Helicobacter pylori-dependent NF-kappa B activation in newly established Mongolian gerbil gastric cancer cell lines

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Mongolian gerbils are an ideal animal model to explore the role of *H. pylori* on cancer development. However, there have been no established adenocarcinoma cell lines from this model animal. In the present study, we have established cancer cell lines from a primary gastric cancer tissue of a Mongolian gerbil. The derived cells could be stably attached with *H. pylori*, revealed under a scanning electron microscope, and easily transplanted to the nude mice. Rapid phosphorylation of IκB, Erk1/2, and AKT of these cells was observed by Interleukin-1 beta stimulation, and luciferase reporter gene assay on transcriptional activation of Nuclear Factor kappa B after challenging with either *H. pylori* NCTC11637 or its isogenic *cagE*-knockout mutant, *H. pylori* revealed the *cagE*-dependent NF-κB transcriptional activation. The newly established cancer cell lines from the *in vivo* gastric carcinogenesis model animal, the Mongolian gerbil, can be used to develop effective therapeutic strategies against gastric cancer, especially in exploring the effect of *H. pylori*, and thus might greatly contribute to gastric cancer prevention and treatment in humans. (*Cancer Sci* 2005; 96: 170–175)

Helicobacter pylori (*H. pylori*) infection has been proven to facilitate gastric disorders[1–3] and gastric cancer development through epidemiological studies and *in vitro* experimental studies.[4–11] As for *in vivo* experimental animal models, Mongolian gerbils (*Meriones unguiculatus*) have been used to study the virulence of *H. pylori* infection upon gastric carcinogenesis[12–18] because of their high susceptibility to infection with *H. pylori*. After the infection, the glandular stomachs of gerbils exhibit acute gastritis such as erosion, foveolar cell hyperplasia, and varying degrees of multifocal cystic dilatation and infiltration of inflammatory cells. Long-term persistent infection in gerbils results in chronic active gastritis including intestinal metaplasia and glandular atrophy, generating severe dysplastic proliferation of the gastric glands into submucosa with interruptions of the lamina muscularis mucosa. The histological changes and gastric disorders induced by *H. pylori* infection in Mongolian gerbils resemble those in humans very well.[15]

Though the precise mechanism of generating histological changes after *H. pylori* infection has not been satisfactory clarified, one of the typical features of *H. pylori* infection has been focused on the activation of signaling pathways in the target host cell. Some virulent factors of *H. pylori* are documented to link with the *cag* pathogenicity islands in the genome,[19] of which CagA protein is translocated from bacteria into host cells through the type IV secretion system,[20,21] comprised of transporters including CagE protein encoded by *cagE*. Tyrosine residues of translocated CagA are phosphorylated in host cells, which can perturb signal transduction interacting with a host cell protein SHP-2 that connects receptor tyrosine kinases and activates Ras/MAPK signaling, resulting in deregulation of the pathway activation.[11,20,21] In addition, it has also been documented that *H pylori* adhesion can directly activate TRAF2 or TRAF6 to induce nuclear localization of NF-κB, resulting in transcriptional activation.[22] Taken together, these results suggest that deregulation of these signaling pathways is associated with the generation of gastric disorders. However, the actual meaning of these alterations in transcriptional activities in gastric *carcinogenesis* remains unclear. The concept of responsible virulence depending on CagA function is supported by epidemiological studies including a meta-analysis of the positive relationship between *H. pylori* CagA seropositivity and gastric cancer.[10] The majority of the Japanese population have the ESS-type[11] of CagA, a strong virulence factor of *H. pylori*. Nevertheless, gastric cancer occurs in 0.5% of the *H. pylori*-infected Japanese population per year.[9] Epidemiological studies indicate that not only the strain diversity of *H. pylori*, but also the effects of overproduction of pro-inflammatory cytokine and environmental factors might serve to develop gastric cancers.

A series of studies using Mongolian gerbils with chemical carcinogen and *H. pylori* infection has demonstrated that *H. pylori* infection apparently enhances gastric carcinogenesis.[12–15] Persistent infection with *H. pylori* can enhance to produce all histological types (well-differentiated, poorly differentiated, and signet ring cell type) of gastric carcinomas in the glandular stomach of this animal, while subsequent eradication at an early stage of inflammation reduced such enhancing effects.[14,15] The promotion and progression of gastric cancers were apparently related to the time course of the inflammatory status after infection with virulent *H. pylori*. However, it is not known how the initiation or progression of cancers is caused by such infection, because there have been no established cancer cell lines from an *in vivo* experimental carcinogenesis model with apparent histories of *H. pylori* infection, available to explore the mechanism of its virulence.

