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Association of cholera toxin with *Vibrio cholerae* outer membrane vesicles which are internalized by human intestinal epithelial cells

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ABSTRACT

Cholera toxin (CT) is the major virulence factor of pathogenic *Vibrio cholerae*. The present study demonstrates that a fraction of CT is associated with the outer membrane vesicles (OMVs) released by *V. cholerae*. Atomic force microscopy (AFM) and also transmission electron microscopy (TEM) of purified OMVs from toxigenic *V. cholerae* O395 revealed spherical shaped vesicles of size range 20–200 nm. Immunoblotting of purified OMVs with polyclonal anti-CT antibody and GM1-ganglioside dependent ELISA suggest that CT is associated with OMVs. CHO cell assay indicated that OMV associated CT is physiologically active. OMVs labeled with fluorescent dye interacted with intestinal epithelial cells via the CT-receptor and were internalized increasing the cAMP level. Thus OMVs may represent an important vehicle in delivering CT to epithelial cells.

1. Introduction

The acute diarrheal disease cholera remains a significant public health problem, due to its ability to spread rapidly and kill a high proportion of those affected. *Vibrio cholerae*, the etiologic agent of cholera, is a highly motile non-invasive Gram-negative bacterium which colonizes the small intestine and produces and secretes a potent enterotoxin called cholera toxin (CT), the major virulence determinant primarily responsible for the disease cholera [1].

Cholera toxin, composed of one A and five B subunits, are synthesized in the cytosol in unfolded form and translocated across the inner membrane via Sec dependent pathway to the periplasmic space where appropriate folding occurs leading to 3D-structure of CT. CT then translocates across the outer membrane of *V. cholerae* via type-II secretion system consisting of multi-protein complexes [2,3]. Biological action of CT initiates by binding of the pentameric B-subunit to the intestinal epithelial cells through GM1 receptor. A conformational change is induced when A subunit enters the cell via receptor mediated endocytosis which in activates the adenylate cyclase in the cell membrane and increases the cAMP production in intestinal epithelial cells [4].

Outer membrane vesicles or OMVs are discrete, closed outer membrane blebs produced naturally by growing cells and do not arise due to cell lysis or cell death [5]. Electron microscopic studies reveal that vesicles emerge as spherical bodies having a bilayer membrane and electron-dense luminal content. Biochemical analysis indicated that OMVs consist only of outer membrane (OM) and periplasmic components and do not contain inner membrane (IM) and cytoplasmic fractions [5]. OMVs has been found in a variety of Gram-negative bacteria [6,7] and several studies demonstrated that OMV play a role as protective transport vesicles, delivering toxins, enzymes and DNA to eukaryotic cells [5]. As early as 1967, formations of OMVs were demonstrated in growing cells of *V. cholerae*. The thin-section electron micrographs of *V. cholerae* cells revealed the bulging out and pinching off the cell wall in the form of vesicles encapsulating soluble periplasmic cargo [8,9]. Recently mucosal immunization by *V. cholerae* OMVs has been shown to induce long term protective immune response against this pathogen in a mice model [10] .

So far no reports of association of cholera toxin (CT) with OMVs are available. The present study is the first report which demonstrates that CT is associated with OMVs in a physiologically active form and binding and internalization of *V. cholerae* OMVs by intestinal epithelial cells is mediated by CT.

2. Materials and methods

2.1. Bacterial strain and growth condition

A streptomycin resistant *V. cholerae* O395 (O1 Classical, Ogawa, CT+) strain used in the present study was maintained at −70 °C in Luria–Bertani medium containing 20% (v/v) glycerol and were grown in LB medium with streptomycin (1 mg/ml, Sigma–Aldrich, USA) at 37 °C.

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2.2. Epithelial cell culture

Human intestinal epithelial cell line Int407 (NCCS, Pune, India) and Chinese Hamster Ovary (CHO) cells (NCCS, Pune, India) were grown and maintained in minimal essential medium (MEM; GIBCO-BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum (GIBCO-BRL) containing penicillin–streptomycin and gentamycin in presence of 5% CO_2 at 37 °C.

2.3. Isolation of outer membrane vesicles and fluorescent labeling

The vesicles were isolated from late-exponential phase (8 h) *V. cholerae* cultures using the procedure adopted from Schild et al. [10]. Briefly, cells from 1liter culture were harvested by centrifugation (4500×*g*, 15 min, 4 °C) and the supernatant was filtered through 0.45 μm pore size membrane (Millipore, USA) to remove residual cells. An aliquot of the filtrate was tested for the presence of viable *V. cholerae* cells on LB agar. In all cases, no colonies were detected. Protease inhibitor cocktail (Sigma–Aldrich, USA) was then added to the filtrate to prevent protein degradation and kept at 4 °C. Vesicles were recovered by ultracentrifugation (140 000×*g*, 4 h, 4 °C) using a Sorvall T-865 rotor, washed with 0.1 M phosphate-buffered saline (PBS) and suspended in PBS. The protein concentration was determined using Bradford reagent (BioRad Laboratories, USA). OMVs were stored at −80 °C and used within a week. For fluorescence labeling, vesicles were incubated (1 h, 25 °C) with fluorescein isothiocyanate (FITC; Sigma–Aldrich, USA; 1:1), pelleted (56 000×*g*, 30 min), washed and suspended in PBS.

