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Advances in Brief

Helicobacter Pylori Membrane Protein 1: A New Carcinogenic Factor of *Helicobacter Pylori*[1]

Masami Suganuma, Miki Kurusu, Sachiko Okabe, Naoko Sueoka, Masaru Yoshida, Yoshio Wakatsuki, and Hirota Fujiki[2]

Saitama Cancer Center, Saitama 362-0806, Japan [M. S., M. K., S. O., N. S., H. F.], and Division of Clinico Bio-Regulatory Science, Graduate School of Medicine, Kyoto University, Kyoto 606-8707, Japan [M. Y., Y. W.]

Abstract

Considering a suspected link between *Helicobacter pylori* infection and human stomach cancer, a new *H. pylori* gene for membrane protein 1 (HP-MP1) was recently cloned. Because HP-MP1 induces release of inflammatory cytokines and tumor necrosis factor-α acts as both initiator and tumor promoter, we studied the possible involvement of HP-MP1 in carcinogenesis of *H. pylori*. Two cell lines, BALB/3T3 cells as control and v-Ha-*ras*-transfected BALB/3T3 cells (Bhas 42 cells) as putative initiated cells, were each transfected with *HP-MP1*, *urease B* genes, or vector alone. All of the Bhas/mpl clones showed strong expression of tumor necrosis factor-α gene and produced tumors in 100% of nude mice. Two Bhas/ure clones showed weak tumorigenicity; the other Bhas and BALB clones showed none. Results indicate strong carcinogenic activity of HP-MP1 in cooperation with viral Ras protein and weak activity of urease B.

Introduction

HP-MP1[3] is a M_r 16,000 protein, the gene of which was cloned recently (1) from genomic DNA of *H. pylori* strain SR 7791. Yoshida *et al.* (1) reported that HP-MP1 protein attached to the inner membrane of *H. pylori* as homodimer but that its function in the bacteria was not clear. We paid special attention to the results showing that HP-MP1 and urease induced release of various inflammatory cytokines, such as TNF-α, IL-1α, IL-8, and macrophage inflammatory protein 1α from human monocytes (1, 2) and also noted that anti-HP-MP1 antibody was detected in the sera of *H. pylori*-infected patients (1). Considered together, the results suggested that HP-MP1 might be a new proinflammatory mediator in stomach infected with *H. pylori*.

Our study on tumor promotion with TNF-α-deficient mice had revealed the TNF-α and other inflammatory cytokines stimulate tumor promotion mediated through activation of nuclear factor κB (3, 4).

Among various inflammatory cytokines, we demonstrated recently (3) that TNF-α is the first instigator in tumor promotion and that the sequence of cytokine network for tumor promotion appears to be from TNF-α through IL-1 and IL-6. On the basis of this, we believe that among various inflammatory cytokines, TNF-α is the essential tumor promoter (5). If so, the TNF-α-inducing activity of HP-MP1 would have a function as a cancer mediator. In the light of this, we asked how could the cytokine-inducing activity of HP-MP1 be linked to carcinogenesis?

Our first indication of TNF-α as an endogenous tumor promoter was finding that TNF-α induced clonal growth of v-Ha-*ras*-transfected BALB/3T3 cells (Bhas 42 cells), whereas it did not induce growth of BALB/3T3 cells without v-Ha-ras (6). Furthermore, we and other investigators found that Bhas 42 cells were useful tools to demonstrate *in vitro* the tumor-promoting activity of some proteins, such as leukemia-related protein MTG8 (ETO; Ref. 7) and hepatitis C virus core protein (8). Considering the accumulated results, we transfected *HP-MP1* gene into both Bhas 42 and BALB/3T3 cells. We found that Bhas/mpl clones expressed *TNF-α* gene more strongly than did BALB/mpl clones, with the Bhas/mpl clones inducing cell transformation. The carcinogenicity of the transformed clones was further confirmed by soft agar colony formation and tumor development in nude mice. Bhas 42 and BALB/3T3 cells were similarly transfected by *urease B* gene of *H. pylori* or vector alone. Two Bhas/ure clones, which expressed *TNF-α* gene, showed weak tumorigenicity in nude mice, but none of the other clones of either cell type showed any significant transformation.

This study shows that TNF-α protein, induced by both HP-MP1 and urease B, resulted in cell transformation in cooperation with viral Ras protein, strongly suggesting a new carcinogenic mechanism of human stomach cancer development associated with *H. pylori* infection.

