

## STOMACH

p27<sup>kip1</sup> regulates the apoptotic response of gastric epithelial cells to *Helicobacter pylori*

H Eguchi, S Carpentier, S S Kim, S F Moss

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See end of article for authors' affiliations

Correspondence to:  
Dr S F Moss, Division of  
Gastroenterology,  
Department of Medicine,  
Rhode Island Hospital, 593  
Eddy St, APC 414,  
Providence, RI 02903,  
USA; smoss1@pol.net

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**Background:** *Helicobacter pylori* infection increases the risk of gastric cancer but the molecular mechanisms responsible are not well understood. Gastric cells chronically exposed to *H pylori* in vitro develop resistance to apoptosis associated with low levels of p27, a cyclin dependent kinase inhibitor and haplo insufficient tumour suppressor gene that is downregulated in gastric cancer.

**Aim:** To determine whether the low level of p27 protein is responsible for the resistance to apoptosis of gastric cancer cells.

**Methods:** The effects of increasing the expression of p27 protein were examined by transiently and stably transfecting a plasmid encoding full length p27 mRNA into apoptosis resistant gastric cancer cell lines with low p27 expression that were derived from AGS gastric cancer cells by chronic *H pylori* coculture followed by dilutional cloning.

**Results:** Low p27 expression in the apoptosis resistant derivative cell lines was associated with an approximate 30% decrease in p27 mRNA and an 80% decrease in p27 protein that was not due to increased proteasome dependent degradation of p27 protein. Transient or stable transfection with p27 constructs partially restored the sensitivity of the apoptosis resistant cells to 5-fluorouracil and *H pylori* induced apoptosis without altering spontaneous apoptotic cell death.

**Conclusions:** These results demonstrate that p27 positively regulates, at least in part, the apoptotic response of gastric epithelial cells to *H pylori*. Low gastric p27 may promote gastric carcinogenesis associated with *H pylori* infection by inhibiting apoptotic pathways.

p27<sup>kip1</sup> is a cyclin dependent kinase whose major target is the cyclinE/CDK2 complex that governs cell cycle transition from late G1 to S phase.<sup>1</sup> In addition to its function in cell cycle control, p27 has also been implicated in the regulation of apoptosis, differentiation, drug resistance, cell migration, and in the response to inflammatory stimuli,<sup>1–2</sup> possibly as a transcriptional regulator.<sup>3</sup> Mice lacking one or two functional copies of p27 are predisposed to tumours induced by exogenous carcinogens<sup>4</sup> and expression of p27 is reduced in many human malignancies.<sup>5</sup> In addition, low levels of p27 protein are associated with a poor prognosis in several types of cancer, including gastric cancer.<sup>6–8</sup>

Downregulation of p27 in cancers is probably due to increased proteasomal degradation of p27 following ubiquitination,<sup>9</sup> which is the major pathway that normally regulates the abundance of p27 protein during physiological cell cycle progression.<sup>1</sup> However, the level of p27 protein may also depend on other mechanisms, including transcriptional regulation and promoter methylation, mRNA degradation, cytoplasmic sequestration of p27 protein, and by non-proteasomal proteolysis (reviewed by Philip-Staheli and Kemp<sup>1</sup>).

Most cases of gastric cancer develop in patients who have been colonised by the Gram negative bacterium *Helicobacter pylori*.<sup>10</sup> Infection with *H pylori* is associated with a 2–5-fold increased risk of developing non-cardia gastric cancer<sup>11–13</sup> and eradication of *H pylori* may prevent gastric cancer in high risk populations with chronic gastritis.<sup>14</sup> Direct evidence linking *H pylori* to gastric cancer has come from the demonstration of gastric cancer in susceptible rodent models following experimental infection by *H pylori*, without additional cocarcinogen exposure.<sup>15–17</sup> Certain *H pylori* determinants and host susceptibility factors that predispose to *H pylori* associated gastric cancer have been identified in recent years, including genes within the *cag* pathogenicity island of *H pylori*<sup>18–19</sup> and host

single nucleotide polymorphisms for cytokines and cytokine receptors<sup>20–22</sup> but the cellular and molecular mechanisms responsible for gastric carcinogenesis following *H pylori* infection remain poorly understood.

It is well established that chronic *H pylori* infection increases epithelial cell turnover in the gastric mucosa; proliferating and apoptotic cell numbers decrease to normal levels following eradication of *H pylori*.<sup>23</sup> Chronic *H pylori* infection also decreases expression of gastric epithelial cell p27, both in chronic gastritis and in gastric intestinal metaplasia.<sup>24–25</sup> Furthermore, in animal<sup>26</sup> and cell culture<sup>24</sup> models of chronic *H pylori* infection, long term exposure of gastric epithelial cells to *H pylori* leads to the emergence of epithelial cells that are relatively apoptosis resistant. An attenuated apoptotic response to *cag* positive *H pylori* strains has also been reported in some<sup>27–28</sup> but not all<sup>29</sup> studies of *H pylori* infection in humans, perhaps mediated through activation of the antiapoptotic transcription factor nuclear factor κB.<sup>30</sup> We have previously suggested<sup>24</sup> that the reduction in p27 expression by *H pylori* may be responsible for inducing a state of apoptosis resistance in gastric epithelial cells, thereby promoting gastric tumorigenesis.<sup>31</sup> However, this hypothesis is dependent on demonstrating that p27 promotes apoptosis in gastric cells.

