Vacuolation induced by cytotoxin from *Helicobacter pylori* is mediated by the EGF receptor in HeLa cells

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Abstract The bacterial toxin VacA produced by *H. pylori* induces large vacuoles in several types of cultured cells such as HeLa cells or gastric cells. To determine the mechanism of vacuolation induced by this toxin we employed several inhibitors of membrane trafficking and endocytosis. The development of vacuolation induced by VacA in HeLa cells were prevented by bafilomycin A1 and low temperature conditions that inhibited vesicle transport or endocytosis. Formation of large vacuoles was also inhibited by an antibody against EGF receptor, which was previously shown to be internalized by endocytosis, but not by an anti-transferrin receptor antibody. Moreover, proteins of 58 and 37 kDa, corresponding to fragments of VacA, were recognized by an anti-*H. pylori* antibody after immunoprecipitation with anti-EGF receptor of cell extracts from HeLa cells treated with VacA, but not from untreated HeLa cells. We suggest that VacA may enter cells by endocytosis mediated by the EGF receptor. These are the first data indicating that the EGF receptor may be significant in the development of vacuolation caused by VacA.

Key words: VacA; *Helicobacter pylori*; EGF receptor; Bacterial toxin; Vacuolation; Vacuole

1. Introduction

Helicobacter pylori (*H. pylori*) is a micro-aerophilic, Gram-negative, spiral bacterium that infects more than 50% of the human population [1]. *H. pylori* plays a major role in the development of gastritis, peptic ulcer disease, and gastric carcinoma [2]. It is known that *H. pylori* produces a vacuolating toxin, VacA [3], and several other cytotoxins such as CagA [4], NapA [5], and PicB [6].

VacA is translated as a 140-kDa pre-pro protein and is cleaved at the N-terminal 33 amino acid leader sequence in the periplasm and at the C-terminal 55-kDa peptide domain in the outer membrane, and then is released from the organism as a 95-kDa mature protein [7]. The released VacA can be cleaved into two fragments of molecular weights 58 kDa and 37 kDa [7,8], but this protein forms complexes of more than 1000 kDa under non-denaturing conditions [9]. It has been reported that VacA has a 'flower' like oligometric structure [10,11]. It is known that VacA induces large vacuoles in cultured cells [12,13] and in animal cells in vivo [14]. Previous studies have shown that the vacuoles in HeLa cells caused by VacA are enriched in rab7, a small GTP-binding protein associated with the late endosomal component [12,15]. Dominant negative rab7 mutation prevents vacuolation caused by VacA. It is also known that vacuolation is inhibited by bafilomycin A1, which is a specific V-type ATPase inhibitor [12], or by addition of an anti-V-type ATPase antibody [16]. In addition, it has been reported that bafilomycin A1 can inhibit the transition from early to late endosome [17] and it is being investigated whether the late endosome is a key component in vacuolation caused by VacA.

Many bacterial toxins are internalized into eukaryotic cells. For example, it is known that diphtheria toxin enters cells and then enzymatically modifies a specific intracellular target [18]. It has been reported that VacA is associated with the plasma membrane of eukaryotic cells and is then internalized [19]. The important subunit of VacA for internalization seems to be the C-terminal 58-kDa fragment [19]. However, the mechanism by which the toxin enters the cells is not clear and the receptor or ligand on the plasma membrane has not been defined.

In this study, we have investigated the mechanism of internalization of VacA in cultured cells and the receptor on the membrane of host cells that binds with VacA.

