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Helicobacter pylori Vacuolating Cytotoxin (VacA) Disorganizes the Cytoskeletal Architecture of Gastric Epithelial Cells

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***Helicobacter pylori* colonization of the gastric mucosa induces peptic ulcer disease and interferes with ulcer healing. Re-epithelialization is an essential component of ulcer healing. It requires cell migration and proliferation which are dependent on the cell cytoskeleton. Most *H. pylori* strains produce a toxin (VacA) that induces multiple structural and functional changes in epithelial cells. In this study, we investigated the effects of VacA on the gastric epithelial cell cytoskeletal architecture. Exposure of rat gastric epithelial cells to purified VacA from *H. pylori* 60190 significantly inhibited actin stress fiber formation (83 ± 5% reduction; $p < 0.0001$) and disorganized microtubule pattern (90 ± 8%; $p < 0.001$). Furthermore, VacA treatment significantly reduced tyrosine phosphorylation of focal adhesion kinase (FAK) (by 45 ± 6%; $p < 0.002$) and its expression in focal adhesions (73 ± 8%; $p < 0.0001$). These findings suggest that *H. pylori* VacA interferes with cytoskeleton-dependent cell functions and with the transmission of signals related to cell spreading and growth.** © 1999 Academic Press

Key Words: **gastric RGM1 cells; actin cytoskeleton; focal adhesion kinase; tyrosine phosphorylation.**

Despite intensive clinical and basic research efforts, the cellular and molecular mechanisms by which *H. pylori* induces peptic ulcers or interferes with ulcer healing are as yet not well understood. One putative virulence factor of *H. pylori* is a ~90 kDa vacuolating cytotoxin (VacA) (1–5). The most prominent effect of this toxin is its capacity to induce vacuolation in epithelial cells *in vitro* (6). In addition, VacA alters the intracellular trafficking of proteins such as procathepsin (7), increases permeability of polarized epithelial monolayers (8), inhibits the process of antigen presentation (9) and forms anion-selective channels in lipid bilayers (10).

Our previous study *in vivo* demonstrated that VacA delays healing of gastric ulcer in rats, inhibits re-epithelialization, and worsens the quality of mucosal scar (11). Our more recent study *in vitro* demonstrated that VacA inhibits gastric epithelial cell proliferation and interferes with epidermal growth factor (EGF)-activated signal transduction, essential for gastric mucosal healing (12–14). Studies focussed on epithelial restitution *in vitro* have demonstrated that gastrointestinal cell migration depends on actin polymerization (stress fiber formation) (15). It is also well established that both cell migration and cell proliferation are dependent on the cell cytoskeleton (16).

The cytoskeleton consists of microfilaments, microtubules, and intermediate filaments, which have contractile properties and participate in various cellular functions (17). Cytoplasmic microfilaments (comprised of actin) can assemble, disassemble and form cross-linked bundles, which are anchored to other cellular components, including the cell membrane. Microtubules are important cytoplasmic structures that are involved in intracellular transport and are essential for cell division and differentiation. The intermediate filaments consist of fibrous proteins, which exhibit mechanical functions in stiffening of cells and in organizing intracellular organelles for coordinated activity (16).

The key events regulating cell structure and shape, cell motility and proliferation include polymerization of actin, formation of actin stress fibers, and focal adhesions (18). Epithelial "barrier" function is also dependent on an intact cell cytoskeleton (19). Several bacterial protein toxins are known to cause alterations in cytoskeletal architecture. For example, *C. botulinum* C2 toxin and Clostridial iota toxin modify actin micro-

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filaments directly, whereas other toxins enzymatically modify intracellular proteins that are involved in the regulation of the cytoskeleton (20–23). Thus far, the possible role of VacA in altering cytoskeletal architecture has not been examined. Therefore, in this study, we examined whether VacA alters the cytoskeleton of gastric epithelial (RGM1) cells derived from normal rat gastric mucosa.