Currently, there is disagreement on the role of p27 in the regulation of apoptosis. Most reports support the role of p27 in promoting apoptosis. For example, gastric cancers with low p27 expression (as determined by immunohistochemistry) have low levels of apoptosis and are associated with a relatively poor prognosis compared with those cancers expressing higher p27.<sup>7</sup> Overexpression of p27 increases apoptosis in several human non-gastric cancer cell lines.<sup>32–34</sup>

**Abbreviations:** GFP, green fluorescent protein; 5-FU, 5-fluorouracil; PBS, phosphate buffered saline

and in human and rat fibroblast lines.<sup>35</sup> Furthermore, inhibiting expression of p27 by overexpression of *skp2*, the ubiquitin ligase F-box substrate recognition unit of p27, results in resistance to actinomycin D induced apoptosis in gastric cancer cells.<sup>36</sup>

However, overexpression of p27 has been demonstrated to *inhibit* apoptotic responses to hypoxia and serum deprivation in small cell lung cancer cells<sup>37</sup> and to inhibit cytotoxic drug induced apoptosis in leukaemia cells, perhaps through generation by activated caspases of p27 cleavage products with antiapoptotic function.<sup>38, 39</sup> In addition, p27 null fibroblasts and mesangial cells undergo excessive apoptosis following growth factor deprivation.<sup>40</sup> Thus it is conceivable that p27 may normally exert antiapoptotic effects in certain situations.

Because previous studies have shown conflicting results, and because of the potential importance of p27 in the pathogenesis of *H pylori* associated gastric cancer, we examined the role of p27 in the regulation of apoptosis in gastric cancer cells by utilising gastric cell lines with low p27 expression that are resistant to apoptosis. We demonstrate here that p27 is proapoptotic in gastric cancer cells and that the effect of *H pylori* in decreasing p27 makes gastric epithelial cells more resistant to apoptosis.

## METHODS

### Cell culture and establishment of *H pylori* resistant gastric epithelial cell lines

AGS human gastric epithelial cells (CRL-1739; American Type Culture Collection, Manassas, Virginia, USA) were maintained in an atmosphere of 5% CO<sub>2</sub> at 37°C, in Ham's F12 medium supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, California, USA) without antibiotics, in 75 cm<sup>2</sup> tissue culture flasks (BD Biosciences, San Jose, California, USA). *H pylori* resistant AGS cell derivatives were selected by coculture with progressively increasing concentrations of *H pylori* strain 60190 (ATCC 49503) from an initial bacterium to epithelial cell ratio of 20:1 to 10 000:1 over a three month period.<sup>24</sup> From these derivatives, two single cell clones designated HS3C and HS3L were picked by limiting dilution assays and expanded for further analysis.

### *H pylori* strains and culture conditions

*H pylori* strain 60190, a *cagA* positive and *vacA* positive strain isolated from a patient with dyspepsia was maintained on trypticase soy agar containing 5% sheep blood (BD Biosciences) incubated at 37°C in 8% CO<sub>2</sub>, for a minimum of two and a maximum of four passages from frozen stocks. Inocula for coculture were diluted from suspensions that had been prepared from 48 hour subcultures and adjusted by comparison of absorbance to McFarland standards. *H pylori* bacteria were added to gastric epithelial cells at a ratio of 200:1 in all experiments.

### 5-Fluorouracil (5-FU) treatment

Cells were treated with 10 mM 5-FU for 24 hours, as described previously.<sup>24</sup>

### Protein extraction and western blotting

Protein extraction and western blotting were performed as described previously<sup>41</sup> using 30 µg protein samples and mouse monoclonal antibodies to p27<sup>kip1</sup> (clone 57; BD Biosciences) and to Skp2 (p45<sup>skp2</sup>, clone SKP2-2B12; Zymed Laboratories Inc., South San Francisco, California, USA).

### Measurement of apoptosis

Apoptosis was evaluated qualitatively by caspase-3 cleavage on western blot and quantified by counting the percentage of altered nuclei after Hoechst 33342 staining (Sigma, St Louis,

Missouri, USA). For nuclear staining, both cells that had spontaneously detached from the dishes and attached cells released by trypsinisation were collected, washed with phosphate buffered saline (PBS), and pooled. Collected cells were fixed with 2% paraformaldehyde for 10 minutes at 37°C, stained with Hoechst 33342, and examined under a fluorescent microscope with an excitation filter of 350 nm and an emission filter of 460 nm. Apoptosis was defined as the presence of nuclear condensation or fragmentation. Samples were prepared in quadruplicate and at least 200 cells per slide were examined without knowledge of the experimental manipulation. Data are expressed as the mean percentage of cells showing evidence of nuclear fragmentation. In some experiments, apoptosis was also quantified by the percentage of cells with sub-diploid DNA content by flow cytometry, as described previously.<sup>42</sup>

### Colony formation assay

AGS, HS3C, and HS3L cells were suspended in 0.3% agar with complete medium (Ham's F12 supplemented with 10% fetal bovine serum), plated at a density of 5 × 10<sup>4</sup> cells in a 60 mm dish which had been coated with 0.5% base agar, and maintained at 37°C. During the experiment, 0.5 ml of fresh complete medium was added every five days. On day 21, the number of colonies greater than 0.2 mm in diameter was recorded at four points in each dish.

