Postnatal Changes and Effects of Glucocorticoid on MUC5AC mRNA Expression in the Rat Stomach

Toru TANAKA* and Satoru TANI

Faculty of Pharmaceutical Sciences, Josai University; 1–1 Keyaki-dai, Sakado, Saitama 350–0295, Japan.
Received March 22, 2003; accepted April 14, 2003

Mucus is an important factor in gastric mucosal protection against acid, pepsin and various factors such as alcohol and nonsteroidal anti-inflammatory drugs. MUC5AC is a gel-forming mucin secreted from gastric surface mucous cells. However, little is known about expression of the MUC5AC gene. We examined developmental changes in rat MUC5AC mRNA expression and the effect of glucocorticoid on MUC5AC mRNA expression in infant rat gastric mucosa. Expression levels of MUC5AC mRNA in the stomach of 0 to 30-d-old and 8-week-old (adult) rats were evaluated by reverse transcription polymerase chain reaction (RT-PCR) and by *in situ* hybridization. We also examined pepsinogen C (PgC) and F (PgF) mRNA expression by RT-PCR. The expression of MUC5AC mRNA increased from 10 d of age, which was about one week earlier than that of PgC mRNA. The expression of PgF mRNA decreased as that of PgC mRNA increased. The injection of hydrocortisone induced PgC mRNA expression in the infant rat stomach, whereas MUC5AC and PgF mRNA expression decreased. These results suggest that developmental changes of MUC5AC mRNA expression differ from those of Pgs, and are not induced by glucocorticoid.

Key words MUC5AC; mRNA; stomach; development; glucocorticoid; pepsinogen

Gastric mucus is an important factor for protecting the gastric mucosal epithelium against digestive juices. The cDNAs of the human mucin core peptides have been partially or fully cloned, and MUC1—8 has been detected in various organs.[1—9] Mucins are classified according to their biochemical characteristics as membrane-associated and secreted types. The secreted mucins are further classified as gel-forming and soluble types. MUC5AC and MUC6 are mainly found in the stomach, and are produced in surface mucous and mucous neck cells, respectively.[10,11] However, the expression of these genes in rat gastric mucosa is not well understood.

Pepsinogen C (PgC) and F (PgF) mRNA expression increases and decreases, respectively during the weaning period.[12] After this period, MUC5AC that is a secretory gel-forming mucin plays an important role as the gastric barrier to protect gastric mucosal epithelial cells from acid, pepsin and food. On the other hand, glucocorticoid induces remarkable changes in the infant rat stomach, namely a simultaneous increase in the potential peptic activity of pepsinogen and a change from the infant to the adult pepsinogen isozyme.[13,14]

The present study clarifies the postnatal changes and effects of hydrocortisone treatment in rat gastric MUC5AC mRNA expression by reverse transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization. We also compared the regulation of MUC5AC, PgC, and PgF mRNA expression in the infant rat stomach.

MATERIALS AND METHODS

Chemicals Hydrocortisone acetate and proteinase K were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sense and antisense primers were customize synthesized by Proligo Japan KK (Kyoto, Japan). RNase-free DNase and pGEM®-T Easy Vector Systems were obtained from Promega Co. (Madison, WI, U.S.A.). M-MLV Reverse Transcriptase, Oligo-$(dT)_{12—18}$ Primer, RNase inhibitor, dNTP Set, and *Taq* DNA Polymerase, recombinant were from Invitrogen Corp. (Carlsbad, CA, U.S.A.). T7 and SP6 RNA Polymerase, DIG RNA Labeling Mix, Blocking Reagent, Anti-Digoxigenin-AP Fab fragments, Nitroblue tetrazolium chloride, and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt were obtained from Roche Diagnostics GmbH (Mannheim, Germany). All other reagents were of the highest commercial quality available.

