Outer membrane vesicles enhance the carcinogenic potential of *Helicobacter pylori*

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**Introduction**

*Helicobacter pylori* is the oldest known human pathogen (1) and also the most successful, colonizing more than half the world’s population (2). Infection is persistent and always associated with a host inflammatory response (3). This is a major risk factor in the development of gastric disease including distal gastric cancer (4). Altered gastric epithelial cell turnover is considered one mechanism by which this bacterium exerts a carcinogenic effect, and there is increasing evidence that *H. pylori* mediates this response indirectly via the induction of host inflammatory mediators (5). However, *H. pylori* can also have a direct and potentially carcinogenic effect on epithelial cells in the absence of inflammatory mediators, an effect usually attributed to the well-characterized virulence factors CagA and VacA.

CagA is a strain-specific *H. pylori* gene that is recognized as a marker for strains that confer increased risk for gastric cancer (6). Introduction of the CagA protein via a bacterial type four secretion system (7) can have a direct effect on epithelial cells, disrupting cell signalling pathways and cell polarity (8–10). Processes considered essential to maintaining a normal epithelium. In contrast, the vacA gene is conserved among all *H. pylori* strains. However, significant polymorphism exists (11) and it is *H. pylori* strains carrying the s1m1 vacA genotype that produce a protein best known for its ability to induce cell vacuolation. VacA is endocytosed into epithelial cells and traffics to late endocytic compartments, where it forms anion-selective channels in the endosomal membrane (12). The diffusion of weak bases such as ammonia (which is a by-product of *H. pylori* degradation of urea) into these compartments causes them to swell, resulting in vacuolation (13). The general consensus is that this has a negative effect on the cell, and VacA is considered a toxin (14).

There is evidence that VacA-positive strains of *H. pylori* release the cytotoxin in soluble form and associated with outer membrane vesicles (OMV). These small membranous structures are constitutively shed from the surface of *H. pylori* in vitro and in vivo (15,16) and we are particularly interested in the role of OMV in the progression of disease since *H. pylori* is non-invasive and only a small proportion of the many organisms found within the mucus layer adhere to epithelial cells (17). OMV, which contain the surface identity of the donor bacterium (15,18), are rapidly internalized into cultured gastric epithelial cells (16), where they cause interleukin-8 release in a dose- and time-dependent manner, and interfere with proliferation (19). The aim of this study was to determine the direct effect of *H. pylori* OMV on epithelial cells, in particular, their ability to stimulate genomic instability as assessed by the cytokinesis-block micronuclei assay (20).

**Materials and methods**

**Bacterial strains and culture**

Two well-characterized *H. pylori* clinical isolates were used in this study. *Helicobacter pylori* 60190 is a vacA s1m1 strain (11), producing a cytotoxin that induces extensive vacuolation in cultured epithelial cells (21). An isogenic mutant, 60190:v1, in which the vacA gene has been disrupted preventing formation of the cytotoxin (22), was also used. Tx-30a is a vac:s2m2 strain (11) associated with reduced transcription and protein expression of the cytotoxin (23,24) and produces a VacA protein that fails to induce vacuolation in vitro (21). Bacteria were cultured in 2.8% (wt/vol) *Brucella* broth (Becton Dickinson and Company, Sparks, MD) supplemented with 5% (vol/vol) fetal bovine serum (Invitrogen, Auckland, New Zealand) for 72 h at 37°C under microaerobic conditions and with constant rotation (120 rpm).

**Collection of OMV**

At 72 h incubation, bacteria were removed from the broth cultures by two centrifugations (10 000 g, 15 min, 4°C). The culture supernatants were then ultracentrifuged (100 000g, 2 h, 4°C) to recover OMV. The resultant OMV pellet was washed twice in sterile phosphate-buffered saline (PBS) before being assayed for protein content using a modification of the Lowry procedure (25). Samples were stored at −20°C until required.

