Changes in the Incorporating Activity of $^{35}$S-Sulfate into Gastric Sulfated Glycoproteins in the Rat with Erosions by Restraint and Water Immersion Stress

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Abstract—The synthetic activity of rat gastric sulfated glycoproteins (SGP) in vitro was investigated at various time intervals after water immersion stress using $^{35}$S-sulfate as a precursor. More than 90 percent of the total radioactivity was incorporated into mucosal SGP, and the rest was incorporated into glycosaminoglycans in the gastric muscular layer. The incorporation of $^{35}$S-sulfate into SGP increased at 2 hr and decreased at 6 hr after the onset of stress. The incorporating activity again increased markedly at 12 hr and then recovered to the normal level at 24 hr after the onset of stress. An anti-ulcer agent, $N$-($N$-acetyl-$\beta$-alanyI)-L-histidine aluminum complex (AAHA), significantly increased the SGP synthetic activity at 12 hr and at 24 hr after the onset of stress. It was indicated from the elution patterns on the DEAE-cellulose column that AAHA increased the amount of highly sulfated glycoproteins compared with the stress control at 12 hr after the onset of stress. The uronic acid content in the gastric muscular layer of the rat was unchanged with stress. These results in the in vitro experiment indicate that the SGP synthetic activity does not decrease with stress load, but rather increases at 2 hr and at 12 hr after the onset of stress when a sufficient amount of $^{35}$S-sulfate is supplied. Accordingly, it is suggested that SGP facilitates the restoration of the gastric mucosal damage caused by stress.

It has been reported by many investigators that erosions and hemorrhages of the gastric mucosa produced by anti-inflammatory drugs or stress load were associated with impaired mucus production, and the alterations in the production of mucus may account for the decrease in its protective capacity (1–6). It is thought that the increased biosynthesis of gastric glycoproteins and glycosaminoglycans may be predominant factors in the protection and healing of gastric ulcers (7). Glycoproteins including sulfated glycoproteins (SGP) were isolated from gastric juice and gastric mucosa (8–13), and their biosynthesis were investigated using radioactive precursors (14–16). They were synthesized in the glandular cells and secreted into the lumen. These glycoproteins, especially SGP, have been studied with special reference to their efficacy as inhibitors of proteolysis by pepsin. It has been observed that the sulfated mucosubstances were decreased in various types of experimental ulcer models and was recovered by the administration of a certain anti-ulcer agent (15, 17–19). Recently, Azuumi et al. (5) suggested that the decrease of gastric mucus macromolecular glycoproteins including neutral and acidic glycoproteins with or without sulfate may be a causative factor in the etiology of ulcers in aspirin-induced gastric damage. In order to study the SGP involvement in stress-induced ulcer in rats, we have now investigated the correlation between the development of erosions and SGP synthesis in the glandular stomach with a lapse of time. We also investigated the effect of an anti-ulcer agent, $N$-($N$-acetyl-$\beta$-alanyI)-L-histidine aluminum...
Materials and Methods

Induction of gastric lesions by restraint and water immersion stress: Rats (male Donryu strain weighing 240–280 g) were deprived of food, but allowed access to water for 24 hr prior to the experiments. The animals were placed in a stress cage and immersed in a water bath (22±0.5°C) (20). Stress began at 10:00 A.M. in the all experiments. The animals were then sacrificed by a blow on the head at time intervals of 2, 6, 12 and 24 hr after the onset of stress. Each stomach was excised immediately and cut along the greater curvature. The stomach content was washed with a cold saline solution. After the mucosal damages were observed, the forestomach was removed and the glandular stomach was cut into two pieces, and they were used for the incubation experiment. AAHA (1,000 mg/kg body weight) dissolved in distilled water was given orally 30 min before stress.

Incubation of the gastric tissue with labeled precursors: The glandular stomach was preincubated for 30 min at 37°C under 95% O₂/5% CO₂ in 20 ml of the Krebs-Ringer bicarbonate buffer containing antibiotics (penicillin, G 65U/ml, Meiji Seika; streptomycin, 65U/ml, Kaken Chemical) at pH 7.4 and then incubated for another 6 hr at 37°C in the presence of 100 µCi of H₂³⁵SO₄ (carrier free, Radioisotope Association) and/or 50 µCi of D-[6-³H]glucosamine hydrochloride (34.6 Ci/mmol, Amersham). After the incubation at 37°C, the incubation medium was removed, and the labeled tissue was washed three times with 5 ml of the cold Krebs-Ringer bicarbonate buffer.

Extraction of sulfated glycoproteins (SGP): Extraction of SGP was carried out according to the method described by Azuumi et al. (5); The labeled tissue was placed in a boiling water bath for 3 min to inactivate the enzymes, and 2 ml of 50 mM Tris-HCl buffer containing 2% Triton X-100, pH 7.2, was added to the labeled tissue. The labeled tissue was then homogenized in the same buffer using a motor-driven glass homogenizer. The homogenate was centrifuged at 10,000×g for 30 min at 4°C. The pellet obtained was used for the determination of glycosaminoglycans. The supernatant was dialyzed exhaustively against running tap water and then distilled water. The dialysate was applied to the Bio Gel A-1.5 m column (100–200 mesh, 1.4×55 cm, Bio Rad Laboratories) equilibrated with 50 mM Tris-HCl buffer containing 2% Triton X-100, pH 7.2, and eluted with the same buffer. Fractions of 2.3 ml were collected and assayed for radioactivity and hexose.

Isolation of SGP by the DEAE-cellulose column: SGP containing the high molecular glycoprotein fraction (peak I) obtained by gel filtration was further applied to the DEAE-cellulose column (DE-52, 0.5×9.5 cm, Whatman) equilibrated with 50 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.2. The column was washed with 30 ml of the same buffer and eluted with a linear gradient of 0–0.4 M NaCl in the same buffer. Fractions of 2.3 ml were collected and assayed for radioactivity and hexose.

