Establishment and Characterization of Two Cell Lines from N-Methyl-N-nitrosourea-induced Mouse Glandular Stomach Carcinomas

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We previously reported the induction with N-methyl-N-nitrosourea (MNU) of mouse glandular stomach carcinomas showing a gastric phenotype but variation in histologic appearance, as with human gastric carcinomas. In the present study, we established two cell lines, designated MGT-40 and MGT-93, from MNU-induced mouse glandular stomach carcinomas. These cell lines are keratin-positive and grow as epithelial monolayers in culture, requiring transforming growth factor α, epidermal growth factor or insulin/transferrin for optimal growth in addition to 10% fetal bovine serum. Retention of the differentiated phenotype for gastric surface mucous cells has been confirmed by cathepsin E immunohistochemistry and reverse transcriptase-polymerase chain reaction for mouse spasmolytic polypeptide. Neither transplantability in nude mice nor colony formation on soft agar was observed, except in one subline. Chromosome analysis revealed aneuploidy with modal chromosome numbers ranging from 58 to 78 and no specific structural abnormalities. This is the first report of cell lines derived from mouse glandular stomach carcinomas. They should prove useful for studies of the mechanisms of regulation of growth and differentiation.

Key words: Mouse cell line — N-methyl-N-nitrosourea — Cathepsin E — TGFα — Glandular stomach carcinoma

Human gastric carcinomas are extremely heterogeneous with regard to histology and differentiation. A large bank of cultured cell lines reflecting this diversity of tumor phenotypes may thus be necessary to elucidate underlying mechanisms. Establishment of gastric epithelial cell lines, however, is limited by culturing difficulties and while some have been obtained from human stomach carcinomas,1–4 most were derived from metastatic lesions in the peritoneal cavity, lymph node and liver and were relatively undifferentiated. Primary culture of fetal5 and adult normal rat gastric epithelium has been used for functional studies of tissue-specific products, but no immortalized cell lines have been reported except for one from N-methyl-N′-nitro-N-nitrosoguanidine-induced rat gastric carcinoma.6 With regard to the mouse stomach, an immortalized gastric surface epithelial cell line was established from transgenic mice harboring SV40 large T-antigen gene,7 but counterparts from glandular stomach adenocarcinomas have not been reported.

We previously described induction of mouse glandular stomach carcinomas by N-methyl-N-nitrosourea (MNU).8 We here report establishment of two cell lines from two relatively early mouse glandular stomach carcinomas and we describe their growth and differentiation characteristics as determined from immunohistochemical and reverse transcriptase-polymerase chain reaction (RT-PCR) studies.

Materials and Methods

Source of cell lines Glandular stomach carcinomas were induced in mice by administration of MNU as described previously.9 Briefly, male 7-week-old BALB/c mice were given MNU in their drinking water at a concentration of 120 ppm for 24 weeks and then returned to normal tap water. BDF1 strain animals similarly received the MNU solution as their drinking water for 18 weeks and then returned to normal tap water. At the end of the fifty-second experimental week, animals were killed and autopsied under ether anesthesia. Two independent glandular stomach carcinomas were induced in BALB/c and BDF1, designated MGT-40 and MGT-93, respectively, were selected for culture.

Cell culture Both tumors were small flat lesions, 3–5 mm in diameter and localized in the pylorus. Fresh tumor tissues were cut into small pieces and minced in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical, Tokyo) with sterile scissors and washed with DMEM. The tumor pieces were treated with Dispase (50 U/ml; Godo Shusei, Tokyo) for 30 min at 37°C and large tumor pieces were allowed to settle. Supernatant fluid containing cell clumps was collected after centrifugation at 100g and cell pellets were resuspended in DMEM containing 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY) plus serum expander MITO (0.1%, Collaborative Biomedical Products, Bedford, MA), 100 units/ml penicillin, and 100 µg/ml streptomycin (GIBCO) and cul-
tured on dishes coated with collagen type I (Iwaki, Tokyo) in a humidified 5% CO₂ incubator at 37°C with weekly changes of the medium. After one month, growing colonies were harvested with trypsin/EDTA and passaged several times. Any remaining fibroblasts were removed by mechanical scraping and a differential attachment selection method, after which cultures of MGT-40 and MGT-93 cells forming typical epithelial monolayers were obtained. MGT-40 E-1, E-16 and E-25 cells were obtained from MGT-40 cells by single-cell dilution cloning using 96-well cellulture plates. MITO contains insulin, transferrin, epidermal growth factor (EGF), endothelial growth supplement, dexamethasone, triiodothyronine, hydrocortisone, progesterone, testosterone, and 17β-estradiol. The absence of *Mycoplasma pulmonis* contamination of MGT cell lines was confirmed by a direct agar method. The absence of two viruses (mouse hepatitis virus and Sendai virus) was also confirmed by a PCR method with the assistance of the Experimental Animal Research Center of Japan (Tokyo).

