

MICROBIAL PATHOGENICITY

Inhibition of cell proliferation and induction of apoptosis by *Helicobacter pylori* through increased phosphorylated p53, p21 and Bax expression in endothelial cells

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Microcirculation plays a crucial role in mucosal physiological function as well as repair of gastric mucosal damage. Endothelial cell damage is known to disturb microcirculation and suppress angiogenesis. Therefore, the direct effect of *Helicobacter pylori* on endothelial cells *in vitro* was investigated with *H. pylori* water extract. The effect of *H. pylori* water extract on cell proliferation and apoptosis of human umbilical vein endothelial cells (HUVECs) was evaluated. The ratio of BrdU-positive HUVECs in both *cagA/vacA*-positive and -negative *H. pylori* water extract-treated groups was significantly lower at 24 h than that in the control group, but *Escherichia coli* water extract did not affect the proliferation of these endothelial cells. Apoptosis was induced by *H. pylori* water extracts after incubation for 24 h in a *cagA/vacA*-independent manner. In the mitochondrial permeability transition assay, tetramethylrhodamine methyl ester was accumulated in mitochondria of HUVECs. Western blot analysis showed no difference in the level of total p53 protein in *H. pylori* water extract-treated and non-treated cells, but the level of phosphorylated p53 protein was increased in the treated cells at 15 and 60 min after addition of the extract. Reverse transcription (RT)-PCR products for p21 and Bax were elevated in the *H. pylori* water extract-treated cells. p21 levels began to increase 0.5–1 h after addition of the extract, whereas Bax increased in the period 0.5–2 h. *H. pylori* induced a disturbance of cell proliferation and apoptosis in the vascular endothelial cells which may contribute to gastric mucosal injury and to delayed healing of gastric lesions.

Introduction

Helicobacter pylori infection delays gastric ulcer healing and the eradication of the infection has been shown to heal gastric ulcers as fast as proton pump inhibitors [1–4]. Generally, healing of gastric ulcers requires both epithelial cell restitution and the proliferation of mesenchymal cells, including vascular endothelial cells. Although many *in-vivo* and *in-vitro* experimental studies have explored the mechanisms of pathogenesis of *H. pylori* infection, most have focused on impairment of gastric epithelial cell integrity and function [5–7]. However, gastric mucosal microcirculation

is known to regulate proliferation of mesenchymal cells and play an important role in the healing of gastric ulcers or other gastric mucosal injuries [6–9].

Angiogenesis at the base of gastric ulcers has been shown to be inhibited by *H. pylori* infection [10, 11], suggesting that *H. pylori* infection has implications not only for the disturbance of gastric epithelial cells, but also for angiogenesis in the gastric mucosa. Endothelial cell damage by *H. pylori* may lead to disturbance of microcirculation and suppression of angiogenesis, thereby contributing to delayed ulcer healing. However, the direct effects of this bacterium on endothelial cells have been poorly characterised.

To investigate whether *H. pylori* directly damages the endothelial cells, its effect on cell proliferation and apoptosis needs to be revealed. Previous reports of the

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effects of *H. pylori* on proliferation and apoptosis of epithelial cells described the cellular mechanisms that are mediated by several gene products including p53, p21, p27, Bak, Bax or Bcl-2 [12–18]. Of these gene products, p53, p21 and p27 are involved in cell cycle arrest [12], and Bak, Bax or Bcl-2 are closely related to apoptosis of the cells [13]. Exploration of the expression of these gene products may be useful for confirming the experimental observations and for a better understanding of the cellular mechanisms underlying the effects of *H. pylori* on the endothelial cells. Therefore, the effects of *H. pylori* water extract on proliferation and apoptosis of endothelial cells, and the mechanisms, were investigated.

Materials and methods

Water extracts of H. pylori and Escherichia coli

Water extracts were prepared from two subtypes of *H. pylori*, strains ATCC43504 (*cagA/vacA*-positive) (obtained from American Type Culture Collection, Manassas, VA, USA) and C0002 (*cagA/vacA*-negative) (obtained from Otsuka Assay Laboratory, Tokyo, Japan). Bacteria were inoculated on to Brucella agar and cultured in air with CO₂ 10% at 37°C for 72 h. Colonies were harvested with sterile cotton swabs and suspended in distilled water (10⁹–10¹⁰ cfu/ml).