**Cell culture**

The AGS cell line (ATCC CRL-1739) derived from a human gastric adenocarcinoma was used in this study. AGS cells were routinely cultured in F-12 Nutrient Mixture (HAM) (+-glutamine) supplemented with 10% (vol/vol) fetal bovine serum and 1% (vol/vol) penicillin-streptomycin-glutamine supplement (all from Invitrogen) at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were seeded on 12-, 24- or 96-well plates (depending on the cell numbers required for each assay) and cultured for 24 h before OMV addition. Initial seeding concentrations were calculated to ensure that cells were sub-confluent at the time of OMV addition, and 100–200 μg of total OMV protein was added per 1 × 105 AGS cells.

**Cell viability and proliferation**

Cell viability was assessed by propidium iodide staining after 48 h incubation with *H. pylori* OMV. Culture media from two duplicate wells were removed and replaced. The adherent AGS cells were detached with 1% trypsin–ethylenediaminetetraacetic acid, the well washed with PBS and combined fractions added to the pooled media. The cells were incubated with 2 μg/ml propidium iodide for 10 min, and the fluorescence of 10 000 cells was measured with a bivariate flow cytometer (Becton Dickinson, Mountain View, CA). The effect of OMV on proliferation of AGS gastric epithelial cells was determined using a colorimetric assay that examines the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into the cellular DNA of proliferating cells (Roche Diagnostics, Mannheim, Germany). AGS cells were cultured overnight in 96-well plates and then incubated a further 24 h in the presence of OMV. The BrdU assay was performed as per manufacturer’s directions. Briefly, BrdU labelling solution was added to individual wells for 2 h. Following removal of the media, fixative solution was added to each well for 30 min at room temperature and then replaced with BrdU antibody (diluted 1:100) for 2 h. After washing, a substrate solution was applied for 15 min at room temperature before the reaction was stopped by the addition of 1 M H2SO4. Absorbance was read at 450 nm on a SpectraMax plate reader.

**Abbreviations:** AO, acridine orange; BrdU, 5-bromo-2′-deoxyuridine; GSH, glutathione; OMV, outer membrane vesicles; PBS, phosphate-buffered saline.
Cytokinesis-block micronuclei assay

AGS cells were cultured overnight on glass coverslips in 12-well plates, incubated for a further 24 h with OMV and then incubated for a further 24 h in media containing 5 μg/ml cytochalasin B. The coverslip-bound cells were fixed (2% vol/vol glutaraldehyde in PBS) for 30 min and stained using Diff-Quick. At least 1000 binucleate cells were counted on each slide and micronuclei were scored using the criteria described by Fenech (20).

Immunolabelling of OMV

Immunofluorescence microscopy was used to visualize intracellular OMV. AGS cells were cultured on glass coverslips in 24-well plates overnight and then incubated for a further 24 h with OMV. Following fixation (4% paraformaldehyde in 60 mM piperazine-N,N'-bis-[2-ethanesulfonic acid] buffer, pH 6.8) for 45 min, the cells were washed extensively in piperazine-N,N'-bis-[2-ethanesulfonic acid] buffer (five 5 min washes) before being blocked [5% (wt/vol) bovine serum albumin] for 60 min at room temperature. Following a further three 5 min washes, the cells were permeabilized [0.5% (vol/vol) Triton X-100] for 20 min and washed again (3 x 5 min) before being incubated overnight at 4°C with a murine IgG1 subclass monoclonal antibody to H. pylori Lpp20 (26). Primary antibody binding was detected using a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody. The cells were mounted with 0.1% (wt/vol) of p-phenylenediamine dihydrochloride and examined using epifluorescence microscopy (AxioVision 4.5, ApoTome software, Carl Zeiss, Oberkochen, Germany).

Vacuole staining

AGS cells were cultured overnight on coverslips and then incubated for a further 24 h with OMV in F12 medium without the addition of fetal calf serum. Following two washes with medium, cellular vacuolation was visualized by epifluorescence microscopy of cells stained with acridine orange (AO). Briefly, the cells were stained with AO (5 μg/ml) for 30 min, washed another four times and resuspended in F-12 medium. AO staining in the cytoplasm and nucleus was detected using a fluorescein isothiocyanate filter, whereas lysosomal/vacuole staining was detected using a Texas Red filter. All steps were carried out in the dark.

