Effect of Glucocorticoid on Expression of Rat MUC5AC mRNA in Rat Gastric Mucosa *in Vivo* and *in Vitro*

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The gastric mucus is an important factor in the gastric mucosal protection from acid, pepsin and various reagents (alcohol, aspirin, *etc.*). MUC5AC is the mucin secreted from surface mucous cells, and belongs to the gel-forming mucin. We examined the regulation of rat MUC5AC (rMUC5AC) mRNA by glucocorticoid *in vivo* and *in vitro*, comparing it with that of pepsinogen (Pg) mRNA. By adrenal gland resection, rMUC5AC and Pg mRNA levels and Pg content in rats significantly decreased to 70%, 46% and 42% of those in the sham operated controls, respectively. With the treatment of hydrocortisone (1, 5 and 50 mg/kg), Pg mRNA level and Pg content in adrenalectomized rats was restored. On the other hand, the rMUC5AC mRNA level exceeded the control with 1 or 5 mg/kg injections of hydrocortisone, but drastically decreased to 18% of sham operation levels with it (50 mg/kg). Similar results were obtained in normal rats with the treatment of hydrocortisone (50 mg/kg). Mucus and DNA content of cultured rat gastric epithelial cells were not affected by hydrocortisone, but rMUC5AC mRNA level was significantly decreased in a dose-dependent manner.

From the *in vivo* and *in vitro* results, at least a physiological concentration of glucocorticoid was necessary in the expression of rMUC5AC mRNA. However, high dose of hydrocortisone directly suppressed the expression of rMUC5AC mRNA. These results suggested that hydrocortisone might directly cause the suppression and indirectly the enhancement of the mucin biosynthesis.

Key words MUC5AC; pepsinogen; hydrocortisone; mRNA; rat

The gastric mucus is an important factor for protection of the gastric mucosal epithelium against digestive juice. The cDNAs of the human gastric mucin core peptides have been partially or fully cloned, and the existence of MUC1-8 was confirmed in various organs.[1] Mucins are classified into two types according to their biochemical characteristics, membrane-associated and secreted mucins. Moreover, the secreted mucins are divided into two subgroups, gel-forming and soluble mucins. MUC5AC and MUC6 are mainly found in the stomach, and are produced in surface mucous cells and mucous neck cells, respectively.[2] Because of the difficulty in estimating the mucin molecular species, the uptake of the ^{14}C or ^{3}H labeled glucosamine into the sugar chain has usually been examined as the index of gastric mucus secretion and biosynthesis.[3]

On the other hand, ulcerogenic side effects of glucocorticoids on the gastric mucosa, and inhibition of gastric mucus secretion and biosynthesis was determined in an early study.[4] The enhancement of mRNA expression and biosynthesis of the pepsinogen (Pg) which is one of the aggressive factors in a gastric ulcer, was later reported using adrenalectomized and glucocorticoid administered rats.[5] In spite of the importance of MUC5AC as a secretory gel-forming mucin in the gastric barrier, regulation of MUC5AC mRNA expression by glucocorticoid in the stomach has not yet been shown.

In this study, we examined the rat MUC5AC (rMUC5AC) mRNA level as an index of the gastric mucin biosynthesis. We demonstrated the effects of hydrocortisone on the expression of rMUC5AC mRNA in the adrenalectomized and normal rat stomach, comparing them with those on the expression of Pg mRNA. We also examined the direct effect of hydrocortisone using the cultured rat gastric epithelial cells.

MATERIALS AND METHODS

Chemicals Hydrocortisone acetate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Biotinylated soybean agglutinin (SBA) and horseradish peroxidase-avidin D (HRP-avidin D) were from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.). Dispase I and bovine serum albumin (BSA) (fraction V) were from Godo Shusei Co. (Tokyo, Japan) and Sigma Chem. Co. (St. Louis, MO, U.S.A.). Immobilon polyvinylidene difluoride (PVDF) transfer membrane was from Japan Millipore, Ltd. (Tokyo, Japan). Antisense oligodeoxynucleotides for rMUC5AC and Pg mRNA were customized by Greiner Japan (Tokyo, Japan), as follows: rMUC5AC, 5′-GGTGGTGAGCTGGTGTGGGTTGT-3′, which was estimated by Inatomi *et al.*,[6] and for Pg, 5′-TGTGTCTACAATGCCTTGGCAGCCCTG-3′, which was estimated by Ichihara, *et al.*,[7] then labeled by ^{32}P with the Oligonucleotide 5′-End Labeling System (Du Pont NEN Res. Pro., Boston, MA, U.S.A.). All other reagents were of the best commercial quality available.