Materials and Methods

Plasmids and Transformed Clones. *HP-MP1* gene cloned from *H. pylori* SR 7791 and *urease B* gene from *H. pylori* NCTC 11637 were subcloned into the His tag expression vector, pET28c(+) (Novagen, Madison, WI), as reported previously (1). The *HP-MP1* and *urease B* genes were separately inserted into the mammalian expression vector pcDNA3.1/hygro(+) (Invitrogen, Carlsbad, CA), which contains human cytomegalovirus immediate-early promoter and *hygromycin-resistant* gene, resulting in the expression plasmids, pcDNA-mpl and pcDNA-ure. These two plasmids were each transfected into both v-Ha-*ras*-transfected BALB/3T3 cells (Bhas 42; Ref. 9) and BALB/3T3 A31-1-1 cells (JCRB0601) by lipofection protocol using DIMRIE-C reagent (Life Technologies, Inc., Rockville, MD). The two types of cells were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). After 48-h culture, hygromycin B (Sigma Chemical Co.) at a concentration of 400 μg/ml was added to the medium, and this was further cultured for 2 weeks. As a negative control, pcDNA3.1/hygro vector alone was transfected into both types of cells. Twenty hygromycin B-resistant foci were picked from each set of transfections and were then grown separately to study the properties of transformed clones. Finally, three groups of Bhas clones, Bhas/mpl, Bhas/ure, and Bhas/vec, and three groups of BALB clones, BALB/mp1, BALB/ure, and BALB/vec, were obtained.

Expression of *TNF-α* Gene. Total RNA was isolated from five clones randomly selected from the three Bhas groups and five clones from the three BALB groups by Isogen reagent (10). TNF-α mRNA was determined using a semiquantitative RT-PCR method, as reported previously (11). PCR amplification was conducted in the presence of [α-^{32}P]dCTP, and *glyceraldehyde-3-phosphate dehydrogenase* gene was used as a control. The primers used were

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[2] To whom requests for reprints should addressed, at Saitama Cancer Center, Ina, Kitaadachi-gun, Saitama 362-0806, Japan. E-mail: hfujiki@cancer-c.pref.saitama.jp.

[3] The abbreviations used are: *HP-MP1*, *Helicobacter pylori* gene for membrane protein 1; TNF-α, tumor necrosis factor α; IL, interleukin; RT-PCR, reverse transcription PCR.

5′-AGCCCACGTCGTAGCAAACCACCAA-3′ (sense) and 5′-ACACCCATTCCCTTCACAGAGCAAT-3′ (antisense) for *TNF-α* gene, and 5′-TGGCATTGTGGAAGGGCTCATGAC-3′ (sense) and 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′ (antisense) for *glyceraldehyde-3-phosphate dehydrogenase* gene. TNF-α mRNA was expressed as relative fold expression in comparison with that of nontransfected controls, Bhas 42 and BALB/3T3 cells. All of the experiments were repeated twice.

Determination of TNF-α Protein. Each (3×10^6 cells) of the Bhas and BALB clones in growing stage was extracted with lysis buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 10 mM sodium PP_i, 2 mM sodium vanadate, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride]. After 14,000 rpm centrifugation for 15 min, each supernatant (about 350 mg) was subjected to TNF-α ELISA kit (Genzyme Co., Cambridge, MA). The concentration of TNF-α in cell lysates was expressed as pg/mg protein. Experiments were duplicated.

Expression of *HP-MP1* and *Urease B* Genes in Bhas and BALB Clones. Expression of *HP-MP1* and *urease B* genes was determined by RT-PCR. Briefly, total RNA (1 μg) was reverse transcribed by murine leukemia virus reverse transcriptase (Roche Molecular Systems, Inc., Branchburg, NJ) using $oligodeoxythymidylate_{16}$. PCR amplification was performed with AmpliTaq DNA polymerase in the PCR reaction mixture for 30 cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 60 s). The PCR products were subjected to electrophoresis in 1.0% agarose gel and stained with ethidium bromide. Primers used in this experiment were 5′-TTGCAAAATCGCAGTGCGTAT-3′ (sense) and 5′-GGCTATAGG GACTTTCGCATCGC-3′ (antisense) for *HP-MP1* gene and 5′-CGGTGAAGGTTTGATCGTAACG-3′ (sense) and 5′-CAAAGTGTCTGTGTGGATAGCGACT-3′ (antisense) for *urease B* gene.