2. Material and methods

2.1. Materials

Brucella broth was purchased from Difco Laboratories (Detroit, MI, USA). RPMI 1640 medium was obtained from Gibco Laboratories (Grand Island, NY, USA). Bafilomycin A1, neutral red, and β-cyclodextrin were purchased from Wako Chemicals (Osaka, Japan). Fetal bovine serum was obtained from Dai-nihon Pharmaceuticals (Osaka, Japan). Antibiotic-antimycological solution was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-EGF receptor antibody directed against the extracellular domain was obtained from Monosan (the Netherlands). Mouse IgG was obtained from Vector Laboratories (Burlingame, CA, USA). Anti-transferrin receptor antibody was obtained from Austral Biologicals (Son Ramon, CA, USA). Protein G-agarose was obtained from Calbiochem (Cambridge, MA). Anti-*Helicobacter pylori* antibody was obtained from Kirkegaard Perry Laboratories (Gaithersburg, MD). Anti-EGF receptor antibody directed against the intracellular domain was obtained from Transduction Laboratories (Lexington, KY, USA). Fluorescein-labeled protein G was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Bacterial culture and preparation of VacA

H. pylori (ATCC 43504, American type culture collection) was grown with shaking at 70 rpm in brucella broth containing 5% fetal bovine serum under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2) at 37°C for 24 h. The cultures were centrifuged at 3000 rpm, the medium was removed, and the bacteria were resuspensed at 10^7 cfu/ml in brucella broth containing 0.3% β-cyclodextrin without fetal bovine serum. Then organisms were cultured with shaking at 70 rpm under microaerophilic conditions at 37°C for 2 days. The cultures were centrifuged and the supernatants were precipitated with saturated ammonium sulfate (50% saturation). The pellets were re-suspended in phosphate-buffered saline, and were stored at −30°C until use.

2.3. Cell culture and assay for cell vacuolation

HeLa cells were cultured in RPMI 1640 containing 10% fetal bovine

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serum in a 96-well culture plate at 37°C for 3 days. The cells were incubated with crude extracts containing VacA prepared as described in Section 2.2 in RPMI 1640 with 10% fetal bovine serum at 37°C. The inhibitor bafilomycin A1 was added simultaneously to the medium, while antibodies were added 30 min before VacA addition. The total number of cells and the number of vacuolated cells were counted. Neutral red uptake was assessed as described by Papini et al. [12]. Neutral red was incorporated into the cells for 5 min after treatment with VacA and then the dye was extracted with 40% HCl-0.37% EtOH, and was detected by UV absorbance at 550 nm. In a time course experiment, the cells were incubated with VacA for the indicated times, and then were treated with neutral red for 5 min.

2.4. Immunoprecipitation and Western blot analysis

HeLa cells were treated with toxin fractions containing VacA in RPMI 1640 for 6 h, and were then harvested with ice cold RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.1 mM Na_2Vo_4, 50 mM NaF, 1 mM PMSF, 1 mM leupeptin, and 10 μg/ml trypsin inhibitor) and homogenized using a syringe. The cell lysates were pretreated with protein G-agarose solution and centrifuged at 15 000 rpm for 20 min. The supernatants were treated with anti-EGF receptor directed against the internal domain of the receptor, and were incubated with protein G-agarose solution. After centrifugation, the pellets were solubilized in SDS-PAGE sample buffer, and aliquots were loaded onto the gels [20]. The proteins thus obtained were transferred to a nylon membrane, and were incubated in PBS with 0.1% Tween 20, and 3% non-fat milk containing anti-*H. pylori* antibody. After washing, the proteins were detected by HRP-labeled protein G using an ECL detection kit (Amersham-Pharmacia, Buckinghamshire, UK).

3. Results

3.1. Microscopy of large vacuoles induced by VacA

As shown in Fig. 1, vacuoles were induced by the crude extract containing VacA obtained from the culture medium of *H. pylori* as described in Section 2. Vacuolated cells comprised more than 80% of all cells at low magnification (Fig. 1a,c).