Perl's stain for ferric iron

AGS cells were cultured on coverslips overnight and then further incubated for up to 24 h with OMV before being fixed (2% glutaraldehyde in PBS) for 30 min. After washing, the fixed cells were stained with Perl's solution (1% potassium ferrocyanide in 1% HCl) for 1 h at room temperature (27). Staining was intensified using the DAB+ substrate-chromagen (DAKO Corporation, Carpinteria, CA) (27). Perl's stained ferric iron was visualized by microscopy.

Glutathione levels

AGS cells were cultured in 24-well plates overnight then incubated with OMV for 24 h, with and without the addition of 5 mM glutathione (GSH) ester (Sigma, St Louis, MO). The medium was removed and the cells washed twice in PBS before trypsin was added to the wells to lift the cells. The non-adherent cells were collected, centrifuged at 1200 r.p.m. for 5 min and the resultant cell pellets lysed with a buffer containing 0.5% (vol/vol) Triton X-100, 100 mM sodium phosphate buffer (pH 8) and 5 mM ethylenediaminetetraacetic acid. A stock solution of monobromobimane prepared in acetonitrile was added to final concentration of 1 mM and the samples were left at room temperature (in the dark) for 15 min. Trichloroacetic acid was added to a final concentration of 6.25% before the cell lysates were micro centrifuged for 2 min. The supernatants were analysed by high-performance liquid chromatography with fluorescence detection (excitation 394 nm, emission 480 nm), using GSH (Sigma) as a standard. The protein concentration of the cell lysates was measured by Lowry assay and GSH expressed relative to cell protein.

Fig. 1. Effect of Helicobacter pylori OMV on proliferation and micronuclei formation in AGS cells. AGS cells were incubated for 24 h in medium alone or with H. pylori OMV. (a) AGS cell proliferation was assessed by examining the incorporation of BrdU and results are presented as percentages of the control. (b) Micronuclei formation (indicated by arrowhead) counted in 1000 untreated binucleate AGS cells or following exposure to H. pylori OMV or bacteria. (d) Dose response to 60190 (closed circles) and 60190 isogenic vacA mutant (open circles) OMV. Data are expressed as percentages. Values are the means ± standard error of three experiments. *P < 0.05 versus control, except in (d), which is a representative experiment showing the mean ± standard error of three counts.
Protein carbonyl enzyme-linked immunosorbent assay

AGS cells were cultured overnight in 24 well then incubated for an additional 24 h with OMV. The cells were collected (see above) and lysed by a freeze–thaw process. Protein carbonyls were determined using the Lowry assay. Protein carbonyls were detected using an ELISA kit (BioCell, Auckland, New Zealand) as described previously (28). The carbonyl content of each sample was determined with reference to a standard curve.

Statistical analysis

Values are shown as the mean ± standard error of at least three independent experiments. Data were analysed by one-way analysis of variance using SigmaStat version 3.10 (Systat Software).

Results

Helicobacter pylori OMV (100–200 µg total protein) were incubated with AGS cells (10⁵) for 24 h. Consistent with our earlier report (19), there was no significant effect on cell viability (data not shown) but significantly reduced proliferation (Figure 1a). To investigate whether genomic damage was occurring at these sublethal doses, we quantified micronuclei formation in the AGS cells. Micronuclei are produced in dividing cells when chromosome breakage occurs within the cell nucleus and these chromosomes are not correctly distributed during cell division (20). A significant increase in the number of cells with micronuclei was observed in AGS cells incubated with OMV from strain 60190 (Figure 1b and c). A dose dependency showed significant micronuclei formation at 50 µg of OMV protein (Figure 1d). Disruption of the vacA gene, which prevents cytotoxin formation (22), abrogated OMV-induced formation of micronuclei indicating a VacA-mediated effect (Figure 1c). This was supported by the absence of micronuclei formation in AGS cells treated with OMV from the non-toxigenic H.pylori strain Tx-30a (Figure 1c). Micronuclei formation was also observed when AGS cells were incubated with H.pylori

