effects [25]. Moreover, APN levels and AdipoR expression were lower in patients with severe periodontitis than in healthy subjects [26,27].

APN is an important immunomodulatory cytokine, and serum APN levels are closely associated with metabolic syndromes, coronary heart disease, kidney disease, emphysema, and rheumatoid arthritis [28–31]. Measurement of salivary APN levels is easy and non-invasive and would be useful for many clinical studies. However, the nature and the precise forms of APN in human saliva are not well understood. In the present study, we showed the multimeric isoforms of APN using non-reducing Western blotting and identified for the first time a super high-molecular-weight band of APN in saliva distinct from that in serum.

2. Materials and methods

2.1. Study subjects

Saliva samples from 52 healthy individuals (37 males and 15 cfemales) were used in the study. The study protocol was reviewed and approved by the ethics committee of Osaka University, and the study was conducted according to the Declaration of Helsinki. All

Abbreviations: APN, adiponectin; HMW, high molecular weight; SHMW, super high molecular weight.

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subjects gave written acknowledgement of informed consent for participation.

2.2. Measurement of APN and hemoglobin in saliva

Subjects were instructed to rinse their mouths with cold water 5–10 min before saliva collection. After 5–10 min, the subjects were given sterile gauze and instructed to chew 60 times/min for 2 min and then spit into a 50-mL centrifuge tube. The samples were extracted from the gauze wads by centrifugation at 3000 rpm for 15 min. The supernatant was measured and stored at −80 °C until analysis. AlphaLISA® kits (Perkin Elmer®, MA, USA) were used to determine salivary APN (#AL209) levels according to the manufacturer's recommendations. The protocols have previously been optimized by the manufacturer for the optimal concentration of acceptor beads and biotinylated antibody concentrations. Salivary hemoglobin was detected using a clinical investigation kit (Hemoglobin detection kit for fecal occult blood; Wako Pure Chemical Industry, Osaka, Japan).

2.3. Western blotting

Western blotting was used to measure the trimer, hexamer, and HMW APN isoforms in serum and saliva samples. Saliva samples were incubated with a non-reducing sample buffer containing 2% SDS, 10% glycerol, and 10 mmol/L Tris–HCL (pH 6.8) at room temperature for 10 min; separated by 8% (non-reducing condition) and 16% (reducing condition) SDS–PAGE; transferred to PVDF membranes (GE Healthcare, Piscataway, NJ, USA); and blotted with anti-human APN antibody. The immune complex was detected using an ECL Advance Western Blot Detection System (GE Healthcare, Buckinghamshire, UK). Two independent antibodies against human APN from Millipore (MAB3604; Millipore, Bedford, MA) and Oriental Yeast (8B3; Oriental Yeast Co., Ltd. Tokyo, Japan) were used. Immunoreactive protein bands were scanned and quantified using ImageJ 1.45s software (National Institutes of Health, USA). The amount of super HMW APN (SHMW APN) protein was determined by subtracting the intensity of less than 250 kDa from the total intensity under the non-reducing condition, followed by normalization to the total APN protein under the reducing condition. The formula used for estimating non-HMW APN was as follows:

$$\text{Total APN(reduction condition)} \times \left(1 - \frac{\text{APN of less than250 kDa(non-reducing condition)}}{\text{Total APN(non-reducing condition)}}\right)$$

2.4. Statistical analysis

Data are presented as mean ± SEM. Differences in continuous variables were compared using Student's test. A p value of <0.05 was considered significant.

3. Results

3.1. Subject characteristics

Subject characteristics are summarized in Table 1. Age and body mass index (BMI) were significantly higher and body fat percentage was lower in males than in females. No significant differences in saliva volume and APN levels were observed between the two groups.

Table 1
Participants characteristics in this study.

	Male	Female	p value
Number	37	15	
Age, years	42.5 ± 9.6	31.0 ± 7.2	<0.001
Body massindex, kg/m^2	22.9 ± 2.0	19.5 ± 2.5	<0.001
Fat mass, %	20.8 ± 4.0	26.6 ± 6.0	<0.001
Saliva volume in 2 min, mL	4.8 ± 1.4	4.5 ± 1.6	0.62
APN, ng/mL	7.1 ± 8.0	8.5 ± 8.0	0.54

Data are presented as means ± SD.

3.2. Detection of APN protein by non-reducing SDS–PAGE

To observe the band pattern of APN, human serum samples were subjected to SDS–PAGE, followed by Western blotting as reported previously [32]. The antibody 8B3 detected two bands under the non-reducing condition, one of which was a faster migrating major band of approximately 150 kDa (Fig. 1A, hexamer) representing an APN hexamer, and the other was a slower migrating minor band (Fig. 1, multimer) of approximately 250 kDa representing an HMW multimer. These two bands could also be detected using the antibody MAB3604 (data not shown). This result indicates that these bands are specific to APN.