E. coli competent strain DH5 α (Takara, Shiga, Japan) was cultured at 37°C for 24 h in LB broth (Bacto tryptone 10 g, Bacto yeast extract 5 g, NaCl 10 g, 5 N NaOH 200 μ l and amphotericin B 500 μ g/L) and centrifuged at 3000 rpm for 10 min. The pellet was rinsed with sterile distilled water and resuspended in an appropriate volume of distilled water (10⁸–10⁹ cfu/ml).

Suspensions were held at room temperature for 20 min before centrifugation at 12 000 rpm for 15 min. The supernates were passed through a 0.2- μ m syringe-adapted filter to remove high mol. wt material, consisting mainly of membrane vesicles and whole flagella. The protein concentration was measured by the Bradford method [19].

Endothelial cells

Human umbilical vein endothelial cells (HUVECs; ATCC) were seeded on to collagen-coated polyethylene dishes (Sumitomo Bakelite, Tokyo, Japan) and cultured in EBM-2 medium (Bio Whittaker, Walkersville, MD, USA), supplemented with the EGM-2 bullet kit (Bio Whittaker) containing long R insulin-like growth factor-1, human epidermal growth factor and human fibroblast growth factor-B and vascular endothelial growth factor in humidified air with CO₂ 5% at 37°C for 4 days before experiments. Cultured HUVECs were used for experiments between passage 5 and 8.

Detection of cell proliferation

An artificial wound was made on HUVEC complete monolayer cell sheets with a rotating silicon tip resulting in a cell-free round area with a constant size (2 mm²) [20]. *H. pylori* water extract (0, 50, 100, 200 μ g/ml) or *E. coli* water extract (200 μ g/ml) was added to the cell sheets and cultured for 24 h. 5-Bromodeoxyuridine (BrdU) was added 22 h after wounding and incubation continued for a further 2 h. Proliferating cells were detected by indirect immunohistochemical methods with anti-BrdU monoclonal antibody (MAb). The BrdU labelling index – number of BrdU positive cells/total number of cells per unit area (0.02 mm²) – was calculated in control, *H. pylori* water extract- and *E. coli* water extract-treated groups (n = 3).

Detection of apoptosis

HUVECs were cultured for 24 h in the presence of *H. pylori* water extract (0, 50, 100, 200 μ g/ml) or *E. coli* water extract (200 μ g/ml). Apoptotic cells were detected by a terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labelling (TUNEL) method with a fluorescent apoptosis detection system kit (Promega, Madison, WI, USA). The stained cells were recorded on a fluorescence microscope (Axiophot ZVS 3C75DE; Carl Zeiss, Goleta, CA, USA) and Ektachrome Dyna 400 films (Eastman Kodak, Rochester, NY, USA). To calculate the percentage of apoptotic cells, four random fields (0.2 mm²) were examined in a blind manner in each preparation (n = 3).

Mitochondrial permeability transition assay

To monitor the changes in mitochondrial permeability from the mitochondrial permeability transition (MPT) changes, HUVECs were cultured with *H. pylori* water extract (0, 200 μ g/ml) for 24 h then loaded with tetramethylrhodamine methyl ester (TMRM) 0.5 μ mol/L for 10 min at 37°C. Nuclear condensation was evaluated by staining with Bisbenzimidazole H 33342 Fluorochrome (Calbiochem-Novabiochem, La Jolla, CA, USA). Stained cells were observed with an Axiophot fluorescence microscope (Carl Zeiss).

