CHARACTERIZATION AND APPLICATION OF A Gastric Surface Mucous Cell Line GSM06 ESTABLISHED FROM TEMPERATURE-SENSITIVE SIMIAN VIRUS 40 LARGE T-ANTIGEN TRANSGENIC MICE

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ABSTRACT—It has been indicated that transgenic mouse harboring a temperature-sensitive simian virus 40 large T-antigen gene is useful for establishing cell lines from tissues that have proved difficult to culture in vitro. The gastric surface mucous cell line GSM06 was established from a primary culture of gastric fundic mucosal cells of the transgenic mice. GSM06 cells showed temperature-sensitive growth in culture and expressed large T-antigen at a permissive temperature (33°C) but not at a nonpermissive temperature (39°C). At 39°C, the cells produced periodic acid-Schiff positive glycoconjugates that formed a mucous sheet like the gastric surface mucosa in the stomach. Insulin markedly increased the production of glycoconjugates. In addition, proprotein-processing endoprotease furin suppression retarded cell growth, but accelerated cell differentiation. An air-liquid interface promoted the differentiation of GSM06 cells in a reconstruction culture with nitrocellulose membrane and collagen gel. The gastric surface mucous cell line GSM06 with unique characteristics, therefore, should be useful as an in vitro model of the gastric mucosa for physiological and pharmacological investigations. Moreover, experiments using immortalized cells established in vitro and having specific functions may offer an alternative to experiments using living animals and thereby offer a solution to this ethical issue.

Keywords: GSM06, Transgenic mouse, Gastric surface mucous cell, Temperature-sensitive simian virus 40 large T-antigen, Cell differentiation

Gastric mucosal cells protect the stomach from various aggressive factors such as gastric acid, digestive enzyme and pathogens. It has been shown that the cells produce protective factors including glycoproteins, bicarbonate and prostaglandins (1, 2). The use of in vitro gastric mucosal cell culture systems has been of central importance in the development of cellular and molecular biology of gastric mucosa. Although there have been many reports on the primary culture of normal gastric mucosal cells (3, 4), almost all of these cell cultures rapidly lose their differentiating phenotype. On the other hand, although there are a large number of cell lines derived from gastric carcinomas (5 – 7), only a few of these retain any of the properties of the gastric mucosa. Some viral and cellular oncogenes have the ability to establish continuous proliferation in primary culture cells (8). Previous papers indicated that stabilization of cell type-specific functions in various immortalized cell lines was introduced by the simian virus 40 (SV40) large T-antigen gene (9, 10). It has also been shown that immortalization of cells due to a temperature-sensitive simian virus 40 (tsSV40) large T-antigen gene results in more or less stable cell type-specific functions and that the oncogene product is rapidly degraded at the nonpermissive temperature (39°C) but functions at the permissive temperature (33°C) (11). Moreover, Yanai et al. (12) and Jat et al. (13) reported that transgenic mice that harbor the tsSV40 large T-antigen gene are useful for establishing cell lines from tissues that have proved difficult to culture in vitro. So far, several investigators have established various cell lines with specific functions from different organs by using transgenic mice (14); e.g., hepatocyte cells (12), renal cells (15), colonic epithelial cells (16), tracheal epithelial cells (17), bone...
marrow stromal cells (18, 19), vascular smooth muscle cells (20, 21), lung epithelial cells (22) and vestibular hair cells (23). Recently, we succeeded in establishing the gastric surface mucous cell line GSM06 from a primary culture of gastric mucosal cells of transgenic mice harboring the tsSV40 large T-antigen gene (24). The cell line, interestingly, produced mucous layers on the cell surface such as normal gastric mucosa (24–31). To our knowledge, no gastric mucosal cells in culture produce mucus layers on the cell surface except GSM06 cells. In this review, I would like to introduce the characterization and application of gastric surface mucous cell line GSM06 revealed by these studies to date.

Transgenic mouse harboring tsSV40 large T-antigen gene
Transgenic mice harboring the tsSV40 large T-antigen gene derived from temperature-sensitive A58 were produced by the injection of BamHI DNA fragments of whole tsSV40 large T-antigen gene DNA (pSVtsA58) into the pronuclei of fertilized eggs of C57BL/6 mice (11). To express ubiquitously among different tissues in the body, the promoter of the SV40 large T-antigen gene itself was used. The transgenic mice induced colloid plexus tumor and died within 5 months.