Soft Agar Colony Formation of Bhas and BALB Clones. Five clones from the three Bhas groups (Bhas/mpl, Bhas/ure, and Bhas/vec) and five from the three BALB groups (BALB/mpl, BALB/ure, and BALB/vec) were tested for anchorage-independent growth in soft agar. Each clone (5×10^2 cells) was cultured in MEM containing 0.22% soft agar in a 35-mm diameter dish in duplicate (12). The numbers of large colonies (>0.4 mm in diameter) determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were counted 3 weeks after plating. The parental Bhas 42 and BALB/3T3 cells were similarly cultured.

Tumorigenicity of Bhas/mp1 and Two Other Bhas Clones in Nude Mice. Three clones (each 5×10^6 cells) from the three Bhas groups (Bhas/mpl, Bhas/ure, and Bhas/vec) and the parental Bhas 42 cells were implanted s.c. into two sites/one nude male mouse (5 weeks of age; BALB/cAJcl-nu; The Jackson Laboratory; Ref. 7). Thus, one Bhas clone was implanted into a total of six sites for the three nude mice. Tumor development was measured once a week for 4 weeks. Average tumor volume was expressed as mean ± SD of tumors by calculation as $(\text{short axis})^2 \times (\text{long axis})/2$.

Results and Discussion

Expression of *TNF-α* Gene and Determination of TNF-α in Bhas and BALB Clones. Three groups of Bhas clones and three groups of BALB clones were obtained by transfection with pcDNA3.1/hygro vector containing *HP-MP1* gene, *urease B* gene, or vector alone. Then five clones were randomly selected from each group and named as follows: Bhas/mp1: 52, 53, 54, 57, and 63; Bhas/ure: 52, 55, 57, 61, and 65; or Bhas/vec: 53, 57, 68, 74, and 75; and BALB/mp1: 9, 11, 13, 16, and 19; BALB/ure: 2, 7, 9, 17, and 19; or BALB/vec: 3, 9, 12, 17, and 18. Expression of *TNF-α* gene in Bhas and BALB clones was determined using a semiquantitative RT-PCR method. All of the five Bhas/mp1 clones significantly expressed *TNF-α* gene, with expression levels ranging from 12.2–27.0-fold higher than basal levels of the parental Bhas 42 cells (Fig. 1*A*). Two Bhas/ure clones (61 and 65) of five expressed *TNF-α* gene 15.5-fold and 15.1-fold higher than basal levels did, but the three other clones