Western blotting for total p53 and phosphorylated p53

HUVECs were cultured with *H. pylori* water extract (200 μ g/ml) for 15 min and 1 h and scraped into a lysis buffer – 150 mM NaCl, NP40 1.0%, deoxycholate (sodium salt) 0.5%, SDS 0.1%, 50 mM Tris-HCl (pH 8.0). Cell lysates were sonicated for 2 s to reduce sample viscosity. After removing unbroken cells by centrifugation at 10 000 rpm for 5 min, the supernate was boiled in Laemmli loading buffer [21] and separated by SDS-PAGE on an acrylamide 10% gel

(BioRad Laboratories, Tokyo Japan). Proteins were electroblotted on to polyvinylidene difluoride membranes, which were then blocked in skimmed milk 2%. Membranes were then incubated overnight at 4°C with mouse polyclonal antibody to whole p53 (Immuno-Biological Laboratories, Gunma, Japan) or rabbit polyclonal antibody to phosphorylated p53 (Serin 15) (Cell Signaling Technology, Beverly, MA, USA) and specifically bound primary antibody was detected with a horseradish peroxidase-labelled anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by enhanced chemiluminescence. The membrane was drained of excess developing solution, wrapped in Saran Wrap and exposed to X-ray film.

RNA extraction, reverse transcription (RT)-PCR

Expression of p21 and Bax mRNA was determined by reverse transcription of total RNA followed by PCR analysis (RT-PCR). Total RNA from HUVEC was isolated by TRIzol Reagent (Life Technologies, Frederick, MD, USA) and reverse transcribed with Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Gibco-BRL). The specific primer set used for human p21 was 5'-GCCGAAGTCAGTTCCTT-3' (forward) and 5'-TCATGCTGGTCTGCCGC-3' (reverse), and for human Bax was 5'-TGGCAGCTGACATGTTTCTGAC-3' (forward) and 5'-TCACCCAACCACCC TGGTCTT-3' (reverse). p21 was amplified in 36 cycles of 30 s at 95°C for denaturing, 1 min at 52°C for annealing and 1 min at 72°C for extension. PCR for Bax was performed for 28 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C. Human β -actin served as a positive control. Primers used in this amplification were human β -actin sense primer (5'-AACACCCCAGC CATGTACGTAG-3') and antisense primer (5'-GTGT TGGCATAGAGGTCTTTACGG-3'). PCR products were separated by electrophoresis in an agarose 2% gel and visualised by ethidium bromide staining. Product size (bp) was determined with DNA MW Marker 5 (Nippon Gene, Tokyo, Japan) as a standard size marker.

Results

Inhibition of endothelial cell proliferation by H. pylori water extract

The ratio of BrdU-positive HUVECs in both *cagA/vacA*-positive and -negative *H. pylori* water extract-treated groups was significantly lower ($p < 0.05$) than in the control group (Figs. 1 and 2). This reduction occurred in a dose-dependent manner, and there were no different results with either type of water extract (*cagA/vacA*-positive or -negative). On the contrary, as shown in Fig. 2, addition of *E. coli* water extract provided a labelling index similar to the control, suggesting that it did not affect proliferation of these endothelial cells.

Induction of apoptosis in endothelial cells by H. pylori water extract

Nuclear condensation was observed in the *H. pylori* water extract-treated HUVECs after incubation for 24 h. Furthermore, the ratio of TUNEL-positive to TUNEL-negative cells was greater in the *H. pylori* water extract-treated cells than in untreated cells (Fig. 3). As with inhibition of cell proliferation, these effects were observed in a dose-dependent manner ($p < 0.05$) and were independent of *cagA* or *vacA* (Fig. 4).

In the MPT assay, TMRM accumulated in mitochondria of HUVECs as shown in Fig. 5. In most of the apoptotic cells with concentrated nucleus in the *H. pylori* water extract-treated group, TMRM fluorescence was not detected in the cytoplasm, suggesting that the apoptotic induction by *H. pylori* water extract was mediated by a mitochondrial pathway.

Western blot analysis of total p53 and phosphorylated p53

Western blot analysis showed no difference in the level of total p53 protein at any point during the experiment. However, the level of phosphorylated p53 protein was clearly increased in the *H. pylori* water extract-treated group at 15 and 60 min after addition of the extract (Fig. 6).

Expression of mRNA for p21 and Bax in H. pylori water extract-treated cells

RT-PCR products representing p21 and Bax transcripts were clearly elevated in the *H. pylori* water extract-treated cells (Fig. 7). p21 levels began to increase 0.5–1 h after addition of the extract, whereas Bax increased 0.5–2 h after addition of the extract.