Animals, Hydrocortisone Treatment and Tissues Male and female Wistar rats purchased from Tokyo Laboratory Animals, Inc. (Tokyo Japan) were mated and housed under controlled conditions (temperature, 23 °C; light from 0800 to 2000 h) and provided with standard rat chow and water.

Newborn rat pups were sacrificed under ether anesthesia at 0, 5, 10, 15, 20, 25, and 30 d after birth. Adult rats (8 weeks after birth) served as controls. Five doses of hydrocortisone (1, 5, 10, 25, 50 mg/kg) and vehicle (olive oil) were subcutaneously injected into the 5-d-old rats. After 2 d (7-d-old), the animals were sacrificed under ether anesthesia.

The stomach was fixed immediately after removal in 4% paraformaldehyde in 50 mM diethylpyrocarbonate (DEPC)-treated phosphate buffer (PB), pH 7.4, and then placed in 30% sucrose in 50 mM DEPC-treated PB. The stomach was transferred into Tissue-Tek O.C.T. Compound (Sakura Finetech U.S.A., Inc., Torrance, CA, U.S.A.), gradually frozen in liquid nitrogen and stored at −80 °C for *in situ* hybridization. The glandular portion of the stomach was immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation using acid guanidinium thiocyanate phenol chloroform.[15]

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Total RNA treated with RNase-free DNase for 30 min at 37 °C was denatured and reverse transcribed to single-stranded cDNA by an incubation for 60 min at 37 °C with reaction mixture (100 U of Moloney murine leukemia virus

* To whom correspondence should be addressed. e-mail: tanakato@josai.ac.jp

reverse transcriptase, 0.5 μg oligo-$(dT)_{12-18}$ primer, 0.5 U RNase inhibitor, 0.5 mM of each dNTP, 10 mM dithiothreitol, 75 mM KCl, and 3 mM $MgCl_2$ in 50 mM Tris–HCl buffer, pH 8.3. Rat sense and antisense primer sequences were designed based on GenBank entries (rat MUC5AC (product size: 534): sense, 5′-GGCCAATGCGGCACTTGTACCAAT-3′; antisense, 5′-GTCATCTGGACAGAAGCAGCCCTC-3′; rat PgC (product size: 421): sense, 5′-CCAACCTGTGGGTGTCTTCT-3′; antisense, 5′-TAGAGGTTCTTGTCCACGCC-3′; rat PgF (product size: 462): sense, 5′-GATCATTGCCTGTGATGGTG-3′; antisense, 5′-GGTCAGCTTCCTGGATGAAA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (product size: 306): sense 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′; antisense, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′). PCR conditions consisted of denaturation at 95 °C for 3 min followed by cycles of 95 °C, 45 s; 55 °C, 30 s; 72 °C, 1 min (MUC5AC, 24 or 27 cycles; PgC, 21 or 24 cycles; PgF, 20 cycles; GAPDH, 23 cycles). The PCR reaction mixture contained 0.2 mM of each dNTP, 0.5 μM of each primer, 0.25 U of *Taq* polymerase, 50 mM KCl, and 1.5 mM $MgCl_2$ in 20 mM Tris–HCl buffer, pH 8.3. PCR products were resolved by electrophoresis on 2% agarose gels containing ethidium bromide, and visualized under UV light. Images obtained using a digital camera were analyzed by NIH Image software.

