Blockage of HSP 90 modulates Helicobacter pylori-induced IL-8 productions through the inactivation of transcriptional factors of AP-1 and NF-κB

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Abstract

Helicobacter pylori infection leads to significant inflammations in the gastric mucosa, which is closely associated with development of gastric cancer. Heat shock protein 90 (HSP 90) has been revealed to be critical for intracellular signaling that participates in inflammatory response as well as carcinogenesis. In this study, we investigated a regulatory role of HSP 90 in H. pylori-induced IL-8 production. Our results showed that H. pylori stimulated significant phosphorylation of HSP 90 and the phosphorylation was diminished by administration of HSP 90 inhibitor, geldanamycin (GA). Treatment of GA completely inhibited H. pylori-induced IL-8 production due to deactivation of ERK1/2 and NF-κB. These results subsequently lead to inactivation of AP-1 and NF-κB, which are known to be major transcriptional factors of IL-8. Our data provide important insights that HSP 90 is involved as a crucial regulator in H. pylori-induced IL-8 production and its inhibitor could be potentially used for the inhibition of H. pylori-provoked inflammation.

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The concept that chronic inflammation plays a crucial role in the development and progression of several gastrointestinal cancers has been generally agreed [1–3]. Especially, cancers originated from stomach such as mucosa-associated lymphoid tissue lymphoma (MALT lymphoma) and gastric adenocarcinoma are closely associated with chronic, persistent inflammation provoked by Helicobacter pylori infection [4–6]. Though the obvious molecular evidence that inflammation by H. pylori is capable of inducing cancer and the procancerous microenvironment favorable for survival and growth of tumor cells needs to be accumulated more, the explainable factors fostering the neoplastic processes of H. pylori infection include the enhancement of cell proliferation and apoptosis inhibition, induction of neoplastic mutation by oxidative stress, and the production of cytokines and growth factors providing the environment for cell migration and angiogenesis [7–15].

Helicobacter pylori-associated inflammation is characterized by severe infiltration of neutrophils and mononuclear cells in the gastric mucosa. Accumulation and activation of these cells is related to the local production of cytokines. Recent studies have demonstrated that mucosa levels of IL-1β, IL-6, IL-8, and TNF-α were significantly higher in H. pylori positive patients [16,17]. Among the cytokines induced in the gastric mucosa colonized by H. pylori, IL-8 is one of the major proinflammatory cytokines, first isolated from monocytes as a neutrophil chemoattractant and an 8.5 kDa CXC
chemokine, which is also chemotactic for T lymphocytes, monocytes, and eosinophils [18,19]. IL-8 plays a crucial role in the initiation and maintenance of inflammatory response and recently has been identified to function as proangiogenic or carcinogenic factor based on the findings that gastric cancer cells in surgical specimens overexpressed IL-8 compared with corresponding normal mucosa [20] and the IL-8 mRNA level directly correlated with the vascularity of the tumor [21]. Furthermore, transfection of gastric carcinoma cells with IL-8 gene enhanced their tumorigenic and angiogenic potential in nude mice [22].

There are various motifs within the 5′-flanking region of human IL-8 promoter with the potential to bind a number of important transcriptional factors in a cell- and stimulus-dependent manner. Previous studies reported that the transcriptional factor NF-κB, AP-1, and NF-IL-6 were all involved in IL-8 production induced by H. pylori infection [23–25]. H. pylori stimulates NF-κB activation via phosphorylation and subsequent proteolytic degradation of the IκB, and the activated NF-κB translocates into nucleus where it regulates IL-8 gene transcription. Keates et al. [26,27] reported that MAPKs also play a critical role in IL-8 production via documenting that H. pylori activate MAPK signaling pathway and consequently lead to enhancing IL-8 promoter binding activity of activator protein-1 (AP-1).

The 90 kDa heat shock protein, HSP 90, is a major molecular chaperone of the cell that appears to have particular significance to cellular regulatory processes. Recent approaches have revealed that most of target proteins of HSP 90 are protein kinases or transcription factors which play important roles in cellular carcinogenesis [28,29]. The benzoquinone ansamycins, herbinycin A, and geldanamycin (GA), were described as specific inhibitors of HSP 90 by blocking the binding of ATP to HSP 90, which leads to destabilization of HSP 90 complexes with its client proteins rendering them available for proteosomal degradation. The ability of GA to deplete of multiple oncogenic client proteins such as Raf1, ErbB2, and mutant p53 was found to correlate with its anti-proliferative activity, making it a plausible candidate for use in cancer treatment [30–34]. Currently, several groups documented that HSP 90 plays a critical role in inflammatory response and treatment of its inhibitor resulted in a reduced immune response as indicated by a decrease of proinflammatory mediator production [35–37].