In this study, we first present the establishment of new gastric cancer cell lines with different invasive activities, designated MGC1 and MGC2, from one original cancer tissue induced in the Mongolian gerbil, an analogous model of *H. pylori* infection to humans, to explore the essential biological features and the mechanism for enhancement and progression of *H. pylori*-induced gastric carcinomas.

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Materials and Methods

Tumor development. The protocol for gastric cancer induction was previously described in detail.[14] In brief, specific pathogen-free male, 7-week-old Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Seac Yoshitomi, Ltd, Fukuoka, Japan) were housed in steel cages on hardwood chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. They were given rodent food CRF-1 (Oriental Yeast Co. Ltd, Tokyo, Japan) irradiated with 30 Gy gamma rays and autoclaved distilled water *ad libitum*. MNU (Sigma Chemical Co. St. Louis, MO, USA) was dissolved in distilled water at a concentration of 40 p.p.m. and freshly prepared three times per week or administered in drinking water in light-shielded bottles *ad libitum*. MNU was thus given in the drinking water for 10 weeks. At week 11, *Hp* strain ATCC 43504 (*cagA*$^+$, *vacA*$^+$) samples containing 1.0×10^8 colony-forming units were delivered intragastrically using an oral catheter. Finally, at week 50, gerbils were sacrificed under deep ether anesthesia, laparotomized, and tissue samples were taken from occurring tumors. The animal care committee of the Aichi Cancer Center Research Institute approved the experimental design, and the animals were cared for in accordance with institutional guidelines.

Primary culture procedure. For primary culture, an advanced gastric cancer tissue with perineural invasion occurring in a gerbil was subjected to primary culture. The fresh tumor tissue was washed extensively with phosphate-buffered saline, cut into approximately 1-mm^3 cubes and minced in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan). Small pieces of the tumor were then treated with Dispase (50 units/mL, Godo Shusei, Tokyo, Japan) for 30 min at 37°C, and large tumor pieces were allowed to settle. Supernatant fluid containing cell clumps was collected after centrifugation at 100×*g*, and cell pellets were re-suspended in RPMI 1640 containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) plus serum expander MITO (0.1%, Collaborative Biomedical Products Bedford, MA, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco), then cultured on dishes coated with collagen type I (Iwaki, Tokyo, Japan) or non-coated plastic dishes in a humidified 5% CO_2 incubator at 37°C with weekly changes of the medium. One month thereafter, growing colonies were harvested with trypsin/EDTA and passaged several times. Fibroblasts were removed with mechanical scraping and the differential attachment selection method.

The absence of contamination with Mycoplasma pulmonis or the two viruses, mouse hepatitis virus and sendai virus, was confirmed with the assistance of the Experimental Animal Research Center of Japan (Tokyo) with a direct agar method or a PCR method. For counting growth activities, Cells were plated at 1×10^5 cells/dish in 35-mm plastic dishes in RPMI 1640 supplemented with 10% FBS in the absence of MITO. The cell number was counted in triplicate with a hemocytometer 1, 3, 5, 7, 9, and 11 days after seeding.

Tumorigenicity in nude mice. Exponentially growing cells were harvested with trypsin-EDTA, washed with RPMI 1640, and resuspended in Hank's balanced salt solution (HBSS, Sigma, St. Louis, MO). For subcutaneous inoculation, a total of 1×10^6 cells suspended in 0.2 mL of HBSS were injected into the subcutaneous tissue of the left femurs of seven-week-old athymic nude mice (BALB/c nu/nu) weighing 18–20 g, to investigate the tumorigenicity of MGC1 or MGC2 cells. The mice were killed 5 weeks after inoculation, and the tumors were excised and subjected to histological examination.

Histochemical & immunohistochemical analysis. Original primary tumors and transplanted tissues were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4 μm. Cells harvested with trypsin/EDTA on slide glasses, original tumor tissue slices, and transplanted tissue slices were examined for hematoxylin and eosin (H&E) staining, Alcian blue-periodic acid Schiff staining (AB-PAS) as well as immunohistochemistry for cytokeratin.

