Exosome-Like Vesicles with Dipeptidyl Peptidase IV in Human Saliva

Yuko OGAWA, a Masami KANAI-AZUMA, b Yoshihiro AKIMOTO, b Hayato KAWAKAMI, b and Ryohei YANOSHITA a,a

a Institute of Medicinal Chemistry, Hoshi University; Shinagawa-ku, Tokyo 142–8501, Japan; and b Department of Anatomy, Kyorin University School of Medicine; Mitaka, Tokyo 181–8611, Japan.

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Saliva contains a large number of proteins that participate in the protection of oral tissue. We found, for the first time, small vesicles (30–130 nm in diameter) in human whole saliva. Vesicles from saliva were identified by electron microscopy after isolation by gel-filtration on Sepharose CL-4B. They resemble exosomes, which are vesicles with an endosome-derived limiting membrane that are secreted by a diverse range of cell types. We performed a biochemical characterization of these vesicles by amino acid sequence analysis and Western blot analysis. We found that they contain dipeptidyl peptidase IV (DPP IV), galectin-3 and immunoglobulin A, which have potential to influence immune response. The DPP IV in the vesicles was metabolically active in cleaving substance P and glucose-dependent insulinoctopic polypeptide to release N-terminal dipeptides. Our results demonstrate that human whole saliva contains exosome-like vesicles; they might participate in the catabolism of bioactive peptides and play a regulatory role in local immune defense in the oral cavity.

Key words exosome; human whole saliva; dipeptidyl peptidase IV; galectin-3; substance P

During the last decade, a novel mechanism of protein release has been recognized that involves small (30–100 nm) membrane vesicles termed exosomes.1–3 Exosomal vesicles are secreted following the fusion of multivesicular late endosomes with the plasma membrane. While the range of exosomal proteins depends on cell type, these vesicles commonly carry cell-surface proteins and cytoskeletal proteins. Several physiologic roles have been assigned to exosomes, including the expulsion of obsolete membrane constituents, exchange of cellular material and intercellular communication. Exosome production has been observed in a variety of cell types in vitro, including reticulocytes,4 cytotoxic T lymphocytes,5 B lymphocytes,6 dendritic cells7 and neoplastic intestinal epithelial cells.8 Recent studies have reported that such vesicles are present in some physiological fluids, such as bronchoalveolar lavage9 or urine.10 However, there is still little evidence of whether exosomes are produced in vivo.

Saliva is considered to provide the first line of oral cavity defense against bacterial and viral attack. Human whole saliva contains a potent mixture of diverse components such as mucin, immunoglobulin A (IgA), proline-rich proteins and defensins, which are produced in three major paired salivary glands (parotid, submandibular and sublingual) and several minor glands. Although comprehensive proteome analyses of whole saliva have been reported recently,12,13 the protein constituents of saliva are not fully understood.

The membrane-associated serine protease dipeptidyl peptidase IV (DPP IV), which is identical to the lymphocyte surface glycoprotein CD26, cleaves dipeptides from the N-terminus of peptides with a proline or alanine residue in the penultimate position.14 CD26/DPP IV is highly expressed on fibroblasts, epithelial and endothelial cells, and specific leukocyte subsets. The extracellular protease domain of CD26/DPP IV, which is produced by proteolytic cleavage of the membrane-bound form of CD26/DPP IV, also exists in a soluble form in plasma.5 Recently, it was reported that DPP IV is released from intestinal epithelial cells into the extracellular milieu as a constituent of exosome-like vesicles.9 More recently, our previous report revealed that DPP IV is released into snake venom in an unprocessed form,16 suggesting that the DPP IV is associated with membrane. In addition, we have found exosome-like vesicles that carry DPP IV in snake venom.17 DPP IV activity has been found in human saliva,18 but the mechanism of its release has not been elucidated. Snake venom is highly modified saliva that is produced by special glands of certain species of snakes. Therefore, we hypothesized that exosome-like vesicles carrying DPP IV could be present in human saliva. We demonstrate for the first time a population of vesicles in human whole saliva similar in size to the previously described exosomes. We found that these vesicles contain DPP IV, galectin-3 and IgA.

MATERIALS AND METHODS

Materials Gly-Pro-4-methyl-coumaryl-7-amide (Gly-Pro-MCA) was purchased from Peptide Institute Inc. (Osaka, Japan). Sepharose CL-4B was purchased from Sigma. Goat anti-human DPP IV antibody was purchased from R&D Systems, Inc. (Minneapolis, MN, U.S.A.). Rabbit anti-galectin-3 polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc, and mouse anti-actin monoclonal antibody (MAB1501R) was obtained from Chemicon (Temecula, CA, U.S.A.). HRP-conjugated rabbit anti-goat IgG, HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat antimouse IgG were obtained from Zymed laboratories (South San Francisco, CA, U.S.A.). Substance P and glucose-dependent insulinoctopic polypeptide (GIP) were purchased from Peptide Institute Inc. (Osaka, Japan). 99%-grade α-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Fluka (Bush SG, Switzerland).