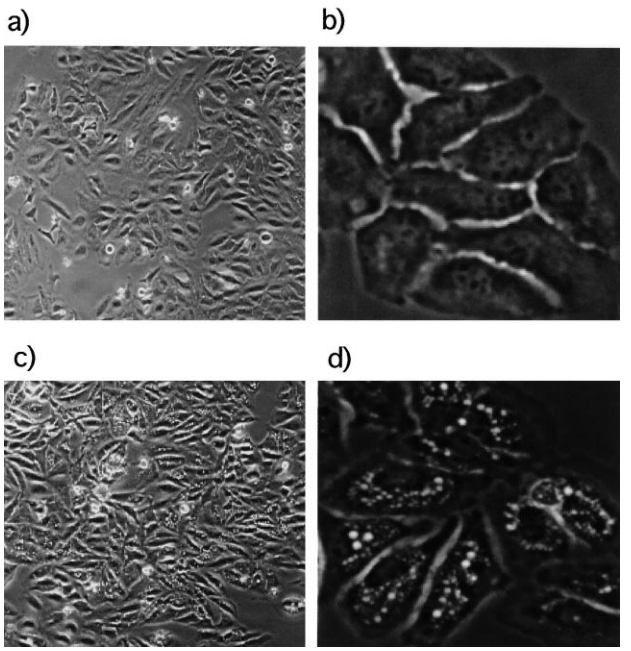

Fig. 1. Vacuole formation induced by VacA. HeLa cells were incubated with (c,d) or without (a,b) VacA crude extract from bacterial culture medium for 24 h and photographed in high magnification (a,c) or low magnification (b,d).

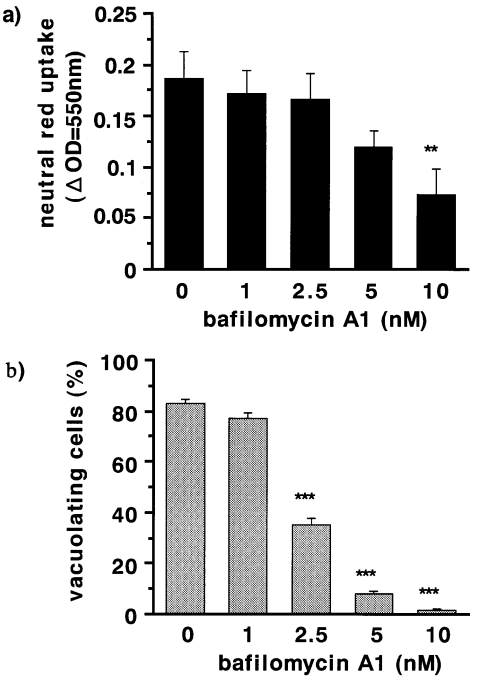

Fig. 2. Effect of bafilomycin on formation of large vacuoles. HeLa cells were treated with bafilomycin A1, and incubated with VacA for 24 h. A neutral red uptake assay and counting of cells were analyzed as described in Section 2. Each column represents mean ± S.E. ($n = 6$). Statistical analysis is Scheffes's test. $^{**}P = 0.01$ vs. cont., $^{***}P = 0.001$ vs. cont.

3.2. Effect of bafilomycin A1 on formation of large vacuoles

As reported previously, neutral red uptake (Fig. 2a) or vacuolation of cells (Fig. 2b) induced by VacA were prevented by bafilomycin A1, a V-type ATPase inhibitor that can inhibit the transition to late endosome from early endosome, or to the lysosome from late endosome.

3.3. Effect of temperature on vacuolation

To determine the role of vesicle transport in the development of vacuolation, we investigated whether the vacuolation

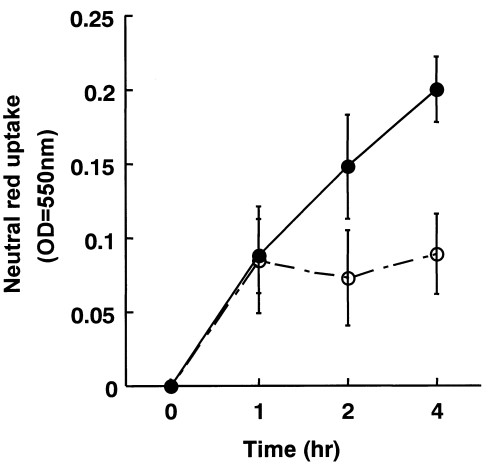

Fig. 3. Effects of temperature on the development of vacuolation. HeLa cells were incubated with VacA in conditions at 37°C (●) or at 10°C (○) for the indicated time, and the activity was analyzed by neutral red uptake assay as described in Section 2. Each point represents mean ± S.E. ($n = 8$). Statistical analysis is Student's *t*-test. $^{**}P = 0.01$ vs. cont.