Scanning electron microscope analysis. Cell samples were fixed in 1% glutaraldehyde (in 0.05 M cacodylate buffer, pH 7.2), and dehydrated through a graded ethanol series. Following dehydration in 100% ethanol, the alcohol was replaced with isoamyl acetate. Specimens were dried in 100% isoamyl acetate in a critical point drying apparatus (HCP-1, HITACHI, Tokyo), coated with gold, and observed under a scanning electron microscope (S-2250 N, HITACHI, Tokyo) at 20 kV.

Phosphorylation after cellular stimulation with recombinant IL-1 beta. Anti-phospho I kappa B (pIκB) (Ser32), antiphospho Akt (Ser473) Ab, and anti phospho-p44/42 MAPK (Thr 202/Tyr204) mAbs, were purchased from Cell Signal (Beverly, MA), anti-α Tubulin (Clone DM1A) from Sigma (Amersham, Uppsala, Sweden).

MGC cell lines (1×10^6) were incubated for different time periods with recombinant human IL-1β 10 ng/mL at 37°C in RPMI. Cells were washed once with cold PBS, and then lyzed at 4°C in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and complete Protease Inhibitor Cocktail from Roche Applied Science, Indianapolis, IN). 10-μg whole cell lysates in Laemmli sample buffer were separated by 12% SDS-PAGE under reducing conditions, transferred to polyvinylidene difluoride membranes (Amersham, Uppsala, Sweden) and immunoblotted with indicated Abs. Bound Abs were visualized using Western Lightning Chemiluminescence Plus (PerkinElmer Life Sciences, Boston, MA).

Luciferase assay on transcriptional activation of Nuclear Factor kappa B. 2×10^5 cells were cotransfected in a 24-well culture dish with two expression plasmids, a luciferase reporter gene under the transcriptional control of NF-κB element (pNF-κB luciferase; BD Science, San Diego, CA), and a transfection efficiency indicator (pRL-CMV; Promega, Madison, WI) using Lipofectamin 2000 (Invitrogen). After 24-h incubation in RPMI containing 10% FCS, cells were challenged to be infected with 1×10^5 of NCTC11637 or its isogenic *cagE* knockout mutant (*cagE* KO) *H. pylori* for 12 h. For constructing *cagE* KO, a portion of the genes encoding the *cagE* gene was amplified by PCR, and the amplified-fragment was inserted into *Eco*RV restriction enzyme site of pBluescriptSK+ (Stratagene, La Jolla, CA). A kanamicin resistance gene cassette (Km) was inserted into *Bsm*F1 site of the insert DNA for *cagE* gene. The plasmids (1–2 μg) were used for inactivation of chromosomal genes by natural transformation as previously described.[23] Inactivation of the genes was confirmed by PCR amplification followed by Southern blot hybridization. NF-κB luciferase reporter gene assay was performed with a Dual Luciferase Assay System (Promega) and luminometer according to the supplier's instructions.

Results

Establishment of MGC1 and MGC2 cells. A gerbil had advanced gastric cancer with strong progression and perineural invasion (Fig. 1a–e). Two weeks after the primary culture, aggregated tumor cell colonies surrounded by fibroblasts were found at the bottom of the culture dishes. After serial passages while decreasing the number of fibroblastic cells, the remaining few fibroblasts were removed with mechanical scraping and the differential attachment selection method. Cells were replaced entirely by the tumor cells, and the obtained cancer cells were designated as MGC cells (Fig. 1f). Next, single-cell dilution cloning of these MGC cells using a 96-well culture plate was performed, and finally, cultures of two different types of cell lines forming typical epithelial monolayer were obtained. These two cell colonies, designated MGC1 and MGC2, respectively, have been cultured for more than 12 months with up to 60

