MAJOR ARTICLE

Preferential Induction of Transforming Growth Factor–β Production in Gastric Epithelial Cells and Monocytes by *Helicobacter pylori* Soluble Proteins

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Background. The cytokines induced by *Helicobacter pylori*, as well as the intricate balance of proinflammatory and anti-inflammatory cytokines, are relevant to the outcomes of *H. pylori* infection. Transforming growth factor (TGF)–β and interleukin (IL)–10 are 2 vital anti-inflammatory cytokines that regulate mucosal immunity in various inflammatory and infectious diseases.

Methods. To elucidate whether host-bacteria interaction can influence TGF-β and IL-10 production, we investigated the expression of TGF-β and IL-10 in various mammalian cell lines preincubated with *H. pylori* and other enteric bacteria.

Results. The amount of TGF-β protein, but not IL-10, was significantly increased after stimulation with *H. pylori*, but other enteric bacteria did not induce TGF-β production. Different *H. pylori* strains isolated from patients with gastritis, peptic ulcer, gastric cancer and strains with *cagA* or *vacA* isogenic mutations showed similar effects on TGF-β induction, indicating that this effect was a constitutional characteristic of *H. pylori* and independent of *cagA* and *vacA* status.

Conclusion. The results imply the presence of a protein factor (termed "TGF-β–inducing protein") that induces production of TGF-β. In view of the multiple effects of TGF-β, we conclude the TGF-β–inducing protein of *H. pylori* might mediate the immune response and contribute to the pathogenesis of *H. pylori* infection.

Helicobacter pylori infects about half of the world's population. The majority of infected patients have asymptomatic gastritis, and 10%–15% develop peptic ulcer, gastric carcinoma, or B cell mucosa-associated lymphoid tissue (MALT) lymphoma [1]. The variable clinical outcomes of *H. pylori* infection are attributed to variations in bacterial virulence factors as well as differences in host immune responses [2]. In particular, the immune response against *H. pylori* virulence factors might provide a direct linkage to the development of gastroduodenal diseases [3].

In the early stages of infection, *H. pylori* induces the production of chemokines, as well as proinflammatory cytokines [4]. The induction of chemokines or proinflammatory cytokines attracts neutrophils, monocytes, macrophages, or dendritic cells, which then migrate to the inflammatory area and activate a number of innate inflammatory responses. Neutrophils participating in gastric inflammation are related to the clearance of *H. pylori* [5]. Mucosal macrophages and dendritic cells, the monocyte-derived cells, can capture and digest pathogenic antigens, soon after infection by the pathogen [6]. Furthermore, specific antibodies against *H. pylori* that are detectable in patients' serum may help eradicate the bacterial infection in gastric mucus [6, 7].

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With respect to cell-mediated immunity, evidence has shown that $CD4^+$ T cells play an important role in protective immunity against *H. pylori* [8, 9]. However, *H. pylori* infection is not eliminated in patients with detectable Th1 and antibody responses. It is presumed that the pathogen has evolved a number of strategies to circumvent protective immune responses. In this regard, studies have shown that *H. pylori* can induce apoptosis of various types of cells, including gastric epithelial cells, macrophages, and T cells [10–12]. Moreover, the local cytokine milieu, particularly the intricate balance between proinflammatory and anti-inflammatory cytokines, can influence T cell development, the efficacy of immune responses, and gastric pathology [13, 14]. An early and persistent Th1-dominated CD4 response appears to be critical in the prevention of chronic infection but may lead to more severe gastric inflammation [15]. In contrast, the development of chronic infection is linked to a weak or absent *H. pylori*–specific Th1 response and to the presence of Th2-type cytokines; but only minimal gastritis was found under such conditions [16]. Among known Th2 cytokines, interleukin (IL)–4 and IL-5 were virtually absent from gastric lymphocytes from patients infected with *H. pylori* [17–19].

Recently, transforming growth factor (TGF)–β and IL-10 have been reported to exert potent anti-proliferative and anergy-inducing effects on CD4 cells [20]. Such pathogen-stimulated IL-10 or TGF-β production might play a vital role in the prevention of infection-induced immunopathology or prolongation of persistence, by suppressing Th1 responses [21, 22]. However, only few studies have investigated the effects of *H. pylori* on TGF-β and IL-10 production and have reported controversial results [23–31]. We investigated whether *H. pylori* infection could modulate the production of TGF-β and IL-10 in various mammalian epithelial cells. We show that *H. pylori* may secrete some soluble protein(s) to induce TGF-β production in gastric epithelial cells and monocytes and that this ability is a constitutional characteristic of *H. pylori* independent of *cagA* status and disease status. We assumed that *H. pylori* might use this capability to escape from or interfere with T cell functions and inflammatory responses.