Human Whole Saliva Sample Preparation and Gel Filtration Human whole saliva was collected from healthy volunteers in our laboratory with informed consent. One milliliter of saliva was filtered through a 0.2 μm cellulose acetate filter to remove bacteria from the sample and used for determination of DPP IV activity. For separation of saliva by gel-filtration, 20 ml of whole saliva from one individual was
filtered and concentrated up to approximately 1 ml using a Centricon YM-100 centrifugal filter device (Millipore Corporation, U.S.A.) with a 100-kDa exclusion. The concentrated filtrate was subjected to gel-filtration on a Sepharose CL-4B column (1.5 x 50 cm) equilibrated with 20 mM Tris–HCl (pH 7.4) containing 150 mM NaCl. The void fractions were collected and concentrated approximately 100 fold using a Centricon YM-100. This concentrated solution was used for further characterization.

**Immunoelectron Microscopy** Immunoelectron microscopy was performed as described previously.19) The concentrated solution of void fractions prepared as described above was mixed 1:1 with 4% paraformaldehyde in phosphate-buffer (pH 7.2) and then applied to 200-mesh Formvar-carbon-coated nickel grids. After blocking with 1% BSA and washing with PBS, the grid was incubated with primary antibody against DPP IV (1:100), overnight at 4°C, washed with PBS, and then exposed to colloidal gold (12 nm)-conjugated donkey anti-goat IgG antibody (1:10) for 1 h at room temperature (Jackson ImmunoResearch, West Groove, PA, U.S.A.). After washing, the grid was refixed in 1% glutaraldehyde-PBS, contrasted with 2% uranyl acetate, pH 7, and embedded with 2% methylcellulose/0.4% uranyl acetate, pH 4. After drying, the grids were examined with a transmission electron microscope (JEM-1010; JEOL, Tokyo, Japan).

**Enzyme Activity Assay** DPP IV activity was assayed as described previously.16) In brief, an assay mixture contained 50 μl of 0.4 mM Gly-Pro-MCA, 100 μl of 100 mM Tris–HCl buffer (pH 8.5) and 50 μl of enzyme solution. After incubation for 20 min at 37°C, 2.8 ml of 1 M sodium acetate (pH 4.2) was added to terminate the reaction. The fluorescence intensity of liberated 7-amino-4-methyl-coumarin was measured at 460 nm with excitation at 380 nm. Protein concentration was determined using the BCA reagent (Pierce, U.S.A.).

**Western Blot Analysis** The proteins in the concentrated void fractions prepared as described above were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer method. Non-specific binding sites were blocked by incubating the membrane in 100 mM Tris–HCl buffer (pH 7.4) and 5% skim milk for 1 h at room temperature. The void fractions (Fr. 22—26) were concentrated and obtained mixture was desalted, concentrated and then eluted with 1 μl of matrix solution (5 mg/ml CHCA in 50% acetonitrile (ACN)/0.1% TFA) and spotted onto a sample plate. Samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on an Axima CFRplus instrument (SHIMADZU, Corp., Kyoto, Japan) in positive reflection mode with 20 kV acceleration voltage.

**RESULTS**

**Exosome-Like Vesicles in Human Whole Saliva** DPP IV activity was detected in whole saliva from all five (three male and two female) individuals tested. The activity ranged from 0.22 to 0.44 nmol/min/mg protein (0.31 ± 0.04 nmol/min/mg protein). Whole saliva from one female individual out of this group was fractionated by gel-filtration on a Sepharose CL-4B column (Fig. 1). Almost all of the DPP IV activity was found in void fractions. A similar result was observed for saliva from another individual (data not shown). The void fractions (Fr. 22—26) were concentrated and observed by electron microscopy, which revealed numerous vesicles varying in size from 30 to 130 nm in accordance with previous findings.4—8) (Fig. 2A). Immunolabeling with anti-human DPP IV antibody revealed that DPP IV was associated with the vesicles (Fig. 2B).

**Identification of Proteins from Exosome-Like Vesicles by Amino Acid Sequence Analysis and Western Blot Analysis** The proteins from these vesicles were analyzed by SDS-PAGE (Fig. 3A). The protein pattern obtained was

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**Fig. 1.** Gel Filtration Chromatography of Human Whole Saliva

Gel filtration chromatography was performed on a Sepharose CL-4B column. Dipeptidyl peptidase IV activity (open triangles) was measured as described in Materials and Methods.

**Fig. 2.** Electron Microscopy of Exosome-Like Vesicles from Human Whole Saliva

(A) Electron microscopy of the concentrated void fractions by Sepharose CL-4B gel-filtration. (B) Immunoelectron microscopy. Immunogold labeling was performed with anti-human DPP IV antibody (arrowhead). Scale bar, 200 nm.
different from that of whole saliva. Bands higher than 100 kDa were enriched in the vesicles. The N-terminal amino acid sequences of the four major bands at 110 kDa, 80 kDa, 52 kDa and 25 kDa were MKTPWKVXGGLGAA, KSPIFGXEEVN, EVQLVESGGLVQPG and DI-VMTQSPSSLSASV, respectively. By BLAST search, these bands were found to represent DPP IV, poly-immunoglobulin receptor (pIgR), immunoglobulin heavy chain and immunoglobulin light chain, respectively. Western blot analysis revealed that the vesicles were positive for DPP IV, actin and galectin-3 (Fig. 3B). While DPP IV could be produced in the periodontal pathogen *Porphyromonas gingivalis* in the oral cavity,20) N-terminal amino acid sequence and Western blot analysis using anti-human DPP IV antibody confirmed that the 110 kDa protein was human DPP IV.