Preparation of Adrenalectomy and Treatment with Hydrocortisone Male Wistar rats weighing about 200 g were used in experiments. Adrenalectomy was performed following the method of Grollman.[8] After adrenalectomy, rats were maintained with free access to food and saline instead of tap water for one week. Three doses of hydrocortisone (1, 5 and 50 mg/kg in olive oil) were subcutaneously injected into the animals once a day for 3 d. At the end of the treatment, rats were starved for 24 h and were sacrificed under ether anesthesia.

Extraction of RNA and Northern Blot Analysis Total RNA was extracted from the glandular portion of the stomach or cultured epithelial cells by the acid guanidium thiocyanate–phenol–chloroform method.[9] Northern blot hybridization was carried out according to the standard

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method.[10] Briefly, the electrophoresis of 5—10 μg of total RNA was carried out in 1.0% agarose formalin gel. RNA in gel was transferred onto a sheet of nylon membrane (Geen Screen; NEN Life Sci. Pro., Inc., Boston, MA, U.S.A.) by capillary action. The membrane was prehybridized for 4 h at 58 °C in a prehybridization buffer. After hybridization for 18 h at 58 °C with antisense rMUC5AC or Pg ^{32}P-oligodeoxynucleotide, the membrane was washed twice with 0.1% sodium dodecylsulfate (SDS) in 2×SSC (1×SSC; 16.6 mM sodium chloride, 16.6 mM sodium citrate) buffer at room temperature for 5 min, and twice with 0.1% SDS in 0.1×SSC buffer at 50 °C for 15 min. After exposing a sheet of X-ray film to the membrane, the obtained autoradiogram was analyzed by NIH Image software, in which values were corrected by the amount of rRNA.

Culture of Rat Gastric Epithelial Cells and Treatment with Hydrocortisone Culture of rat epithelial cells was prepared by our method as previously described.[11] Briefly, everted sacs of the stomachs were filled with medium, immersed in incubation medium containing Dispase I (1 kU/ml), and gassed with 95% O_2 and 5% CO_2 at 37 °C for 1 h. Mucosal cells were gently isolated from the surface of the gastric mucosa by pipetting. Cells obtained (2×10^6 cells/dish) were inoculated on plastic dishes (35 mm diameter) covered with collagen gel, and cultured in a Dulbecco's modified eagle medium–Ham's F12 (1 : 1) medium (GIBCO BRL/Life Tech., Eggenstein, Germany) supplemented with 10% fetal calf serum (Biofluids, Inc., Rockville, MD, U.S.A.) and 100 μg/ml gentamicin sulfate at 37 °C for 24 h in a CO_2 incubator. After the 24 h culture, hydrocortisone was added to final concentrations of 0.1, 1 and 10 μg/ml and incubated at 37 °C for 24 h in a CO_2 incubator.

Measurement of Pepsinogen The homogenate of the glandular portion of the stomach (10%) with 50 mM phosphate buffer (pH 7.5) was centrifuged at 10000×***g*** for 10 min after freezing and thawing. The supernatant was used to measure peptic activity by the method of Anson and Mirsky,[12] and protein concentrations by the method of Bradford (Bio-Rad Protein Assay; Bio-Rad Lab. Co., Richmond, CA, U.S.A.).[13]

Measurement of Mucin and DNA in Cultured Epithelial Cells A dot blot analysis was used for the measurement of mucin. Samples or various concentrations of purified gastric mucin in 4 M guanidine hydrochloride were applied in a microfiltration apparatus (Immunodot, Atto Co., Ltd., Tokyo) and adsorbed onto a sheet of PVDF-membrane by aspiration. After blocking the blotted sheet with 3% BSA in TBS-T (0.05% tween 20 in 50 mM tris HCl buffered saline (pH 7.3)) at room temperature for 30 min and washing it with TBS-T, the sheet was incubated with 5 μg/ml biotinylated-SBA in 0.1% BSA and 1 M NaCl in TBS-T for 30 min and washed 6 times with TBS-T at room temperature for 5 min. The same procedure was carried out for HRP-avidin D. Enzyme activity was measured with 0.03% H_2O_2 and 0.02% 3-amino-9-ethylcarbazole in 50 mM acetate buffer (pH 5). DNA concentration of the cultured epithelial cells was measured using a method of fluorescence enhancement of 4′,6-diamidino-2-phenylindole (DAPI).[14]

Statistical Analysis The results were expressed as mean±S.E. Data were analyzed using Student's *t*-test for paired comparison. The statistical significance of the differences in the dose-response was determined by analysis of variance.