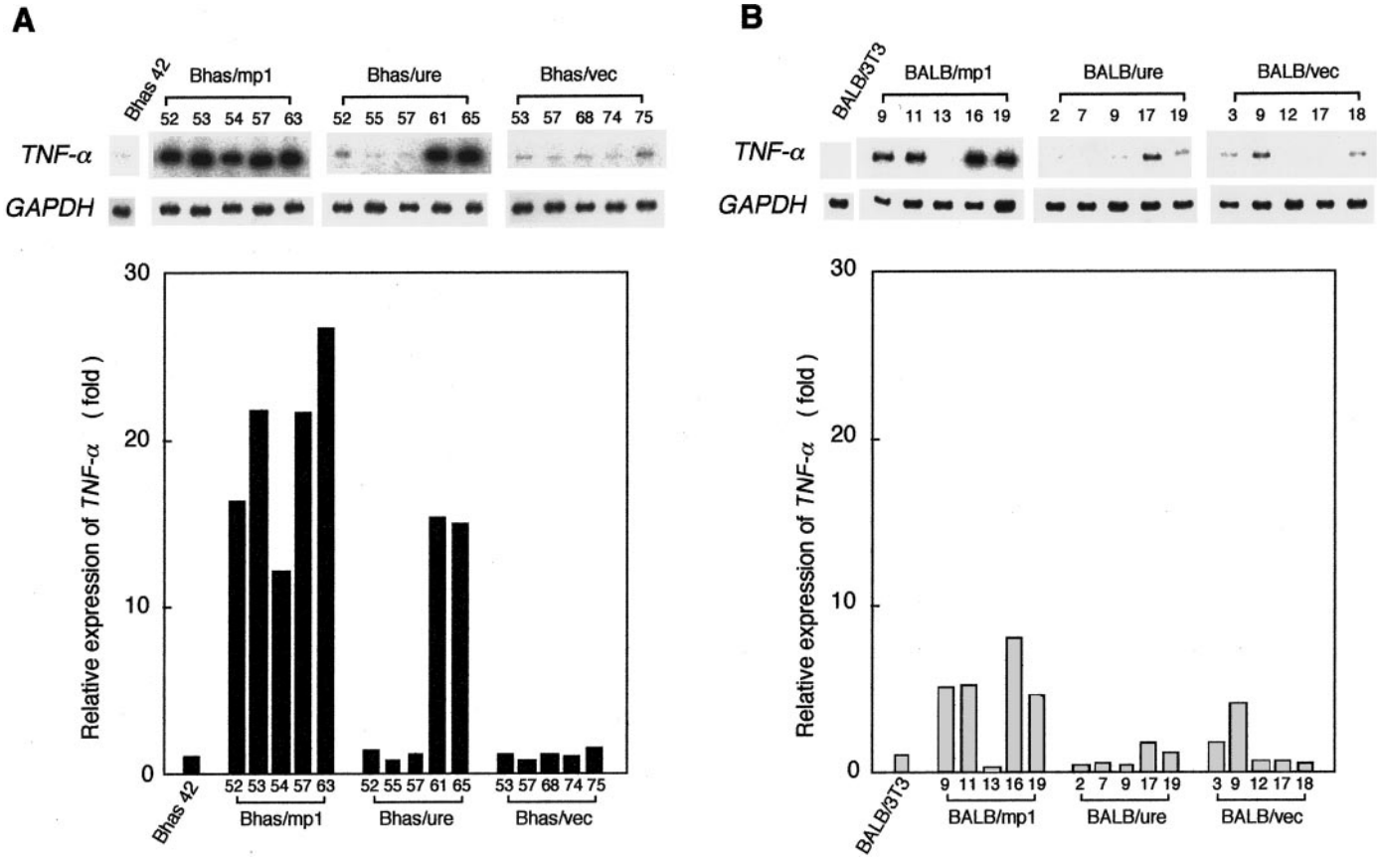

Fig. 1. Expression of *TNF-α* gene in Bhas and BALB clones transfected with *HP-MP1* and *urease B* genes and pcDNA3.1/hygro(+) vector alone. TNF-α mRNA was determined using a semiquantitative RT-PCR, as described in "Materials and Methods." TNF-α mRNA of Bhas (*A*) and BALB (*B*) clones was expressed as relative fold expression in comparison with that of nontransfected Bhas 42 and BALB/3T3 cells. Experiments were repeated twice.

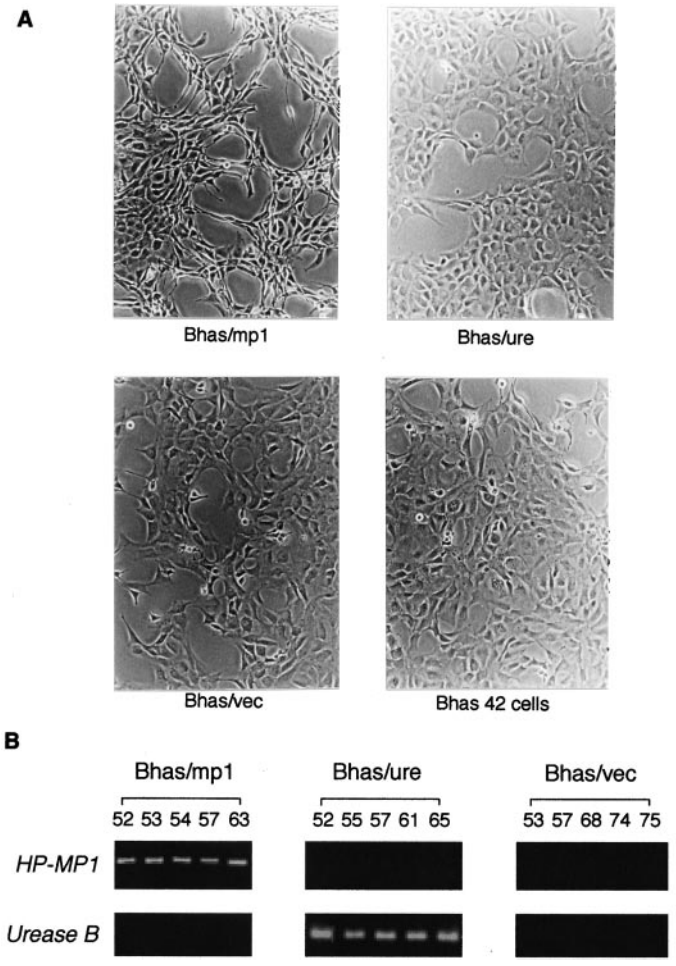

Fig. 2. Morphological changes of Bhas/mpl clone compared with those of Bhas/ure and Bhas/vec clones and parental Bhas 42 cells (*A*). All of the five Bhas/mpl clones induced morphological changes of transformation. Confirmation of *HP-MP1* and *urease B* gene expressions in Bhas/mpl, Bhas/ure, and Bhas/vec clones (*B*).