MATERIALS AND METHODS

Bacterial strains. The *H. pylori* strains were obtained from ATCC (ATCC 43504) or freshly isolated from gastric biopsy specimens at National Taiwan University Hospital. Clinical isolates were cultured on blood agar plates under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) [32]. After isolation, a single colony was subcultured. The strains were then preserved at −70°C in Brucella broth (Difco Laboratories) supplemented with 15% glycerol (vol/vol). *H. pylori* strains isolated from 15 patients with chronic gastritis, 15 patients with gastric ulcer, 14 patients with duodenal ulcer, and 15 patients with gastric cancer were randomly selected from more than 300 clinical isolates for further studies. All these strains were determined to be *cagA*-positive by PCR. The *cagA*- or *vacA*-negative isogenic mutants were kindly provided by Professor Jin-Town Wang [32]. Before the infection of epithelial cells, pure cultures of *H. pylori* were recovered from stocks cultured on blood agar plates under microaerophilic conditions at 37°C for 3 days. The culture plate was washed with 2 mL PBS, and the amount of bacteria suspended in PBS was determined by measuring the optical density at 600 nm.

Bacterial strains including *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas aeruginosa, Shigella flexneri,* and group B *Salmonella* were obtained from National Taiwan University Hospital and were identified by standard microbiological techniques.

Epithelial cell cultures. Different epithelial cell lines were obtained from the ATCC, including gastric cancer cells (human stomach adenocarninoma [AGS] cells, N87, SUN-1, SUN-16, Hs578T, T-47D, L48, and TSGSH), colonic cancer cells (colo 320), breast cancer cells (NIC-H157), and hepatoma cells (HepG2). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum and 50 μg/mL penicillin-streptomycin.

Infection procedure. Epithelial cell lines were seeded in 24-well culture plates in a volume of 1 mL per well and grown at 37°C in a 5% CO_2 atmosphere to reach confluence. Prior to infection, each well was washed twice in 1 mL of antibiotic-free cell culture medium. Bacterial cells were harvested, washed with PBS and then resuspended in the same medium. The bacteria were added to the cultured cells at an MOI of 400. After incubation for 16 h at 37°C in a 5% CO_2 atmosphere, the concentrations of TGF-β or IL-10 in the supernatant were measured at indicated time points. In addition, an uninfected control was included in each experiment.

***Preparation of soluble extract of* H. pylori.** *H. pylori* was harvested, washed and resuspended in PBS (4×10^7 cfu/mL). Soluble extract was prepared in PBS by sonication, centrifuged at 13,400*g* for 10 min and passed through a 0.20-μm filter. The protein concentration of soluble extract was determined by the bicinchoninic acid method with bovine serum albumin as a standard.

Preparation of peripheral blood mononuclear cells (PBMCs) and primary gastric epithelial cell lines. Monocytes were isolated from buffy coat prepared from a *H. pylori*–negative volunteer from our laboratory staff. The plasma of blood samples was carefully removed, PBMCs were prepared with the use of Ficoll-Hypaque density gradient centrifugation and resuspended in RPMI 1640 with 5% inactivated fetal calf serum. Cells were incubated in Petri dishes at 37°C for 1 h. Nonadherent cells were removed by several washes with PBS. The ad-

herent cells were cultured in RPMI 1640 with 10% fetal calf serum at 37° in an atmosphere with 5% CO_2 and 95% humidity.

Primary cultures of human gastric epithelial cells were established from gastric biopsies taken at gastroscopic examinations, as detailed elsewhere [33]. In the experiments to determine TGF-β induction, the concentrations of both primary gastric epithelial cell lines and PBMCs were adjusted to 10^5 cells/mL.

Size exclusion assays. The supernatants from *H. pylori* were poured into a centrifugal filter device (Amicon Ultra-15; Millipore) with 100-kDa ultrafiltration membranes. The device was centrifuged at 3300*g* for 30 min, and the supernatant in the upper layer or lower layer was separately collected. The supernatant in the lower layer was later centrifuged again at 1200*g* for 30 min with a 50 kDa Vivaspin concentrator device (Viva-Science) with a 50-kDa ultrafiltration membrane. The protein concentrations of supernatants were measured with a BCA protein assay kit (Pierce), and their TGF-β–inducing activities were determined.