**Growth characteristics in vitro** Cells were plated at 1×10³ cells/dish in 35 mm plastic dishes, in DMEM supplemented with 10% FBS plus MITO. The cell number was counted in triplicate with a hemocytometer 4 days after seeding. To compare cell proliferation on collagen-coated and plastic dishes, 1×10³ cells/dish were plated in 35 mm dishes of both types, in DMEM with 10% FBS in the absence of MITO. The numbers of cells were counted in triplicate 3 days after seeding. To evaluate the influence of growth factors on cell proliferation in the cell lines, DMEM with 10% FBS in the absence of MITO was used as a control medium. Cells 1×10³ of each line were plated into 35 mm plastic dishes in control medium alone or supplemented with various growth factors, and the numbers of cells were counted 4 days thereafter. Concentrations of growth factors used were; insulin/transferrin (each, 5 µg/ml), EGF (10 ng/ml), transforming growth factor α (TGFα, 10 ng/ml). All the reagents were obtained from Sigma (St. Louis, MO).

**Analysis of differentiated phenotype by immunohistochemistry** Portions of the original primary tumors induced by MNU were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4 µm. Cells cultured for more than 40 generations were harvested with trypsin/EDTA and auto smears were made by centrifugation with a Cytospin (Sakura Seiki, Tokyo). To examine cellular differentiation, tissue slices and Cytospin preparations were examined for immunohistochemistry and mucin histochemistry. For mucin histochemistry, paradoxical concanavalin A (class III mucin staining)⁹ and galactose oxidase-Schiff (GOS)¹⁰,¹¹ staining techniques were performed as described previously. For immunohistochemistry, anti-pepsinogen isozyme 1 (Pg1)¹² and anti-cathepsin E¹³ antibodies were prepared as described previously. The polyclonal anti-Pg1 antibody was kindly donated by Dr. Chie Furihata (Department of Molecular Oncology, Institute of Medical Science, University of Tokyo, Tokyo) and polyclonal anti-cathepsin E antibody was also kindly donated by Dr. Kazumasa Miki (First Department of Internal Medicine, Faculty of Medicine, University of Tokyo). Tissue sections were deparaffinized, rehydrated, and incubated with fresh 3% hydrogen peroxide in methanol. Smears were fixed in cold methanol for 5 min. Specimens were treated sequentially with normal goat or horse serum, primary antibodies, rabbit anti-rat Pg1 (1:15,000) or rabbit anti-rat cathepsin E (1:10,000), secondary antibodies, biotin-labeled goat anti-rabbit or horse anti-mouse immunoglobulin G, avidin-biotin-peroxidase complex.¹⁴ The sites of peroxidase binding were visualized using diaminobenzidine. Sections were lightly counterstained with hematoxylin for microscopic examination.

