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Review

Tumor promotion by TNF-α-inducing protein and its receptor nucleolin in human gastric carcinogenesis by *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori is classified as the definitive carcinogen for stomach cancer in humans: It induces inflammation in cancer microenvironment of the stomach associated with induction of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), an endogenous tumor promoter. The finding of tumor promoting activity by *Helicobacter pylori* membrane protein 1 (HP-MP1) made it possible to identify *HP0596* gene from the complete genome sequence of *H. pylori* strain 26695. The HP0596 protein, consisting of 172 amino acids with 19 kDa, was named TNF-α-inducing protein (Tipα), a new carcinogenic factor of *H. pylori.* To study the mechanism of Tipα, we prepared His-tagged recombinant Tipα protein (rTipα), the His-tag attached to N-terminal methionine, and deletion mutant of Tipα (rdel-Tipα) lacking the N-terminal 6 amino acids including 2 cysteines. The rTipα protein formed an active homodimer, and rdel-Tipα was an inactive monomer. Crystal structure of rdel-Tipα, however, showed a heart-shaped homodimer without covalent bonds, indicating the importance of N-terminal region. Nucleolin was identified as a receptor of Tipα using the pull-down assay with anti-FLAG antibody in a mixture of mouse gastric cancer cell lysates and rTipα-FLAG. Specifically, the homodimer of rTipα directly binds to two-thirds of C-terminal nucleolin on cell surface. Binding inhibitors of nucleolin, such as AS1411 and lactoferrin, are discussed as tools of gastric cancer treatment in humans.

KEYWORDS: TNF-α, HP-MP1, Tipα, del-Tipα, crystal structure, nucleolin, AS1411, lactoferrin, vaccine

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that does not invade gastric tissue but remains in the gastric lumen. Since the discovery of *H. pylori*, research on gastric cancer etiology has rapidly developed [1]. Initially, Bizzozero reported “spiral bacteria” colonized in the human stomach in 1905 [2]. In 1984, Marshall and Warren published their findings on the association of *H. pylori* infection with chronic gastritis and peptic ulcer [3]. In 1994, the Working group of the International Agency for Research on Cancer (IARC, WHO) classified *H. pylori* as the definitive carcinogen for humans (Class 1), based on epidemiological studies [4]. Marshall and Warren were awarded the 2005 Nobel Prize in Physiology or Medicine, for their findings on *H. pylori* and its role in gastritis and peptic ulcer. 9% of worldwide cancer mortality caused by gastric cancer is associated with long-term infection by *H. pylori* [5]. Thus it is now well accepted that *H. pylori* infection is the strongest recognized risk factor associated with chronic inflammation for gastric adenocarcinoma [1]. Virulence factors of *H. pylori*, such as *cytotoxin-associated* gene A (cagA), *cytotoxin-associated* gene Pathogenicity Island (cag PAI), vacuolating cytotoxin A (vac A), and urease have been studied, and the strongest association of *cag PAI* gene with the occurrence

of peptic ulcers and cancer has been reported [6-13]. There are reports that Japan and Korea have the highest incidence rates of gastric cancer in the world [14-16], and that the high frequency of cag PAI^{+} *H. pylori* is nearly 100% in clinical isolates in Japan and Korea [17, 18]. The evidence strongly suggests that the presence of such virulence factor genes is closely linked to stomach cancer rates. However, Correa and Piazuelo have indicated that the knowledge on this subject is incomplete: Other genes may be involved in other populations [19].

Two-stage chemical carcinogenesis experiments with initiators and tumor promoters have allowed us to investigate a new carcinogenic factor of *H. pylori*: *H. pylori* is not directly mutagenic, but may become mutagenic through some inflammatory mediator or impairment of the mismatch repair pathway [20-22], for example, upregulation of cytidine deaminase altered the *TP53 tumor suppressor* gene in gastric epithelial cells [23]. As for tumor promoter in *H. pylori* carcinogenesis, we refer to our study on chemical tumor promoters and endogenous tumor promoters, as follows: When the classic tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is applied on mouse skin initiated with 7,12-dimethylbenz[a] anthracene (DMBA), TPA activates protein kinase C (PKC) and induces tumors [24, 25]. Another chemical tumor promoter, okadaic acid, which is a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A) - and does not activate PKC - induces tumors on mouse skin as potently as TPA [26, 27]. Both TPA and okadaic acid induce clonal growth of initiated cells containing a mutation at the second nucleotide of codon 61 in the c-Harvey-*ras* gene [28, 29]. Although the biochemical mechanisms of TPA and okadaic acid are discrete in the cells, it is of great importance to note that topical applications of TPA and okadaic acid both induced the expression of *tumor necrosis factor-α* (*TNF-α*) gene on mouse skin [30] (Fig. 1), and that human TNF-α significantly stimulated transformation of BALB/3T3 cells initiated with 3-methylcholanthrene (MCA) [31]. Since TNF-α is a pleiotropic and proinflammatory cytokine, released mainly from activated macrophages [32, 33], we named TNF-α as an endogenous tumor promoter [34]. Furthermore, we next identified a new gene product: TNF-α-inducing protein (Tipα), deduced from *H. pylori* genome, which acts as a carcinogenic factor inducing endogenous tumor promoters, such as TNF-α, IL-1, IL-6, IL-8 and chemokines in the stomach [21, 35-44].

In this paper, we first review our study on tumor promotion on mouse skin, rat glandular stomach and rat liver because it engendered a new concept

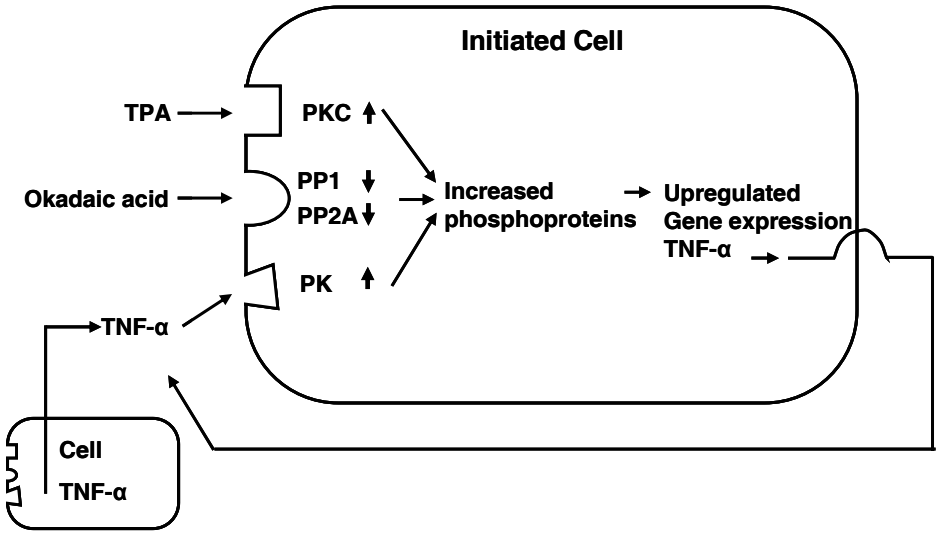

Fig. 1. Schematic illustration of tumor promotion on mouse skin by TPA, okadaic acid, and TNF-α in initiated cell and cancer microenvironment.