Gel filtration and SDS-PAGE. Fractionation to collect the protein with TGF-β–inducing activity (~50–100 kDa) was applied on a Sephadex G200 column (Amersham Biosciences). The protein was monitored by Coomassie Plus Bradford assay with absorbance set at 595 nm. Gel filtration was carried out at room temperature with the buffer containing 1x PBS (pH, 7.4). Every 5 fractionations (5 μL/fraction) collected by gel filtration were mixed, and the TGF-β–inducing ability was examined in AGS cells. The TGF-β–producing fraction was then subjected to 10% SDS-PAGE electrophoresis. The gels were later stained with Coomassie blue and their molecular equivalent masses were determined by comparing them with standard markers.

Quantitation of TGF-β and IL-10. TGF-β and IL-10 proteins in the supernatant were measured with ELISA kits purchased from Promega and used according to the manufacturer's instructions.

Statistical analysis. Triplicate results of 3 experiments ($n = 9$) are expressed as mean values ± SEM. Statistical differences were determined with the software program Schoolstat (White Ant Occasional Publishing) using overall analysis of variance and the independent *t* test.

RESULTS

***Induction of TGF-β production in human gastric epithelial cell lines exposed to* H. pylori.** To examine the effects of *H. pylori* on TGF-β production by gastric epithelial cells, *H. pylori* were cultured with different human gastric cancer cell lines in the initial experiments. As shown in figure 1*A*, quantification of TGF-β in supernatants after incubation for 16 h demonstrated a significant increase in TGF-β, compared with cells unexposed to *H. pylori* ($P < .05$). Three clinical isolates of *H. pylori* isolated from patients with gastritis, duodenal ulcer, or gastric cancer were tested in AGS cells to determine the response time for TGF-β production. The expression of TGF-β increased 5 min after *H. pylori* infection, reached its maximal