As a possible mechanistic link between H. pylori infection and perpetuated inflammation or carcinogenesis, we hypothesized that HSP 90 might be principally involved. Therefore, in the current study, in order to know the implication of HSP 90 in H. pylori infection, we checked the changes of HSP 90 and IL-8 after H. pylori infection and evaluated the effect of HSP 90 inhibitor, geldanamycin, on H. pylori-induced IL-8 production with its molecular mechanism.

Materials and methods

Cell line and bacterial strain. Human gastric epithelial AGS cells were cultured in RPMI1640 (Gibco-BRL, Grand Island, New York) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS in a humidified 5% CO₂ atmosphere. A cagA- and vacA-positive standard strain of Helicobacter pylori (ATCC43504) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). H. pylori were recovered from frozen stock by seeding on a blood agar plate composed of 37, 15 mg/ml, and 7% sheep blood at 37°C for 5days under microaerophilic conditions (5% O₂, 10% CO₂) generated with campy pouch (Becton–Dickinson Microbiology Systems, Sparks, MD). For inoculation of the bacteria, H. pylori were resuspended in PBS to an A₅₅₀ of 1.2U, which corresponds to a bacterial concentration of 5 × 10⁸ CFU/ml and co-cultured with AGS cells at the concentration of 5 × 10⁴ CFU/ml.

Antibodies and Western blotting. For the Western blot analysis, primary antibodies specific for HSP 90, phospho-ERK, phospho-JNK, phospho-c-jun, phospho-Erk, NF-κB p65, and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, California) and antibody corresponding to phospho-p38 was purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA). Total proteins were extracted from H. pylori-infected cells, electrophoresed on SDS-PAGE gels, and transferred to PVDF membranes using a semi-dry transfer system ( Hoeffer Pharmacia Biotech, San Francisco, CA). Non-specific binding sites were blocked by incubation with 5% nonfat dried milk in TBST (10 mM Tris-CI, pH 8.0, 150 mM NaCl, and 0.1% Tween 20 (v/v)) at room temperature for 1h. The membrane was incubated in a 1:1000 dilution of primary antibody in blocking solution overnight at 4°C, followed by probing with 1:2000 diluted HRP-conjugated secondary antibody. The immunocomplex was detected using ECL detection kit (Amersham–Pharmacia Biotec).

Two-dimensional electrophoresis. Protein separation by isoelectric point was performed with an isoelectric focusing system (IPGphor, Amersham Biosciences). Immobiline DryStrips (18 cm, nonlinear pH 3–10, Amersham Biosciences) were rehydrated for 16h at room temperature in a hydration buffer (8 M urea, 0.5% CHAPS, 10 mM DTT, and 302% BPB). The hydrated strip containing 500 μg protein sample was kept at 20°C on an electric field programmed as follows: 100 V for 2h; 200 V for 1h; 500 V for 1h; 1000 V for 1h; and 8000 V for 6.5h. After IEF, the IPG gel strip was incubated for 15min with 1% DTT in equilibration buffer (1.5 M Tris–HCl buffer, pH 8.8, containing 6 M urea, 22% glycerol, and 2% SDS) and equilibrated in the same buffer containing 2.5% iodoacetamide instead of DTT for 15 min. Separation of proteins was carried out with a horizontal SDS–PAGE system (20 × 20 cm, Hoeffer Pharmacia Biotech, San Francisco, CA). The 2D gel was used for Western blotting.

Immunofluorescence staining. Dispersed single cells (2 × 10⁶ cells/well) were grown in six-well cell culture plates. After 24h culture, cells were fixed in ice-cold methanol for 5min at a –20°C freezer and permeabilized with 1% Triton X-100/PBS for 10 min at room temperature. After washing with TBST, the cells were blocked with 5% BSA for 30min and probed with anti-NF-κB p65 antibodies (1:100 diluted in 5% BSA, Santa Cruz Biotechnology) for 2h, Cy3-conjugated or FITC-conjugated secondary antibodies were used to visualize under an inverted fluorescence microscope (Olympus, BX50F, Japan). Nuclei of the cell were stained with DAPI to address the subcellular localization.