of endogenous tumor promoters, such as TNF-α [27, 34, 44, 45]. Next, we deal with a new *H. pylori* gene encoding the TNF-α-inducing protein (Tipα) that induces proinflammatory cytokines, i.e., endogenous tumor promoters such as TNF-α, in the stomach [34, 36, 37, 39, 43]. The study on the interaction of both active recombinant Tipα (rTipα) and the inactive deleted form of rTipα (rdel-Tipα) with mouse gastric cancer cells MGT-40 revealed the specific binding of rTipα to the cells, its internalization into the cells, and secretion of Tipα from *H. pylori* clinical isolates [39]. The crystal structure of rdel-Tipα shows that rdel-Tipα has a novel elongated structure and forms a heart-shaped homodimer through non-covalent bonds [46]. Nucleolin on cell surface is identified as a Tipα receptor, and the significant role of nucleolin is studied with gastric cancer cell lines [47]. The binding inhibitors of nucleolin can be used in cancer treatment. This review discusses tumor promotion of *H. pylori* carcinogenesis by the carcinogenic factor Tipα and its receptor nucleolin, to demonstrate the significant role of endogenous tumor promoters in human cancer development.

TNF-α as an endogenous tumor promoter in rodent carcinogenesis

The study of tumor promotion by TPA provides significant evidence on signal transduction. In addition to TPA, teleocidin and aplysiatoxin - which are structurally different from TPA but also activate PKC - are found to be tumor promoters as potent as TPA on mouse skin [48, 49]. Since tumor promotion by TPA was found only on mouse skin, the TPA pathway by activation of PKC still has classical limits in tumor promotion, such as organ and tissue specificities [45]. However, potent inhibitors of PP1 and PP2A - such as okadaic acid, dinophysistoxin-1, calyculin A, microcystin-LR and nodularin - can overcome this limitation of tumor promotion for *in vivo* animal experiments [45, 49]. Specifically, okadaic acid, dinophysistoxin-1 and calyculin A are now known to be tumor promoters on mouse skin that are as potent as TPA [26, 50, 51]. Okadaic acid in drinking water induced tumor promotion in rat glandular stomach initiated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) [52], and microcystin-LR and nodularin were confirmed as tumor promoters in rat liver initiated with diethylnitrosamine [53, 54]. Considering these findings, we can now present new evidence showing that inhibition of PP1 and PP2A induces tumor promotion in various organs. We next moved on to a question about the presence of endogenous tumor promoters active in various organs.

TNF-α was originally identified as a serum factor to induce hemorrhagic necrosis of transplanted solid tumors in mice [32, 33]. If inflammatory reaction is induced by upregulation of *TNF-α* gene expression, mediated through activation of NF-κB at the molecular level, then TNF-α is an endogenous tumor promoter [34]. Our further study revealed a fascinating link between chemical tumor promoters and TNF-α, based on our results showing that TNF-α induces transformation of BALB/3T3 cells initiated with MCA [31], and that TNF-α-deficient (TNF-$\alpha^{-/-}$) mice are refractory to tumor promotion by both TPA and okadaic acid on mouse skin initiated with DMBA [55, 56]. This shows that TNF-α is the essential cytokine in tumor promotion [34, 45, 57] (Fig. 1), and IL-1 and IL-6 have also been proposed as additional endogenous tumor promoters [58]. Among various inflammatory cytokines, we found that the sequence of cytokine network for tumor promotion appears to be from TNF-α through IL-1 and IL-6 [59], indicating that TNF-α is an instigator of cytokines in tumor promotion [59]. It is well known that *H. pylori* infection induces inflammation in cancer microenvironment of the stomach associated with induction of TNF-α, IL-1, IL-6 and IL-8, and that the mucosal levels of these cytokines are significantly higher in *H. pylori* positive patients than in negative patients [60, 61]. Tatematsu and his associates first conducted two-stage carcinogenesis experiments in the glandular stomach of Mongolian gerbis initiated with MNNG or with *N*-methyl-*N*-nitrosourea, followed by inoculation with *H. pylori.* They reported that *H. pylori* infection significantly enhances glandular stomach carcinogenesis, which is a typical model of human gastric cancer [62-64]. This allows us to raise two important questions: What is the new carcinogenic factor gene in *H. pylori* genome and what kinds of endogenous

tumor promoters are subsequently induced by this new carcinogenic factor of *H. pylori* in human stomach.

Helicobacter pylori membrane protein 1 as a new carcinogenic factor

Helicobacter pylori membrane protein 1 (HP-MP1) has a molecular weight of 16 kDa; it is structurally unrelated to virulence factors of *H. pylori*, including cagA, vac A, and urease. The *HP-MP1* gene was cloned from genomic DNA of *H. pylori* strain SR 7791 [65]. Wakatsuki and his associates reported that HP-MP1 protein attaches to the inner membrane of *H. pylori* as a homodimer and then induces release of various inflammatory cytokines, such as TNF-α, IL-1α, IL-8, and macrophage inflammatory protein 1α from human monocytes. In addition, anti-HP-MP1 antibody has been in the sera of *H. pylori*-infected patients [65]. Although the function of HP-MP1 in the bacteria has not been clearly elucidated, we focused on the release of cytokines, such as TNF-α, from the cells treated with HP-MP1.