**Analysis of differentiated phenotype by RT-PCR** Expression of mouse spasmyloytic polypeptide (mSP), a marker of surface mucous epithelial cells of the stomach, was examined by nested primer RT-PCR. Total RNA was extracted from cells cultured for more than 40 generations by the modified acid guanidinium isothiocyanate-phenol-chloroform method using ISOGEN (Nippon Gene, Tokyo) reagents according to the manufacturer’s instructions. To remove any contaminating DNA, the extracted total RNA preparation was treated with RNase-free DNase 1 (Boehringer Mannheim, Mannheim, Germany). First-strand cDNA was synthesized from purified RNA using SuperScript II reverse-transcriptase (GIBCO BRL) and the target cDNA sequence was amplified in a reaction mixture (10 µl) at pH 8.8 containing 50 mM KCl, 1.5 mM MgCl₂ and 10 mM Tris-HCl, 0.1 µM of each primer, 0.1 mM dNTP, 0.5 units of Taq DNA polymerase (Stratagene, La Jolla, CA), and 1 µl of template cDNA. Primer sequences for mSP were as follows; outer sense, 5'-GAG AAA CCT TCC CCC TGT CGG-3', outer antisense, 5'-TGG GAA GAA ACA CCA GGG-3', inner sense, 5'-TGG CTG AGG CTG ACA CCCCAC-3', inner antisense, 5'-CTG TGG GAA GAA ACA CCA GGG-3'. nested-primer PCR was performed with 40 cycles in a thermal cycler (MJ Research Inc., Watertown, MA) consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. Mouse stomach was used as a positive control. For mRNA expression of pepsinogen C in two cell lines, northern blot analysis was performed as described previously.¹⁵ cDNA probe was kindly donated by Dr. Kenji Takahashi (School of Life Science, Tokyo University of Pharmacy and Life Science, Tokyo).

**Tumorigenesis and anchorage-independent growth** To examine tumorigenesis of the cell lines, aliquots of 1×10⁷
cells were resuspended in 0.2 ml of Hanks' balanced salt solution and injected subcutaneously into nude mice. Development of subcutaneous tumors was assessed 2 months after injection. To examine colony-forming ability in soft agar, a bottom layer of 0.6% agar was prepared with complete medium and Bacto-Agar (Difco Laboratories, Detroit, MI), placed in plastic dishes, and allowed to solidify. Cell suspensions of 3,000 viable cells/ml in complete medium were prepared and agar was added to each suspension (1:1) to form a 0.3% agar solution. One milliliter of each agar/cell solution was placed over the lower layer of agar, allowed to solidify, and incubated at 37°C in a 5% CO₂ atmosphere. Colony formation was determined by phase contrast microscopy 2–4 weeks after seeding.

**Chromosome analysis** Chromosome analysis was performed on metaphase cells. Cells were treated with 0.01–0.02 µg/ml colcemid for 2 h and subjected to hypotonic conditions for 30 min, then fixed in methanol : acetic acid (3:1). Air-dried slides were G-banded for analysis. At least twenty metaphases were examined for each cell line.

**Statistical analysis** The statistical significance of differences in growth was analyzed by using Student’s t test, with \( P<0.05 \) as the criterion of significance.

**RESULTS**

**Original adenocarcinomas in glandular stomach of mice** Incidences of MNU-induced glandular stomach carcinomas induced by these experimental protocols were 32.3% (10/31) and 36.4% (8/22) respectively, in BALB/c and BDF1. Primary MGT-40 and MGT-93 tumors induced in glandular stomachs of mice were well differentiated and poorly differentiated tubular adenocarcinomas, respectively (Figs. 1A and 2A). The depth of invasion was the submucosa and mucosa, both being classified as early gastric carcinomas. Immunohistochemically in the MGT-40 tumor, GOS and cathepsin E were cytoplasmically positive and class III mucin partly positive, but Pg1 was negative (Fig. 1, B and C). In the MGT-93 tumor, GOS and cathepsin E were cytoplasmically positive, and class III mucin was negative in poorly differentiated portions but partly positive in moderately differentiated portions, while Pg1 was negative (Fig. 2, B and C). Therefore the gastric phenotype of the original tumors was mainly surface mucous and partly pyloric glandular in both tumors.

**Morphological characteristics of cell lines** The sublines E-16 and E-25 of MGT-40, and the MGT-93 line formed typical epithelial monolayers while the subline E-1 of MGT-40 exhibited an elongated shape (Fig. 3, A–D). They
Fig. 2. Light micrographs of the primary MGT-93 (A–C) tumor induced by MNU, stained with HE (A), anti-cathepsin E antibody (B) and class III mucin (C). The lesion is a poorly differentiated adenocarcinoma. ×200.