To compare the multimeric isoforms of APN, saliva and serum samples were subjected to SDS–PAGE, followed by Western blotting. The antibody 8B3 detected one band under the non-reducing condition, a SHMW band (Fig. 1, arrow) above the band for the HMW multimer in serum.

A previous report indicated that salivary APN levels are 1000 times lower than serum APN levels [23]. To evaluate the effect of dilution, serum samples were diluted and treated with anti-human APN antibody 8B3 (Fig. 1, right). In diluted serum samples, both hexamer and multimer bands became weaker without the appearance of additional bands, indicating that the SHMW band in saliva cannot be attributed to low APN levels.

3.3. APN protein expression in saliva samples contaminated with occult blood

Given that salivary APN levels are 1000 times lower than serum APN levels [23], samples contaminated with blood must be excluded. Accordingly, saliva samples were subjected to an occult blood assay. In the alphaLISA assay, occult blood-positive saliva samples had significantly higher APN levels than occult blood-negative saliva samples (Fig. 3A). In Western blotting, the occult blood-positive samples showed significantly higher levels of APN

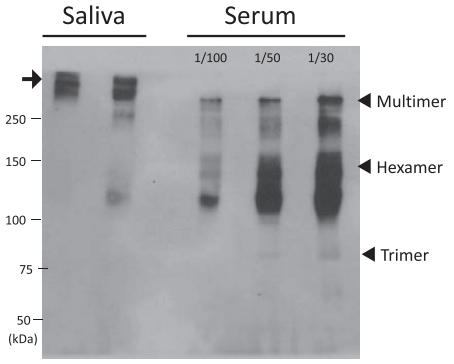

Fig. 1. Western blotting of diluted serum samples subjected to non-reducing SDS–PAGE and comparison with saliva samples. Saliva samples were applied in 20-μL aliquots. Serum was diluted to different concentrations and applied in 10-μL aliquots.

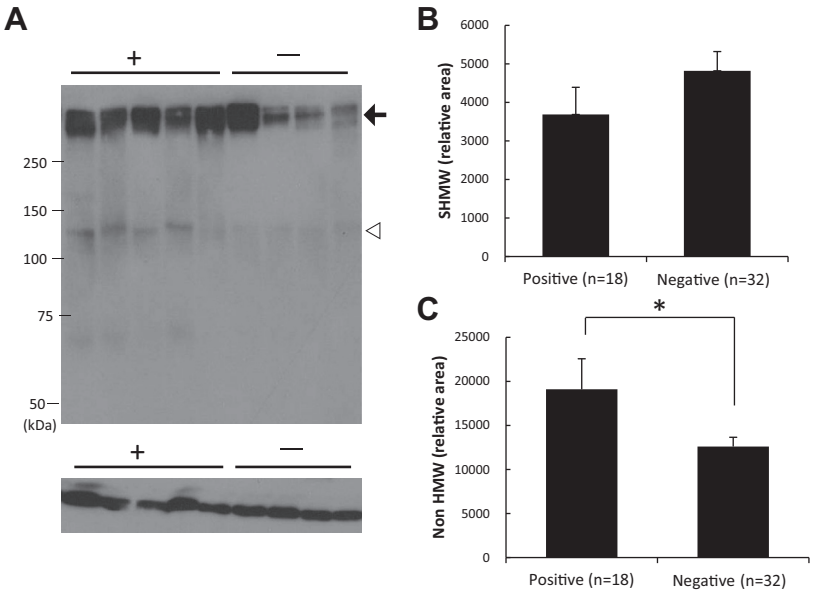

Fig. 2. (A) Representative examples of saliva samples with and without occult blood contamination treated with anti-human adiponectin (APN) antibody and subjected to non-reducing (upper) and reducing (lower) Western blotting. (B) No significant difference in high-molecular-weight (HMW) APN was found between occult blood-negative and -positive samples. (C) Non-HMW APN was calculated as the difference between HMW and total APN. Occult blood-positive samples showed increased non-HMW expression.