did not express it. The expression levels of all of the five Bhas/vec clones were at basal levels. Four BALB/mp1 clones expressed *TNF-α* gene from 3.3-fold to 6.3-fold higher than basal levels of the parental BALB/3T3 cells, but the expression level of one clone, BALB/mp1-13, was less than that of the parental cells (Fig. 1*B*). The expression levels of BALB/ure and BALB/vec clones were marginal. The results indicate that, compared with its transfection into BALB/3T3 cells, transfection of *HP-MP1* gene into Bhas 42 cells strongly induced TNF-α mRNA expression in cooperation with v-Ha-*ras* gene, with expression levels varying slightly among the Bhas/mp1 clones. Only two Bhas/ure clones (61 and 65) expressed *TNF-α* gene significantly; the other three did not express it even in the presence of v-Ha-*ras* gene.

Although production of TNF-α protein in Bhas/mp1 clones was relatively low, TNF-α protein was detected in three of five Bhas/mp1 clones. Specifically, Bhas/mp1-54, -57, and -63 clones produced TNF-α with concentrations of 32.1, 18.9, and 12.2 pg/mg protein, respectively. However, TNF-α protein was under detectable levels in cell lysates of all of the Bhas/ure clones, including 61 and 65, and Bhas/vec clones.

Transformation of Bhas/mp1 Clones. Five Bhas/mp1 clones induced morphological changes of transformation, such as spindle shape and multilayer/criss-cross formation (Fig. 2*A*), whereas Bhas/ure and Bhas/vec clones did not induce morphological changes. Before going on to additional experiments, we first confirmed expression of *HP-MP1* and *urease B* genes in Bhas/mp1, Bhas/ure, or Bhas/vec clones. Fig. 2*B* shows that all of the five Bhas/mp1 clones expressed *HP-MP1* gene but not *urease B* gene, whereas the five Bhas/ure clones expressed *urease B* gene but not *HP-MP1* gene. The five Bhas/vec clones did not express either *HP-MP1* or *urease B*. Furthermore, the presence of HP-MP1 protein in Bhas/mp1 clones and that of urease B protein in Bhas/ure clones was confirmed by Western blotting using anti-HP-MP1 antibody and anti-urease B antibody (data not shown).

Next, we tested the transforming activity by anchorage-independent growth in a semisolid medium of soft agar. Five Bhas/mp1 clones produced large colonies in soft agars, with an average colony number of 20 ± 10.1 (Table 1), whereas those of Bhas/ure clones, Bhas/vec clones, and the parental Bhas 42 cells were 4.1 ± 6.1, 2.3 ± 1.5, and 2.0, respectively. Moreover, none of the BALB clones (BALB/mp1, BALB/ure, and BALB/vec) and none of the parental BALB/3T3 cells induced any significant colony formation. These results clearly indicated that HP-MP1, a TNF-α-inducing protein of *H. pylori*, transformed BALB/3T3 cells only in cooperation with viral Ras protein.

Tumorigenicity of Bhas/mp1 Clones in Nude Mice. To examine malignant phenotypes of Bhas/mp1 clones, three Bhas/mp1 clones (52, 57, and 63) were implanted s.c. into six sites of three nude mice, two sites/mouse. Fifteen days after implantation, the three Bhas/mp1 clones produced tumors at 17 of 18 injected sites (94.4%) and reached 100% (18 of 18) within 20 days (Fig. 3*A*). Tumors of Bhas/mp1 clones grew rapidly, with average tumor volume reaching 4.3 ± 1.8 cm^3 after 28 days (Fig. 3*B*). In contrast, tumors developed in only 33.3% of sites in the three Bhas/ure clones 28 days after implantation, and one clone (57) developed no tumors. In the other two clones (61 and 65), tumors were found at six sites, with an average tumor volume of 2.4 ± 5.6 cm^3. Thus, these experiments with Bhas groups showed that the clones that had expressed *TNF-α* gene significantly were clearly tumorigenic in nude mice within 28 days after implantation.