MATERIAL AND METHODS

Bacterial strains and growth conditions. VacA was purified from *H. pylori* strain 60190 (ATCC 49503), a wild-type cytotoxin-producing strain, as described previously (24). As a control, broth culture supernatant of *H. pylori* strain 60190 v1, in which the *vacA* gene has been disrupted by insertional mutagenesis, was processed in the same manner (25). Earlier studies have shown that VacA is strongly activated by a short exposure to acidic solutions in the pH 1.5-5.5 range, and this activation persists even after subsequent neutralization (26). In all the present experiments, purified VacA preparations and identically prepared VacA-negative preparations were acidified to pH 3.5 by the addition of HCl. Phosphate buffered saline (PBS) acidified to pH 3.5 (volume equal to VacA) was used as an additional control. Addition of acidified PBS to buffered tissue culture medium (50 μl added to 2.0 ml medium) did not significantly alter the pH, which remained at pH 7.4.

Gastric cells. RGM1, a cell line derived from normal gastric mucosa of the rat (28) was obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan). RGM1 cells were routinely grown in DMEM/F12 (1:1 ratio) medium supplemented with 20% FBS, 1% glutamine, antibiotics and antifungal agents. Cell cultures were prepared by seeding 3×10^6 cells/dish to attain 70% confluence. After 24 hours, the medium was replaced with fresh medium, and cells were incubated with medium containing either VacA at a final concentration of approximately 1.0 μg/ml or control Vac-negative preparation for 1, 4, 24 or 48 hours. The optimal dose of VacA was determined based on dose-dependent studies (0.1-5.0 μg/ml data not shown). After incubation, cells were washed in cold PBS, lysed, and analyzed as described below.

Distribution and localization of actin, β-tubulin, and cytokeratin. RGM1 cells were grown to sub-confluence on collagen I-coated cover slips and treated as described above. After respective treatments, cells were washed in PBS, fixed and permeabilized with 3.7% paraformaldehyde at room temperature for 20 minutes and post fixed in acetone for 5 minutes at −20°C. F-actin was detected by using fluoroscein labelled-phalloidin (F432; Molecular Probes, Inc., Eugene, OR). Distribution of β-tubulin and cytokeratin in RGM1 cells was studied by immunostaining using mouse monoclonal antibodies (T4026, β-tubulin Ab dilution 1:100; C7159; cytokeratin Ab dilution 1:500; Sigma Chemical Co., St. Louis, MO) followed by specific FITC conjugated secondary anti-mouse antibody (dilution 1:200; Sigma Chemical Co., St. Louis, MO). Slides were examined under a Nikon microscope with epifluorescence and photographed. Two investigators unaware of the code evaluated quantitatively the coded slides. The number of stress fibers per cell was counted under 400× magnification. A minimum of five fields and at least 50 cells per slide were counted. The results are expressed as percent of control (cells incubated with medium containing acidified PBS).

Determination of focal adhesion kinase (FAK) protein and tyrosine phosphorylation level. Focal adhesion kinase (FAK) protein level was determined by Western blot analysis following the procedure previously described (13). Tyrosine phosphorylation levels of FAK protein was determined by immunoprecipitation with specific antibody and immunoblotting with anti-phosphotyrosine antibody according to the method previously described (13). Briefly, after various treatments cells were lysed in ice-cold lysis buffer (20 mmol/L Tris-HCl pH 7.5, 50 mmol/L NaCl, 50 mmol/L NaF, 30 mmol/L sodium pyrophosphate, 5 mol/L EGTA, 10% glycerol, 1% Triton X-100, 1 mmol/L PMSF, 1 mmol/L sodium vanadate and 5 μg/ml aprotinin) and clarified by centrifugation at 14,000 rpm for 10 min. The protein concentration of the lysate was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of proteins were incubated with anti-FAK antibody (Santa Cruz Biotech, Santa Cruz, CA) immobilized onto protein A Sepharose for 2 h at 4°C under gentle rotation. Beads were washed extensively with lysis buffer and immunecomplexes eluted by heating for 5 min at 95°C in 2× Laemmli buffer and microcentrifuged. The supernatant was subjected to SDS-PAGE (7.5%) followed by immunoblotting with anti-phosphotyrosine antibody (Santa Cruz Biotech, Santa Cruz, CA).