Discussion

Several studies have described the effect of *H. pylori* infection on the structure and function of endothelial cells [22–25], most focusing on the activation of leucocytes by the bacteria, resulting in leucocyte migration and attachment to the endothelial cells and subsequent disturbance of microvascular circulation [26–30]. In-vitro studies [31] have shown that *H. pylori* induced the expression of interleukin (IL)-8 in gastric endothelial cells and another study demonstrated the mechanism of *H. pylori*-induced neutrophil-dependent vascular protein leakage of the stomach, which is regarded as another indirect effect of *H. pylori*. These observations also addressed the link between *H. pylori* infection and the extra-gastric manifestations of these infections in man [32]. The debate over whether *H. pylori* directly modulates the biological response of endothelial cells raises important questions, because endothelial cells regulate microcir-

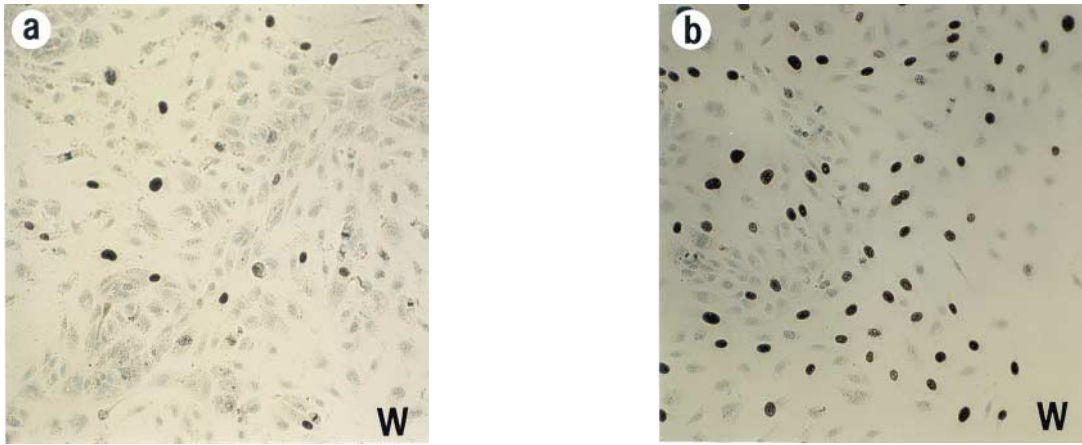


Fig. 1. Inhibition of cell proliferation of HUVECs by *H. pylori* water extracts. BrdU immunohistochemistry showing the effect of *H. pylori* water extract from ATCC43504 (*cagA/vacA*-positive strain, protein concentration 200 $\mu\text{g/ml}$) on HUVECs. BrdU-positive cells were lower in the *H. pylori* water extract-treated group (a) than in non-treated group (b); W, wound.