**Degradation of Bioactive Peptides by Exosome-Like Vesicles** Since the exosome-like vesicles from whole saliva contained DPP IV, we investigated whether the vesicles were active in the degradation of bioactive peptides such as substance P and GIP. Substance P was first cleaved at Pro2-Lys3 and subsequently hydrolyzed at Pro4-Gln5 (Fig. 4). No other peptide fragments were observed. GIP was cleaved at the Ala2-Glu3 position resulting in GIP(3—42) (data not shown). These results demonstrate that DPP IV-like proteolytic activity in the exosome-like vesicles hydrolyzes substance P and GIP.

**DISCUSSION**

We showed for the first time that exosome-like vesicles with a typical size are present in human whole saliva. It has been known that the two main pathways by which membrane vesicles are released from cells involve the formation of exosomes20) or microvesicles.21) Microvesicles are formed by plasma membrane vesiculation or blebbing, and are typically larger than exosomes, with diameters ranging from 0.1 to >1 μm. The size distribution of the vesicles from human whole saliva matched that of exosomes from other sources. Exosome-like vesicles from whole saliva contain DPP IV, galectin-3, IgA, pIgR and actin. This protein profile is consistent with exosomes, which contain cell-surface and cytoskeletal proteins. Proteomic analysis is in progress to further characterize these vesicles.

Whole saliva is a complex mixture derived from not only major and minor salivary glands, but also crevicular fluid, as well as from contaminating bacteria and cellular debris. At present, the origin of the vesicles in whole saliva is unclear, but it has been found that non-neoplastic epithelium cells from salivary glands release exosomes *in vitro*.11) In addition, galectin-3 and DPP IV are expressed in ductal epithelium cells in salivary glands.22,23) Furthermore, pIgR is present in epithelial cells lining the mucosal surface and transport IgA across mucosal epithelial cells.24) Taken together, these findings suggest that exosome-like vesicles in whole saliva might originate from ductal epithelial cells of salivary glands. However, further study will be necessary to clarify which salivary glands and which cells release the vesicles.

Saliva contains a large number of proteins that participate in the protection of the oral tissues; for instance, lysozyme, lactoferrin, lactoperoxidase, immunoglobulins, agglutinin and mucins. In addition, several peptides with bacteria-killing activity have been identified. These include histatins, defensins and the only human cathelicidin, LL-37.25) Although enzyme activity of DPP IV has been detected in human saliva,18) its biochemical characterization has not been performed. The present study revealed that DPP IV in saliva is present in exosome-like vesicles. The N-terminal sequence of the 110 kDa protein suggests that DPP IV on the exosome-like vesicles is an unprocessed form. This is consistent with a form of DPP IV in snake venom.16) It is noteworthy that DPP IV activity may actually limited to exosome fraction of saliva. The expression of DPP IV on vesicles from saliva is in accordance with previous studies on exosomes from intes-
tinal epithelium cells and epididymal fluid. In this report, the vesicles from human saliva are shown to cleave substance P and GIP. DPP IV seems to be responsible for degradation of these peptides. However, we cannot rule out the possibility that other proteases(s) in the vesicles would also participate in the degradation activity of substance P and GIP. Substance P and GIP have been detected in human saliva. Furthermore, salivary glands express chemokines such as SDF-1, RANTES and CXCL10, which share the X-Pro motif at the N-terminus. It is known that some of these are released from salivary epithelial cells. Collectively, DPP IV in the vesicles from saliva might contribute to regulation of concentration of these peptides in the oral cavity.

CD26/DPP IV functions not only as a degradation enzyme of bioologically active peptides, but also as a regulator of immune response. Recombinant soluble CD26/DPP IV enhanced the proliferative response of peripheral blood lymphocytes to stimulation with soluble Ag, a tetanus toxoid, through binding of CD26/DPP IV to caveolin-1 on antigen-presenting cells. Galectin-3 induces death of naïve T lymphocytes. Pathogens captured by IgA enhanced the proliferative response of peripheral blood lymphocytes and GIP. DPP IV seems to be responsible for degradation of these peptides. However, we cannot rule out the possibility that other protease(s) in the vesicles would also participate in the activation and differentiation of naïve T lymphocytes. Soluble Galectin-3 can promote cell adhesion of L-selectin-activated lymphocytes to dendritic cells. Extracellular galectin-3 can promote cell adhesion of L-selectin-activated lymphocytes to dendritic cells. Taken together, exosome-like vesicles in saliva might have the potential to influence the immune system in the oral cavity. However, further studies are needed to clarify the role of these exosome-like vesicles in the oral cavity.

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REFERENCES