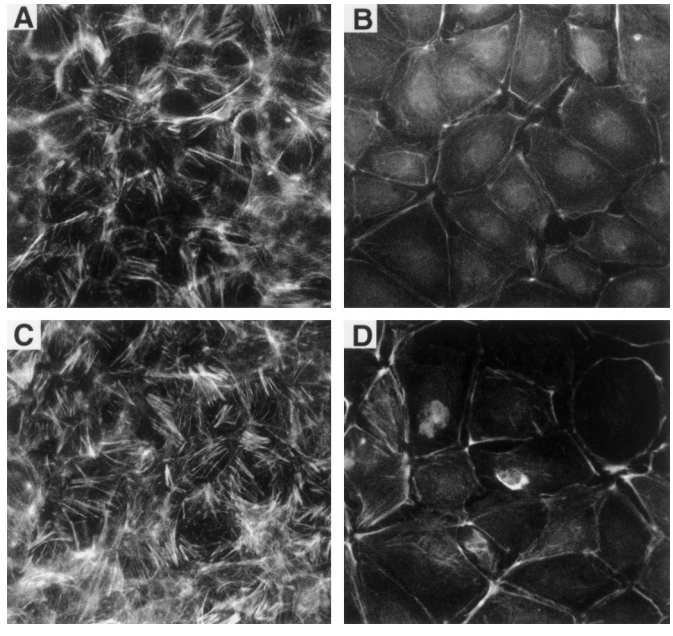

FIG. 1. Effects of *H. pylori* VacA on actin cytoskeleton in gastric epithelial cell monolayers. RGM1 cells were grown to confluence on collagen I-coated cover slips, then treated with either 1) medium containing acidified PBS (control) (Panels A & C: 24 and 48 h respectively) or 2) medium containing VacA for 24 h and 48 h (B & D respectively). After these incubations, actin filaments were labeled with fluorescein-conjugated phalloidin using the procedure described in the Methods Section. Control cells at 24 and 48 h (Panels A & C) demonstrate numerous dense actin stress fibers. VacA treatment resulted in a significant decrease in actin stress fibers at 24 h (Panel B; 65% ± 3% reduction), and at 48 h (Panel D; 83% ± 5% reduction) (400×). Treatment with Vac-negative preparation showed dense actin stress fibers similar to control cells.

Focal adhesion kinase (FAK) distribution. Distribution of FAK in RGM1 cells was studied by immunostaining. RGM1 cells were grown to sub-confluence on collagen-I coated cover slips and subjected to various treatments. Cells were fixed, permeabilized and immunostained using rabbit polyclonal antibody (1:100 dilution; Santa Cruz Biotech, Santa Cruz, CA) followed by specific FITC conjugated secondary anti-FAK rabbit antibody (1:200 dilution; Sigma Chemical Co, MO). Quantification was performed by counting the number of focal adhesions per cell. The results were compared with control cells incubated with medium containing acidified PBS.

Statistical analysis. All data are reported as mean ± SD. Statistical significance of differences between mean values was assessed by