Furthermore, Bhas/vec clones and Bhas 42 cells did not induce any tumors within 28 days. Tumors of Bhas/mp1 clones strongly showed malignant phenotypes, such as large tumors with rapid growth (Fig. 3*B*), apparent invasion from s.c. tissue into peritoneum, and strong angiogenesis.

The potent tumorigenicity of the three Bhas/mp1 clones in nude mice correlated well with the potency of anchorage-independent growth (Table 1), indicating that cooperation of HP-MP1 with viral Ras protein, specifically, induces transformation of BALB/3T3 cells. The potency of HP-MP1 for tumorigenicity was much stronger than that of urease B, and the qualitative difference of carcinogenic features between Bhas/mp1 and Bhas/ure clones is now under investigation.

HP-MP1 protein is structurally not related to virulence factors of *H. pylori*, such as urease, the vacuolating cytotoxin (VacA), and the cytotoxin-associated gene A antigen (CagA) (13–16). And using viral Ras protein, we were able to show carcinogenic activity of HP-MP1 in BALB/3T3 cells. Putting these together, we find that transforma-

Table 1 *Carcinogenicity of Bhas/mp1 and Bhas/ure clones*

Clones and cells	Average no. of soft agar colonies	Tumorigenicity No. of sites with tumors/ no. of injected sites
Bhas/mp1	20.0 ± 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.

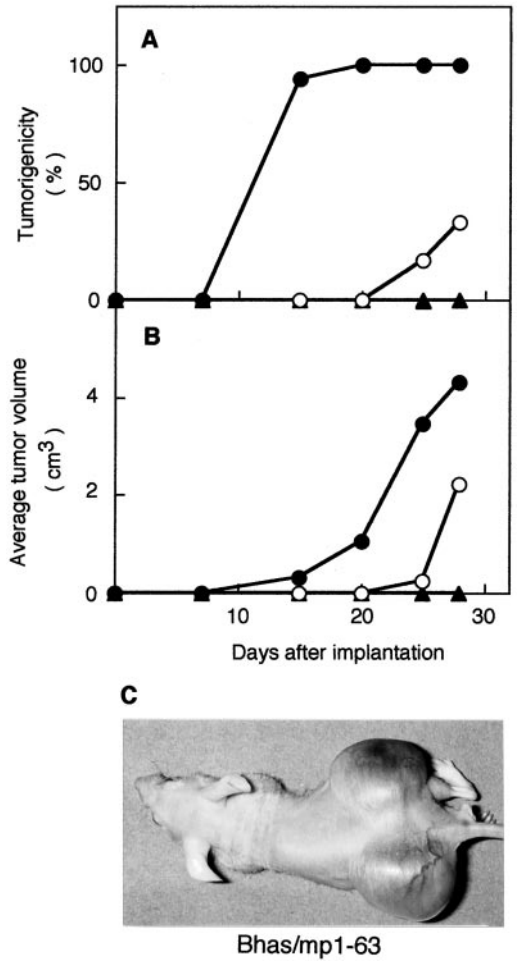

Fig. 3. Tumorigenicity of Bhas/mp1 and Bhas/ure clones in nude mice. Percentages of tumor development (*A*) and average tumor volume (*B*) of the mice implanted with Bhas/mp1 (●), Bhas/ure (○), and Bhas/vec clones and Bhas 42 cells (▲). Tumor-bearing mouse implanted with Bhas/mp1-63 clone 28 days after (*C*).

tion is caused by two main carcinogenic mechanisms: TNF-α is induced by HP-MP1 and consequently activates nuclear factor-κB (17); and the presence of putative activated Ras protein activates mitogen-activated protein kinase (18). Moreover, the expression of viral Ras protein in v-Ha-*ras*-transfected BALB/3T3 cells can be functionally replaced by enhanced c-Ha-*ras* p21 expression in the stomach cancers of humans, based on evidence that levels of c-Ha-*ras* p21 in extracts of human stomach adenocarcinomas were higher than those from extracts of their normal counterparts and also higher than those of c-Ki-*ras* and c-Na-*ras* (19). Thus, we think that this carcinogenic process resembles multistage carcinogenesis in humans. In the light of this, the crucial inquiry into the link between stomach cancer and *H. pylori* infection (20) should be investigated looking at expression of *HP-MP1* gene and coexpression of *TNF-α* gene.

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