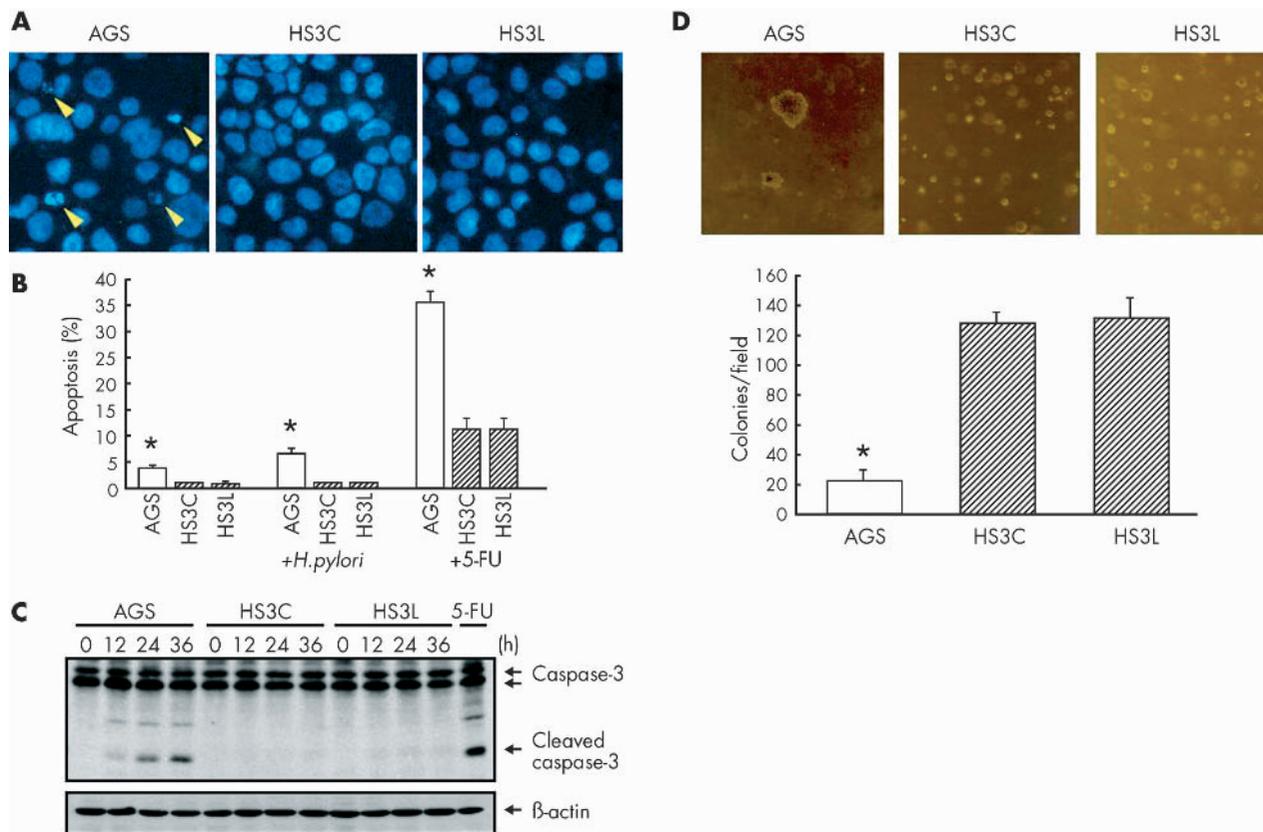
### Immunocytochemistry for p27

Cells were trypsinised, washed in PBS, and fixed with 2% paraformaldehyde in PBS for five minutes at room temperature. After fixation, cell pellets were permeabilised in 70% cold ethanol and then stored at 20°C until analysis. Cells were then washed in PBS with 0.2% Tween 20 and incubated for one hour with 100 µl of monoclonal antibody to p27 diluted 1:50 in PBA (PBS with 0.2% Tween 20 and 1% bovine serum albumin). Cells were then washed twice with PBA and incubated for one hour with 100 µl of phycoerythrin conjugated antimouse IgG (1:50; BD Biosciences). As negative controls, some cells were incubated with only the secondary phycoerythrin conjugated antibody. After secondary antibody incubation, cells were washed twice in PBA and examined under a fluorescent microscope with an excitation filter of 488 nm and an emission filter of 575 nm.

### mRNA quantification using real time reverse transcription PCR

Total RNA from exponentially growing AGS or HS3C cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and treated with RNase free DNase I (Roche Diagnostics Corporation, Indianapolis, Indiana, USA) to remove contaminating genomic DNA. Reverse transcription of RNA was performed in a final volume of 20 µl containing 4 µl of 5 × reverse transcription buffer (250 mM Tris HCl, 40 mM MgCl<sub>2</sub>, 150 mM KCl, 5 mM dithiothreitol, pH 8.5), 250 µM each deoxynucleotide triphosphate, 40 units of RNase inhibitor, 3.2 µg random hexamers, 20 units of avian myeloblastosis virus reverse transcriptase (all from Roche), and 250 ng/10 µl of total RNA. Samples were incubated at 25°C for 10 minutes and 42°C for one hour, and the reverse transcriptase was then inactivated by heating at 99°C for five minutes and kept on ice until use.