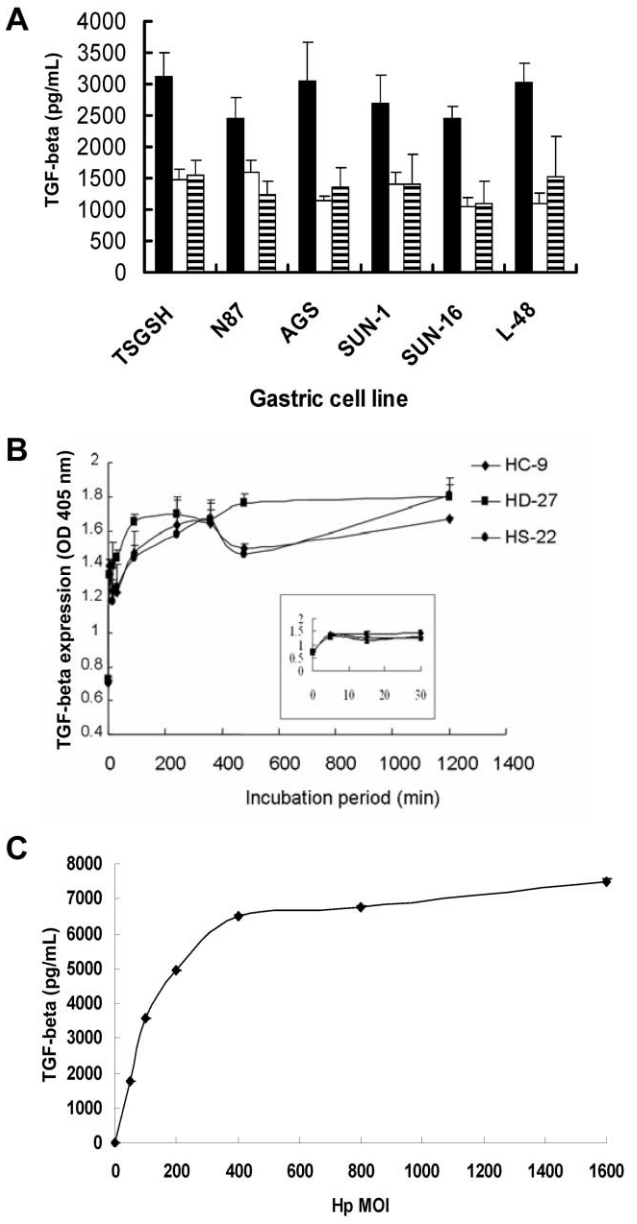

Figure 1. Transforming growth factor (TGF)–β production of human gastric epithelial cells induced by *Helicobacter pylori* (Hp). Results of 3 experiments are shown, expressed as mean values ± SEM. *A,* TGF-β production in human gastric cancer cell lines cultured with *H. pylori. Black bars, H. pylori* soluble extract; *white bars,* supernatants of cells treated with PBS; *striped bars,* supernatants of cells without any treatment. *B,* Time-dependent TGF-β production, as measured by ELISA, in human stomach adenocarninoma (AGS) cells exposed to clinically isolated *H. pylori* strains from patients with gastritis (HC-9), duodenal ulcer (HD-27), and gastric cancer (HS-22). *C,* Relationship between TGF-β production in AGS cells with different MOI values for *H. pylori.*