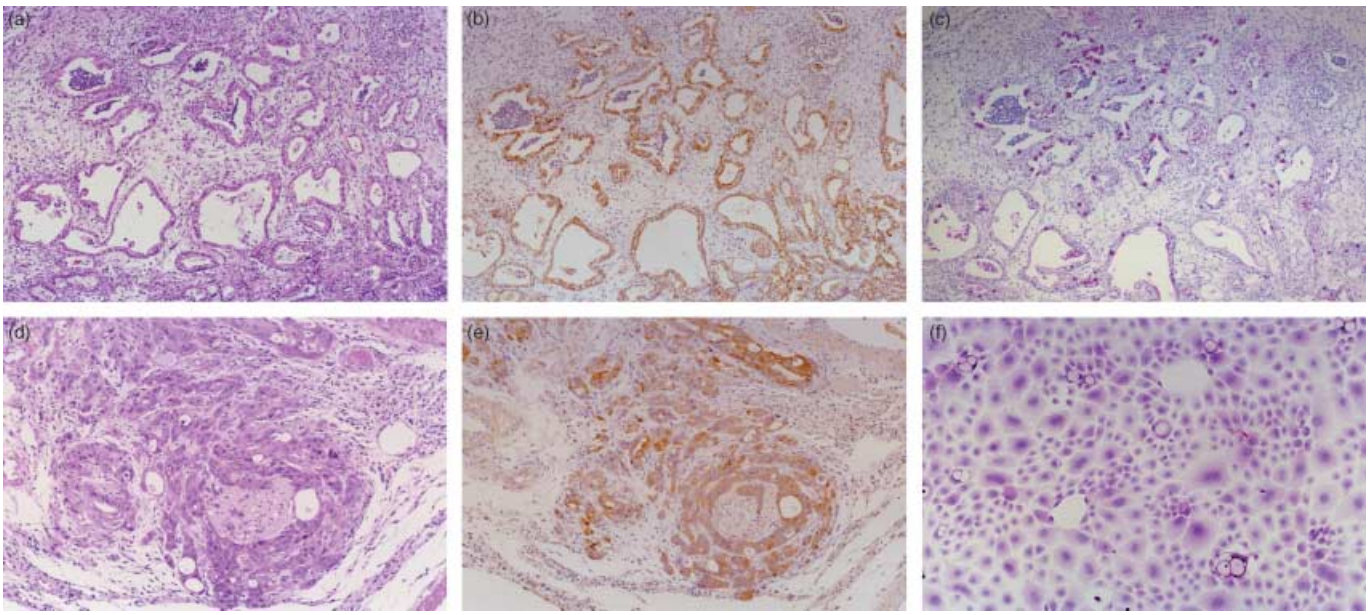

Fig. 1. (a) Original gastric cancer tissue occurring in a Mongolian gerbil. The excised cancer tissue was subjected to the following primary culture procedure. (× 10 H&E). (b) Mongolian gerbil gastric cancer tissue comprised of epithelial cells stained with immunohistochemical staining for cytokeratin. (× 10 Cytokeratin). (c) Alcian blue-periodic acid Schiff staining clearly reveals an aberrant staining pattern in the cancer tissue. (× 10 AB-PAS). (d) High-power view of a deep invasive lesion of the gastric cancer. Proliferating and invasive gastric cancer cells surrounding neural tissue show the perineural invasion. (× 40 H&E). (e) Surrounding gastric cancer cells are stained with cytokeratin, in sharp contrast to the neural tissue without such staining. (× 40 Cytokeratin). (f) Cancer cells derived from the Mongolian gerbil gastric cancer tissue, before single colony dilution cloning. (× 80 H&E).