Real time quantitative polymerase chain reaction (PCR) was performed using an iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, California, USA) in a 25 µl reaction mix containing 5 µl of diluted reverse transcriptase samples (0.25 ng of equivalent total RNA) and pDNA3-p27 plasmid cDNA in serial dilution,<sup>43</sup> 12.5 µl of SYBR Green PCR Master Mix (Applied Biosystems, Foster



**Figure 1** *Helicobacter pylori* resistant cells have low spontaneous apoptosis and are also resistant to 5-fluorouracil (5-FU) induced apoptosis. (A) AGS parental cells and *H. pylori* resistant clones HS3C and HS3L were cultured alone, in the presence of *H. pylori*, or in the presence of 10 mM 5-FU for 24 hours. Cells were then harvested and apoptotic cells were counted by Hoechst staining. Representative nuclear morphologies of exponentially growing cells are shown. Yellow arrowheads indicate apoptotic cells. (B) Summary data demonstrate the mean (SD) per cent apoptotic cells in response to *H. pylori* or 5-FU. \* $p < 0.05$ , AGS compared with HS3C or HS3L cells. (C) Cells were cocultured with *H. pylori* for the indicated times, and proteins were harvested for western blotting for full length and cleaved caspase-3. 5-FU treated AGS cells were used as a positive control (last lane). Equal amount of protein loading was verified by  $\beta$ -actin. (D) HS3C and HS3L show increase anchorage independent growth in soft agar. \* $p < 0.05$  compared with HS3C or HS3L cells. Cells ( $5 \times 10^4$ ) were seeded, cultured for 21 days, and the number of colonies counted. Data are representative of experiments performed three times and are shown as mean (SD). Representative morphologies of each cell type are shown in the upper panels.

City, California, USA), 6.5  $\mu$ l deionised distilled water, and 0.5  $\mu$ l of 10  $\mu$ M of each p27 primer (forward, 5'-AGGACACGCATTGGTGA-3'; reverse, 5'-TAGAAGAATCGTCGGTTCAGGT-3'). Thermocycling was carried out for 45 cycles, with denaturation at 95°C for 15 seconds, and annealing and extension at 68°C for 30 seconds. Standard dilutions were prepared from the pcDNA3-p27 plasmid cDNA as follows:  $3.1 \times 10^6$ ,  $3.1 \times 10^5$ ,  $3.1 \times 10^4$ ,  $3.1 \times 10^3$ ,  $3.1 \times 10^2$ , and  $3.1 \times 10^1$  copy numbers, and used to relate the threshold cycle to the log input amount of template. All samples were run in triplicate. The relative amounts of p27 transcripts were determined using the standard curve method and were normalised to 18S rRNA (forward primer, 5'-GGACCAGAGCGAAAGCATTGCC-3'; reverse primer, 5'-TCAATCTCGGGTGGCTGAACGC-3').

### Transient and stable transfection of p27

Transient transfection of a p27 expression vector was performed using a cationic liposome reagent, TransIT-LT1 (Mirus Corp., Madison, Wisconsin, USA), according to the manufacturer's protocol. Briefly,  $3 \times 10^5$  of HS3C or HS3L cells were seeded in six well plates and cotransfected with 3  $\mu$ g of full length cDNA of human p27 inserted in pcDNA3 plasmid<sup>43</sup> or an empty vector, together with 1  $\mu$ g of cDNA of green fluorescent protein (GFP) inserted in pcDNA3 as a reporter for the detection of transfected cells.<sup>37</sup> After 24 hours of incubation, *H. pylori* or 5-FU was added and cells incubated

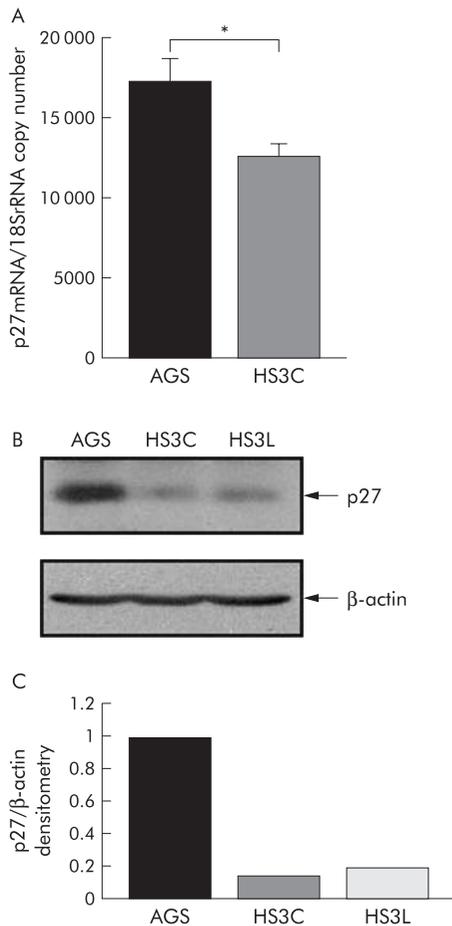
for a further 24 hours. Cells were then harvested, stained with Hoechst 33342, and at least 200 GFP positive cells were examined in quadruplicate for nuclear fragmentation by fluorescence microscopy. In order to obtain cells stably expressing p27, full length cDNA of human p27 inserted in pcDNA3 plasmid or the empty vector pcDNA3 was transfected into HS3C or HS3L cells using LipofectAMINE 2000 (Invitrogen). Transfected cells were selected in the presence of 900  $\mu$ g/ml of G418 for 21 days. G418 resistant colonies were then pooled, expanded, and analysed for p27 expression by western blot. Pools that showed a significant increase in p27 expression were used for subsequent studies.

### p27 degradation assay

To evaluate the turnover of p27, AGS, HS3C, and HS3L cells were cultured in the presence of 100  $\mu$ M cycloheximide (Sigma) to inhibit de novo protein synthesis, thereby allowing evaluation of post-translational events such as degradation in regulating the protein level.<sup>44</sup> Cells were harvested 0, 1, 2, and 3 hours after addition of cycloheximide, and 30  $\mu$ g of whole cell lysates were loaded on sodium dodecyl sulphate-polyacrylamide gels for p27 immunoblotting.

### Statistics

Differences in apoptosis and colony forming ability between cell types were determined by the Mann-Whitney U-test, with  $p < 0.05$  used for the level of statistical significance.