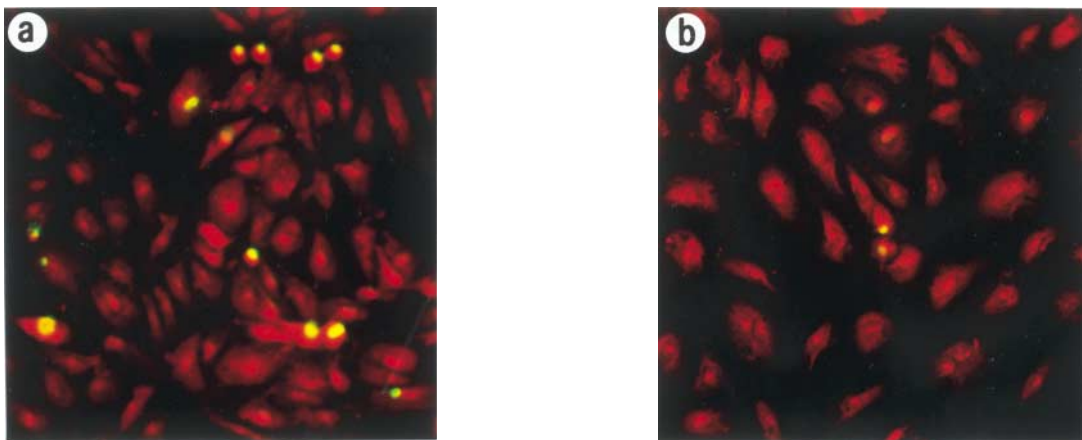


Fig. 3. Apoptosis of HUVECs induced by *H. pylori* water extracts, TUNEL method staining. Apoptotic cells (yellow fluorescent cells) were increased in the *H. pylori* water extract (ATCC43504, *cagA/vacA*-positive strain, protein concentration 200 $\mu\text{g/ml}$)-treated group (a) compared with the non-treated group (b).

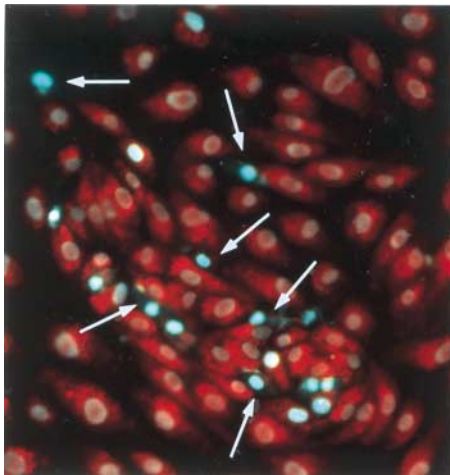


Fig. 5. MPT assay of HUVECs treated with *H. pylori* water extracts demonstrating mitochondrial injury of HUVECs by addition of *H. pylori* water extract (ATCC43504, *cagA/vacA*-positive strain, protein concentration 200 $\mu\text{g/ml}$). MPT were not incorporated in the cytosol of apoptotic cells. The concentrated nucleus in apoptotic cells was tinged by Bisbenzamide H 33342 Fluorochrome with blue fluorescence (shown by white arrows). MPT-negative apoptotic cells were increased in the *H. pylori* water extract-treated group.

ulation and disturbance of endothelial cell proliferation impairs angiogenesis during the healing of ulcerative lesions or mucosal damage. Consequently, the present study aimed to clarify whether *H. pylori* itself affects the viability and proliferation of endothelial cells, thereby disturbing the healing process of ulcerative lesions or mucosal damage in the stomach.

The extract prepared from two different strains of *H. pylori* specifically suppressed the proliferation of cultured human endothelial cells, because this phenomenon was caused only by extracts prepared from *H. pylori* but not those prepared from *E. coli*. The *H. pylori*-specific inhibition of endothelial cell proliferation supports clinical observations of delayed gastric ulcer healing during *H. pylori* infection. After infection-induced destruction of the gastric surface epithelium, *H. pylori* might directly affect the endothelial cells. Although *cagA* and *vacA* have been suggested to be the primary virulence factors for gastric mucosal damage and inducing chemical mediators from gastric cells [33, 34], the observation from the present study that *H. pylori*-induced inhibition of the cell prolifera-

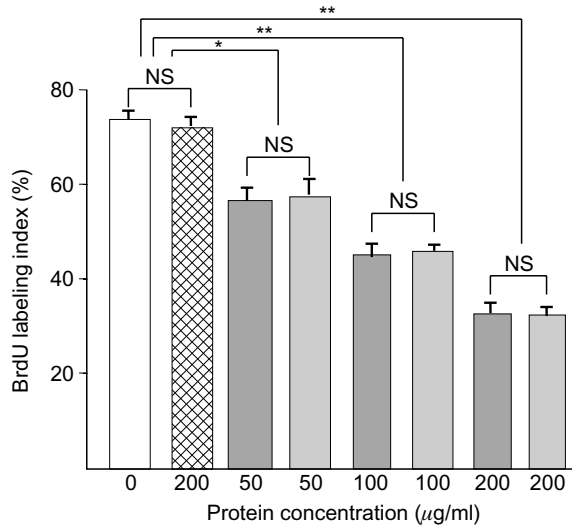


Fig. 2. BrdU labelling index of HUVECs with various concentration of *H. pylori* water extracts. BrdU labelling index decreased in a dose-dependent manner by addition of *H. pylori* water extract, and those of *H. pylori* water extract-treated groups were significantly lower than in the non-treated group. There was no difference between the effects of water extract from *cagA/vacA*-positive (ATCC43504) (■) and -negative (C0002) (□) strains. (⊞), *E. coli*; (□), control.