RT-PCR for IL-8 mRNA. Total RNA was extracted by using TRIzol reagent (Life Technologies, Milan, Italy) and 2 μg of total RNA was reverse transcribed according to the manufacturer’s instructions of M-MLV Reverse transcriptase (Promega). The PCR was
performed by using the Premix Ex Taq kit (Takara, Japan) with specific primers as follows: 5'-GAA TGT CCG AAC TTT AAT TT-3' and 5'-TTT ATA ACT ATT CAA TCA AT-3' for IL-8, 5'-TTG TTG CCA TCA ATG ACC CC-3' and 5'-TGA CAA AGT GGT CGT TGA GG-3' for GAPDH. The PCR was carried out 28 thermal cycles of 94°C for 1 min, 38°C for 1 min, 38°C for 1 min 30 s. The product was resolved on 1% agarose gel and stained with ethidium bromide.

Electrophoretic mobility shift assay. The nuclear fractions for electrophoretic mobility shift assay (EMSA) were prepared using NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL) following the manufacturer's protocol. Sequences of double stranded oligonucleotides used for EMSA were as follows: 5'-AGT TGA GGG GAC TTT CCC AGG C-3' for NF-κB, and 5'-CGC TTG ATG AGT CAG CCC GAA-3' for AP-1, and the oligonucleotides were labeled with Biotin 3'-End DNA Labeling Kit (Pierce, Rockford, IL). EMSA reaction was performed with LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) according to the manufacturers' instructions.

Enzyme-linked immunosorbent assay. Immuno reactive human IL-8 was measured in culture supernatant of AGS cells by using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (HyCult Biotechnology, Netherlands). AGS cells were grown in six-well cell culture dish, incubated in a presence or absence of GA overnight, and then co-cultured with H. pylori for various times indicated. Two hundred microliters of the culture supernatant was used for analysis of IL-8 production.

Results

H. pylori stimulated phosphorylation of HSP 90 in human gastric epithelial AGS cells

We evaluated the effect of H. pylori infection on expression profile of heat shock proteins, and the results showed that H. pylori significantly reduced expression of HSP 70 and stimulated phosphorylation of HSP 90 in gastric mucosa cells (Fig. 1A). Thus, we further studied the biological significance of H. pylori-induced HSP 90 phosphorylation and potency of geldanamycin, a specific HSP 90 inhibitor, able to inhibit phosphorylation of the chaperone and to block H. pylori-induced inflammatory signaling. Fig. 1A using 2DE-Western blot analysis showed that treatment of H. pylori led to significant increase of an acidic HSP 90 isoform, well documented in previous report that the acidic isoform was provoked by protein phosphorylation [38]. As opposed to significant phosphorylation of HSP 90, the signals corresponding to HSP 70, HSP 60 did not show any change of shift profiles by H. pylori infection (Fig. 1A). Interestingly, the phosphorylation of HSP 90 provoked H. pylori significantly diminished by GA treatment (Fig. 1B). These findings suggest that H. pylori induced phosphorylation of HSP 90 and HSP 90 inhibitor diminished the phosphorylation of HSP 90 induced by H. pylori infection, implying the possibility that the phosphorylation of HSP 90 might have contributed to pathophysiological consequence caused by H. pylori and its inhibitor could improve the pathological results, even though the biological significance of phosphorylation of HSP 90 is still unclear.

HSP 90 inhibitor, GA, dramatically inhibited IL-8 production induced by H. pylori

Helicobacter pylori treatment in human gastric epithelial cells dramatically induced IL-8 production in a time-dependent manner (Fig. 2A). The maximal induction of IL-8 appeared at 8 h in AGS cells after H. pylori treatment, and IL-8 production in MKN-45 cells showed gradual increase up to 24 h on this experiment. To analyze whether GA could prevent the H. pylori-induced IL-8 production, human gastric epithelial AGS cells were pretreated with GA overnight prior to H. pylori inoculation. The significant induction of the cytokine was completely inhibited by pretreatment of GA (Fig. 2B). Even administration of 1 μM GA inhibited IL-8 production comparable with that of non-treated control cells. Decreased productions of IL-8 after GA treatment were due to the attenuated expressions of IL-8 mRNA (Fig. 2C). The finding suggested that HSP 90 might be involved in IL-8 signaling induced by H. pylori and GA treatment could suppress the IL-8 production, indicating that attenuation of IL-8 production by GA pretreatment might contribute to prevent IL-8-induced inflammatory response.