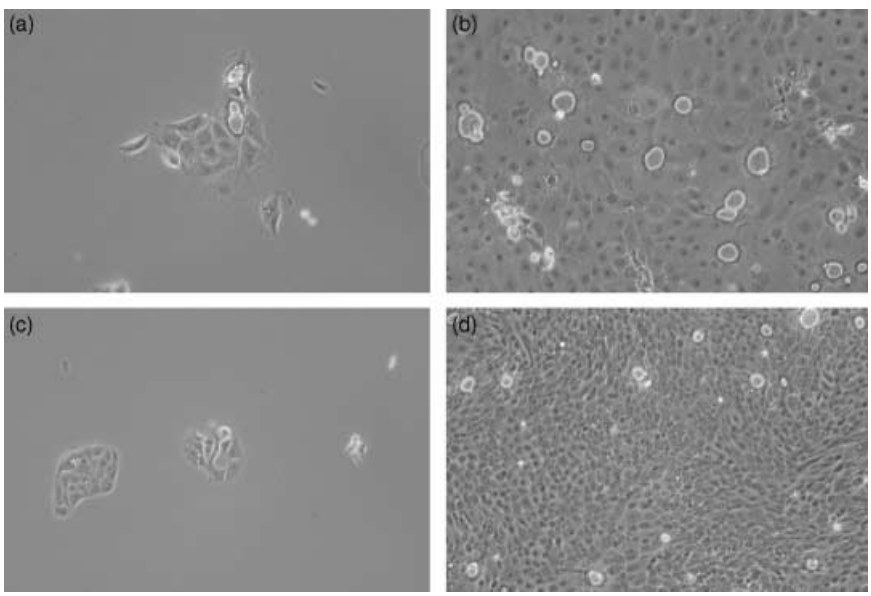

Fig. 2. (a) MGC1 cells derived after single-cell dilution cloning. MGC1 cells show large cytoplasm with a high nucleus/cytoplasm ratio. (× 80). (b) MGC1 cells at confluence show a monolayer sheet of polygonal-shaped epithelial cells. (× 80). (c) MGC2 cells derived after single-cell dilution cloning. They reveal rather small cytoplasm and a much higher nucleus/cytoplasm ratio. (× 80). (d) MGC2 cells at confluence with small cytoplasm and higher growth activity. (× 80).

passages, without further changes in morphology or growth rate.

Morphology of MGC cells in culture. The subconfluent culture of MGC1 cells shows a rather large cytoplasm with a monolayer sheet of polygonal-shaped epithelial cells with a rather high nucleus/cytoplasm ratio. The subconfluent culture of MGC2 cells, on the other hand, reveals a rather small cytoplasm, and much higher nucleus/cytoplasm ratio growing with a cobblestone appearance and rosette formation (Fig. 2a–d). MGC2 cells had 3–4-fold greater growth activity compared to MGC1 cells.

Tumorigenicity in nude mice. Inguinal subcutaneous transplantation of MGC1 and MGC2 cells was attempted using five nude mice. When MGC1 cells were inoculated s.c. into the nude mice, the cells grew slowly to form mainly cystic tumors, which were detected within 4 weeks following inoculation. Four of the five transplanted lesions evidenced stable transplantation, showing cystic lesions consisting of a monolayer of MGC1 cells. Histologically, the tumors were comprised of rather large, pleomorphic, hyperchromatic cells (Fig. 3a–c). Transplanted MGC2 cells show rather high invasiveness of the host tissue, and perineural invasion has been found. Inoculated MGC2 cells grew rapidly to form subcutaneous solid tumors that were detected within 2 weeks after inoculation. In all mice the MGC2 cells showed stable transplantation and

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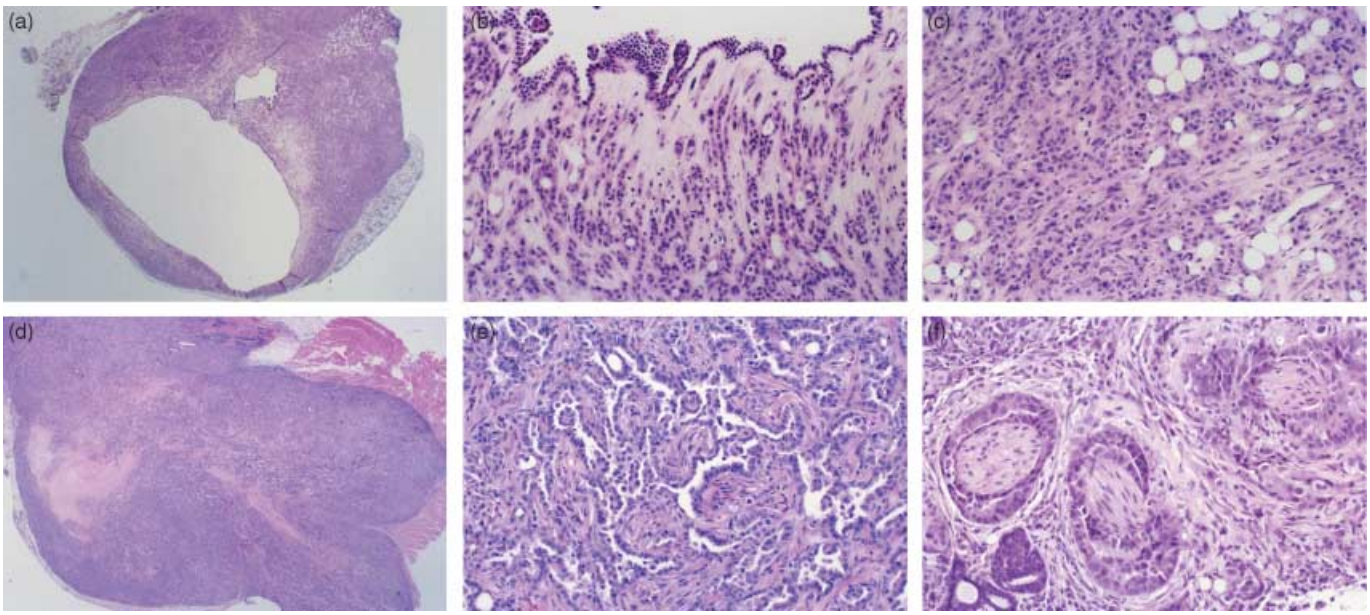