To study the function of HP-MP1 protein, the *HP-MP1* gene was cloned from *H. pylori* SR 7791, and *urease B* gene as a control was cloned from *H. pylori* NCTC 11637. They were separately inserted into the mammalian expression vector pcDNA3.1/hygro (+), resulting in the expression plasmids pcDNA-mp1 and pcDNA-ure. Two plasmids and pcDNA3.1/hygro (+) vector were all transfected into both Bhas 42 cells (v-Ha-*ras* transfected BALB/3T3 cells) [66], as putative initiated cells, and BALB/3T3 cells, as a control. Their clones were named as follows: Bhas/mp1, Bhas/ure and Bhas/vec of Bhas clones, and BALB/mp1, BALB/ure and BALB/vec of BALB clones. All of the five Bhas/mp1 clones significantly induced *TNF-α* gene expression, with levels ranging from 12.2 - 27.0 fold higher than basal levels of the parental Bhas 42 cells; only two out of five Bhas/ure clones expressed *TNF-α* gene 15.1 - 15.5 fold higher than basal levels; the expression levels of all of the five Bhas/vec clones were at basal levels. In contrast to those, *TNF-α* gene expression level of BALB/mp1 clones was relatively weak, and those of BALB/ure and BALB/vec clones were marginal [36]. Moreover, Bhas/mp1 clones significantly produced TNF-α protein with concentrations of 12.2 - 32.1 pg/mg protein, whereas TNF-α in cell lysates of Bhas/ure and Bhas/vec clones was under detectable levels. All the results clearly demonstrated that transfection of *HP-MP1* gene into Bhas 42 cells strongly induces *TNF-α g*ene expression and TNF-α production in cooperation with v-Ha-*ras* gene [36].

Five Bhas/mp1 clones induced morphological changes of transformation, such as spindle shape and multilayer/criss-cross formation, whereas Bhas/ure and Bhas/vec clones did not induce any morphological changes. Moreover the five Bhas/mp1 clones produced large colonies in soft agar with average colony number of 20.1 ± 10.1, whereas those of Bhas/ure, Bhas/vec clones and the parental Bhas 42 cells were 4.1 ± 6.1, 2.3 ± 1.5, and 2.0, respectively (Table 1). These results clearly showed that HP-MP1 protein transforms BALB/3T3 cells only in cooperation with viral Ras protein. Next, three Bhas/mp1 clones were implanted s.c. into six sites of three nude mice, two sites/mouse, to check for tumorigenicity: All three Bhas/mp1 clones produced tumors 100% (18 of 18 injected sites) within 20 days of implantation, and tumors of Bhas/mp1 clones grew rapidly. In contrast, three Bhas/ure clones produced tumors only in 33.3% of sites, and Bhas/vec clones and Bhas 42 cells did not induce any tumors (Table 1). The clones that had induced *TNF-α* gene expression were tumorigenic in nude mice within 28 days of implantation [36]. The potent tumorigenicity of three Bhas/mp1 clones in nude mice correlates well with the potency of soft agar colony formation.

The study on HP-MP1 for the first time presented the significant features of the new carcinogenic factor HP-MP1, as follows: 1) HP-MP1 protein induces the production of TNF-α as an endogenous tumor promoter in Bhas 42 cells, and consequently transforms the cells. 2) The v-Ha-Ras protein in Bhas/mp1 clones plays an important role of c-Ha-Ras p21 protein, representing initiation in the gastric cancer of humans. The results are well supported by previous reports that the levels of c-Ha-Ras p21 protein in extracts of human gastric adenocarcinomas are higher than those from extracts of their normal counterparts, and also higher than those of c-Ki-Ras and c-Na-Ras [67];

Table 1. Carcinogenicity of Bhas/mp1 and other clones.

Clones and cells	Average No. of soft agar colonies	Tumorigenicity No. of sites with tumors/ No. of injected sites
Bhas/mp1	20.1 ± 10.1	18/18 (100%)
Bhas/ure	4.1 ± 6.1	6/18 (33.3%)
Bhas/vec	2.3 ± 1.5	0/18 (0%)
Bhas 42	2.0	0/6 (0%)
BALB/mp1	0.5 ± 0.9	ND[a]
BALB/ure	0.3 ± 0.4	ND
BALB/vec	0.2 ± 0.4	ND
BALB/3T3	0	ND

[a]ND, not determined.

and the enhanced expression of c-Ha-*ras p21* was also confirmed during *H. pylori*-mediated gastric carcinogenesis in humans at mRNA and protein levels [68]. The results suggest that *H. pylori* infection increases the production of c-Ha-Ras p21 protein in the early stage of gastric carcinogenesis.

TNF-α-inducing protein (*Tipα*) gene in *H. pylori* geneome

In light of our significant evidence that HP-MP1 protein shows carcinogenic activity [36], we tried to generalize our concept, by looking at other genes similar to *HP-MP1* in various *H. pylori* strains. We first found *HP0596* gene from *H. pylori* strain 26695 *in silico* from the complete genome sequence [69]: *HP0596* gene (Accession number AE000573) was 94.3% homologous to *HP-MP1* gene [37]. The deduced amino acid sequence of the HP0596 protein was revealed to be a protein of 172 amino acids with 19 kDa, based on the database. HP0596 protein was found in all tested extracts of various *H. pylori* strains, using Western blot analysis with antibody against an oligopeptide (18 amino acids, 11 - 28 of N-terminal domain plus cysteine). HP0596 protein with 38 kDa protein was more common than 19 kDa protein in the absence of dithiothreitol (DTT), but only 19 kDa protein was found in the presence of DTT. The extracts of three other strains, cag pathogenicity island deletion mutant of 26695 (26695ΔPAI), ATCC43504 and SS1 - like strain 26695 - all contained both 38 kDa and 19 kDa proteins in the absence of DTT. Moreover, the extracts of four *H. pylori* clinical isolates obtained from patients with ailments such as gastritis, gastric ulcer, duodenal ulcer, and gastric cancer showed 38 kDa protein dominant without DTT, and only 19 kDa protein with DTT [37]. These results indicate that all *H. pylori* strains synthesize HP0596 protein with 19 kDa and then form a homodimer. In the results, we found that HP0596 protein is almost identical to HP-MP1 of strain SR 7791. Recently the investigation of *HP0596* gene knockout revealed a significant decrease in the levels of *H. pylori* colonization in mice, as measured by real-time PCR [70]. Considering the evidence, we designated HP0596 protein as the TNF-α-inducing protein (Tipα), since it strongly induces both *TNF-α* gene expression and *in vitro* transforming activity. We thus conclude that the *Tipa gene* family includes *HP-MP1* gene [36], *Tipα* gene [37], and *jhp0543* gene of strain J99 [71].