Geldanamycin completely suppressed H. pylori-activated MAPK signaling pathway

It has been reported that H. pylori activated several cellular signaling pathways able to lead to transcription
of genes which were involved in inflammation response, apoptosis, or proliferation of gastric epithelial cells [7–15]. One of the well-documented enzymes that participated in these signal transduction is MAPK that plays an important role in *H. pylori*-induced IL-8 production. Because GA appeared to suppress *H. pylori*-induced IL-8 expression in Fig. 2, we analyzed whether GA participates in inhibition of *H. pylori*-activated MAPK pathways. *H. pylori* maximally triggered ERK activation at 15 min following *H. pylori* infection, gradually decreased time dependently (Fig. 3A). While Elk, one of the transcriptional factors regulated by ERK, did not show an enhanced phosphorylation by *H. pylori* in spite of ERK activation, the phosphorylation of c-Jun was induced from 15 min and kept up to 2 h after *H. pylori* treatment (Fig. 3A).

GA treatment on *H. pylori*-infected epithelial cells suppressed the phosphorylation of ERK and c-Jun induced by *H. pylori* (Fig. 3B). In additionally, *H. pylori* also diminished the activation of p38 and JNK (data not shown). On the contrary, Elk slightly increased phosphorylation level by GA in *H. pylori*-infected cells. Taking together, *H. pylori* induced MAPK signaling and pretreatment of GA completely suppressed the MAPK signaling pathway.

**Helicobacter pylori stimulated translocation of NF-κB p65 subunit to nucleus and treatment of GA inhibited the translocation of NF-κB**

Since NF-κB is the major transcriptional factor regulating IL-8 transcription, we documented the effect of GA on NF-κB activation. NF-κB was activated via phosphorylation and subsequent proteolytic degradation of the IκB, and the active complex of NF-κB p65 and p50 translocates to nucleus, resulting in up-regulation of target gene transcription in the nucleus. So at first, we detected the activation of NF-κB by using Western blot of NF-κB p65 subunit in nucleic fraction compared total NF-κB p65 (Figs. 4A and B) and immunofluorescence stain to view the subcellular localization (Fig. 4C). The results showed that *H. pylori* stimulated translocation of NF-κB p65 subunit into nucleus and the maximal translocation of the p65 subunit was detected at 1–2 h after *H. pylori* treatment in spite of equal amount of total NF-κB p65 (Fig. 4A).

The translocation of the protein was completely blocked by GA pretreatment for 16 h (Fig. 4C). Concomitant with the immunoblot analysis, NF-κB p65 subunit abundantly expressed in the cytoplasmic portion of non-treated control cells contrary to the nuclei...
staining of DAPI and H. pylori treatment significantly stimulated translocation of NF-κB p65 subunit to nucleus. The translocation of the protein was inhibited in GA-treated gastric epithelial cells.

Geldanamycin attenuated H. pylori-induced transcriptional activation of AP-1 and NF-κB as major transcriptional factors of IL-8

Fig. 2 demonstrated that GA completely suppressed H. pylori-induced IL-8 production, and Figs. 3 and 4 showed that the GA treatment efficiently inhibited MAPK ERK42/44 signaling and NF-κB translocation into nucleus. Since MAPK ERK42/44 and NF-κB are known as critical regulators responsible for IL-8 transcription, we tested the direct influence of GA on H. pylori-induced transcriptional activity of AP-1 and NF-κB as major transcriptional factors of IL-8 using by EMSA (Fig. 5). H. pylori enhanced DNA binding activity of NF-κB and AP-1 at 1–2 h following H. pylori treatment. However, pretreatment of GA decreased the retardation of gel mobility through the inhibition of DNA binding activities of NF-κB and AP-1 complex, indicating repression of transcriptional activity of these transcriptional factors. Especially, GA treatment reduced NF-κB DNA binding activity up to 71% compared to control of 1 h treatment of H. pylori (Fig. 5B). Though stimulation of H. pylori for 1 h did not change the AP-1 binding activity by GA treatment, the transcriptional factor binding activity after 2 h treatment of H. pylori decreased significantly from 300% to 242% (Fig. 5C). Thus, the attenuation of IL-8 production by GA may be due to reduction of H. pylori-induced transcriptional activities of AP-1 and NF-κB.