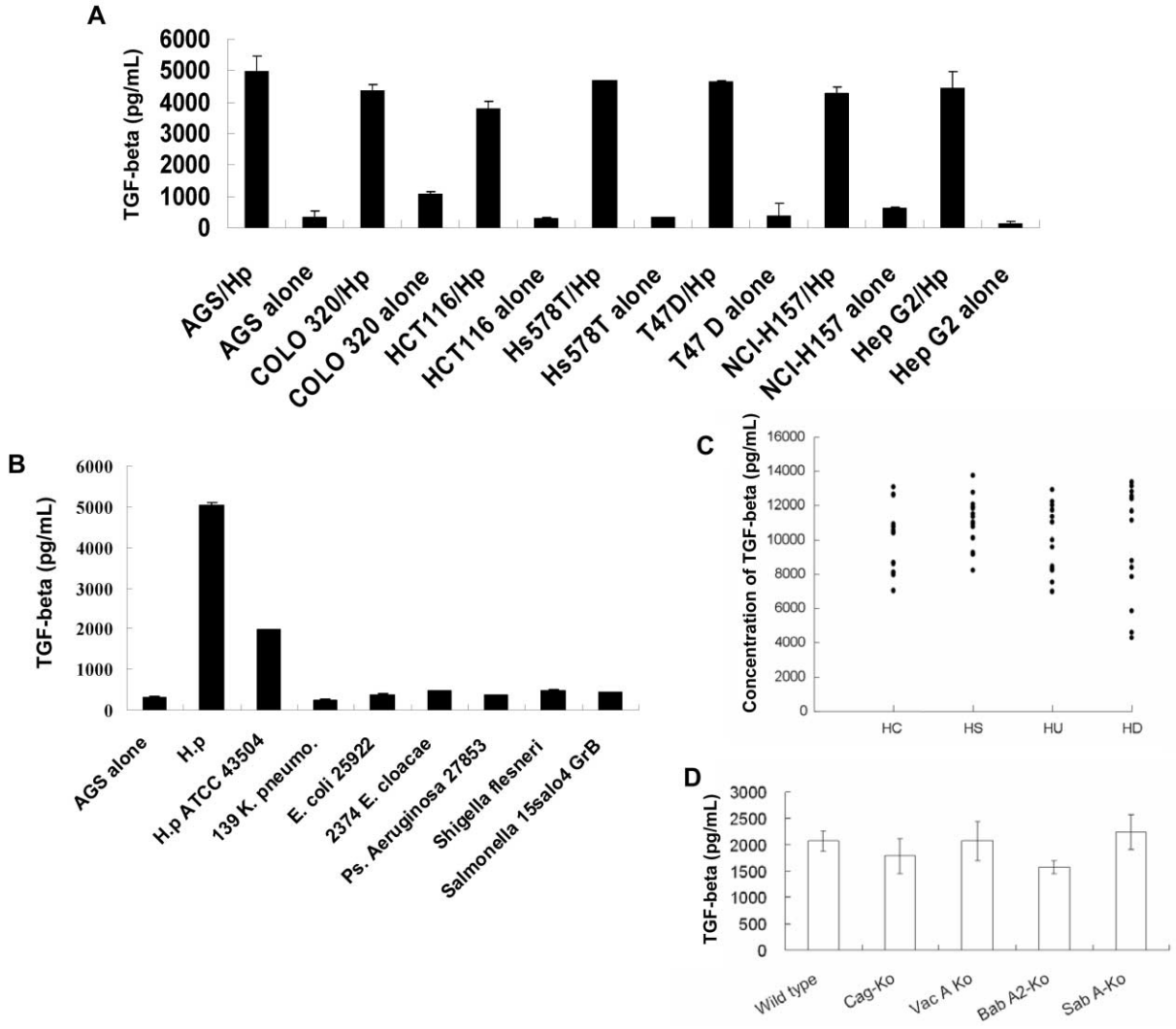

Figure 2. Influence of disease status and virulence factors *cagA* and *vacA* on induction of transforming growth factor (TGF)–β production by *Helicobacter pylori*. Results of 3 experiments are shown, expressed as mean values ± SEM. *A,* Variations in the ability to induce TGF-β production for cell lines exposed to *H. pylori* (MOI, 400). PBS alone was the negative control for each cell line. *B,* TGF-β concentrations in supernatants obtained after incubation of human stomach adenocarninoma (AGS) cells exposed to *H. pylori* and other enteric bacteria (*Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas aeruginosa, Shigella flexneri,* and group B *Salmonella;* MOI, 400). *C,* TGF-β production in supernatants of cultures of AGS cells and clinical isolates of *H. pylori* from patients with gastritis (HC), duodenal ulcer (HS), gastric ulcer (HU), and gastric cancer (HD). *D,* TGF-β production in supernatants of cultures of AGS cells with wild-type *H. pylori* and 5 knockout (KO) strains of *H. pylori.*

level at 90 min, and remained at this high level till the end of the experiment (16 h after infection) (figure 1*B*). Production of TGF-β in AGS cells was noted to depend on the size of the inoculum; the plateau was found to be at an MOI value of 400 (figure 1*C*). On the basis of these results, the following analyses of *H. pylori*–induced TGF-β production were performed using an MOI value of 400 and an incubation period of 16 h, unless otherwise stated.

Induction of TGF-β production by* H. pylori*, according to* cagA, vacA *and the disease status. *H. pylori* could significantly increase the production of TGF-β in various mammalian nongastric cell lines ($P < .05$) (figure 2*A*). In contrast, there was no alteration in the IL-10 level after culture of *H. pylori* with various mammalian cell lines (data not shown). The induction of TGF-β in epithelial cells was specific to *H. pylori*, because other enteric bacteria (*E. coli, K. pneumoniae, E. cloacae, P. aeruginosa, S. flexneri*, and group B *Salmonella*) did not induce TGF-β production (figure 2*B*). There was no strain variability with respect to TGF-β production: cultures of AGS cells with various *H. pylori* strains isolated from patients with gastritis, duodenal ulcer, gastric ulcer, or gastric cancer showed no significant difference in the mean level of TGF-β (figure 2*C*).

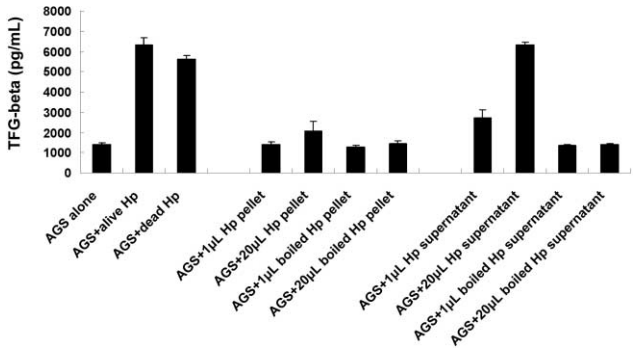

Figure 3. Influence of *Helicobacter pylori*–derived soluble proteins on the production of transforming growth factor (TGF)–β from the gastric cancer cell lines. Human stomach adenocarninoma (AGS) cells were stimulated with live and dead *H. pylori,* boiled and unboiled soluble fractions (supernatants), and boiled and unboiled insoluble fractions (*H. pylori* cell pellets). Results of 3 experiments are shown, expressed as mean values ± SEM.

Furthermore, TGF-β production was similar between *cagA*-negative isogenic mutants and *cagA*-positive strains (figure 2*D*).

***Induction of TGF-β by soluble proteins of* H. pylori.** Stimulation of AGS cells with soluble and insoluble fractions of live and dead *H. pylori* showed that only the insoluble fraction could not induce TGF-β production (figure 3). To further determine whether the modulatory factors present in *H. pylori* preparations were protein or nonprotein factors, the soluble lysate was pretreated with boiling. After boiling, the capability of induction of TGF-β was significantly decreased (figure 3). Furthermore, digestion of the supernatant with proteinase K completely stopped TGF-β production, indicating that soluble proteins account for the inducing effect (data not shown).