***In Situ* Hybridization of MUC5AC** Frozen sections were washed in DEPC-treated phosphate buffered saline (PBS), pH 7.3 twice for 15 s, digested with 1.0 μg/ml proteinase K for 30 min at 37 °C and fixed with 4% paraformaldehyde in 50 mM DEPC-treated PB, pH 7.4. After washing with PBS for 1 min, the sections were incubated with 0.2 M HCl in DEPC-treated water and then washed in PBS for 1 min. The sections were immersed in 0.1 M triethanolamine-HCl, pH 8.0 for 1 min followed by 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, washed in PBS for 1 min, and then immersed in a graded ethanol series (70, 80, 90%) for 15 s each. The sections were immersed in 100% ethanol for 15 s twice and dried for 20 min. The MUC5AC PCR product was subcloned into a vector and sequenced using T7 and M13 promoters to confirm the MUC5AC sequence. Finally, 1 ng/μl digoxigenin (DIG)-labeled anti-sense and sense RNA probes were synthesized using T7 and SP6 RNA polymerases, and DIG RNA Labeling Mix, then diluted in hybridization buffer and dropped onto the tissue sections. The sense RNA probe was the negative control. The sections were covered with Parafilm and incubated for 16 h at 60 °C in a humid chamber. The covers were removed by soaking the slides in 5×SSC (1×SSC; 16.6 mM sodium chloride, 16.6 mM sodium citrate) at 60 °C. The sections were immersed in 2×SSC containing 50% formamide for 30 min at 60 °C, washed with 2×SSC for 20 min and then with 0.2×SSC for 20 min at 60 °C twice each. The sections were incubated for 5 min in buffer-1 (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.01% Tween 20), immersed in 1.5% blocking reagent in buffer-1 for 1 h at 37 °C and then washed in buffer-1 for 5 min. Thereafter, the sections were incubated with alkaline phosphatase-conjugated anti-DIG antibody diluted 1 : 1000 in buffer-1 for 16 h at 4 °C. The sections were washed in buffer-1 for 15 min twice and in buffer-2 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 3 min. A color reagent (330 μg/ml Nitroblue tetrazolium chloride, 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate in buffer-2) was added, and the sections were incubated until a signal was visible. The reaction was stopped by adding 10 mM Tris–HCl, pH 7.6, 1 mM EDTA, and the sections were dehydrated with an ethanol series, cleared in xylene, mounted, and viewed under a light microscope.

Statistical Analysis The results are expressed as means±S.E. The statistical significance of the differences in the dose–response was determined by analysis of variance.

RESULTS

Developmental Changes in MUC5AC, PgC and PgF mRNA Expression Levels in the Rat Stomach The expression levels of MUC5AC, PgC and PgF mRNA in the stomach of 0- to 30-d-old and 8-week-old (adult) rats were evaluated by RT-PCR (Fig. 1) and the distribution of cells expressing MUC5AC mRNA was determined by *in situ* hybridization (Fig. 2). MUC5AC mRNA expression was confirmed in gastric surface mucous cells by *in situ* hybridization from 5 d of age. MUC5AC mRNA expression gradually increased until 10 d of age. MUC5AC mRNA expression increased the most from 10 d of age, which was about one week earlier than that of PgC mRNA. The values of MUC5AC and PgC mRNA expression were 50% maximal at about 18.5 and 25.5 d, respectively. PgF mRNA expression decreased as that of PgC increased. The findings of MUC5AC mRNA expression closely correlated between RT-

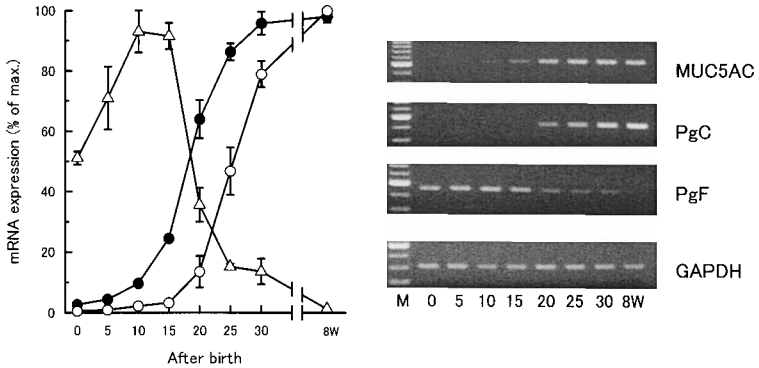

Fig. 1. Developmental Changes MUC5AC, PgC and PgF mRNA Expression Levels in the Rat Stomach

MUC5AC (closed circles), PgC (open circles) and PgF (open triangles) mRNA levels are shown as a ratio (%) of the maximal value at the ratio to GAPDH. Right panels show representative results of RT-PCR of MUC5AC, PgC, PgF and GAPDH in the rat stomach. Each value is mean±S.E. of 3 experiments.