tion was *cagA/vacA*-independent supports the idea that other unidentified *H. pylori* water-soluble proteins are critical mediators of its cytotoxic effects. Furthermore, the effects of whole cells of *H. pylori* (ATCC 43504; *cagA/vacA*-positive strain) on the cell proliferation were examined in a preliminary way and similar results to those obtained by the water extract were found (data not shown), suggesting that some secreted non *cagA/vacA* proteins may be involved in this cytotoxic effect.

Several other mechanisms by which *H. pylori* might directly suppress the proliferation of vascular endothelial cells were investigated. A previous study by Shirin *et al.* [14] indicated that *H. pylori*-induced inhibition of

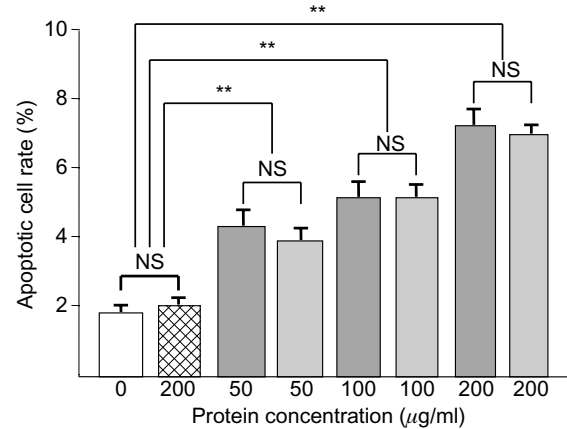


Fig. 4. The ratio of apoptotic HUVECs with various concentrations of *H. pylori* water extracts. The ratio of apoptotic cells increased after addition of *H. pylori* water extract in a dose-dependent manner. This effect was independent of *cagA/vacA*-positive (ATCC43504) (■) or -negative (C0002) (□) source of the extract. (⊞), *E. coli*; (□), control.

the G1-S cell cycle transition in the AGS gastric cancer cell line is associated with reduction of p27 levels but not with reduced p53 or p21. Furthermore, *H. pylori* clinical isolates accelerated the G1 to G2-M transition by a mechanism independent of p53, p21 or mdm-2 gene involvement [15, 16]. Critically, neither of these studies examined alterations in the level of phosphorylated p53. The results of the present study indicated that there was no change in the total levels of p53 induced by the *H. pylori* water extract, but instead there was an increase in phosphorylated p53. Several factors may be involved in the difference between the results of the present study and previous studies that gave negative results for the involvement of this tumour suppressor gene. The involvement of p53 phosphorylation in the effect of *H. pylori* water extract on endothelial cells is supported by the accompanying increase in p21 mRNA expression.