Fig. 3. (a) MGC1 cells transplanted subcutaneously into nude mice grew slowly to form cystic tumors, consisting of a monolayer of rather large, pleomorphic, hyperchromatic cells. (× 10 H&E). (b) High-power view of Fig. 3a. Cystic tumor formation with monolayer of MGC1 cells. (× 40 H&E). (c) High-power view of Fig. 3a. Tumor formation with MGC1 cells. (× 40 H&E). (d) MGC2 cells transplanted subcutaneously into nude mice grew with stable transplantation and glandular-like configurations. The tumors were composed of rather small, pleomorphic, hyperchromatic cells arranged in a solid pattern. (× 10 H&E). (e) High-power view of Fig. 3d. Tumor tended to form glandular-like constructs with MGC2 cells. (× 40 H&E). (f) Transplanted MGC2 cells with perineural invasion. MGC2 cells grew invasively under the transplanted tissue, with perineural invasion and invasion to the muscle layer. (× 40 H&E).

glandular-like configurations. The tumors were composed of rather small, pleomorphic, hyperchromatic cells arranged in a solid pattern (Fig. 3d–f). They grew invasively under the transplanted tissue, evidencing perineural invasion of the muscle layer (Fig. 3f).

Stimulation of cells with recombinant IL-1 beta. To assess the reactivity of MGC cells to pro-inflammatory cytokines, the phosphorylation status of signaling molecules was evaluated in MGC cells with recombinant human IL-1β 10 ng/mL during different time periods. IL-1β stimulation resulted in increased phosphorylation of IκB, Erk1/2 (p44/42 MAPK), and AKT (Fig. 4). Three independent experiments with recombinant human IL-1β 10 ng/mL activation gave essentially the same results. As shown in Fig. 4, maximal phosphorylation of IκB was seen 5 min after stimulation of IL-1β, then gradually decreased but was re-up-regulated 45 min after stimulation. Maximal phosphorylation of Erk1/2 was observed at 10 and 15 min, and the level gradually decreased with time. By comparison, increased phosphorylation of Akt was seen at 5 and 10 min, and remained elevated at the endpoint.

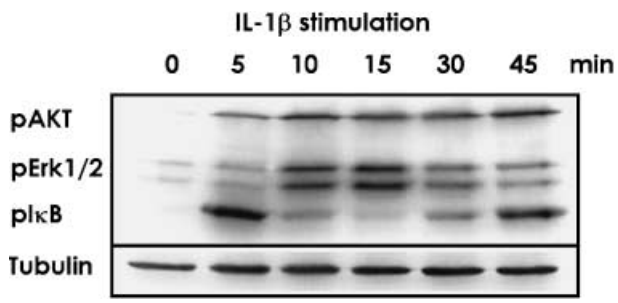

Fig. 4. MGC cells were stimulated with recombinant IL-1β 10 ng/mL. Phosphorylation status of pIκB, pErk1/2 and pAKT was confirmed with western blotting. Anti-α Tubulin was treated as internal control. Maximal phosphorylation of IκB was seen 5 min after stimulation of IL-1β, then gradually decreased, but was re-up-regulated 45 min after stimulation. Maximal phosphorylation of Erk1/2 was observed at 10 and 15 min, and the level gradually decreased with time. By comparison, the increased phosphorylation of Akt was seen at 5 and 10 min, and was still elevated at the endpoint.

***H. pylori* attached to MGC cell and induced NFkB activation.** To study NF-κB activation following *H. pylori* infection on these newly established rodent gastric cancer cells, a luciferase reporter gene under the control of the *cis*-acting enhancer element NF-κB (NF-κB-luciferase) was used. Initial experiments were performed in triplicate with the established cells 24 h after cotransfection with 1 μg of pNF-κB luciferase and 0.1 μg of pRL-CMV, and activation with culture media with and without the presence of 10 ng/mL lipopolysaccharide. A 1.7-fold higher transcriptional activation was seen in both MGC1 and MGC2 cells.