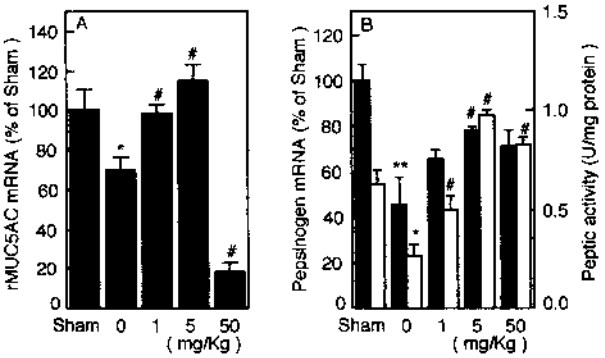

Fig. 1. Effects of Hydrocortisone on rMUC5AC and Pg mRNA Levels and Pg Content in Adrenalectomized Rats

Hydrocortisone (0, 1, 5 and 50 mg/kg; s.c.) was injected into adrenalectomized rats once a day for 3 d one week after the operation. Each mRNA level (closed columns) is shown as % of sham operation level, and Pg content (U/mg protein) as peptic activity[12] (open columns). Each value is the mean±S.E. of 4 experiments. $*p<0.05$, $**p<0.01$ (V.S. sham) and $\#p<0.05$ (V.S. 0 mg/kg) (ANOVA).

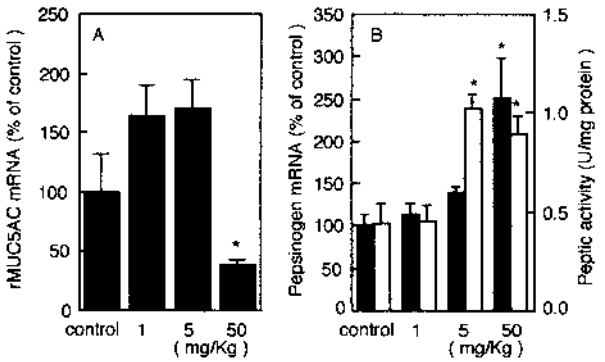

Fig. 2. Effects of Hydrocortisone on rMUC5AC and Pg mRNA Levels and Pg Content in Normal Rats

Hydrocortisone (0, 1, 5 and 50 mg/kg; s.c.) was injected into adrenalectomized rats once a day for 3 d. Each mRNA level (closed columns) is shown as % of the control, and Pg content (U/mg protein) as peptic activity[12] (open columns). Each value is the mean±S.E. of 4 experiments. $*p<0.05$ (ANOVA).

RESULTS

Effects of Hydrocortisone on rMUC5AC and Pg mRNA Level and Pg Content in Adrenalectomized Rats Hydrocortisone was subcutaneously injected into adrenalectomized rats once a day for 3 d one week after the operation. rMUC5AC and Pg mRNA levels and Pg content were significantly decreased by the adrenalectomy to 70%, 46% and 42% of sham operation levels, respectively (Fig. 1). With the injections of hydrocortisone (1, 5 and 50 mg/kg), Pg mRNA level and Pg content were restored (Fig. 1B). On the other hand, the rMUC5AC mRNA level recovered to sham operation levels by injection of hydrocortisone (1 and 5 mg/kg), but was drastically decreased to 18% by the injection of hydrocortisone (50 mg/kg) (Fig. 1A). With the injections of hydrocortisone (1 and 5 mg/kg) into normal rats, the rMUC5AC mRNA level increased to 164% and 171% of control, respectively. However, with its injection at 50 mg/kg, this level decreased to 38% (Fig. 2A).

Effects of Hydrocortisone on rMUC5AC mRNA Level

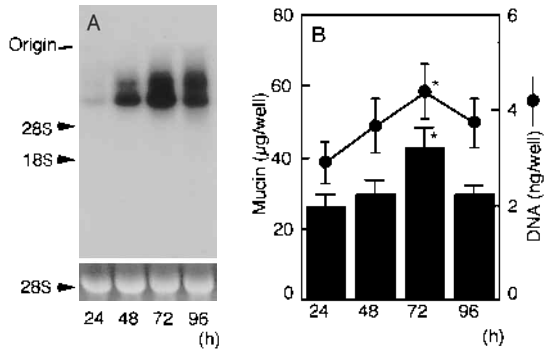

Fig. 3. Changes of rMUC5AC mRNA Level, and Mucin and DNA Content in the Rat Gastric Epithelial Cells during Culture

(A) Top panel is a Northern blot of rMUC5AC mRNA, and lower panel is ethidium bromide dyeing of 28 s. (B) Mucin (closed columns) and DNA content (closed circles) were measured according to the method in the text. Each value is the mean±S.E. of 5 experiments. $*p<0.05$ (V.S. 24 h).