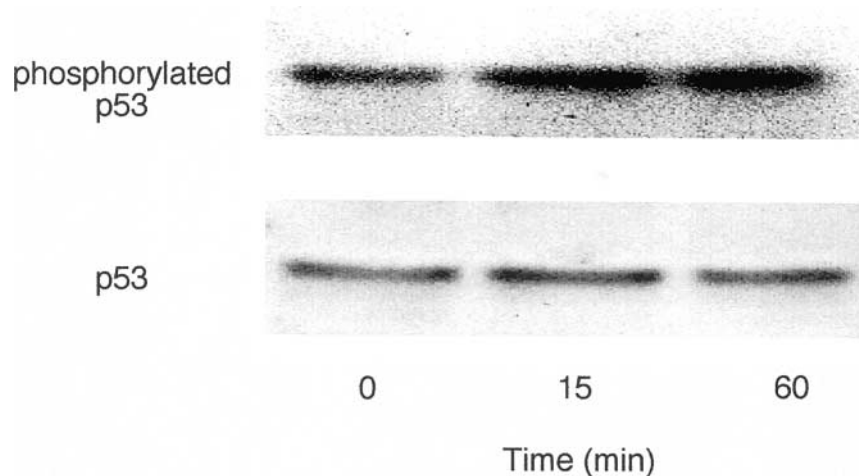


Fig. 6. Western blot analysis of total and phosphorylated p53 levels in the extracted proteins from HUVECs treated with or without *H. pylori* water extract (ATCC43504, *cagA/vacA*-positive strain, protein concentration 200 µg/ml) and anti-p53 and anti-phosphorylated p53 antibody. The whole p53 protein was expressed consistently during the time course, the expression of phosphorylated p53 was increased at 15 and 60 min after addition of the water extract (no *H. pylori* water extract was added at '0 min', meaning a basal expression of p53 protein). This result was confirmed by five separate experiments.

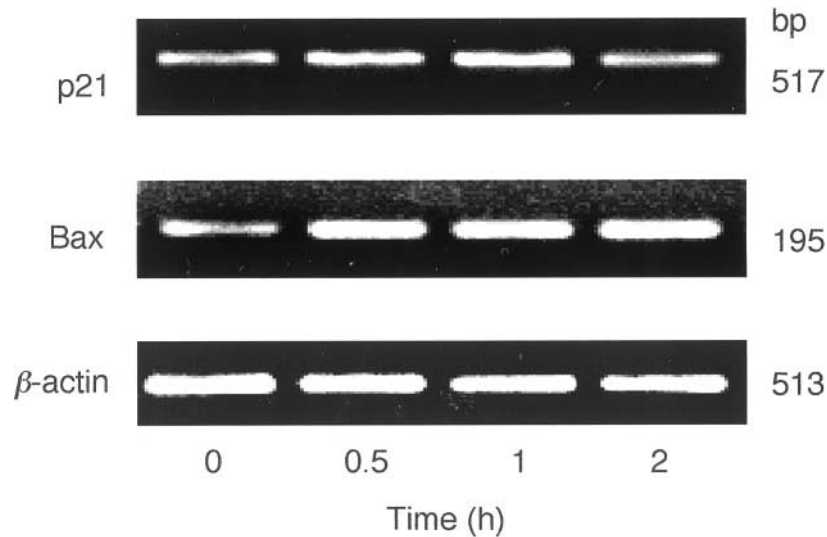


Fig. 7. RT-PCR analysis of p21 and Bax mRNA levels in the HUVECs with or without *H. pylori* water extract (ATCC43504, *cagA/vacA*-positive strain, protein concentration 200 $\mu\text{g/ml}$) stimulation. PCR products of p21 and Bax c-DNA were increased at 0.5 and 1 h, while the intensity of the bands for β -actin was unchanged, suggesting that p21 and Bax gene expression were up-regulated by the *H. pylori* water extract. This result was confirmed by five separate experiments.

The present study also provided evidence that the *H. pylori* water extract dose-dependently induced apoptosis in HUVECs. The results of the MPT assay suggested that the compromised mitochondrial permeability was associated with this phenomenon. Bax is one of the Bcl-2 family [35] and known to be a key protein relating to apoptosis through mitochondrial injury. Furthermore, as its expression is known to be partially regulated by p53 activity [36], the finding that *H. pylori* water extract facilitated phosphorylation of p53 protein correlated well with up-regulated Bax expression, i.e., the results demonstrated that the *H. pylori*-induced apoptosis was mediated by the Bax-related mitochondrial injury. A recent study [17] has described induction of Bax expression and repression of Bcl-2 during mitochondrial-dependent apoptosis, yet only Bak (one of the bcl-2 family) plays a role in apoptosis in the AGS cancer cell line [18]. In addition, Fas–Fas ligand interaction has been implicated in *H. pylori*-induced apoptosis [37]. Establishing the relevance of this pathway, which works through activated monocytes, would require an in-vivo model. The results of the present study raise the possibility that *H. pylori*-induced endothelial cell apoptosis may not only enhance mucosal injury through extravasation of inflammatory cells, but might also retard mucosal repair through inhibition of angiogenesis. Future in-vivo studies should clarify the clinical implications of *H. pylori*-induced apoptosis of vascular endothelial cells in gastric mucosal lesions.

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