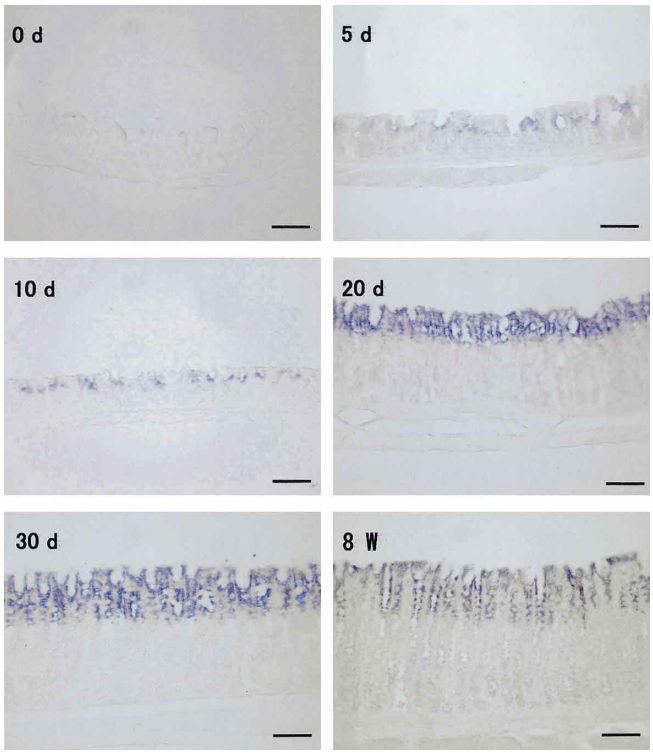

Fig. 2. Microphotographs of Growing Rat Glandular Stomach

Cells expressing MUC5AC mRNA were detected by *in situ* hybridization. Bars=100 μm.

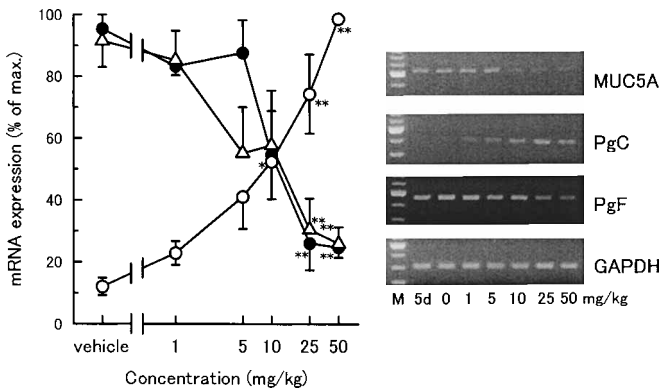

Fig. 3. Effects of Hydrocortisone on MUC5AC, PgC and PgF mRNA Expression in Infant Rat Stomach

Hydrocortisone (0, 1, 5, 10, 25, 50 mg/kg; s.c.) was injected into 5-d-old rats. MUC5AC (closed circles), PgC (open circles) and PgF (open triangles) mRNA levels are shown as a ratio (%) of the maximal value at the ratio to GAPDH. Right panels show representative results of RT-PCR of MUC5AC, PgC, PgF and GAPDH in the rat stomach. Each value is mean±S.E. of 3 experiments. $*p<0.05$, $**p<0.01$ (*vs* 0 mg/kg) (ANOVA).

PCR and *in situ* hybridization.