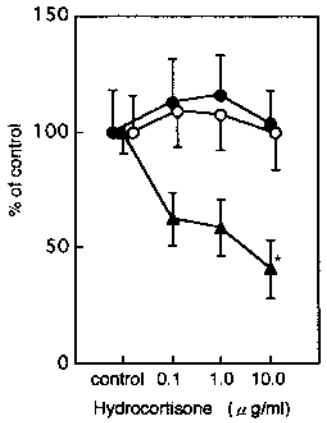

Fig. 4. Effects of Hydrocortisone on rMUC5AC mRNA Level and Mucus and DNA Content in the Cultured Rat Gastric Epithelial Cells

Gastric epithelial cells were cultured for 24 h. After that, hydrocortisone (0.1—10 μg/ml) was added to the medium, and cells were incubated for 24 h. rMUC5AC mRNA level (closed triangle), and mucus (closed circle) and DNA (open circle) content were shown as % of control. Each value is the mean±S.E. of 4 experiments. $*p<0.05$ (ANOVA).

and Mucus and DNA Content in Cultured Gastric Epithelial Cells The changes in mucus content, rMUC5AC mRNA level and DNA content in cultured rat gastric epithelial cells for 96 h are shown in Fig. 3; all three factors reached a peak at 72 h. However, the ratios of mucus/DNA were almost the same during the culture.

Hydrocortisone was added to the culture medium 24 h after inoculation and the cells were incubated for 24 h, after which, mucus content, rMUC5AC mRNA level and DNA content were measured. Mucus and DNA contents were not affected by hydrocortisone, however, the expression of rMUC5AC mRNA was significantly decreased to 41% of the control (Fig. 4).

DISCUSSION

It is well known that glucocorticoid is essential for maturation in gastrointestinal development and it has an ulcerogenic side effect on the gastric mucosa.[4,15] A pharmacological dose of corticosteroids usually inhibits phospholipase A_2 *via* the lipocortin, while there are reports that glucocorticoid does not inhibit prostaglandin synthesis in the gastric mucosa.[16] We therefor examined the effect of hydrocortisone on the expression of rMUC5AC mRNA in the rat gastric mucosa, and compared it with that on Pg mRNA. After adrenalectomy, rMUC5AC and Pg mRNA levels and Pg content were significantly decreased. With the treatment of hydrocortisone (1 and 5 mg/kg) the rMUC5AC mRNA level was restored to the sham operation level, whereas by the treatment of hydrocortisone (50 mg/kg), it drastically dropped to 18% of sham operation level, much lower than that of the adrenalectomized rats. On the other hand, Pg mRNA level and Pg content increased with the treatment of hydrocortisone (1, 5 and 50 mg/kg). Similar results with hydrocortisone treatment were also obtained in normal rats. There is a report that the gastric mesenchyme secretes humoral factors that are induced by glucocorticoids.[17] The reduction of the rMUC5AC mRNA level by the adrenalectomy showed that a physiological concentration of glucocorticoid was necessary at least for the expression of rMUC5AC mRNA. Therefore, increase in the rMUC5AC mRNA levels might be induced by certain factors, one of which is from gastric mesenchyme with the treatment of an appropriate amount of hydrocortisone. The decrease in the rMUC5AC mRNA level by the treatment with hydrocortisone (50 mg/kg) was thought to be the sum total of enhancement and suppression, different from Pg. This discrepancy shows the existence of two mechanisms *in vivo*: hydrocortisone increases or decreases mucin mRNA expression both directly and indirectly.

Based on the above results, we examined whether or not hydrocortisone regulates the expression of rMUC5AC mRNA directly, using cultured rat gastric mucosal epithelial cells. We reported earlier the mucus secretion from cultured rat gastric epithelial cells.[11] In this study, we confirmed that rMUC5AC mRNA was expressed in mucosal cells of our established primary monolayer culture. Hydrocortisone suppressed the expression of rMUC5AC mRNA in mucosal cell culture in a dose-dependent manner, although it did not affect mucus or DNA content. These results suggested that hydrocortisone (0.1—10 μg/ml) directly suppressed the expression of rMUC5AC mRNA in gastric epithelial cells without affecting the cellular viability. There is a report that dexamethasone directly suppressed gastric mucin secretion and expression of MUC1 mRNA on the human stomach *in vitro*.[18] Our study had similar results on this point. In this culture system, epithelial cells continuously expressed rMUC5AC mRNA, although the existence of a trace amount of glucocorticoid in fetal calf serum was not absolutely ruled out.

Taking *in vivo* and *in vitro* results into consideration, the expression of rMUC5AC mRNA might be balanced by indirect enhancement by a certain factor that is regulated by hydrocortisone, from other cells like mesenchyme, and by direct suppression by hydrocortisone. Therefore, pharmacological doses of glucocorticoids increase synthesis of Pg, and inhibit mucin biosynthesis, that is, they induce the reinforcement of an aggressive factor and the declination of a protective factor. Suppression of rMUC5AC in the epithelial cells also seems to be involved in the pathogenesis of the steroid therapy-induced gastric mucosal damage.

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