Induction of TGF-β by the soluble proteins of* H. pylori *in PBMCs and gastric epithelial cells. Although epithelial cells from cancer cell lines can be stimulated to express TGF-β, whether the normal gastric epithelial cells and PBMCs can respond to *H. pylori* is unknown. Therefore, the human primary gastric epithelial cells were incubated with *H. pylori* or the soluble supernatant to monitor the secretion of TGF-β into medium. The results revealed that *H. pylori* and its soluble proteins could induce TGF-β secretion in the gastric epithelial cells and in PBMCs, but *E. coli* could not (figure 4*A* and 4*B*).

Partial characterization of the TGF-β inducing factor(s). TGF-β inducing activity was detectable in the retentate after concentration with a 50–100 kDa–cutoff concentrator (results not shown). After eluates that passed through the 100-kDa filter were reseparated with the gel filtration column, and the TGF-β inducing activities in the fractions were determined (figure 5*A*), SDS-PAGE analysis of fractions with peak TGF-β inducing activity revealed 2 major bands with a molecular mass of ~70 kDa (figure 5*B*).

DISCUSSION

The mechanisms by which *H. pylori* causes chronic infection and interacts with immune systems remain unclear. Proinflammatory cytokines, particularly Th1 cytokines, have long been considered as the main mediators of the immune response to *H. pylori* infection [34]. In view of the recent findings on the critical role of anti-inflammatory cytokines in diverse pathogens [20], this study focuses on the anti-inflammatory cytokines TGF-β and IL-10. We first demonstrated that TGF-β, rather

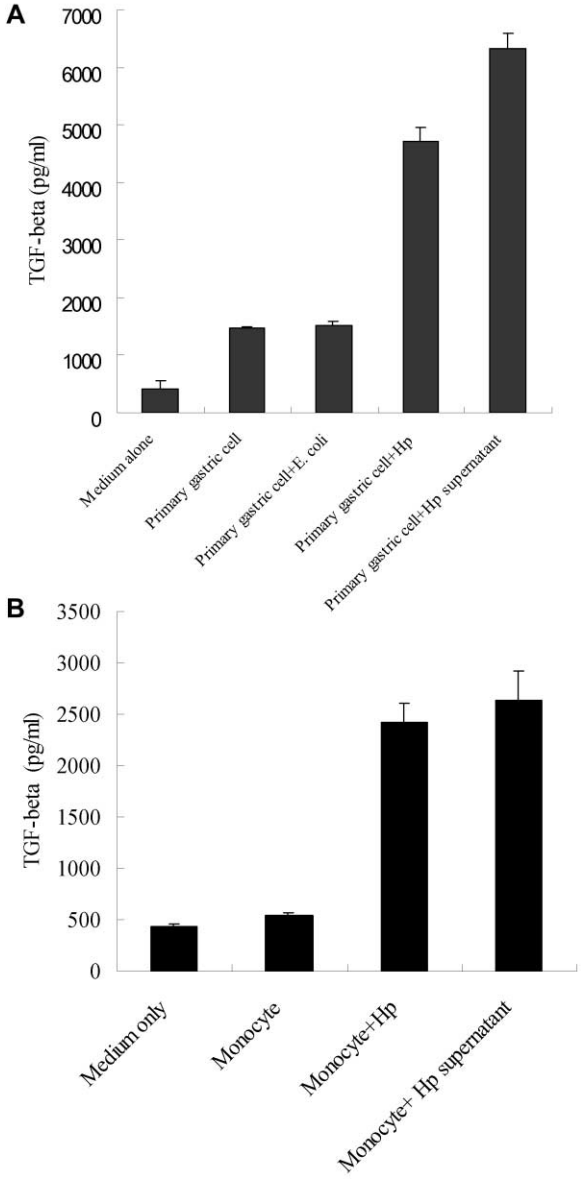

Figure 4. Induction of transforming growth factor (TGF)–β by *Helicobacter pylori* (Hp)–derived soluble proteins in exposed to *Escherichia coli* or *H. pylori* or their soluble proteins. *A,* Induction in primary gastric epithelial cells. *B,* Induction in monocytes. Results of 3 experiments are shown, expressed as mean values ± SEM.