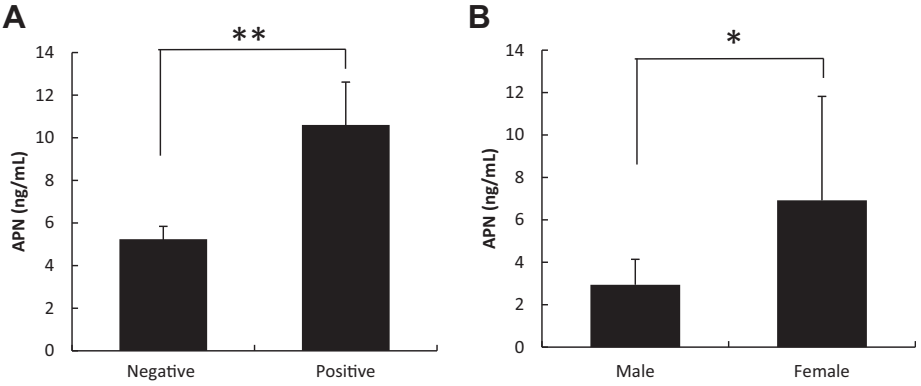

Fig. 3. (A) Salivary adiponectin (APN) levels in occult blood-negative and -positive samples determined by the commercial assay (negative, 30; positive, 22). The occult blood-positive saliva samples had significantly higher APN levels than the occult blood-negative saliva samples. (B) Salivary APN levels in occult blood-negative samples of subjects determined by the commercial assay (male, 12; female, 6). Females had higher salivary APN levels than males. $^{*}p < 0.05$; $^{**}p < 0.01$.

hexamers (Fig. 2A, asterisk), but not of SHMW APN (Fig. 2A), than the occult blood-negative samples. Signal densities of total APN in saliva samples were measured by reducing Western blotting (Fig. 2A, lower panel), and amounts of SHMW APN were calculated. Interestingly, no significant differences were observed in SHMW APN levels between the occult blood-positive and -negative samples (Fig. 2B). In contrast, non-SHMW APN levels were significantly reduced in the occult blood-negative samples (Fig. 2C). These results indicate that occult blood contamination did not affect SHMW levels but increased non-SHMW APN levels.

Next, salivary APN levels were measured using the alphaLISA assay. In all study groups, no significant differences were observed in serum APN levels between males and females (Table 1). To measure SHMV APN precisely in saliva, we excluded occult blood-positive samples. In these saliva samples, APN levels were significantly elevated in females than in males (6.9 ± 4.9 vs. 2.9 ± 1.2 ng/mL, $p < 0.05$) (Fig. 3B), in agreement with the results of previous report for serum samples [33].

4. Discussion

In the present study, we detected SHMW APN in saliva for the first time. SHMW APN is specific to saliva samples and was not affected by occult blood contamination.

Both saliva and blood serum contain similar proteins and RNAs; therefore, saliva is considered a "mirror of the human body" [34]. Saliva provides an easily available, non-invasive diagnostic medium for a rapidly widening range of diseases and clinical situations [35]. Evidence indicates that APN can be produced by cells other than adipocytes, including salivary gland epithelial cells, and is implicated in the regulation of local immune responses [21], such as those that regulate cell survival under energy stress conditions to protect the salivary gland epithelial cells from the inflammatory milieu [36].

Previous studies have reported that APN levels in human saliva measured using the enzyme immunoassay kit were significantly correlated with plasma APN levels [22,23,37]. Specifically, these

studies also showed that salivary APN levels were not correlated with age, sex, BMI, and body fat percentage. Our data are in agreement with those of these earlier studies. Serum APN levels were negatively correlated with BMI, and females were characterized by higher serum APN levels. Interestingly, using a commercially available assay and samples in the occult-native condition, we demonstrated that salivary APN levels were similar to serum APN levels, but the levels were higher in females than in males (Fig. 3).

Using non-reducing Western blotting, the presence of APN isoforms in saliva samples from the healthy subjects was tested. Saliva expressed all APN isoforms, although at low levels. SHMW APN in saliva between serum was detected using five different human APN antibodies (data not shown). APN shares sequence similarity with a family of proteins that are characterized by an N-terminal collagen-like region and a C-terminal complement factor C1q-like globular domain [38–40]. In plasma, hexamer and HMW complex APNs are the major oligomeric forms, and the HMW form of APN has been shown to be more active than the low-molecular-weight form of APN [41]. Our immunoblot analysis of HMW APN showed no differences associated with sex, BMI, or body fat percentage in the healthy subjects. The commercial assay revealed that that occult blood-positive saliva samples contained significantly higher APN levels than occult blood-negative saliva samples, whereas Western blotting revealed that SHMW APN expression remained unchanged and hexamer APN expression was significantly increased. However, SHMW was not affected by bleeding and is associated with oral environments. Subjects positive for *Porphyromonas gingivalis*, one of the major pathogens among chronic periodontitis bacteria [42], have a strong tendency to have lower HMW APN levels in saliva ($p = 0.06$, data not shown), a finding that supports a possible proinflammatory role for salivary HMW APN in oral environments and provides a first step toward a new biomarker in oral disease development. Additional work is required for elucidating the function and mechanism of salivary HMW APN.

Overall, our study shows that salivary APN is affected by occult bleeding; therefore, making commercially available kits insufficient to use marker. Technical approaches for determining salivary HMW APN await further studies and improvements.

Disclosure

The authors declare no personal financial or institutional interest in this article.

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