The cells cotransfected with pNF-κB luciferase and pRL-CMV were challenged to be infected with NCTC11637 or its isogenic *cagE* knockout mutant (*cagE* KO) *H. pylori* for 12 h. Stable attachment on these cells with *H. pylori* NCTC11637 or its isogenic mutant could be observed with a scanning electron microscope (Fig. 5). Results in Fig. 6 show the representative results of three separate experiments conducted under each condition in triplicate. Elevated transcriptional activation of NF-κB was induced after infection with NCTC11637, while infection with *cagE* KO *H. pylori* did not show such an effect (Fig. 6). The *cagE* KO *H. pylori* strain has a breakdown of the Type IV secretion system, and is considered to lack the ability to inject CagA protein into the attached host cells. These results indicate that the results in transcriptional activation of NF-κB in MGC cells differ in the infected *H. pylori*-dependent manner.

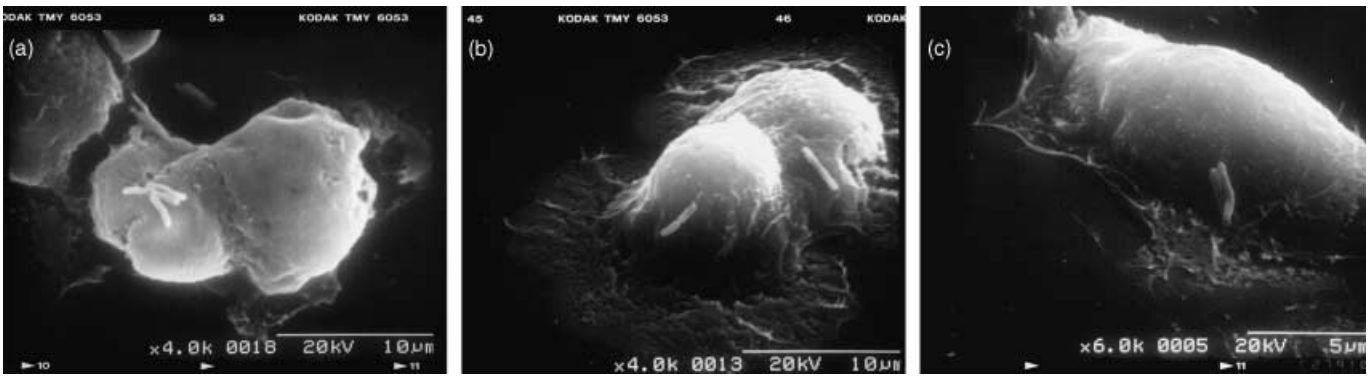

Fig. 5. (a) Scanning electron micrograph (SEM) of MGC cell infected with *H. pylori*. (× 4000). (b) SEM showing MGC cell attached with *H. pylori* on the cell surface. (× 4000). (c) SEM showing MGC cell attached with *H. pylori*. (× 6000).

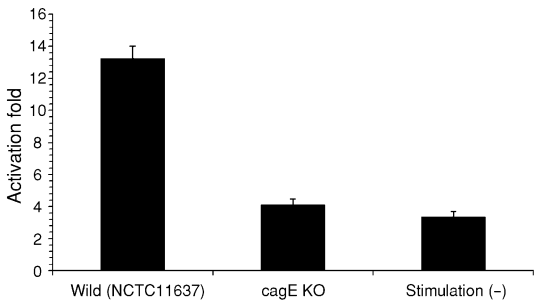

Fig. 6. Transcriptional activation of NF-κB (Luciferase assay on NF-κB activation after being infected with NCTC11637 or its isogenic *cagE* knockout mutant (*cagE* KO) *H. pylori*). Elevated transcriptional activation of NF-κB was induced after infection with NCTC11637, while infection with *cagE* KO *H. pylori* did not show such an effect. Results shown are the average and standard error of three independent experiments.