To try to find the molecular mechanism of Tipα protein, we made His-tagged recombinant Tipα protein (rTipα), consisting of His-tag and 172 amino acids; we used the deleted Tipα protein (rdel-Tipα) as a control, with deletion of 6 amino acids (2 - 7 containing two cysteine residues at C5 and C7) (Fig. 2). The molecular weight of rTipα was 42 kDa without DTT and 21 kDa with DTT; that of rdel-Tipα was 20 kDa with or without DTT

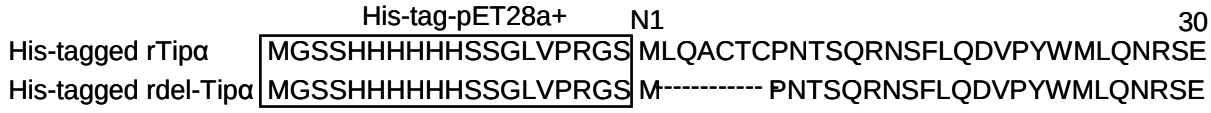

Fig. 2. Partial sequence of His-tagged rTipα and rdel-Tipα. The rdel-Tipα has deletion of 6 amino acids (2 - 7 containing 2 cysteine residues at C5 and C7).

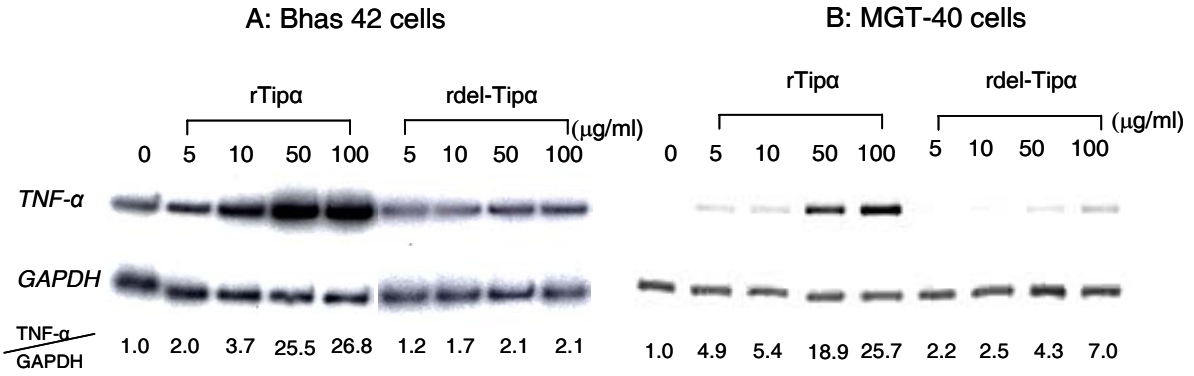

Fig. 3. Induction of *TNF-α* gene expression in Bhas 42 (A) and MGT-40 cells (B) treated with rTipα and with rdel-Tipα.

on Novex-gel electrophoresis [37]. This suggests that Tipα protein forms a homodimer by disulfide bonds between two cysteine residues in N-terminal domain.

TNF-α gene expression with both rTipα and rdel-Tipα was determined in two cell lines, Bhas 42 cells and mouse gastric cancer cell line MGT-40 [72]. Treatments of Bhas 42 cells with rTipα at a concentration of 50 μg/ml induced *TNF-α* gene expression about 26-fold over the basal levels, whereas that with rdel-Tipα even at concentrations up to 100 μg/ml did not significantly induce it in the cells (Fig. 3). The results were similar to those with MGT-40 cells, indicating that rTipα induces the endogenous tumor promoter TNF-α in mouse gastric cancer cells (Fig. 3). Moreover, treatment of Bhas 42 cells with rTipα at a concentration of 50 μg/ml (2.6 μM) strongly induced cell transformation 18.0 foci/well, and TPA (1 μg/ml, 1.6 μM) as a positive control induced 38.0 foci/well. However, that with rdel-Tipα at concentrations up to 100 μg/ml did not induce any transformed foci. In addition, rTipα did not induce transformation of BALB/3T3 cells unless v-Ha-*ras* gene was present. The results are significant evidence that rTipα requires the cooperation of v-Ha-*ras* gene for induction of the endogenous tumor promoter TNF-α as well as tumor promotion [37], and that MGT-40 cells may already have a similar genetic change, e.g., activated c-Ha-*ras* gene, in mouse gastric epithelial cell line [72].

To elucidate the mechanism of *TNF-α* gene expression with rTipα, the activation of NF-κB was examined using Trans^{AM} NF-κB Transcription Factor Assay kit, with DNA binding activity of p65 subunit in whole cell extracts of both Bhas 42 and MGT-40 cells treated with rTipα and with rdel-Tipα. The rTipα dose-dependently increased DNA binding activity of NF-κB, i.e. activation of NF-κB, in both Bhas 42 and MGT-40 cells, whereas rdel-Tipα did not show any significant DNA binding activity of NF-κB in either group of cells (Fig. 4). Following degradation of IκB, NF-κB p65 was clearly translocated into nuclei in MGT-40 cells [37], and then it produced TNF-α in the cells. It is important to note that rTipα is a new carcinogenic factor of *H. pylori* that induces an endogenous tumor promoter, resulting in tumor promotion.

Interaction of rTipα with MGT-40 cells

We previously reported unique features of Tipα, as follows: 1) no similarity to other virulence

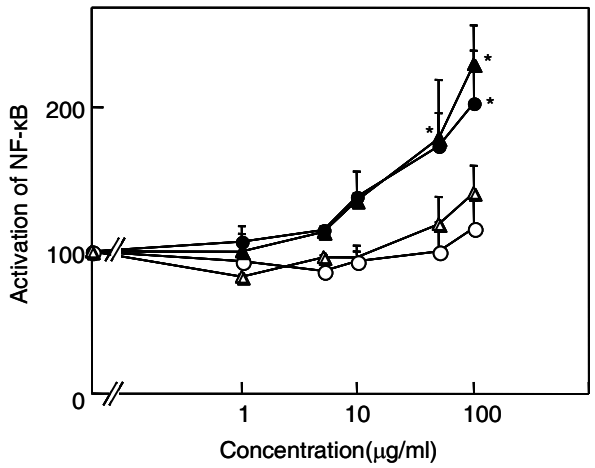

Fig. 4. Activation of NF-κB in Bhas 42 and MGT-40 cells treated with rTipα and with rdel-Tipα. The rTipα dose-dependently increased DNA binding activity of NF-κB in Bhas 42 (●) and MGT-40 cells (▲), whereas rdel-Tipα did not significantly induce it (○ and Δ).

factors of *H. pylori*, 2) secretion from *H. pylori* in a manner independent of Type IV secretion system of cagA, 3) induction of NF-κB activation in cag PAI-independent manner. To understand the mechanism of tumor promotion in gastric cancer cells, we further investigated how rTipα induces tumor promotion in MGT-40 cells. This section includes the specific binding of fluorescein isothiocyanate (FITC)-labeled rTipα to MGT-40 cells, incorporation of rTipα into the cells, localization of rTipα in nucleus, and secretion of large amounts of Tipα from *H. pylori* clinical isolates obtained from gastric cancer patients [39].