Fig. 3. Morphology of the established E-1 (A), E-16 (B), E-25 (C) sublines of MGT-40 and MGT-93 (D) cell lines grown on collagen-coated dishes. Subline E-1 of MGT-40 is spindle-shaped in morphology and the other cell lines form typical epithelial monolayers.
were all keratin-positive although staining of the E-1 subline was relatively weak (data not shown).

In vitro growth characteristics In the presence of MITO, the relative growth rates of the cell lines on plastic dishes were MGT-40, E-1 > E-16 > E-25 > MGT-93 (Fig. 4A). Growth of MGT-93 was very slow and the cell number only doubled during 4 days of culture. In DMEM containing 10% FBS without MITO, no substantial increase in cell number of the cell lines on plastic dishes was observed for any of the cell lines. However, on collagen-coated dishes, sublines E-1, E-16 and E-25 of MGT-40, but not MGT-93, proliferated significantly (Fig. 4B). Growth of all cell lines was significantly stimulated by TGFα (P<0.01), EGF (P<0.01) and, to a lesser extent, insulin/transferrin (P<0.05) (Fig. 5). The growth responses to EGF and TGFα were dose-dependent (data not shown).

Immunohistochemical analysis of cell line phenotypes Since both primary tumors were GOS- and cathepsin E-positive, we examined whether this gastric phenotype was preserved in the established cell lines. Immunohistochemical analysis of cytospin preparations showed the E-25 subline of MGT-40 and the MGT-93 cells to be still cathepsin E-positive (Fig. 6), but negative for GOS as well as class III mucin and Pg1, indicating partial retention of expression of the gastric surface mucous cell phenotype.

RT-PCR analysis of cell line phenotypes Next we examined expression of mSP, a marker of gastric surface mucous cells, at the mRNA level by RT-PCR. The results showed only subline E-25 of MGT-40 to be positive. Enhancement of mSP expression was observed upon removal of MITO from the culture medium, which
resulted in growth inhibition of the cells (Fig. 7). mRNA expression of pepsinogen C could not be detected in any cell lines on northern blot analysis (data not shown).  

Tumorigenicity As shown in Table I, only subline E-1 of MGT-40 formed a tumor in subcutaneous tissue in nude mice with a 33% incidence (1/3). The transplanted tumor was undifferentiated carcinoma (data not shown). This subline E-1 also demonstrated colony-forming ability in soft agar. None of the other cell lines were tumorigenic in nude, SCID or syngeneic mice.

Chromosome analysis The sublines E-1, E-16 and E-25 of MGT-40 were near-triploid with modal chromosome numbers of 66, 58 and 66, respectively. MGT-93 cells were hypotetraploid with 78 XY. Most cells did not contain apparent derivative chromosomes whose origin could not be identified or specific structural abnormalities.

DISCUSSION

Several experimental gastric carcinoma models in rats, hamsters, ferrets and dogs have been reported. The mouse is a particularly useful species for carcinogenesis studies because of the availability of a number of transgenic, mutant, and chimeric strains. Recently, using MNU, we induced mouse experimental gastric carcinomas, which proved more similar to human gastric cancers in terms of their heterogeneous histopathologic features than those typically found in other species. In the present study, we succeeded in establishing two cultured cell lines from relatively early lesions. They are unique for the following reasons. 1) They are the first established cultured cell lines derived from mouse glandular stomach carcinomas; the only previous report con-
cerned an immortalized gastric surface mucous cell line from a transgenic mouse harboring the SV40-large T antigen. They partially retain a differentiated gastric phenotype, in contrast to human gastric carcinoma cell lines, most of which are negative for the specific mucin histochemical and immunohistochemical markers of gastric phenotypes. 3) They show mitogenic responses to EGF and TGF\(\alpha\), which have been implicated in the growth control of gastric carcinoma cells.