**Figure 2** p27 mRNA and protein expression in parental and apoptosis resistant cells. RNA and protein were extracted from exponentially growing cells. (A) Real time quantitative polymerase chain reaction analysis of p27/18S copy number (mean (SD) of triplicate samples). \* $p < 0.02$ . (B) Western blot: equal amount of protein loading was verified by  $\beta$ -actin. (C) Densitometric values for the ratio of p27/ $\beta$ -actin.

## RESULTS

### Establishment and characterisation of *H pylori* resistant cell lines

Two *H pylori* resistant cell clones (HS3C and HS3L) were selected from the HS3 derivatives that were established from AGS gastric epithelial cells by repeated exposure to *H pylori*.<sup>24</sup> We then confirmed that the clones were apoptosis resistant. As shown in fig 1A and 1B, both clones had a smaller percentage of cells showing spontaneous apoptosis or apoptosis in response to *H pylori* or to 5-FU in comparison with parental AGS cells. Following coculture with *H pylori* or after the addition of 5-FU, activation of caspase-3 was detected in apoptotic cells by the appearance of a caspase-3 cleavage product in AGS cells in a time dependent manner, but was not detected in either the HS3C or HS3L clones (fig 1C). HS3C and HS3L cells also showed lower percentages of apoptotic cells during exponential growth (spontaneous apoptosis) and in response to 5-FU (fig 1B). This low percentage of apoptosis in HS3C and HS3L cells was a stable trait observed even after 10 weeks of continual passage (data not shown). To determine whether resistance to apoptosis of HS3C and HS3L cells was associated with a more malignant behaviour, the anchorage independent growth of HS3C and HS3L cells was evaluated in a semi-solid agar medium. As shown in fig 1D, HS3C and HS3L cells formed approximately six times more colonies in soft agar than did AGS cells,

although each colony was smaller in the apoptosis resistant HS3 lines compared with colonies formed by AGS cells. Confirming our data in the heterogenous HS3 derivative cells,<sup>24</sup> expression of p27 mRNA (fig 2A) and p27 protein (fig 2B) was lower in HS3C than in AGS cells. p27 mRNA copy number measured by quantitative real time PCR in triplicate cell culture samples and normalised for 18S rRNA expression was reduced by 26% in the HS3C cell line. The amount of p27 protein determined by densitometry of p27 immunoblotting (after normalisation for  $\beta$ -actin) was decreased by 85% in HS3C, and by 80% in HS3L compared with the parental AGS cells (fig 2B). The amount of p27 protein in *H pylori* resistant cells remained at a low level even after 10 weeks of continual in vitro passage (data not shown).

### Transient transfection of p27 restores sensitivity to apoptotic stimuli

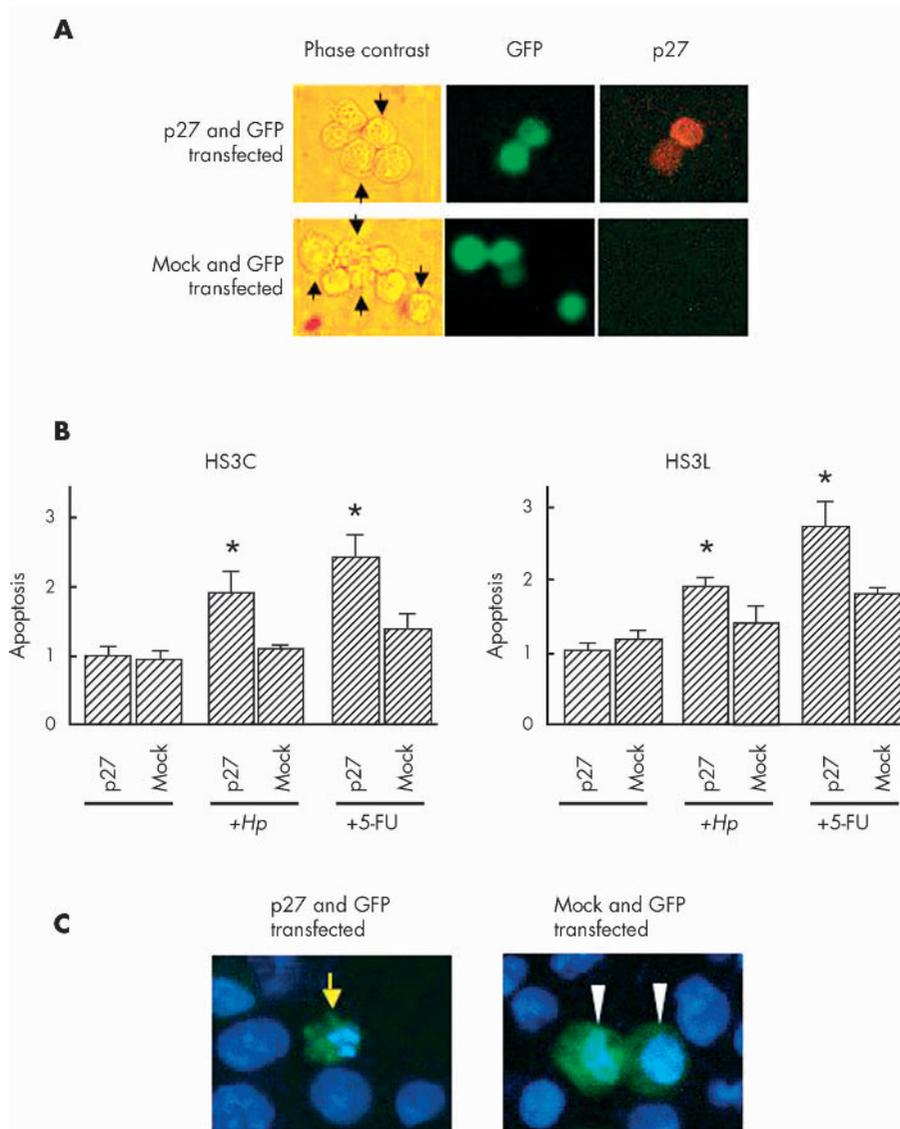
In order to investigate whether the decreased expression of p27 in *H pylori* resistant gastric cells is responsible for their resistance to apoptotic stimuli, the apoptosis resistant HS3C and HS3L cells were transiently cotransfected with a p27 expression vector and a marker plasmid encoding GFP to identify the transfectants (fig 3A). Transfection with p27 did not alter the percentage of spontaneous apoptosis in HS3C or HS3L cells (fig 3B, column 1 and 2 in each graph) but a significantly higher percentage of the transfected cells underwent apoptosis in response to *H pylori* or 5-FU (fig 3B).