Effects of Hydrocortisone on MUC5AC, PgC and PgF mRNA Expression Levels in the Infant Rat Stomach Hydrocortisone (0, 1, 5, 10, 25, 50 mg/kg) was subcutaneously injected into 5-d-old rats. After 2 d (7-d-old), MUC5AC, PgC and PgF mRNA expression levels were evaluated by RT-PCR (Fig. 3). Hydrocortisone significantly and dose dependently increased PgC mRNA expression. However, levels of MUC5AC and PgF mRNA expression were significantly decreased by hydrocortisone. Figure 4 shows *in situ* hybridization microphotographs of MUC5AC mRNA expression. Injected hydrocortisone (50 mg/kg) decreased the number of cells expressing MUC5AC mRNA in the infant rat stomach, and the cells were light in color.

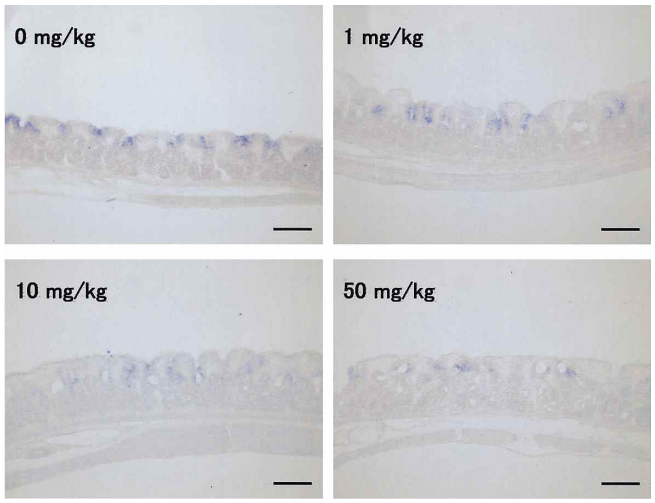

Fig. 4. Microphotographs of Infant Rat Glandular Stomach Treated with Hydrocortisone

Cells expressing MUC5AC mRNA were detected by *in situ* hybridization. Bars=100 μm.

DISCUSSION

Mucin genes that have completely or partially characterized have a central tandem repeat. *In situ* hybridization and RNA analysis are used to quantify and localize mucin gene expression. High levels of MUC5AC mRNA expression in the stomach are suggesting that this mucin plays an important role in forming the mucous barrier.[10,11] However, little is known about the expression of its gene. We examined the developmental changes in rat gastric MUC5AC mRNA expression and the effects of hydrocortisone.

The weaning period alters conditions in the gastrointestinal tract. That is, not only does the source of nutrition change from mother's milk to solid food, but pepsinogen also switches from fetal type (PgF) to adult type (PgC).[12] PgC and F mRNA expression levels and weaning are correlated in the same way as enzymes lactase and sucrase activities in jejunal mucosa.[16] We speculated that MUC5AC mRNA expression is also regulated by various factors during weaning. However, we confirmed that MUC5AC mRNA is expressed in 5-d-old infant rats, and that it obviously increases from 10 d of age. The MUC5AC mRNA expression was about 1 week earlier than PgC mRNA expression. Therefore, it seems likely that mucus gel is formed before weaning to protect the gastric mucosa epithelium from solid food, acid and peptic digestion.

Glucocorticoid is not only essential for gastrointestinal maturation during development, but also has an ulcerogenic side effect on the gastric mucosa.[17,18] Moreover, glucocorticoid triggers the appearance of gastrin receptors in the rat gastric mucosa and a significant increase in antral gastrin.[19] Glucocorticoid stimulates the maturation of H,K-ATPase in the infant rat stomach.[20] Therefore, we compared the effects of hydrocortisone on the expression of MUC5AC and PgC mRNA in the rat gastric mucosa. Hydrocortisone signifi-

cantly and dose-dependently increased PgC mRNA and decreased MUC5AC and PgF mRNA expression in the infant rats. These results indicate that switching from PgF to PgC induced by hydrocortisone stimulates gastric maturation. The decrease in MUC5AC mRNA expression suggests direct hydrocortisone suppression in gastric surface mucosal cells, which is further supported by our previous report.[20] These results suggest that the developmental changes of rMUC5AC mRNA expression differ from that of PgC and PgF, and that they are not induced by glucocorticoid.