Incubation of MGT-40 cells with FITC-rTipα at 4°C for 2h dose-dependently increased the amount of fluorescence in the cells, and the binding of FITC-rTipα to MGT-40 cells was saturated at concentrations of 5.0 - 7.5 μM. Specific binding of FITC-rTipα to MGT-40 cells was inhibited by nonlabeled rTipα (IC_{50} 1.9 μM), and less weakly inhibited by rdel-Tipα (IC_{50} 20.0 μM) [39]. These results clearly indicate that a dimer formation of rTipα with disulfide bonds is required for specific binding to MGT-40 cells, induction of *TNF-α* gene expression [37, 39] and *chemokine* gene expression in MGT-40 cells [38]. Specifically, treatment with rTipα upregulated 10-fold expression of 6 chemokine genes, CXCL1, CXCL2, CXCL5, CXCL10, CCL20 and CCL2 in MGT-40 cells [38]. The expression of CXCL1 and CXCL2 was heightened by TNF-α via NF-κB activation, specifically associated with metastasis [73].

To characterize the significance of cysteine residues more precisely, three mutants of rTipα were made: One cysteine substituted with an alanine at either 5 or 7 position of rTipα (C5A-rTipα and C7A-rTipα), and two cysteines substituted with 2 alanines at both 5 and 7 positions at the same time (C5A/C7A-rTipα) (Fig. 2). C5A-rTipα and C7A-rTipα formed a dimer similar to rTipα, whereas C5A/C7A-rTipα showed only a monomer band on SDS-PAGE in the absence of DTT. Moreover, C5A-rTipα and C7A-rTipα dose-dependently inhibited specific binding of FITC-rTipα to MGT-40 cells (IC_{50} 2.4 μM, and 2.9 μM, respectively), as rTipα did, and C5A/C7A-rTipα inhibited it weakly (IC_{50} 21.0 μM). The results suggest that homodimer formation with a disulfide bond through a cysteine residue is essential for specific binding activity of rTipα to MGT-40 cells, as well as induction of *TNF-α* gene expression [39].

When MGT-40 cells were incubated with FITC-rTipα at 37°C or 4°C, the binding amount of FITC-rTipα on the cells at 37°C was lower than that at 4°C, suggesting that FITC-rTipα internalized into MGT-40 cells in a temperature-dependent manner. To confirm the internalization of FITC-rTipα into the cells, rTipα was labeled with a photostable dye, Alexa Fluor 488. Incubation of MGT-40 cells with Alexa Fluor 488-conjugated-rTipα (AF488-rTipα) showed fluorescence increasing in the cells in dose-dependent and time-dependent manner. The cells incubated with AF488-rdel-Tipα had only slight fluorescence, similar to those incubated with AF488-BSA. The results suggest that AF488-rTipα is incorporated into MGT-40 cells, but the inactive AF488-rdel-Tipα is not. Moreover, the rTipα incorporation into the cells was confirmed by immunocytochemical analysis with anti-rTipα antibody and a confocal laser scanning microscope. Anti-rTipα antibody recognized rTipα protein in both cytosol and nuclei of MGT-40 cells after treatment with rTipα for 1h, indicating the internalization of rTipα into nuclei. The study on cell fractionation into cytosol, membrane and

nuclei after treatment with rTipα for 1h at 37°C, revealed that rTipα was present in each cell fraction, with 13.5-17.5% of rTipα present in nuclei of the cells. However, inactive rdel-Tipα was not found in any nuclei fraction [39].

Since Tipα is produced in various *H. pylori* strains, we think that the amount of Tipα secreted from *H. pylori* must be different depending on the gastric ailment. The difference in production and secretion of Tipα was examined using 28 clinical isolates of *H. pylori* obtained from 17 gastric cancer patients and 11 chronic gastritis patients. All *H. pylori* isolates of Japanese patients produced Tipα, and 98% produced cag PAI. To compare the amount of Tipα in clinical isolates, we expressed secreted 1.0 ng Tipα/10^9 CFU/ml as 1 relative unit. Clinical isolates from cancer patients secreted Tipα at 1.4 - 13.4 relative units, and those from gastritis patients secreted it at 0.8 - 6.7 relative units. Interestingly, *H. pylori* isolated from 3 of 11 gastritis patients who later developed gastric cancer also secreted large amounts of Tipα, similar to those from cancer patients [39] (Fig. 5). The results suggest that secreted Tipα produces a cancer microenviroment during *H. pylori* infection in human stomach.

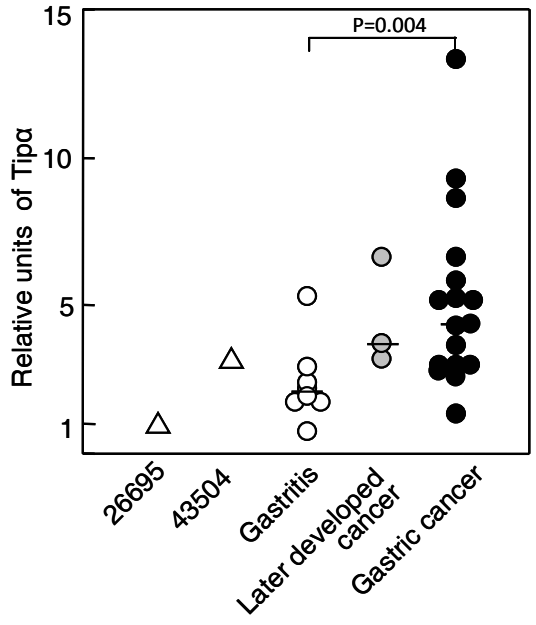

Fig. 5. Secretion of large amounts of Tipα from *H. pylori* clinical isolates.

Crystal structure of rdel-Tipα

Tipα has no obvious homologue in any other species, and bears no similarity to any other *H. pylori* virulence factor. Since Tipα has a unique protein structure, we performed X-ray crystallographic analysis of rTipα. However, the crystal of rTipα would not grow because of the molecule's tendency to aggregate. To overcome the problem, the crystal of rdel-Tipα was used for the experiment, although rdel-Tipα is an inactive monomer in both the presence and absence of DTT, with biological activity 10 - 50 fold weaker than that of rTipα. The crystal structure of rdel-Tipα was determined using multiple isomorphous replacement with anomalous scattering (MIRAS), and the protein-protein interaction was studied to elucidate the importance of the N-terminal region [46].