Establishment and identification
Mouse gastric fundic mucosal cells were isolated from the stomachs of the transgenic mice by pronase E digestion and cultured in Dulbecco’s modified Eagle medium/ Ham F-12 (1:1) medium supplemented with several growth factors in a collagen-coated plastic culture dish at 33°C (24). The cells exhibited high proliferating activity and formed monolayers within a few days. After this, however, for a period of 6 weeks, the cells stopped proliferating, but then began to proliferate again until confluent monolayers were formed. A similar latent period of growth was observed when immortalized liver (11) and tracheal (17) cell lines were established from transgenic mice. After twice cloning using colony formation, GSM06 cells were established.

GSM06 cells grew until confluent monolayers were formed and showed a foveolar epithelial cell-like morphology. Transmission electron microscopic examination revealed that microvilli-like structures were apparent on the cell surface, tight junctions were observed on the boundaries of the cells and electrondense mucous granules were present in the cytoplasm (28). In addition, glycoconjugate layers produced by GSM06 cells were positively stained by the periodic acid-Schiff (PAS) and class I concanavalin A-horseradish peroxidase methods recognizing both surface mucous and mucous neck cell-synthesized glycoproteins (32). The layers, however, were not stained by the class III concanavalin A-horseradish peroxidase procedure that stained only glycoconjugates produced by mucous neck cells (32). The cells showed neither pepsin activity (marker for chief cell) nor H^+K^-ATPase expression (marker for parietal cell). These results strongly suggest that GSM06 cells originate from the surface mucous cells, not from the mucous neck, chief or parietal cells (24).

Cell growth
GSM06 cells proliferated at 33°C and 37°C with a population doubling time of about 29 h. At 39°C, the cells did not grow, but when the temperature of the culture was lowered to 33°C, cell growth was restored, indicating that this temperature-sensitive growth change is reversible. The large T-antigen was produced in the nuclei of the cells only at 33°C (24). A tsSV40 large T-antigen mutant, tsA58, used in our studies contains a single nucleotide mutation, resulting in a peptide that undergoes a conformational change at nonpermissive temperature, which is thought to inactivate its immortalizing capabilities (10). GSM06 cells did not show colony forming activity in a soft agar gel. However, chromosome analysis showed that the chromosome number in the cells was distributed widely. Although the reason is still unclear, GSM06 cells are, in a sense, transformed in culture in vitro (28).

Production of protective factors: characteristics and these roles
Gastric surface mucous cells line the gastric fundic mucosa, form a mucous gel barrier and secrete bicarbonate or prostaglandins (1, 2). PAS-positive glycoconjugates were time-dependently increased on the cell surface and markedly stimulated by insulin, but not by major acid secretagogues such as histamine, carbachol or gastrin-17 (29). However, the limitation of staining with PAS and paradoxical concanavalin A stain to a few cells in a monolayer suggests that the GSM06 cell line might consist of a heterogeneous population in the process of differentiation (31).

Content of total phospholipids, cholesterol, cholesterol sulfate, total sugar and sialic acid gradually increased with time elapsed. The fatty acid composition of phospholipids revealed increased relative levels of oleic acid in phosphatidylcholine and phosphatidylethanolamine and an increased level of plasmenylethanolamine. The level of dolichylphosphate continued to increase in a time-dependent manner. Glycosylation of various proteins was enhanced from day 7. When GSM06 cells were cultured on a membrane culture vessel, transepithelial resistance between the apical and basolateral sides significantly increased. Moreover, production of prostaglandin (PG)E_2 into culture medium was observed over culture periods. Interestingly, great resistance to ethanol, taurodeoxycholate and acetylsalicylic acid was observed when the cells produced such protective factors (25, 28).
Since GSM06 cells were stained by PAS staining, it was expected the cells would synthesize mucin. Unexpectedly, Goso et al. (26) showed that GSM06 cells produce not mucin, but hyaluronan, by analyses using Sepharose CL-2B chromatography; cesium trifluoacetate equilibrium centrifugation; and trypsin, hyaluronidase and chondroitinase ABC digestion (26). Dohl et al. (25) indicated that the amount and size of mucin-like glycoproteins produced by GSM06 cells were different from those of mucin produced by normal mouse gastric mcosa. It has been shown that the major component of the high molecular weight glycoconjugates produced by tracheal epithelial cells on a plastic surface (undifferentiated condition) was hyaluronic acid, whereas the cells cultures on specific cellular support systems such as collagen matrices (differentiated condition) produced high molecular weight glycoconjugates including mucins and mucin-like glycoproteins (33, 34). Therefore, it is considered that GSM06 cells may still be undifferentiated regarding mucin production. Further studies are needed to find experimental conditions under which GSM06 cells produce mucin.