### Stable transfection of p27 also restores sensitivity to apoptotic stimuli

In order to further investigate the role of p27 in apoptosis, p27 cDNA was stably expressed in *H pylori* resistant cells and its effects on apoptosis evaluated. Stably transfected HS3C and HS3L expressed levels of p27 protein similar to those found in AGS cells (fig 4A). Consistent with the transient transfection experiments, although stable expression of p27 in both HS3C and HS3L cells did not alter the spontaneous apoptosis rate (fig 4B, left side), it restored sensitivity to *H pylori* induced apoptosis and increased the sensitivity to 5-FU induced apoptosis (fig 4B, 4C, 4D). In these experiments, neither *H pylori* nor 5-FU influenced the growth of the mock or p27 transfected HS3 clones, as determined by the number of cells remaining attached 24 hours after these treatments.

### Low p27 expression in *H pylori* resistant gastric epithelial cells is not due to increased p27 protein degradation

To determine whether the decrease in p27 protein in HS3C and HS3L cells is accompanied by accelerated proteasomal degradation of p27 protein, parental AGS, HS3C, and HS3L cells were cultured in the presence of the highly specific proteasome inhibitor bortezomib (Millennium Pharmaceuticals, Boston, Massachusetts, USA). Addition of bortezomib at a concentration that resulted in marked accumulation of p27 protein in AGS cells increased p27 protein levels to a lesser degree in HS3C and HS3L cells (fig 5A), indicating that decreased expression of p27 protein in HS3C and HS3L cells compared with AGS cells was not due to increased activation of the proteasome dependent degradation of p27 in these cells. In addition, inhibiting protein synthesis with cycloheximide demonstrated that degradation of p27 was decreased rather than increased in HS3C and HS3L compared with AGS cells (fig 5B). Previous studies have shown that the ubiquitin-proteasome dependent degradation of p27 is regulated mainly by the availability of its ubiquitin ligase *skp2*. However, *skp2* protein levels were decreased in *H pylori* resistant HS3C and HS3L cells compared with parental AGS cells (fig 5C). Taken together, these results



**Figure 3** Transient transfection with p27 restores sensitivity to apoptotic stimuli. HS3C or HS3L cells were transiently transfected with p27 or mock plasmids together with green fluorescent protein (GFP) plasmid in a 3:1 ratio. GFP transfected cells were identified by green fluorescence, p27 expression by immunocytochemistry, and apoptosis was measured by Hoechst staining. (A) HS3C cells transfected with p27 and GFP plasmids stain green (arrows) by fluorescence microscopy, and red by p27 immunocytochemistry. (B) Twenty four hours after transfection with p27 or mock plasmids, cells were sub-cultured alone, in the presence of *Helicobacter pylori* (Hp), or in the presence of 5-fluorouracil (5-FU) for 24 hours. Floating and attached cells were then harvested and the percentage of transfected cells (identified as GFP positive) that had an apoptotic morphology was evaluated by Hoechst staining. Data are expressed relative to apoptosis in mock transfected cells as mean (SD). \* $p < 0.05$  compared with mock transfected cells. (C) Representative photomicrographs show that in response to *H pylori*, p27 transfected cells undergo apoptosis (yellow arrow) whereas mock transfected cells do not (white arrowheads).

suggest that the low level of p27 protein in the *H pylori* resistant HS3C and HS3L cells is not due to accelerated p27 protein degradation by the proteasome in these cells but more likely due to low mRNA abundance.