Discussion

We succeeded in the establishment and initial characterization of adenocarcinoma cell lines (MGC1 and MGC2) from the gastric cancer tissue of a *H. pylori*-infected and MNU-treated Mongolian gerbil. This is the first report of gastric cancer cells established from a Mongolian gerbil, and the cell lines established may be of potential use in the study of *H. pylori*-induced gastric cancers. These cells can easily be cultured, and when the cells were inoculated s.c. into the nude mice, they showed stable transplantation to nude mice and grew firmly, forming solid or cystic tumors. Transplanted MGC2 cells show rather high invasiveness of the host tissue with perineural invasion. Inoculated MGC2 cells grew rapidly to form subcutaneous solid tumors that were detected within 2 weeks after inoculation. Stable attachment on these cells with *H. pylori* could be observed with scanning electron microscope analysis. The hummingbird phenotype was not clearly observed in the electron microscope analysis; however, this phenotype can differ with the infected *H. pylori* status or depending on the type of cell line.[21] The cells under study here can also be easily infected with *H. pylori* ATCC43504 or the Sydney strain of *H. pylori*. Furthermore, the transcriptional activation through NF-κB was apparently elevated in the infected *H. pylori*-dependent manner.

These established cell lines are unique for the following three reasons. First, they are the first gastric cancer cell lines established in the Mongolian gerbil, a candidate for an ideal *H. pylori*-related gastric carcinogenesis model.[24] The derived cells were easily infected with *H. pylori* and could be used for examinations of the influences after *H. pylori* infection.

Second, these cell lines can be used to explore the molecular and cellular biological investigations on the basic mechanisms of glandular cell proliferation. The NF-κB and the signaling pathways that are involved in its activation have great importance for tumor development.[25] In the present study, treatment with recombinant IL-1β attenuated the phosphorylation status of the IκB/NFκB, MAPK, and the PI3K/Akt pathways. α-Tubulin was used as an internal control, and the behavior of phosphorylated AKT, ERK1/2, and IκB were clarified. As a result, the activation of pIκB had distinct peaks at 5 and 45 min in the time course. This may indicate a fluctuation resulting from cross-talk with other signaling pathways such as AKT or ERK, or from the negative feedback of the IκB – NF-κB signaling cascade. Cross-talk of these signaling pathways, arising from the cell-to-cell interactions between epithelial, mesenchymal, and inflammatory cells, might also be examined from the viewpoint of introducing cancer progression. Furthermore, infection with *H. pylori* clearly attenuated the transcriptional activation of NF-κB in these cancer cells, suggesting that the causal effect of *H. pylori* on the cancer cell progression might be mediated not only by Ras/MAPK but also with the NF-κB signaling pathway, partly mediated with the epithelial–mesenchymal interactions.

Third, the newly derived two cell lines exhibit markedly different tumorigenesis and invasive activities into the skin, muscle layer, and perineural cavities of the transplanted tissue. They appeared to show a clearly different morphology associated with the invasive activity in the transplanted tissue. One forms a cystic lesion, with lesser invasion, while the other tends to form expansive tumors with diffuse invasion. The two lines were established from the same gastric carcinoma specimen, and are of the same clonal origin. The cells were stable in terms of both morphology and proliferation after being separated into the two lines. Therefore, it may be appropriate to consider the lines to have been derived from heterogenous cell populations contained in the primary cancer; for example, differentiated and undifferentiated components of the adenocarcinoma, rather than the two types differentiating during the process of establishing the two lines. Searching for the factors that determine the differences between the two might be meaningful. Gene expression analysis might provide some information to explain such morphological and biological differences.[26–29] Since these cell lines are not immortalized cells but derived from actual tumors, they are sure to have properties that reflect the characteristics contained in the original tumor tissue.

The newly established adenocarcinoma cell lines from the *in vivo* gastric carcinogenesis model showed response to *H. pylori* infection or IL-1β stimulation, implying that continuous *H.*

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pylori infection can affect the cell proliferation of the induced gastric cancer. In fact, NF-κB activation was induced with *H. pylori* infection, and AKT, ERK, and IκB activation were observed as a result of IL-1β stimulation. Thus, proliferative response of the cell to *H. pylori* infection should be considered, in terms of the direct actions of *H. pylori* on cancer cells, and the indirect actions through cytokine stimulation by inflammatory cells.

In conclusion, new adenocarcinoma cell lines were purified from the Mongolian gerbil, the standard *H. pylori*-related gastric cancer model. The lines can be used to develop effective therapeutic strategies against gastric cancer, especially in exploring the effect of *H. pylori*, and thus might contribute greatly to gastric cancer prevention and treatment in humans.

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