Retention of the gastric epithelial phenotype was evidenced by the fact of immunohistochemical staining for cathepsin E and mSP expression at the mRNA level. Cathepsin E is an aspartic proteinase, present in stomach, like pepsinogens, and is localized in the surface mucous cells and pyloric gland cells. It is also present in lymphoid tissues such as the thymus, as well as blood cells. mSP is a trefoil peptide, secreted predominantly by gastric mucous cells, and a marker for mucin production. The lack of immunohistochemically detectable pepsinogen isozyme I and class III mucin in the cell lines indicates a surface mucous cell rather than a pyloric gland cell phenotype. The original tumors from which these cell lines were derived expressed not only cathepsin E, but also weakly GOS and partly class III mucin. Loss of GOS and class III mucin expression in cultured cells may occur during establishment of cell lines and may be in line with a phenotypic shift from gastric type to null cell type and subsequently intestinal type with advancing stage, as observed in both human and rat gastric carcinomas.

Interestingly, weak mSP expression of E-25 subline was enhanced to some extent by removal of MITO from the culture medium. This suggests that this subline can differentiate into surface mucous cells on cessation of division. Further studies using insulin, which is known to stimulate mucin production in some cell lines, or differentiation-inducing agents such as sodium butyrate and dimethyl sulfoxide are needed to clarify the optimal conditions for inducing such differentiation.

The requirement of MGT-40 and MGT-93 cell lines for EGF, TGF\(\alpha\), and insulin for optimal growth in addition to 10% FBS and Type I collagen as a substrate indicates that they do not possess growth autonomy. TGF\(\alpha\), a natural ligand for EGF receptors in the stomach, is known to control growth of normal rabbit and canine fundic epithelial cells, and guinea pig gastric mucous cells in primary culture. Yasui et al. reported that EGF and/or TGF\(\alpha\) produced by tumor cells act as autocrine growth factors for advanced gastric carcinomas. The lack of growth autonomy observed in these cell lines, in contrast to various human gastric carcinoma cell lines, presumably reflects their origin from early gastric carcinomas. To elucidate the role of growth factors in these cell lines, their expression of EGF, TGF\(\alpha\) and EGF receptors needs to be examined in detail.

Only subline E-1 of MGT-40 formed a tumor in a nude mouse, and the tumor was histologically an undifferentiated carcinoma. The subline E-1 might have originated from a mesenchymal cell based on its morphology in culture, but was judged to have a stomach carcinoma origin because its chromosome number was almost the same as those of the other sublines and the keratin staining of cultured cells was weakly positive.

In conclusion, our cell lines established from MNU-induced mouse glandular stomach carcinomas demonstrate unique characteristics in terms of differentiated phenotype, responsiveness to growth factors and genetic alterations. Although these cell lines are not tumorigenic in mice, they may be useful for \textit{in vitro} analysis of the regulatory mechanisms of growth and differentiation of gastric carcinoma.

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nitrosoguanidine. *Cancer Res.*, **43**, 1335–1341 (1983).

25) Sauma, S., Huang, F., Winawer, S. and Friedman, E. Colon goblet cells lose proliferative response to TGF alpha as they differentiate. *Int. J. Cancer*, **61**, 848–853 (1995).

26) Yoshida, S., Kasuga, S., Hirao, Y., Fuwa, T. and Nakagawa, S. Effect of biosynthetic human epidermal growth factor on the synthesis and secretion of mucin glycoprotein from primary culture of rabbit fundal mucosa cells. *In Vitro Cell Dev. Biol.*, **23**, 460–464 (1987).

27) Chen, M. C., Lee, A. T. and Soll, A. H. Mitogenic response of canine fundic epithelial cells in short-term culture to transforming growth factor alpha and insulin-like growth factor I. *J. Clin. Invest.*, **87**, 1716–1723 (1991).

28) Rattner, D. W., Ito, S., Rutten, M. J. and Silen, W. A rapid method for culturing guinea pig gastric mucous cell monolayers. *In Vitro Cell Dev. Biol.*, **21**, 453–462 (1985).

29) Yasui, W., Hata, J., Yokozaki, H., Nakatani, H., Ochiai, A., Ito, H. and Tahara, E. Interaction between epidermal growth factor and its receptor in progression of human gastric carcinoma. *Int. J. Cancer*, **41**, 211–217 (1988).