REFERENCES

1) Gendler S., Taylor-Papadimitriou J., Duhig T., Rothbard J., Burchell J., *J. Biol. Chem.*, **263**, 12820—12823 (1988).
2) Gum J. R., Hicks J. W., Toribara N. W., Siddiki B., Kim Y. S., *J. Biol. Chem.*, **269**, 2440—2446 (1994).
3) Gum J. R., Hicks J. W., Swallow D. M., Lagace R. L., Byrd J. C., Lamport D. T. A., Siddiki B., Kim Y. S., *Biochem. Biophys. Res. Commun.*, **171**, 407—415 (1990).
4) Porchet N., Cong N. V., Dufosse J., Audie J. P., Guyonnet-Duperat V., Gross M. S., Denis C., Degand P., Bernheim A., Aubert J. P., *Biochem. Biophys. Res. Commun.*, **175**, 414—422 (1991).
5) Guyonnet-Duperat V., Audie J. P., Debailleul V., Laine A., Buisine M. P., Galiegue-Zouitina S., Pigny P., Degand P., Aubert J. P., Porchet N., *Biochem. J.*, **305**, 211—219 (1995).
6) Meerzaman D., Charles P., Daskal E., Polymeropoulos M. H., Martin B. M., Rose M. C., *J. Biol. Chem.*, **269**, 12932—12939 (1994).
7) Toribara N. W., Roberton A. M., Ho S. B., Kuo W. L., Gum E., Hicks J. W., Gum J. R., Jr., Byrd J. C., Siddiki B., Kim Y. S., *J. Biol. Chem.*, **268**, 5879—5885 (1993).
8) Bobek L. A., Tsai H., Biesbrock A. R., Levine M. J., *J. Biol. Chem.*, **268**, 20563—20569 (1993).
9) Shankar V., Pichan P., Eddy R. L., Tonk V., Nowak N., Sait S. N. J., Shows T. B., Schultz R. E., Gotway G., Elkins R. C., Gilmore M. S., Sachdev G. P., *Am. J. Respir. Cell Mol. Biol.*, **16**, 232—241 (1997).
10) Seregni E., Botti C., Massaron S., Lombardo C., Capobianco A., Bogni A., Bombardieri E., *Tumori.*, **83**, 625—632 (1997).
11) Reid C. J., Harris A., *Gut*, **42**, 220—226 (1998).
12) Kageyama T., Ichinose M., Tsukada-Kato S., Omata M., Narita Y., Moriyama A., Yonezawa S., *Biochem. Biophys. Res. Commun.*, **267**, 806—812 (2000).
13) Furihata C., Kawachi T., Sugiyama T., *Biochem. Biophys. Res. Commun.*, **47**, 705—711 (1972).
14) Tatematsu M., Takahashi M., Tsuda H., Hirose M., Furihata C., Sugimura T., *Cell Differentiation*, **4**, 285—294 (1975).
15) Chomczynski P., Sacchi N., *Anal. Biochem.*, **162**, 156—159 (1987).
16) Henning S. J., *Am. J. Physiol.*, **235**, E451—E456 (1978).
17) Menguy R., Masters Y. F., *Surgery.*, **54**, 19—28 (1963).
18) Henning S. J., *Am. J. Physiol.*, **235**, E451—E456 (1978).
19) Peitsch W., Takeuchi K., Johnson L. R., *Am. J. Physiol.*, **240**, G442—G449 (1981).
20) Wang Z.-M., Aizman R., Grahnquist L., Yasui M., Hemphala A., Celsi S., *Pediatr. Res.*, **40**, 658—663 (1996).
21) Tanaka T., Kobayashi T., Sunaga K., Tani S., *Biol. Pharm. Bull.*, **24**, 634—637 (2001).