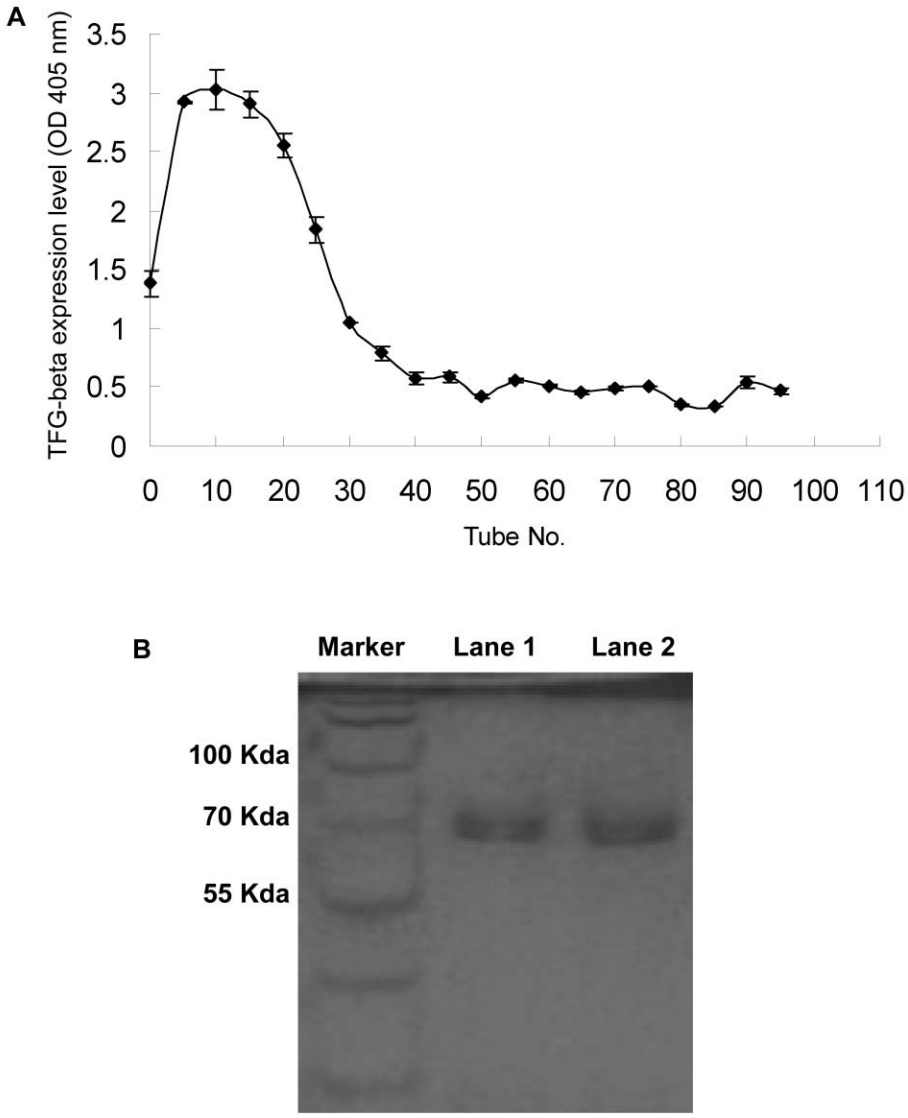

Figure 5. Partial characterization of factors inducting transforming growth factor (TGF)–β by gel filtration and SDS-PAGE. *A,* Gel filtration profile of *Helicobacter pylori* supernatants, showing the fractionation of TGF-β–inducing factors and their ability to induce TGF-β production. Results of 3 experiments are shown, expressed as mean values ± SEM. *B,* SDS-PAGE of gel filtration exclusion chromatography eluted fractions. The molecular weight marker position is shown at left. *Lane 1,* pooled fractions 1–5; *lane 2,* pooled fractions 6–10.

than IL-10, was elicited in vitro in gastric epithelial cells and monocytes by secreted soluble proteins of *H. pylori*. Our results indicate a new interaction between *H. pylori* and epithelial and PBMCs through the induction of TGF-β.