Recently, RGM1, a rat gastric mucosal cell line, was established from rat glandular stomach (35). The cells had mucus-producing cell characteristics including epithelial-like morphology (35), PAS-positive stain (35) and production of PGE$_2$ (36); and they were used as a normal gastric epithelial cell model (36 – 38). In addition, MKN28 (6, 39, 40), MKN45 (6, 40, 41) and KATO-III (6, 7, 42 – 45), gastric carcinoma cell lines, were commonly used as a gastric mucosal cell model. Characteristics of GSM06 and these gastric cell lines are summarized in Table 1.

**Role of protein-processing endopeptidase furin**

Gastric surface mucous cells form a tubular structure by lining the wall of the gastric pit region and the luminal surface region. The cells originate from the granule-free progenitor cells (stem cells) located at the isthmus of the gastric gland unit. The maturation of the cells is well characterized by the appearance of secretory granules, which are initially absent in stem cells (46). It has been indicated that the proprotein processing endoprotease furin may be one of the enzymes involved in the regulation of cell growth and differentiation in pancreatic β cell line MIN6 (47). Konda et al. (30) found that furin-positive cells were layered around the upper one fourth of the gastric glands (stem cell zone) of adult rats. Moreover, they examined the role of furin in the growth and differentiation of surface mucous cells by using the cell line GSM06. At 33°C, the cells exhibited a high level of furin expression with a negligible level of PAS-positive materials and a low level of transforming growth factor α (TGFα), which was produced in surface mucous cells, but not in stem cells (48). In contrast, at 39°C, the cells produced a high level of PAS-positive materials, TGFα and secretory granules, with a negligible level of furin expression. These results were also confirmed by additional experiments with a sense or an antisense furin cDNA. They therefore concluded that furin is instrumental in controlling the growth of the surface mucous cells (30). More recently, Kamimura et al. (49) reported that furin was expressed specifically in pit-region parietal cells of the rat gastric mucosa. Because GSM06 cells express furin but not H’-K’-ATPase (24), they are not considered to be parietal cells.

**Cell injury models**

Evidence has been accumulated to indicate that lipid peroxidation and peptidoleukotrienes in the gastric mucosa participate, at least in part, in the process of ulcer formation.

### Table 1. Characteristics of GSM06, RGM1, MKN28, MKN45 and KATO-III cell lines

|                  | GSM06                             | RGM1                             | MKN28                          | MKN45                          | KATO-III                        |
|------------------|-----------------------------------|----------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Origin           | tsSV40 TG mouse surface mucous cell (24) | normal rat mucus-producing cell (35) | moderately differentiated tubular adenocarcinoma (6) | poorly differentiated adenocarcinoma (6) | signet-ring cell carcinoma (6, 7) |
| Morphology       | epithelial-like (24)             | epithelial-like (35)             | epithelial-like (6)            | piled-up focus (6)             | suspension (6, 7)               |
| Polarity         | + (28)                           | + (35)                           | + (6)                          | − (6)                          | − (6, 7)                        |
| Carcinogenesis   | − (28)                           | − (35)                           | + (6)                          | + (6)                          | + (7)                           |
| Production of mucus | + (24, 27 – 31) hyaluronan (26) dolichylphosphate, etc. (25) | + (35)                           | + (6)                          | + (6)                          | + (6, 42) mucin (43)             |
| PAS stain        |                                  |                                  |                                |                                |                                |
| Production of prostaglandins | PGE$_2$ (28)                  | PGE$_2$ (36)                     | PGE$_2$ (39)                   | ?                              | − (45)                          |
| Mitogens         | permissive temperature (24)      | FBS (35)                         | FBS (6)                        | FBS (6)                        | FBS (6, 7)                      |
|                  | FBS (29)                         | EGF (35, 38)                     | EGF (40)                       | EGF (40)                       | EGF (44)                        |
|                  | EGF (58)                         | TGFα (35, 38)                    | HGF (41)                       |                                |                                |
|                  | adrenomedullin (37)              |                                  |                                |                                |                                |
| Population doubling time (h) | 29 (24)                             | 16 (35)                         | 28 – 32 (6)                     | 30 – 35 (6)                     | 32 – 36 (6, 7)                 |