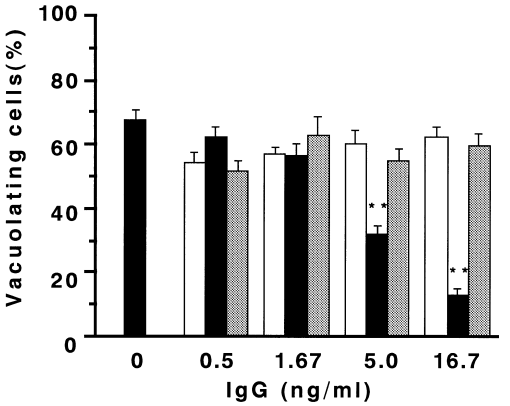

Fig. 4. Effects of antibodies against receptors in plasma membrane on the development of vacuolation induced by VacA. After treating with antibodies for 30 min, HeLa cells were incubated with VacA for 24 h, and then the total number of cells and vacuolated number of cells were counted (open column: mouse IgG; closed column: anti-EGF receptor antibody; dotted column: anti-transferrin receptor antibody). Each column represents mean ± S.E. ($n = 6$). Statistical analysis is Scheffes's test. ***$P = 0.001$ vs. normal IgG.

is prevented by low temperature that can inhibit the vesicle transport. As shown in Fig. 3, neutral red uptake was time-dependently increased by VacA, and was prevented by a low temperature of 10°C.

3.4. *Effect of antibodies against plasma membrane receptors on vacuolation*

To investigate the mechanism of internalization of VacA, we used antibodies directed against plasma membrane receptors, anti-EGF receptor and anti-transferrin receptor antibodies. As shown in Fig. 4, vacuolation of HeLa cells induced by the addition of crude VacA extracts was prevented by addition of anti-EGF receptor antibody directed against the extracellular domain of the receptor, but not by anti-transferrin receptor antibody or normal IgG. The number of vacuolated cells was decreased depending on the concentration of anti-EGF receptor antibody.

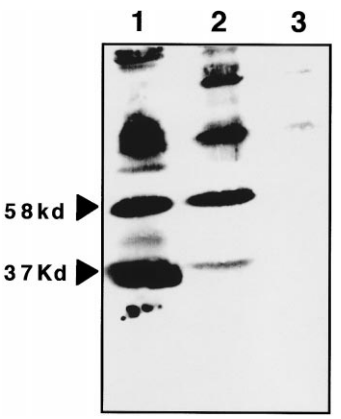

Fig. 5. Association of VacA with EGF-R on the development of vacuolation. Extracts from HeLa cells treated with crude VacA were immunoprecipitated with anti-EGF-R antibody reacting with the intercellular domain of the C-terminal region in EGF-R, and the immunoprecipitate was analyzed by SDS-PAGE and Western blotting using anti-*H. pylori* antibody reacting with VacA. Lane 1: *H. pylori* culture medium precipitated with saturated ammonium sulfate. Lane 2: Immunoprecipitate of HeLa cells treated with the crude fraction containing VacA. Lane 3: Non-treated HeLa cells.

3.5. *Association of VacA with EGF-R in the development of vacuolation*

We investigated whether VacA associated with the EGF receptor using immunoprecipitation and Western blot analysis. Extracts from HeLa cells treated with crude VacA were immunoprecipitated using an anti-EGF receptor antibody reacting with the intracellular domain of the C-terminal region of EGF receptor, and the immunoprecipitate was analyzed by SDS-PAGE and Western blotting using an anti-*H. pylori* antibody reacting with VacA. As shown in Fig. 5, proteins of 58 kDa and 37 kDa reacted with anti-*H. pylori* antibody in the extracts immunoprecipitated with anti-EGF receptor antibody from VacA-treated HeLa cells, but not from untreated HeLa cells.