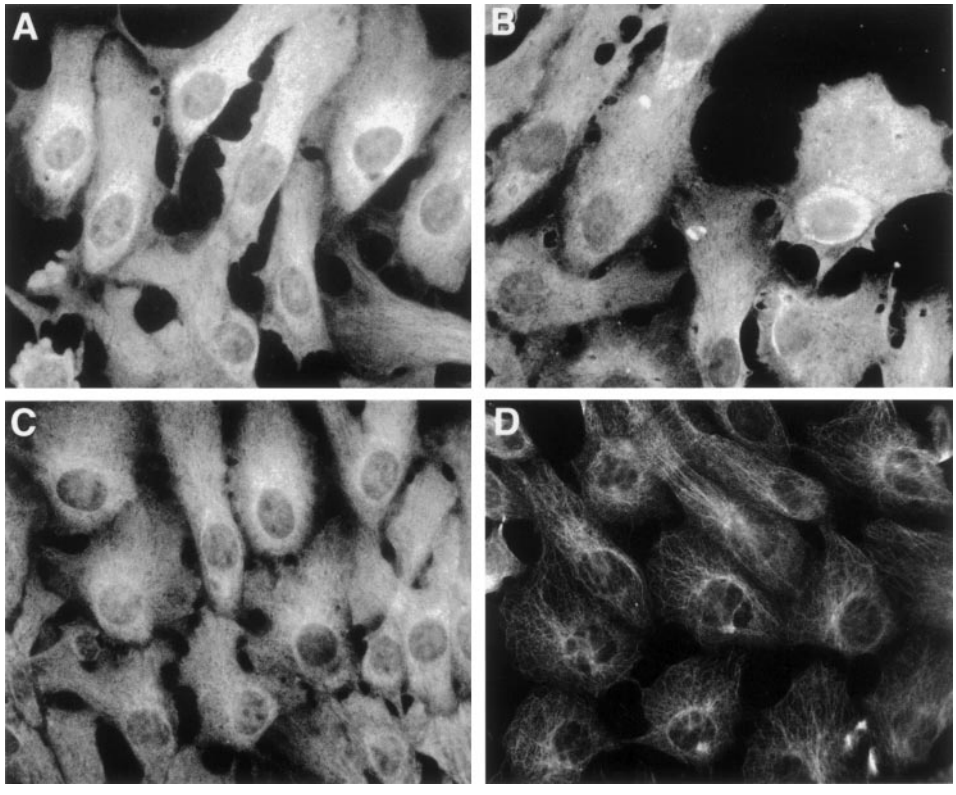

FIG. 2. Beta-tubulin distribution in gastric epithelial cell monolayers. RGM1 cells were grown to confluence on collagen I-coated cover slips, and incubated for 24 and 48 h with either 1) medium containing acidified PBS (control) or 2) medium containing VacA. Cells were immunostained for β-tubulin according to the procedure described in the Methods section. In monolayers incubated with medium alone, cells demonstrate a dense network of microtubules assembled mostly in parallel and radial arrays, tightly packed, often starting from the microtubule organization center (MTOC) or centrosome (Panel A & C: 24 and 48 h respectively). VacA treatment for 24 h (Panel B) did not result in any significant changes in microtubule distribution or intensity of fluorescent signal, but after 48 h microtubules were less dense (Panel D; 90% $\pm$ 8% reduction; $p < 0.001$; vs control) (400×). Treatment with Vac-negative preparation showed dense network of microtubules similar to control cells.

Student's *t* test for unpaired data. A p value of <0.05 was considered statistically significant.

RESULTS

Effect of VacA on F-Actin Distribution in Gastric Epithelial (RGM1) Cells

To determine whether VacA altered cytoskeletal architecture of gastric epithelial cells, rat gastric epithelial cells (RGM1 cells) were incubated with purified VacA or with various controls for varying time intervals. Control cells showed a dense network of actin stress fibers (Figs. 1A & C). Exposure of cells to VacA for 4 h did not cause any significant change in actin distribution. After 24 h of incubation with VacA (Fig. 1B), cells demonstrated a substantial decrease in stress fibers (65% $\pm$ 3% reduction; $p < 0.001$). A similar effect was observed after 48 h of incubation with VacA (Fig. 1D; 83% $\pm$ 5% reduction $p < 0.0001$; vs control). F-actin microfilaments were markedly disorganized throughout the cytoplasm in cells incubated with VacA. In contrast, a Vac-negative control preparation did not cause any significant change in the number or density of stress fibers (not shown).