The rdel-Tipα monomer had an elongated structure with a long axis of about 50 Å and a novel β1-α1-α2-β2-β3-α3-α4 topology (Fig. 6A). The key feature of rdel-Tipα was the 40-Å-long α helix (α1) that extended from the bottom to the top part of the molecule (Fig. 6A). The bottom part of the molecule was made up of a portion helix α1 enveloped by a three-stranded, anti-parallel α sheet, while the top part was made up of a 4-helix bundle comprising the remainder of α1 and α2, α3 and α4. The 4-helix bundle appeared to be stabilized through formation of a hydrophobic interface among the four inner surfaces. By contrast, a large number of hydrophilic residues were present on the outer surfaces of the bundle, and a unique flexible N-terminal region made up a short helix [46].

The quaternary structure of rdel-Tipα was unique because rdel-Tipα monomer A interacts with another monomer B along a crystallographic twofold axis to form a heart-shaped dimer with a hole at its center (Fig. 6B and 6C). The two monomers were linked through electrostatic and hydrophobic interactions that occurred at site I in the flexible N-terminal region and at site II (Fig. 6B and 6D). Figure 6D shows the experimental electron density around His68 from each monomer at site II. At site I, the electron density of the N-terminal 12 amino acids was not visible due to the region's flexibility. The first visible amino acid (Asp, 19D of Fig. 2) in the N-terminal

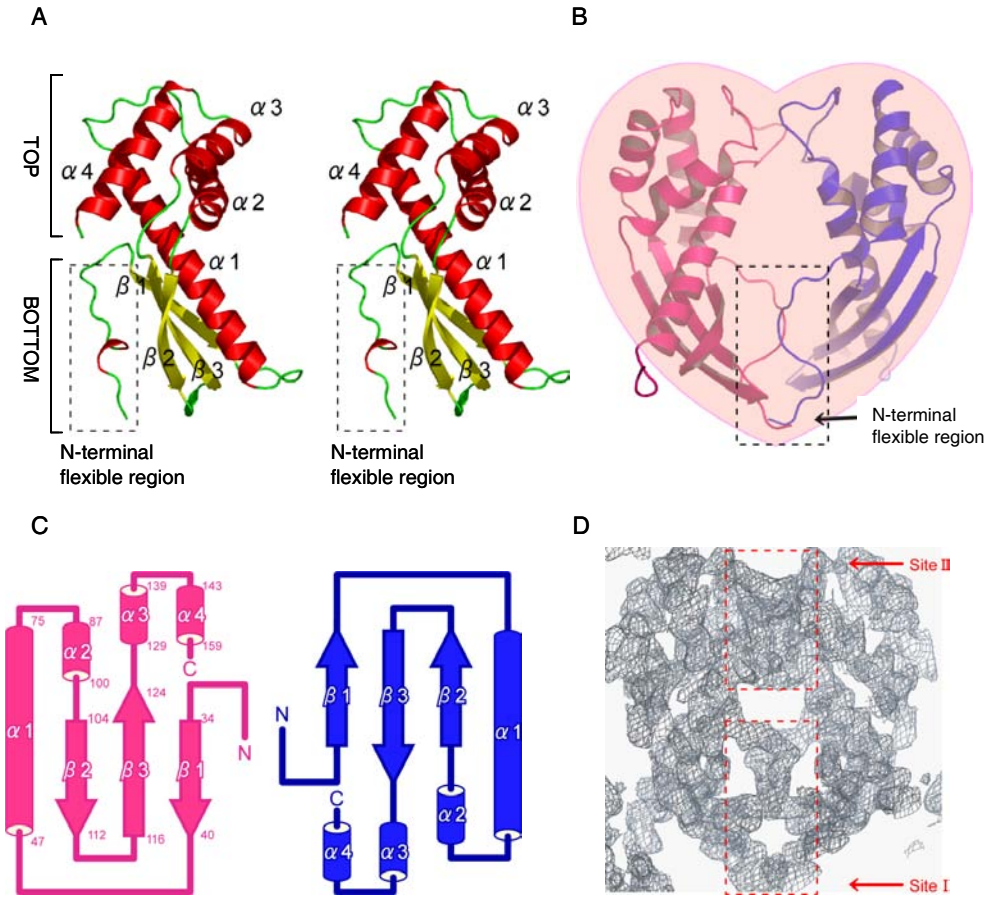

Fig. 6. Overall structure of rdel-Tipα protein. A) Stereochemical structure of rdel-Tipα monomer, B) Tertiary structure of the heart-shaped rdel-Tipα dimer, C) Topology diagram of rdel-Tipα dimer, D) Experimental electron density map as determined by MIRAS contoured at 1σ.

portions of monomers A and B were in close proximity. The hole in the center of the rdel-Tipα dimer had a diameter of about 10 Å and was formed by hydrophilic residues [46]. The structural feature of rdel-Tipα suggests that the structure and oligomerization state of Tipα is maintained by disulfide bridges linking the N-terminal portions of the monomers.

The solution structures of rTipα and rdel-Tipα were also evaluated by comparing their CD spectra, because the CD spectrum in the near-UV region reflects the three dimensional structure of a protein. The results clearly show that the solution structures of rTipα and rdel-Tipα are the same, despite the fact that rTipα contains disulfide bonds and rdel-Tipα does not. Thus, we conclude that rTipα has the same elongated structure - with a 40-Å-long α helix (α1) - as rdel-Tipα does, and also has the same heart-like dimer as rdel-Tipα [46]. Although HP-MP1 is reported to be membrane-bound [65], the crystal structure of rdel-Tipα suggests that neither HP-MP1 nor rTipα has a hydrophobic surface to interact with the lipid layer of the membrane. Thus, we think that rTipα is secreted from *H. pylori* as a soluble homodimer through a secretion mechanism different from the Type IV secretion system [39]. In addition, other groups have independently studied the structures of Tipα (residues 28 - 192) and TipαN34, both of which are truncated forms of Tipα lacking the N-terminal 27 and 33 amino acids, and also reported the significance of two cysteine residues (C5 and C7 of Tipα) in maintaining the dimeric formation [74, 75]. All the results so far indicate that the carcinogenic activity of Tipα is quite different from that of

cagA. The N-terminal structure of rTipα should soon be elucidated, so we can better understand the interaction with other important proteins. It is important to note that Dr. Tsuge recently succeeded in solving the crystal structure of active rTipα (personal communication).