4. Discussion

We investigated the mechanism of internalization of VacA into the cells and the cell surface receptor of host cells binding with VacA. It is already known that the vacuoles in HeLa cells created by VacA are enriched in rab7, a small GTP-binding protein previously shown to be associated with the late endosomal component [12,15]. It has been reported that vacuolation induced by VacA is prevented by bafilomycin A1, a V-type ATPase inhibitor [12,16], and this was confirmed in our study (Fig. 2). It is known that bafilomycin A1 can inhibit membrane trafficking to late endosome from early endosome, or to lysosome from late endosome [17]. Therefore, our results suggested that the development of vacuolation by VacA was related to membrane trafficking from early endosome, and not to the pathway from TGN to late endosome.

It is unclear whether the mechanism of internalization of VacA involves endocytosis or another pathway. It has been suggested that VacA is internalized by a non-endocytotic pathway, because its internalization is slower than that of other proteins. However, VacA has a high molecular weight of 95 kDa under denaturing conditions and forms a more than 1000-kDa complex under non-denaturing conditions [21], so it would not be able to cross the plasma membrane. In this study we found that the increase of neutral red uptake by VacA was rapid and the development of vacuolation was prevented by low temperature conditions that can inhibit endocytotic membrane transport (Fig. 3). As shown in Fig. 4, vacuolation was also prevented by a monoclonal antibody against EGF receptor, which was previously shown to be internalized by endocytosis. These findings suggest that VacA may be internalized into cells by endocytosis.

On the other hand, vacuolation was not prevented by a monoclonal antibody against the transferrin receptor, which was previously shown to be internalized by endocytosis as well as the EGF receptor (Fig. 4). Therefore, it may be that the EGF receptor is important in the internalization of VacA. In the case of other bacterial toxins, it is reported that diphtheria toxin is internalized into cells via a cell surface receptor [18,22]. It is known that this toxin is processed into two fragments and one of the fragments is important for internalization [18,23], as is the case for VacA. Thus, internalization of VacA may mimic that of diphtheria toxin. This study also showed that anti-*H. pylori* antibody reacted with the 57-kDa protein corresponding to one of the fragments of VacA after immunoprecipitation with anti-EGF receptor antibody of extracts from cells treated with VacA (Fig. 5). This result sug-

gests that VacA may be associated with the EGF receptor directly or indirectly, and that this receptor may be a significant factor in the development of vacuolation by VacA.

It is known that the genotype of VacA is related to the pathogenesis of gastric inflammation and peptic ulceration in patients infected with *H. pylori* [24]. Infection with *H. pylori* induces apoptosis and causes atrophic gastritis in animal [25] or man [26]. On the other hand, EGF is one of the most important factors in gastric wound healing [27]. If VacA competes with EGF for receptor binding, the response to EGF may be decreased and the rate of cell turnover may also decrease. The final results may be apoptosis or cell atrophy. As shown in Fig. 4, the vacuolating action of VacA was prevented by anti-EGF receptor monoclonal antibody that can inhibit actions of EGF. Therefore, it is interred that a binding site of EGF in the receptor is near the region with that of VacA and EGF can compete with this toxin for receptor binding. But we have no evidence whether EGF directly competes with VacA for receptor binding.

We investigated the mechanism of vacuolation caused by VacA produced from *H. pylori*. Our study suggested that the pathway from early to late endosome is essential to the development of vacuolation and that VacA enters the cells by endocytosis. The possibility that the EGF receptor is important in the internalization of VacA was also suggested. These are the first data indicating that the EGF-receptor may be involved in the development of vacuolation by VacA, and they are of interest not only with regard to the cell biologic action of bacterial toxins but also to the pathogenesis of *H. pylori* infection.

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