Effect of VacA on Microtubules and Intermediate Filaments in Gastric Epithelial (RGM1) Cells

Immunostaining for β-tubulin in control gastric epithelial cells revealed a dense network of microtubules assembled mostly in parallel and radial arrays, tightly packed, often starting from the microtubule organization center (MTOC) or centrosome (Figs. 2A & C). VacA addition did not significantly alter microtubule architecture at 24 h (Fig. 2B). However, at 48 h, in cells treated with VacA, microtubules were less dense (Fig. 2D; 90% $\pm$ 8% $p < 0.001$; vs control), with fainter fluorescence signals and showed irregular arrangement. In contrast, the VacA- negative control preparation failed to bring about these effects (not shown).

VacA treatment did not cause any apparent changes in cytokeratin (intermediate filament) organization when compared with control gastric epithelial cells

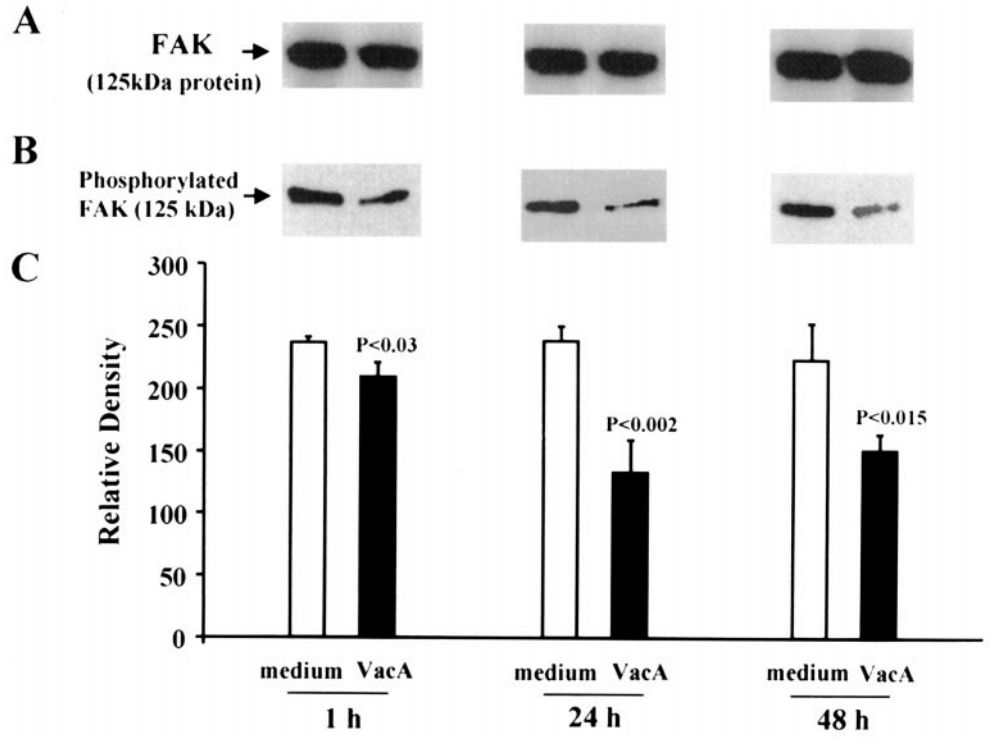

FIG. 3. Focal Adhesion Kinase (FAK) protein and tyrosine phosphorylation levels in RGM1 cells. RGM1 cells were incubated for 24 and 48 h with either 1) medium containing acidified PBS (control) or 2) medium containing VacA at a final concentration of approximately 1.0 μg/ml. FAK protein levels were determined by Western blot analysis. Tyrosine phosphorylation of FAK was determined by immunoprecipitating FAK with specific anti-FAK polyclonal antibody, and the immunoprecipitate was subjected to immunoblotting using anti-phosphotyrosine antibody. The densities of 125 kDa phosphorylated protein bands corresponding to FAK were analyzed using Eagle Eye II. A) Representative Western blot showing FAK protein levels without any significant changes in the presence or absence of VacA. B) Representative Western blot showing significant decreases in FAK phosphorylation level after VacA treatment. The Western blot is a representative of three separate experiments. C) Graph showing the mean ± SD (n = 3) relative densities of FAK phosphorylation levels in gastric epithelial cells.