Nucleolin as cell surface receptor for Tipα

Since specific binding of FITC-rTipα to MGT-40 cells is inhibited by rTipα, C5A-rTipα and C7A-rTipα, with their IC_{50} values about 1.9 - 2.9 μM, the specific receptor of rTipα is probably present on cell surface [39]. To identify the specific rTipα-binding protein, we made rTipα-FLAG, rdel-Tipα-FLAG, and C5A/C7A-rTipα-FLAG for experiments: All carried both an His-tag at the N-terminal region (Fig. 2) and a FLAG-tag at the C-terminal region. The rTipα-FLAG induced *TNF-α* gene expression in MGT-40 cells, whereas rdel-Tipα-FLAG-induced expression was over 10-fold weaker than rTipα-FLAG [47]. Thus, rTipα-FLAG and rdel-Tipα-FLAG were confirmed to have the same biological activity as rTipα and rdel-Tipα.

MGT-40 cell lysates was incubated with rTipα-FLAG and with rdel-Tipα-FLAG *in vitro* at 4°C for 2 h, and separate pull-down assays were done using resin conjugated with anti-FLAG antibody. The polypeptides, which co-immunoprecipitated with rTipα-FLAG but not with rdel-Tipα-FLAG, were subjected to LC-MS analysis after tryptic digestion. Two polypeptides with 88 and 40 kDa had protein sequences identical to that of mouse nucleolin. Thus, we concluded that the polypeptide with 88 kDa is nucleolin and the other polypeptide with 40 kDa is a fragment of nucleolin [47]. The polypeptide with 88 kDa was further confirmed using immunoblot analysis with anti-nucleolin antibody: Nucleolin is known to be present on both nucleolus and surface of the cells [76-81]. We found that a small but significant amount of nucleolin is present in membrane fraction of MGT-40 cells, while most of the nucleolin is still in the nuclear fraction. Localization of nucleolin on cell surface was also found by flow cytometry using anti-nucleolin antibody (anti-NUC295) [47].

Nucleolin has three different structural domains: an N-terminal domain containing highly acidic residues, a central domain containing four RNA recognition motifs, and a C-terminal domain containing Arg-Gly-Gly (RGG) repeats [80]. We next studied the direct interaction of rTipα with nucleolin. His-tag-removed rTipα-FLAG was incubated *in vitro* with recombinant human nucleolin fragment NUC284, which consists of amino acids from 284 to 710 containing four RNA binding domains and Ni^{2+} chelating resins. rTipα-FLAG significantly co-precipitated with NUC284 fragment, but His-tag-removed C5A/C7A-rTipα-FLAG did not co-precipitate with it. And the internalized rTipα-FLAG interacted with nucleolin in human gastric cancer cell line MKN-1, as determined by immunoprecipitation assay with anti-nucleolin antibody [47]. All the results indicate that the homodimer of rTipα directly binds to two-thirds of C-terminal nucleolin, without any scaffold proteins, while the monomer does not.

To understand the role of the rTipα-nucleolin complex on cell surface, we looked at how anti-nucleolin antibody affects the induction of *TNF-α* gene expression in MGT-40 cells treated with rTipα: Treatment with anti-nucleolin antibody (anti-NUC295) dose-dependently enhanced the *TNF-α* gene expression induced by rTipα up to 2-fold, and the treatment also enhanced the incorporation of rTipα into the cytosol of MGT-40 cells. However, treatment with rabbit IgG and anti-nucleolin H250 antibodies - the latter does not recognize nucleolin on cell surface - did not affect the levels of *TNF-α* gene expression induced by rTipα. So we can speculate that the complex of rTipα, nucleolin and anti-NUC295 antibody together internalize into the cells and then induce *TNF-α* gene expression and tumor promotion [47].

Nucleolin on cell surface is a glycoprotein containing *N*- and *O*-glycans [82], and the *N*-glycosylation of nucleolin is essential for localization on cell surface [83]. We think it is important to study the regulation of nucleolin on cell surface by flow cytometry. Treatment of MGT-40 cells with tunicamycin, an inhibitor of the *N*-linked glycosylation of protein, reduced the amount of nucleolin on cell surface by approximately 50%, suggesting down-regulation of nucleolin on cell surface (Fig. 7). This resulted in 50% inhibition of *TNF-α* gene expression in

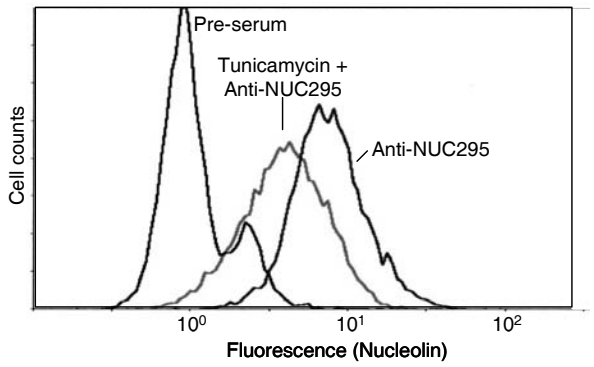

Fig. 7. Cell surface nucleolin on MGT-40 cells visualized using flow cytometry with anti-nucleolin antibody (anti-NUC295).

MGT-40 cells induced by rTipα. The results clearly show that nucleolin on cell surface is a functional receptor of rTipα that incorporates rTipα into the cells [47].

Significant role of nucleolin in gastric carcinogenesis

Nucleolin is an abundant non-ribosomal protein consisting of 710 amino acids in nucleolus. Biochemically, nucleolin is a ubiquitous phosphoprotein which has multifunctions: regulation of ribosomal biogenesis and maturation, control of ribosomal DNA transcription, pre-ribosome packaging, nucleolar chromatin remodeling, DNA recombination, DNA replication, RNA transcription by RNA polymerase I and II, rRNA processing, mRNA stabilization, cytokinesis and apoptosis [80, 81]. Looking at the unique features of nucleolin in carcinogenesis, there are numerous reports that the amount of nucleolin is highly elevated in rapidly proliferating cells, and that nucleolin is a major component of the silver-stained nucleolar organizer region (AgNOR), a histopathological marker of cancer [80, 84, 85]. In addition, cell surface nucleolin acts as a receptor for various ligands, including midkine, lactoferrin, endostatin, and human immunodeficiency virus (HIV) particles [77, 86-89].

We first determined the localization of nucleolin on cell surface of 5 human gastric cancer cell lines: MKN-1 (adenosquamous carcinoma), MKN-45 (poorly differentiated adenocarcinoma), MKN-74 (moderately differentiated adenocarcinoma), AGS (adenocarcinoma) and KATOIII (signet ring cell carcinoma). The cells were fractionated into membrane, cytosol and nuclear fractions, and each fraction was then subjected to SDS-PAGE, followed by immunoblotting with anti-nucleolin antibody. It is important to note that the three polypeptide bands in the membrane fraction had molecular weights of 107, 98, and 81 kDa, and that these bands were similar to those found in nuclear fractions of MKN-45, MKN-74, AGS and KATOIII cell lines. Moreover the amount of nucleolin with 107 kDa in their membrane fractions was almost the same as that of nuclear fractions of the human gastric cancer cell lines. However, MKN-1 cells contained smaller amounts of nucleolin in membrane fraction compared with the nuclear fraction. We think that nucleolin with 107 kDa in both membrane and nuclear fractions is derived from the full length of nucleolin, and that the two bands with 98 and 88 kDa are degradation fragments. Thus, we conclude that a large amount of nucleolin is present in membrane fractions of 4 human gastric cancer cell lines [90].