Several studies in which *H. pylori* and epithelial cell lines have been cultured together yielded contradictory findings: both increased IL-10 production and no change of IL-10 production have been found [23–26]. This discrepancy may be the result of different experimental conditions, such as differences in bacteria strains, cell lines, host-bacteria interaction, and methods of quantitation (eg, use of reverse-transcription PCR or ELISA). In a recent publication, Nakachi et al. [30] found that *H. pylori* infection did not increase the levels of TGF-β mRNA in human gastric epithelial cells. However, Takagi et al. [28] have demonstrated that TGF-β was produced by gastric cancer cells after exposure to *H. pylori*. In agreement with the latter report, our results show that *H. pylori* induced a rapid accumulation of TGF-β in various cell lines. It is important to note that *H. pylori* also stimulated TGF-β production in primary gastric epithelial cells and PBMCs. Because PBMCs comprise a major part of the cellular inflammatory response to *H. pylori* infection, our finding for PBMCs strengthens the importance of the TGF-β in vitro model. Further evidence for the involvement of TGF-β in *H. pylori* infection was found by

immunohistochemical staining of gastric biopsy specimens. Compared with cells from uninfected subjects, cells from *H. pylori*–infected patients have significantly higher TGF-β levels [35]. Moreover, Messa et al. [29] have demonstrated that TGF-β expression dropped remarkably after successful eradication of *H. pylori*. Confirming these observations, our data suggest that *H. pylori* infection can induce TGF-β production in epithelial cells and PBMCs.

The phenomenon of TGF-β induction by *H. pylori* is quite unique, and different from the findings for other commonly isolated enteric pathogens. Only *H. pylori* can influence the release of TGF-β by the gastric epithelial cells. Intriguingly, the target of the TGF-β–inducing protein exists in not only gastric epithelial cells but also other types of cell. It implies that the ability to induce the secretion of TGF-β is specific to *H. pylori*, but the receptor for the modulating factor may be expressed on different cells.

Previous studies have demonstrated that cytokine production is dependent on direct contact between epithelial cells and bacteria [23–28] and is influenced by bacterial strains with an intact *cag* pathogenicity island [36]. The results of our study suggest that the capability to induce TGF-β production can be regarded as a constitutional characteristic of *H. pylori*, because all strains from different disease status show this activity. There was no relation between the inducing activity and the *cagA* or *vacA* status of the strain.

The induction of TGF-β by *H. pylori* probably has biological significance, in view of the central role of TGF-β in immune regulation [37]. As a regulator of site-specific T cell inflammatory response [21], TGF-β is relevant in gastric pathology during *H. pylori* infection. By its immunosuppressive function, TGF-β may protect the gastric mucosa from severe damage caused by gastritis, in one way, and contribute to the persistence of *H. pylori* infection, in another. The role of TGF-β is also elusive in other infectious and inflammatory bowel diseases [38, 39]. Buzoni-Gatel et al. [37] have shown that the regulation of the ileal inflammatory process in *T. gondii* infection is dependent on TGF-β–producing intraepithelial lymphocytes. In colitis, TGF-β production is an essential mechanism of counter-regulation of Th1 cell-medicated mucosal inflammation [39]. In addition, perturbation of TGF-β signaling in animal models is linked to development of severe mucosal inflammation of gastrointestinal tract [40, 41]. In patients with *H. pylori*–related duodenal ulcer, TGF-β production in the metaplastic epithelium of the duodenum is decreased, compared with production in nonmetaplastic epithelium [31]. Pathogen-stimulated TGF-β production in innate cells might prevent infection-induced immunopathology or prolong pathogen persistence by suppressing protective Th1 responses [20].

In accordance with previous reports and our data, we propose a hypothetical explanation of the persistence of *H. pylori* infection in the face of strong immune responses. We speculate that *H. pylori*, through the induction of TGF-β, might construct an immune privileged area in the stomach that mimics the ocular microenvironment, where immune effector responses and inflammation are suppressed [22]. Then the organisms protect themselves in the acidic environment with urease. However, the adhesion would alert the host immune system and induce proinflammatory cytokines and chemokines to trigger the migration of immune cells to the gastric epithelium. To prevent destruction by these immune responses, *H. pylori* secretes TGF-β–inducing protein to induce TGF-β production, and the gastric epithelial cell-derived TGF-β diffuses in the gastric epithelium to protect *H. pylori* from the immediate immune attack at the early stage of infection. Moreover, the secreted TGF-β–inducing protein will interact with macrophages to trigger the second burst of TGF-β secretion, which causes more-extensive immunosuppression. The macrophages expressing TGF-β may interact with T cells to induce anergy. As the *H. pylori* bacteria invade further, they magnify the TGF-β–dependent immune-privileged area. Therefore, although the immune effectors will be attracted by chemokines, immune cells might lose their activity once they enter the TGF-β–inducing, protein-induced, immune-privileged area. The significance of TGF-β and TGF-β–inducing protein for the pathogenesis of *H. pylori*–related diseases remains speculative at present. Nevertheless, it is potent and worth investigating in future studies.

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