Fig. 2. OMV inside AGS cells localize to vacuole membranes. Immunofluorescence microscopy of AGS cells incubated with OMV for 24 h, fixed, permeabilized and stained with an anti-Lpp20 monoclonal antibody. (a) DIC image of 60190 OMV-treated AGS cells with vacuoles (*) and (b) immunolabelled with anti-Lpp20 antibody (arrows). (c) Overlay of images (a and b). (d) DIC image of strain TX-30a OMV-treated AGS cells and (e) immunolabelled with anti-Lpp20 antibody. (f) Overlay of images (d and e).

Fig. 3. Effect of OMV uptake on AO distribution in cells. AGS cells were incubated for 24 h in medium alone or with Helicobacter pylori OMV, stained with AO and examined using epifluorescence microscopy. This revealed discrete orange/red staining of lysosomal compartments in untreated AGS cells, indicative of low lysosomal pH, whereas the diffuse green staining indicated AO staining of the cytosol and nucleus (a, b and c). Large vacuoles were visible in AGS cells treated with VacA-positive OMV. AO staining of these vacuoles was irregular (d and e), with some vacuoles (*) failing to stain (f).
Discussion

The mechanism of carcinogenesis associated with *H. pylori* colonization remains an area of considerable debate. The severity of precancerous lesions shows a strong relationship with *cagA*-positive strains (31) and *s1 vacA* genotypes of *H. pylori* (32), with CagA most probably reflecting the strong inflammatory response that is the hallmark of these infections (33). DNA damage induced by chronic inflammation contributes to gastric cancer in mice (34), and increased DNA damage as a result of chronic inflammation in the gastric mucosae of *H. pylori*-infected humans (35) may trigger progression to intestinal-type gastric cancer (36). *Helicobacter pylori* and their cytosolic extracts and culture supernatants have also been reported to directly cause DNA damage in cultured cells (37–41), independent of the host inflammatory response. Our results show that OMV, which are constitutively shed from the surface of *H. pylori* (15) and rapidly endocytosed *in vivo* and *in vitro* (16), also directly promote genomic instability in cultured gastric epithelial cells. This provides a new role for OMV in the gastric carcinogenesis associated with chronic *H. pylori* infection.

Micronuclei formation was dependent on VacA and also appeared to be associated with altered iron metabolism in the cultured cells. Cytoplasmic vacuoles formed within 4 h of toxigenic OMV being added to AGS cells. VacA localizes to large acidic vacuoles (42) thought to originate from an intermediate compartment between lysosomes and late endosomes (43,44), with vacuolation driven by the presence of a vacuolar-type ATPase proton pump (45) and enhanced by the formation of transmembrane anion-specific channels (46) that increase the permeability of the endosomal membrane to weak bases (47). OMV were still present inside vacuolated AGS cells after 24 h, whereas OMV from a non-toxigenic strain had no apparent effect on the distribution of ferric iron in AGS cells (Tx-30a).}

Fig. 4. Effect of OMV on distribution of ferric iron in AGS cells. AGS cells were incubated for 24 h in medium alone or with *Helicobacter pylori* OMV and intracellular Fe3⁺ distribution was visualized by Perl’s staining reaction of ferric ions with potassium ferrocyanide. (a) Staining, which was located at the perinuclear region of untreated AGS cells (control), was noticeably more diffuse 24 h post-treatment of cells with VacA-bearing OMV (60190), whereas OMV from a non-toxigenic strain had no apparent effect on the distribution of ferric iron in AGS cells (Tx-30a). (b) Staining of cytoplasmic ferric iron, presented as percentages of the control. Values are the means ± standard error of three experiments. *P < 0.05 versus control.