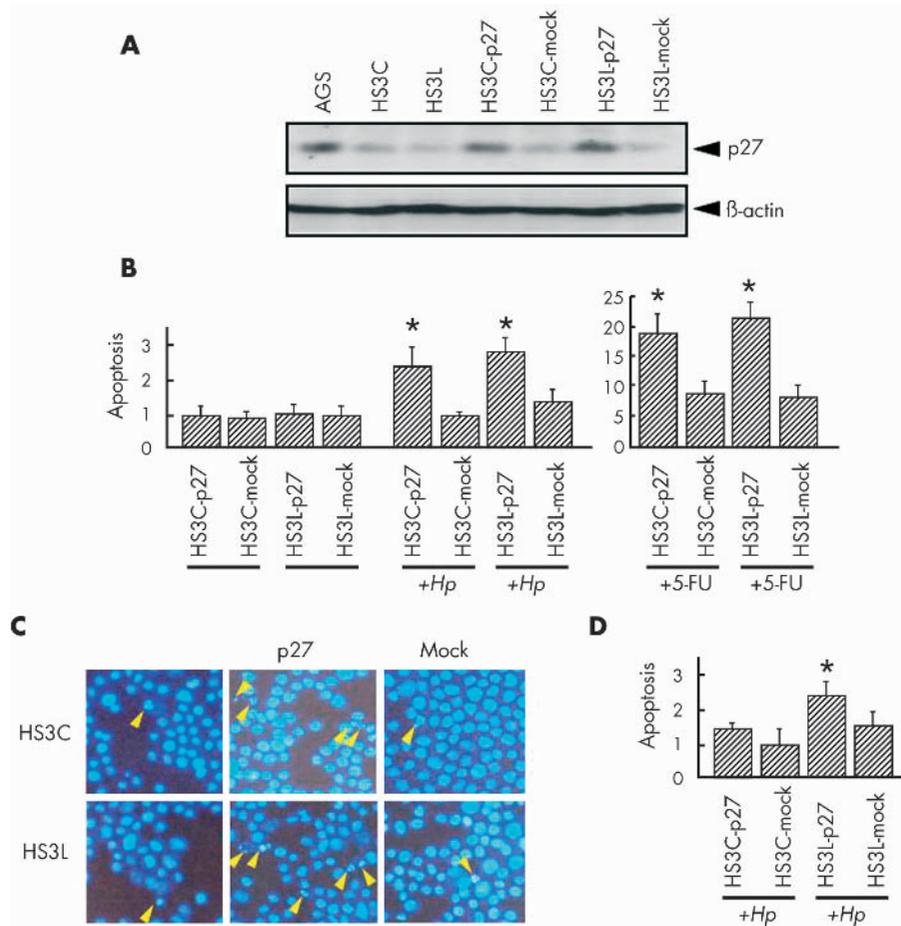
**DISCUSSION**

Our results demonstrate that the experimental manipulation of p27 protein expression in gastric cancer cells modulates their resistance to apoptosis and therefore supports a key role for p27 in the apoptotic response of gastric epithelial cells to *H pylori* and potentially other proapoptotic stimuli. The development of resistance to proapoptotic stimuli may be an important mechanism whereby *H pylori* promotes dysregulated gastric mucosal growth and neoplasia.

Although our results clearly indicate an important role for p27 in the sensitivity to apoptosis of gastric cancer cells, it is

likely that decreased p27 is not the only mechanism contributing to the apoptosis resistant phenotype of the HS3C and HS3L gastric cell lines. Following transfection, HS3C and HS3L cells displayed increased apoptosis in response to *H pylori* and 5-FU but the increase in sensitivity to apoptosis (30–100% increase, depending on the type of transfection and the cell line that was transfected) did not return entirely to the level observed in AGS parental cells (that have an approximately 3–5-fold increased sensitivity to apoptosis compared with its HS3 derivative lines). Furthermore, p27 transfection had no effect on spontaneous apoptosis in the HS3 cell lines which remained lower than that observed in the parental AGS cells.

Differences in expression of some Bcl-2 family members between AGS cells and their heterogeneous apoptosis resistant HS3 derivatives have been reported by us pre-



**Figure 4** Stable transfection of p27 restores the sensitivity to apoptotic stimuli. HS3C or HS3L cells were transfected with p27 or a mock plasmid, selected with G418 for 21 days, and surviving cells were harvested for protein extraction and their sensitivity to apoptosis determined by Hoechst staining and microscopy. (A) Western blot analysis confirmed that HS3C or HS3L cells stably transfected with p27 express p27 protein at a level similar to AGS cells. Equal amount of protein loading was verified by  $\beta$ -actin. (B) p27 transfected cells (HS3C-p27, HS3L-p27) show similar spontaneous apoptosis rates compared with mock transfectants (HS3C-mock, HS3L-mock). After coculture with *Helicobacter pylori* (*Hp*) for 24 hours, or after exposure to 10 mM 5-fluorouracil (5-FU) for 24 hours, stable p27 transfectants display increased apoptosis compared with mock transfectants. \* $p < 0.05$ , p27 transfected cells compared with mock transfected cells. Data expressed as mean (SD) apoptosis relative to mock transfected HS3 cells. (C) Representative morphologies of nuclei (blue) of apoptosis resistant derivatives, p27 transfected derivatives, and mock transfected derivatives following *H. pylori* infection. Arrowheads indicate apoptotic cells. (D) Subdiploid analysis by flow cytometry confirmed that stable transfection of p27 increases sensitivity to *H. pylori* induced apoptosis. \* $p < 0.05$ , p27 transfected HS3L cells compared with mock transfected cells.

viously.<sup>24</sup> We are currently undertaking cDNA microarray analyses to determine global differences in mRNA expression between HS3C and AGS cells with the expectation that such a strategy will identify, from a broader perspective, other genes that are responsible for the apoptosis resistance in gastric cancer cells. Potentially this approach could lead to identification of novel gastric oncogenes and tumour suppressor genes.