(data not shown), indicating that the intermediate filaments are not markedly affected by VacA.

Effect of VacA on Focal Adhesion Kinase Tyrosine Phosphorylation

Since FAK phosphorylation has been shown to accompany the assembly of stress fibers and focal adhesions (29–35), we next examined whether VacA altered FAK phosphorylation. Treatment of gastric epithelial cells with VacA significantly reduced FAK tyrosine phosphorylation when compared with control cells. This effect was detected after 1 h (12% reduction; $p < 0.03$), peaked at 24 h (45% reduction; $p < 0.002$) and was sustained up to 48 h. (33.3%; $p < 0.015$; Figs. 3B & C). FAK protein levels determined by Western blot analysis did not show significant changes (Fig. 3A), indicating that changes in FAK phosphorylation were not due to reduction in their FAK protein levels. The VacA-negative preparation did not cause any significant change in either FAK phosphorylation or FAK protein levels (data not shown).

Effect of VacA on Focal Adhesion Kinase Distribution

Specific inhibition of FAK localization to focal adhesions has been shown to decrease cell motility and proliferation (36). Therefore, we sought to determine the effect of VacA on FAK localization. Treatment of gastric epithelial cells with VacA for 1 or 4 h did not show any significant change in FAK distribution. After 24 h, control cells showed strong FAK fluorescence signal localized to focal adhesions. There was a slight reduction in the signal by 48 h, likely due to cells attaining their confluence (Figs. 4A & C). In contrast, cells treated with VacA for 24 h and 48 h demonstrated significant reduction in FAK fluorescence signals (Figs. 4B & D; 73% ± 8% and 92% ± 5% respectively) indicating significant reduction of FAK in focal adhesions (both $p < 0.0001$ vs control).

DISCUSSION

Gastric mucosal re-epithelialization is essential for repair of acute mucosal injury and for ulcer healing. It

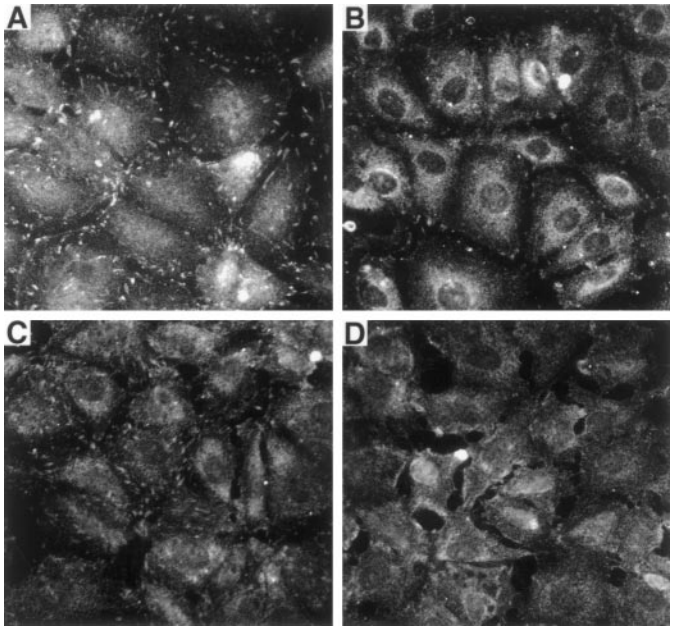

FIG. 4. Focal Adhesion Kinase (FAK) distribution in gastric epithelial cell monolayers. RGM1 cells were grown to confluence on collagen I-coated cover slips, and incubated for 24 and 48 h with either 1) medium containing acidified PBS (control) or 2) medium containing VacA at a final concentration of 1.0 μg/ml. Cells were immunostained for FAK as described in the Methods section. In monolayers incubated with medium alone, cells demonstrate dense localization of FAK to focal adhesions (Panel A & C). In monolayers treated with VacA, cells show a significant reduction in fluorescence signal and loss of FAK localization to focal adhesions at both 24 h (Panel B) and 48 h (Panel D) (400×). Treatment with Vac-negative preparation showed dense localization of FAK to focal adhesions similar to control cells.