Next, the amount of nucleolin in membrane fraction of MGT-40 cells was compared with that in normal epithelial cells of mouse glandular stomach. It was striking to find that the membrane fractions of normal glandular stomach in two different mice contain less than 1% of the nucleolin in a nuclear fraction. We think that the amount of nucleolin in the membrane fraction of normal epithelial cells in mouse glandular stomach is much lower than that in malignant MGT-40 cells, and that nucleolin increases in the membrane fraction during the early stage of carcinogenesis [90].

Cancer treatment by inhibitor of nucleolin

It is reasonable to suppose that an inhibitor of the rTipα-nucleolin complex would provide some benefit to treatment, possibly even prevention of gastric cancer. Based on the significance of nucleolin on cell surface in carcinogenesis, it is vital to screen for inhibitors of nucleolin with anticancer activity [90]. The well-investigated anticancer aptamer named AS1411 is a DNA aptamer of 26-mer unmodified guanine-rich oligonucleotide: It directly binds to nucleolin, resulting in inhibition of nucleolin function and

cancer cell growth *in vitro* and *in vivo* [91, 92]. Based on evidence that AS1411 is now in phase II clinical trials for treatment of acute myeloid leukemia and renal cell carcinoma, we studied the inhibitory effects of AS1411 on cell growth and cell cycle regulation of 5 human gastric cancer cell lines, using 26-mer cytosine-rich oligonucleotide (CRO) as control. The IC_{50} values for growth inhibition with AS1411 were MKN-45 (2.3 μM) > KATOIII (9.5 μM) > AGS (10.0 μM) > MKN-74 (>20.0 μM) = MKN-1 (>20.0 μM), in order of potency.

To study the relationship between the large amounts of nucleolin on cell surface and the incorporation of AS1411 into human gastric cancer cell lines, the incorporation of fluorescence-labeled AS1411 (FITC-AS1411) into the cells was determined by flow cytometry. Incorporation of FITC-AS1411 into MKN-45 and AGS cells with large amounts of nucleolin on cell surface was detected in within 2 h, and that into MKN-1 cells with small amounts of nucleolin on cell surface took even less time. The incorporated FITC-AS1411 was colocalized with nucleolin in cytosol and nucleoli. To understand the inhibitory mechanism of AS1411 on human gastric cancer cell lines, the cell cycle regulation of both MKN-45 cells and MKN-1 cells treated with AS1411 was studied by flow cytometry 1 d after. Treatment of MKN-45 cells with AS1411 increased S-phase cells from 14.4 ± 12.4% to 79.8 ± 4.0%, whereas treatment of MKN-1 cells with AS1411 slightly increased the cells from 17.3 ± 2.9% to 32.9 ± 5.0%. The results suggest that AS1411 induces S-phase cell cycle arrest in MKN-45 and MKN-1 cell lines by inhibiting *de novo* DNA synthesis [90]. Furthermore, treatment of MKN-1 cells with rTipα induced cell migration as potently as did 0.1% serum and fibronectin (10 μg/ml), finally enhancing cell adhesion. However, treatment of the cells with siRNA targeted to nucleolin significantly inhibited cell migration induced by rTipα due to knock down of nucleolin [93]. It is significant to note that AS1411 inhibited the induction of *TNF-α* gene expression in MGT-40 cells treated with Tipα [94].

Nucleolin acts as a receptor for various ligands. Some of the ligands, for example, HIV particle and anti-HIV pseudopeptide HB-19, prevented HIV infection [89]. HB-19 has been shown to induce anticancer activity by inhibiting angiogenesis [95]. Lactoferrin, an iron-binding glycoprotein with 80 kDa, is incorporated into cells through cell surface nucleolin, resulting in inhibition of *H. pylori* infection [77, 96-98]. Like AS1411, lactoferrin inhibited the induction of *TNF-α* gene expression in MGT-40 cells treated with Tipα [94]. Since we recently showed that lactoferrin inhibits migration of MKN-1 cells by inhibiting rTipα internalization [93], we think that inhibitors of nucleolin are promising tools for gastric cancer treatment, because AS1411, HB-19 and lactoferrin did not have any toxic effects on normal cells.

It is worthwhile to note that C57BL/6 mice can be immunized against cancer via the intranasal route with CpG, rTipα + CpG and rdel-Tipα + CpG: The levels of rTipα specific antibodies are significantly higher in rTipα-immunized and rdel-Tipα-immunized mice than in the infection control group. And the number of colonizing bacteria is reduced in rTipα-immunized mice (4.3×10^5 CFU/g) and rdel-Tipα-immunized mice (2.5×10^5 CFU/g), compared with infection control mice (5.7×10^6 CFU/g). Vaccinations with rTipα and rdel-Tipα are known to be effective against *H. pylori* infection [99].

CONCLUSION

Since 1981, we have studied classical tumor promotion using chemical tumor promoters. Inhibitors of protein phosphatases 1 and 2A commonly induced tumor promotion on mouse skin, rat glandular stomach and rat liver initiated with three different carcinogens (initiators), and we found that tumor promotion can also be induced by TNF-α and other proinflammatory cytokines in the cells. Specifically, our studies have revealed that TNF-α induces cell transformation, and that TNF-α is an essential cytokine for tumor promotion in TNF-α-deficient mice (TNF-$\alpha^{-/-}$), because TNF-$\alpha^{-/-}$ mice were refractory to tumor promotion of TPA and okadaic acid on mouse skin. We also investigated the involvement of TNF-α as an endogenous tumor promoter in *H. pylori* carcinogenesis, as an example of clinical tumor promotion. It was exciting to find the *TNF-α-inducing factor* (*Tipα*) gene in *H. pylori* genome, as a carcinogenic

factor. And we further demonstrated the biochemical and biological activity of rTipα, rdel-Tipα and nucleolin on cell surface, as further examples of endogenous tumor promotion. This paper presents a new concept of tumor promotion in *H. pylori* carcinogenesis, especially regarding the interaction of Tipα with its receptor nucleolin.

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CONFLICT OF INTEREST

The authors did not have any conflicts of interest to disclose.

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