2.4. Preparation of outer and inner membrane of V. cholerae

Preparation of the crude cell envelope (CCE) of *V. cholerae* was done as described earlier [11]. The CCE was fractionated into outer and inner membranes by treatment with 1% (w/v) Sarkosyl NL-97(Sigma; 30 min, 25 °C) followed by centrifugation (105 000×*g*, 1 h). The pellet containing outer membrane (OM) was washed and suspended in PBS. The supernatant containing inner membrane (IM) was also collected and both IM and OM fractions were stored at −80 °C.

2.5. Electron microscopy

For ultrastructural analysis of OMVs, a droplet of sample was placed on a carbon coated 400 mesh copper grids (ProSciTech, Australia), stained with 1% uranyl acetate and visualized under electron microscope (TECNAI G2 Spirit Biotwine, SEI, the Netherlands) at 80KV acceleration voltage.

2.6. Atomic force microscopy

OMV preparations (10 μl) were diluted with ultrapure water and immediately placed on a freshly cleaved mica surface (ASTM V1 Grade Ruby Mica from MICAFAB, Chennai, India), incubated (25 °C, 5 min), washed gently with ultrapure water and dried in a desiccators (30 min). AFM Imaging was performed in Acoustic AC (AAC) mode in Picoplus 5500 AFM system (Agilent Technologies, USA) using Silicon cantilever (PPP-NCL from Nano sensors). The images were analysed with Picoscan5.

2.7. SDS–PAGE and immunoblotting

SDS–PAGE (12%) was carried out as described earlier [11] at a constant current (15 mA/cm), and the gel was silver stained [12] for analysis of protein profile of OMVs, OM and IM.

Gels were then transferred in PVDF membranes (Millipore, USA) and immunoblotted using a polyclonal anti-CT rabbit antibody (Sigma) at a dilution of 1:20000 followed by incubation with alkaline phosphatase-conjugated mouse anti-rabbit IgG (GENEI, India) at a 1:1000 dilution in NaCl/TrisTbuffer/5% BSA. The alkaline phosphatase-positive bands were visualized in a developing solution containing 1×5-bromo-4-chloroindol-2-ylphosphate/Nitro Blue tetrazolium (GENEI), 1.5 mmTris/HCl (pH 8.8) and water in the dark at room temperature for 10 min.

2.8. Assays for CT

2.8.1. GM1-ELISA

CT production was estimated in *V. cholerae* culture supernatant and OMV by GM1-ELISA [13] using polyclonal rabbit antiserum directed against purified B-subunit of CT (Sigma–Aldrich, USA) and anti-rabbit immunoglobulinG conjugated with horseradish peroxidase (Santa Cruz, USA). A soluble CT standard curve was generated using 6.25–100 ng/ml CT to estimate the amount of CT in the samples. The color intensity was measured at 492 nm in an ELISA reader (E-max, USA).

2.8.2. CHO cell assay

CHO cells cultured in a 35 mm dish (1×10^6 cells) were incubated for 12 h at 37 °C in MEM/1% FCS with CT (100 ng), O395 OMV (100 μg) or 100 μg O395 vesicles preincubated (30 min, 25 °C) with 100 μgGM1 and cellular morphology was observed by phase-contrast microscopy.

2.8.3. cAMP assay

Int407cells (1×10^4 cells/well) were incubated with 100 μgO395 vesicles, 100 μgO395 vesicles preincubated (30 min, 25 °C) with 100 μgGM1, or 100 ngCT or 100 ngCT preincubated (4 h, 25 °C) with 10 μg GM1. cAMP concentrations were measured using the cAMP Activity Assay Kit (Biovision, CA, USA).

2.9. OMV fusion assay

FITC-labeled vesicles (2.5 μg/well) were incubated with confluent monolayers (1×10^4 cells/well) of Int407 in serum-free media in 96-well plates (Costar) for different time points at 37 °C. All incubation conditions were done in triplicate. Cells were washed twice with PBS and then solubilized in 100 μl 1% Triton X-100 in PBS. Fluorescence was detected over time as indicated on a fluorescent plate reader (Ex-485 nm, Em-520 nm; F-7000FL, Hitachi, Japan) Fluorescence intensity was normalized for fluorescence detected by labeled-OMV in the absence of intestinal epithelial cells.

2.10. Confocal microscopy

Int-407 epithelial cells were cultured overnight on coverslip (4×10^4cells) and incubated with fluorescently labeled vesicles (20 μg/ml) for different time points or vesicles pretreated with 10 μg GM1 (30 min, 25 °C) for 2 h in serum-free media at 37 °C. Cells were washed twice to remove unbound vesicles and were then fixed in 2% paraformaldehyde, mounted with ProLong AntiFade reagent (Molecular Probes), and visualized on a NikonA1R Laser Confocal Microscope (Japan).

3. Results and discussion

3.1. Isolation and characterization of V. cholerae OMVs

Outer membrane vesicles were purified from cell free culture supernatants of *V. cholerae* O395 expressing cholera toxin (CT) using methods adopted from earlier studies [10]. *V. cholerae* cells