Fig. 5. Effect of OMV on GSH levels in AGS cells. AGS cells were incubated for 24 h in media alone or with *Helicobacter pylori* OMV. Intracellular GSH levels were measured by high-performance liquid chromatography analysis of monobromobimane derivatives and expressed relative to protein concentration. Data shown are the means ± standard error of three experiments. *P < 0.05 versus control.

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strains 60190 bacteria, but not those from VacA-deficient bacteria (Figure 1e).

Immunofluorescence microscopy revealed that the internalization of OMV from strain 60190 was associated with the development of large intracellular vacuoles (Figure 2a). These vacuoles were observed as early as 4 h after the addition of OMV, and OMV were observed at the membrane bounding and in the luminal space of the vacuoles (Figure 2b and c). OMV from the isogenic vacA mutant of the same strain (60190v:1) were internalized by AGS cells, but as expected there was no evidence of vacuolation (Figure 2d–f).

We used the lysosomotropic dye AO to assess the stability of the VacA-induced vacuoles. AO is a weak base that preferentially distributes within acidic vacuolar compartments (29). Due to its metachromatic properties, this probe fluoresces red inside lysosomes, where it is highly concentrated, and green in the cytosol, where it is much less concentrated. Accordingly, epifluorescence microscopy revealed cytosolic green fluorescence observed in untreated cells, disrupted by discrete patches of bright red staining (Figure 3a–c). Most vacuoles that developed in OMV 60190-treated AGS cells also stained with AO (Figure 3d and e). However, cells with a decreased number of intact AO-accumulating lysosomes (‘pale’ cells) were detected by their diminished red fluorescence and some vacuoles showed no AO staining at all (Figure 3f). To test the hypothesis that reduced staining was associated with compromised vacuole integrity, we used Perl’s iron stain for measurement of ferric iron. This staining method uses dilute ble blue compound (27). There was no change in AGS cells following 4 h incubation with strain 60190 OMV (data not shown); however, after 24 h a small but significant number of cells showed extensive cytoplasmic staining (Figure 4). This effect was not observed in AGS cells incubated with OMV from strain Tx-30a (Figure 4).
but these vacuoles displayed weak AO staining, and in a small proportion of cells there was increased cytosolic staining of Perl’s reaction product. This may reflect VacA-mediated alkalization of these acidic cellular compartments (48) resulting in the relocation of iron and other cations to the cytosol (49).

Extensive work by Brunk et al. has shown that lysosomes are susceptible to oxidative stress due to the pool of redox-active iron produced during the degradation of iron-containing macromolecules (50), and release of this iron promotes oxidative DNA damage (50,51). Such a mechanism would be consistent with the observations in this study, in particular, micronuclei formation under conditions where there was a loss of ~75% of the reduced cellular GSH. It is not known how GSH depletion occurs, but others have shown previously that the H. pylori bacterium itself is able to lower GSH (52,53), and purified VacA cytotoxin is reported to impair GSH metabolism (54). GSH peroxidases utilize GSH in the breakdown of hydrogen peroxide, and the OMV-mediated decrease in GSH may increase the opportunity for endogenous hydrogen peroxide to promote DNA damage, either alone or in combination with redox-active iron (55,56).

Increasing the concentration of intracellular GSH in cultured cells exposed to VacA-containing OMV reduced micronuclei formation. Interestingly, 50% of normal GSH was sufficient to block micronuclei formation, similar to the levels of GSH depletion observed with VacA-deficient OMV. This indicates a possible threshold value for GSH below which micronuclei formation was favoured. Decreased GSH levels are reported in the gastric biopsies of H. pylori-infected individuals (57) and these are restored when the bacterium is eradicated (58,59). One potential source of oxidative stress is the activation of phagocytic cells during chronic inflammation. However, we have reported increased gastric atrophy in mice with a dysfunctional reduced nicotinamide adenine dinucleotide phosphate oxidase that were co-ported increased gastric atrophy in mice with a dysfunctional reduced nicotinamide adenine dinucleotide phosphate oxidase that were co-

### Supplementary material

Supplementary Figure can be found at http://carcin.oxfordjournals.org/.

### References


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