requires epithelial cell migration in the early stage, as well as epithelial cell proliferation. The present study, using rat gastric epithelial cells (RGM1 cells) originating from normal rat gastric mucosa, demonstrates that the *H. pylori* vacuolating cytotoxin disrupts cytoskeletal architecture, and significantly reduces tyrosine phosphorylation of FAK and its presence in focal adhesions, which are all necessary for the maintenance of cell structural integrity, and for the transmission of signals related to cell spreading and growth.

Previous studies of *H. pylori* VacA have focused on the capacity of this toxin to induce marked vacuolation in epithelial cells. VacA is thought to cause vacuole formation by disrupting normal vesicle trafficking in the cell (6). The specific mechanisms by which the VacA causes these effects are not well understood, but recent studies indicate that VacA interacts directly with a cytoplasmic target molecule (37). In the present study, we demonstrated that VacA disorganizes cytoskeletal architecture in gastric epithelial cells. An effect of VacA on the cytoskeleton may explain the rounding and retraction of HeLa cells that has been observed previously following exposure of these cells to high doses of VacA (27). A recent study has shown that VacA increases selective permeability of polarized epithelial cell monolayers (7). It is well established that epithelial barrier function depends on proper function of the cell cytoskeleton, and our present observation that VacA disrupts cytoskeleton and focal adhesions may provide insight into the underlying mechanism of this effect. In the present study, changes in microtubule architecture were manifested only at 48 hours. Since *H. pylori* infects gastric mucosa chronically and gastric epithelial cells are exposed to VacA for a long period of time, even such late changes have direct pathologic relevance.

Ulcer healing requires interaction of various cellular and connective tissue components (38, 39). A number of growth factors including EGF and TGF-α have been shown to participate in repair of tissue injury by stimulating cell proliferation and migration necessary for re-epithelialization and ulcer healing (40–43). FAK phosphorylation has been shown to accompany cell adhesion and assembly of focal adhesions and stress fibers (30–35). Increased expression and/or activation of FAK have been shown to play a role in cell migration, which plays a crucial role in processes such as embryonic development, and wound healing (44, 45). Conversly, inhibition of FAK phosphorylation has been shown to decrease cell proliferation and motility (46, 47). Therefore, exposure of gastric epithelial cells to VacA may not only interfere with wound healing, but may make the gastric mucosa more susceptible to noxious insults.

While some bacterial toxins, such as CNF1 from *E. coli*, DNT from *Bordetella bronchiseptica* are shown to induce actin stress fibers and focal adhesion formation in Swiss 3T3 and in human umbilical vein endothelial (HUVEC) cells (48, 49), C3-transferase from *Clostridium botulinum*, EDIN from *Staphylococcus aureus* and toxin A from *C. difficile* have been shown to block migration of wounded endothelial cells monolayer by abolishing formation of actin stress fibers and focal adhesions (50).

In summary, our data show for the first time that treatment of gastric epithelial cell (RGM1) monolayers with *H. pylori* VacA disrupts actin stress fiber assembly, microtubule organization, reduces tyrosine phosphorylation of FAK and reduces FAK in focal adhesions. Since cytoskeletal re-arrangements such as stress fiber and focal adhesion formation are crucial for cell migration and proliferation, these findings add new insight into the mechanisms by which *H. pylori* inhibits the repair of gastric mucosal injury and ulcer healing or renders the gastric mucosa susceptible to injury.

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