Figures in the parentheses indicate reference numbers.
caused by necrotizing agents (50–52). However, in the in vitro gastric epithelial cell system using GSM06 cells, lipid peroxides but not peptidoleukotrienes participate in the development of ethanol-induced cell damage (53). The in vitro cell system and the in vivo injury model were compared by evaluating the effects of ebselen (54), an antioxidative agent, on ethanol-induced gastric mucosal injury in these systems. In the in vivo system with mice, ebselen markedly reduced the necrotic lesions, hemorrhage and edema induced in the stomach by absolute ethanol, but failed to completely inhibit the damage of surface epithelial cells. In addition, this compound significantly decreased the elevated lipid peroxide and peptidoleukotriene levels. On the other hand, in the in vitro system using GSM06 cells, ebselen significantly prevented the cytotoxicity induced by ethanol, although the inhibition percentage was only 20%, and completely prevented the increase in lipid peroxide level. These findings indicate that this compound can partly inhibit gastric epithelial cell damage in both model systems and that it protects against gastric mucosal necrotic injury in vivo. Thus, such an in vitro gastric epithelial cell system using GSM06 cells is not comparable with the in vivo gastric ulcer models, which are comprised of several kinds of cells and elicited by a variety of factors. However, the system is capable of serving as a satisfactory substitute for the gastric epithelial cell injury models in experiments that involve the primary culturing of normal gastric mucosal cells (53).

Gastric epithelial restoration has been shown to consist of cell migration and proliferation in a primary cultured gastric restoration model, which was wounded to make a cell-free area of constant size by using a pencil-type mixer with a rotating silicon tip (55, 56). In GSM06 cells with such a wound, gastric epithelial restoration consists of initial cell migration and subsequent cell proliferation at 33°C and 39°C, similar to that of primary cultured gastric epithelial cells. However, the migration speed was slow at 33°C, but fast at 39°C, indicating that cell differentiation resulted in accelerated wound restoration (57). Kato et al. (58) previously showed that insulin enhanced the stimulatory effect of epidermal growth factor on migration and proliferation in the gastric epithelial cell line GSM06, which was wounded to make a cell-free area using a razor blade. Therefore, these models using GSM06 cells seem to provide a gastric mucosal epithelial cell model for studying the wound restoration of gastric mucosa.

Effects of Helicobacter pylori

Helicobacter pylori (H. pylori), a gram-negative, spiral-shaped organism, is now recognized as the causative agent of chronic superficial gastritis and is a major factor contributing to the pathogenesis of peptic ulcer disease (59, 60). Although H. pylori has been reported to stimulate the release of various cytokines from gastric mucosa, it remains unclear whether normal gastric epithelial cells produce these cytokines. Recently, Maekawa and co-workers (31) used GSM06 cells as normal mouse gastric surface mucous cells to determine whether gastric epithelial cells produce proinflammatory cytokines in response to H. pylori. Expression of mRNAs of interleukin-1α, tumor necrosis factor-α and cytokine-induced neutrophil chemotactic factor-2β was enhanced by both intact and sonicated H. pylori, but not by H. pylori lipopolysaccharide. Moreover, the expression of major histocompatibility complex (MHC) class II antigen was introduced by H. pylori more strongly than by interferon-γ, a potent inducer of MHC class II antigen, on immunocytes. These data suggest that gastric epithelial cells may act as antigen-presenting cells and participate in the immune response to H. pylori infection (31).

Reconstruction of gastric mucosa

The gastric surface epithelium is situated at an air-liquid interface because the luminal surface of the alimentary tract is in continuity with the air phase. When GSM06 cells were cultured at the air-liquid interface on a nitrocellulose membrane with collagen gel, the cells became tall columnar and secreted PAS-positive substances on the apical surface (27). Thus, an air-liquid interface promotes the differentiation of gastric surface mucous cells in a reconstruction culture of the gastric surface epithelial layer. This culture system will open new possibilities for studying gastric epithelial cells (27).

Conclusion

Transgenic mouse harboring the tsSV40 large T-antigen gene is useful for establishing cell lines from tissues that have proved difficult to culture in vitro (13). Based on the unique characteristics of the gastric surface mucous cell line GSM06 established from transgenic mice and described herein (Fig. 1), the cell line should be able to serve as a useful model for investigation of physiological functions, exploration of drug targets, tissue transplantation, toxicology of drugs and so on. Moreover, experiments using immortalized cells established in vitro and having specific functions may offer an alternative to experiments using living animals and thereby offer a solution to this ethical issue. Thus far, the GSM06 cell line has been used as a model of gastric mucosal epithelium at more than 20 research centers in the world, and the results of studies using this cell line have begun to appear in academic publications (27, 29–31, 49, 53, 57, 58).

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Fig. 1. Schematic illustration of characteristics and applications of GSM06 cells. The cells proliferated at 33°C but not at 39°C. At 39°C, the surface cells became tall columnar with wide cytoplasm, and many mucous granules were present in the apical cytoplasm. Moreover, GSM06 cells produced periodic acid-Schiff positive glycoconjugates that formed a mucous layer like the surface mucosa of the stomach at 39°C.

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