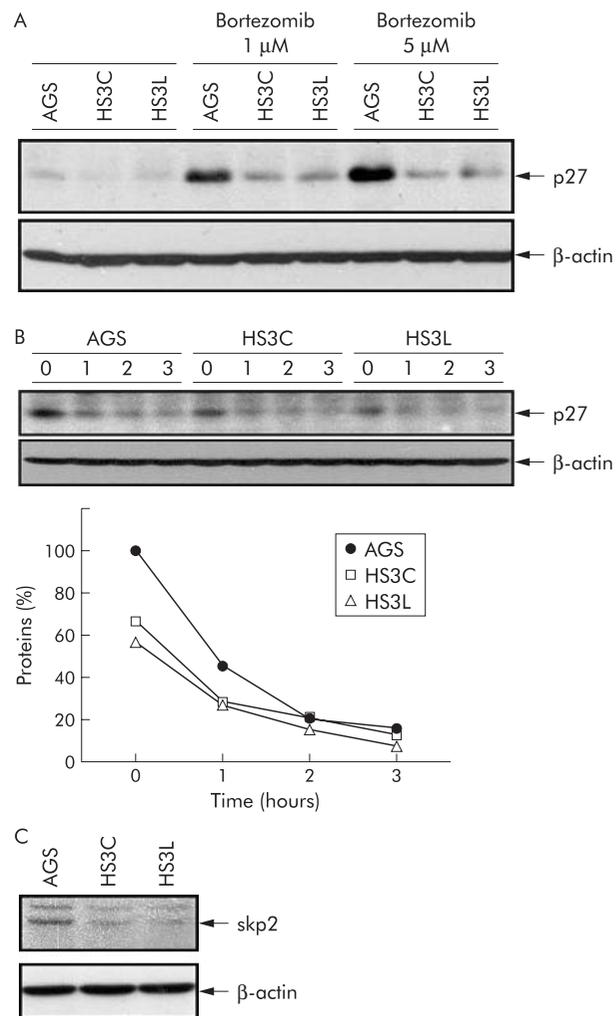
The observation of a proapoptotic role for p27 in gastric cells is consistent with the results of most previous studies that have used a variety of non-gastric cells.<sup>32–35, 37–40</sup> A partial explanation of the apparent antiapoptotic function ascribed to p27 in some studies may be that the effects of p27 on apoptosis may depend on whether the transfected cells have functional phosphorylated Rb protein. In this regard, an apoptotic response to p27 overexpression has been noted only in cancer cells that expressed phosphorylated Rb.<sup>45–46</sup> Because AGS cells and their apoptosis resistant derivative cells HS3 have functional phosphorylated Rb,<sup>24, 42</sup> our findings are in accordance with this hypothesis.

A proapoptotic role for p27 in gastric epithelial cells is also consistent with the gastric phenotype of p27 deficient mice in

which gastric hyperplasia is even more exaggerated following experimental *H. pylori* infection.<sup>47</sup> The extent to which deficient apoptosis or increased proliferation contributes to the hyperplastic phenotype in these animals is currently under investigation in our laboratory.

The emergence of cells with low levels of p27 and an apoptosis resistant phenotype that we observed consequent to the selection pressure of *H. pylori* as a chronic apoptosis inducing stimulus<sup>24</sup> may also occur in other situations. For example, low p27 expression has been associated with resistance to rapamycin induced cell death in the rapamycin resistant RR-3 cell line derived from BC3H1 murine muscle tumour cells.<sup>48</sup>

The mechanisms responsible for decreasing expression of p27 expression in chronic *H. pylori* infection remain unclear. In an acute (short term) exposure model, *H. pylori* increased the degradation of p27 protein by a ubiquitin independent proteasome dependent pathway.<sup>41</sup> However, decreased expression of p27 protein in HS3 cells and its derivatives (by over 80%) was also accompanied by a reduction in p27 mRNA abundance, by about 30–40%, according to our current and previous studies.<sup>24</sup> In contrast with acute *H. pylori*



**Figure 5** Low expression of p27 protein in *Helicobacter pylori* resistant cells is not due to accelerated proteasome dependent degradation. (A) AGS cells or *H pylori* resistant derivative clones were cultured in the absence or presence of 1  $\mu$ M and 5  $\mu$ M bortezomib for 12 hours. Western blot analysis reveals that inhibition of proteasome activity by bortezomib did not restore high levels of p27 protein in the *H pylori* resistant derivatives. Equal amount of protein loading was verified by  $\beta$ -actin. (B) Pulse chase analysis of p27 protein. Protein synthesis was inhibited by adding 100  $\mu$ M cycloheximide, and cellular proteins were harvested at the times (in hours) indicated for p27 protein expression evaluation by western blot. Equal amount of protein loading was verified by  $\beta$ -actin. Lower graph shows densitometric analysis of p27 protein. (C) Skp2 protein expression in exponentially growing cells was measured by western blot. Equal amount of protein loading was verified by  $\beta$ -actin.

exposure, in the HS3 derivatives that were chronically exposed to *H pylori*, we found no evidence for accelerated proteasomal degradation of p27 protein. Therefore, it is possible that both transcriptional and post-translational mechanisms contribute to the reduction in p27 protein in gastric cancer associated with *H pylori* infection. Studies examining expression of p27 protein and p27 mRNA in gastric epithelial cells during progression from a normal gastric epithelium through preneoplastic stages to gastric cancer may help determine how *H pylori* decreases p27 expression in non-transformed gastric epithelial cells and elucidate when and at what level *H pylori* influences p27 expression during the process of gastric carcinogenesis. Similar approaches in an animal model of *H pylori* associated gastric cancer may also be helpful in this regard. Such analyses could also address whether there is any in vivo

correlate to the observation from short term coculture that substances secreted by *H pylori* may also simultaneously increase the level of p27 protein expression.<sup>49</sup>

In summary, our study supports a proapoptotic role for p27 in gastric cancer cells that is likely to explain, at least in part, the association of low levels of p27 with a poor prognosis in this disease.

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## Authors' affiliations

H Eguchi, S Carpentier, S S Kim, S F Moss, Division of Gastroenterology, Department of Medicine, Rhode Island Hospital/Brown University, Providence, RI, USA

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