Discussion

The overall results of our experiment dealing with the inhibitory effect of GA on H. pylori-induced IL-8 sig-
naling are schematically summarized in Fig. 6. After *H. pylori* infection, nuclear translocation of NF-κB p65 subunit followed by increased IL-8 production levels, consistent with increased NF-κB-DNA binding for IL-8 gene transcription, was observed. In addition to regulating NF-κB, *H. pylori* infection triggered MAPK activation, which can activate the other transcription factor such as AP-1, which regulates IL-8 gene transcription. However, in this study, we, for the first time, documented the effect of HSP 90 inhibitor, geldanamycin, on *H. pylori*-induced IL-8 production and provided its molecular mechanism. GA treatment completely inhibited IL-8 production in *H. pylori*-infected gastric epithelial cells. The finding may be due to inactivation of MAPK signaling induced by *H. pylori* infection and decrease of NF-κB translocation into the nucleus, resulting in inactivation of transcriptional factors AP-1 and NF-κB, major transcriptional factors responsible for regulation of IL-8 transcription. Our data provide important insights that HSP 90 involved in *H. pylori*-induced IL-8 production and its inhibitor could have the potential for inhibition of *H. pylori*-provoked inflammation. Thus, this evidence has helped to shed light on anti-inflammatory treatment as well as promising protective therapeutic approach for cancer prevention.

The increasing evidence that *H. pylori* infection is associated with development of gastric cancers raised the need to confirm the basic mechanisms of *H. pylori*-associated carcinogenesis at the molecular level. As one of them, when *H. pylori* are co-cultured with gastric epithelial cells, IL-8 is one of the principal mediators of the inflammatory response to *H. pylori*, and recently has been identified as pro-oncogenic roles able to stimulate mitogenic activity, cell adhesion, metalloprotease activity, and angiogenesis. The overexpression of IL-8 mRNA or protein in the breast cancer was already reported to enhance bone metastasis, presumably through increased adhesion and invasion ability [39]. Human neutralizing antibodies of IL-8 inhibited metalloprotease-2 activity, invasion as well as angiogenesis [40] and tumor necrosis factor-α-induced IL-8 secretion increased in a colon cancer [41]. All these results indicated that IL-8 has significant biological effects on the angiogenesis, carcinogenesis, and progression or metastasis of tumor. In the view of this knowledge, applications or agents that can inhibit the production of IL-8 are thought to be of therapeutic value in the treatment of *H. pylori*-induced gastric inflammation or carcinogenesis.

In this study, we presented new evidence on the molecular mechanisms underlying the inhibitory effect of...
HSP 90 inhibitor on IL-8 production of *H. pylori*-infected gastric epithelial cells. We also documented that HSP 90 inhibitor, GA, effectively down-regulated *H. pylori*-induced ERK activation and inhibited translocation of NF-κB, p65 into nucleus, subsequently resulting in reduction of IL-8 production. Our findings provide important insights into the role of HSP 90 in response to *H. pylori* infection and highlight the preventive effects of its inhibitor on *H. pylori*-associated inflammation. Thus, this evidence has helped to shed light on anti-inflammatory treatment as potential protective therapeutic approach for cancer prevention.

Recently evidence has been accumulated that HSP 90 inhibitor, GA, effectively down-regulated *H. pylori*-induced ERK activation and inhibited translocation of NF-κB, p65 into nucleus, subsequently resulting in reduction of IL-8 production. Our findings provide important insights into the role of HSP 90 in response to *H. pylori* infection and highlight the preventive effects of its inhibitor on *H. pylori*-associated inflammation. Thus, this evidence has helped to shed light on anti-inflammatory treatment as potential protective therapeutic approach for cancer prevention.

Conclusively, we can find that one of the significant chemokines related to the critical pathogenesis of *H. pylori*-associated gastric diseases, IL-8, could be significantly attenuated with the blockade of HSP 90 phosphorylation and HSP 90 might be the promising target for the prevention of *H. pylori*-induced either gastric inflammation or carcinogenesis.

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References