Time course of the incorporation of ³⁵S-sulfate into the gastric tissue and the release of ³⁵S-labeled glycoprotein into the medium: Rats (male Donryu strain weighing 240–260 g) were sacrificed. Each stomach was excised immediately and cut along the greater curvature. The glandular stomach was used for ³⁵S-sulfate incorporation. The incorporation was carried out for 0, 2 and 6 hr as described above, and the homogenate obtained was applied to the Bio Gel A-1.5 m column. The incubation medium was dialyzed. The dialysate was concentrated and applied to the Bio Gel A-1.5 m column. Fractions of 2.3 ml were collected and assayed for radioactivity.

Preparation of glycosaminoglycans: Pronase E (Kaken Chemical) amounting to 50 mg was dissolved in 3.5 ml of 50 mM Tris-HCl buffer containing 10 mM CaCl₂, pH 7.8, and was added to the pellets obtained from centrifuged homogenate. Proteolysis was carried out at 42°C in the presence of a small amount of toluene. After 24 hr, 30 mg of Pronase E in 2.5 ml of the same buffer was further added and incubated for 24 hr at 42°C. The digest was deproteinized by
adding trichloroacetic acid to a final concentration of 10%. The solution was kept at 4°C overnight and centrifuged at 10,000×g for 20 min at 4°C. The supernatant was dialyzed exhaustively against running tap water and then distilled water. The dialysate was concentrated to 5 ml. One percent of cetylpyridiniumchloride (cpc) amounting to 0.3 ml and 66 μl of 3 M NaCl (final concentration of 0.037 M) were added, then incubated for 1 hr at 37°C and kept for a day at room temperature. The cpc precipitate was collected by centrifugation at 2,000×g for 20 min at 25°C. The precipitate was dissolved in 1.0 ml of 4 M NaCl and kept for 1 hr at room temperature. Ice-cold 80% ethanol amounting to 13 ml was added, and it was kept for a day at 4°C. The mixture was centrifuged at 2,000×g for 20 min at 4°C. Ice-cold 75% ethanol amounting to 5.0 ml was added to the pellet and mixed well. The mixture was then centrifuged at 2,000×g for 20 min at 4°C. The mixture was dried under reduced pressure. The pellet was dissolved in 1.0 ml of distilled water and uronic acid was determined.

Analytical methods and measurement of radioactivity: Uronic acid was determined by the carbazole method of Bitter and Muir with glucuronolactone as standard (21). Hexose was determined by the phenol/H₂SO₄ method with galactose as standard (22). The radioactivity of SGP was measured in an Aloka 903 liquid scintillation counter. Statistical analyses were made using Student’s t-test.

Results

Incorporation of labeled precursors into glycoproteins in rat gastric mucosa: Figure 1 shows the elution pattern on the Bio Gel A-1.5 m column. The glycoproteins extracted from non-stressed rat glandular stomach were divided into three fractions. Both ³H-glucosamine and ³⁵S-sulfate were incorporated into macromolecular sulfated glycoproteins which were eluted in the void volume (peak I). Peak I contains both sulfated and non-sulfated macromolecular glycoproteins (23).

Fig. 1. Bio Gel A-1.5 m column chromatography of ³H/³⁵S-labeled glycoproteins prepared from non-stressed rat gastric mucosa. Rat glandular stomach was isolated and incubated with ³H-glucosamine and ³⁵S-sulfate for 6 hr at 37°C. ³H/³⁵S-labeled glycoproteins were extracted with 50 mM Tris-HCl buffer containing 2% Triton X-100 (pH 7.2). The extract was applied to a column of Bio Gel A-1.5 m (1.4×55 cm) and eluted with the same buffer. Fractions of 2.3 ml were collected. Hexose was detected by the phenol/H₂SO₄ method (absorbance at 490 nm ×····×), and radioactivity was counted by a liquid scintillation counter (³H ○····○, ³⁵S ○···○).
The elution pattern of macromolecular glycoprotein fraction (peak I) on a column of DEAE-cellulose (DE-52) is shown in Fig. 2. $^{35}$S-Labeled glycoproteins were eluted with both the equilibrium buffer (peak I-a) and an increasing concentration of NaCl (peak I-b).

Time course of the incorporation of $^{35}$S-sulfate and the release of the $^{35}$S-labeled glycoproteins into the medium: As shown in Fig. 3, $^{35}$S-sulfate was incorporated linearly into the macromolecular glycoprotein fraction (peak I) throughout the experimental period. $^{35}$S-labeled glycoproteins were released into the medium with incubation time. The amount of radioactivity released into the medium was much less than that of the tissue.

Changes in the incorporating activity of $^{35}$S-sulfate into SGP in restrained and water immersed rat: The rat gastric tissue separated at various time intervals after water immersion stress was analyzed for radioactivity in the gastric macromolecular glycoprotein fraction (peak I) after the incubation with $^{35}$S-sulfate. Figure 4 shows changes in the incorporating activity of $^{35}$S-sulfate into SGP per gram tissue with a lapse of time after the onset of stress. The SGP synthetic activity increased at 2 hr after the onset of stress, while gastric injury was not observed macroscopically. Then it decreased at 6 hr, while severe hemorrhagic erosions were visible. It was followed by a rapid increase at 12 hr and recovered to the normal level at 24 hr. AAHA inhibited erosions induced by water immersion stress at 6 hr after the onset of stress; the inhibition was about 50% at
1,000 mg/kg body weight when AAHA was administered orally, but no effect on the SGP synthetic activity was observed. The inhibitory effect of AAHA on erosions at 12 and 24 hr did not increase compared with that at 6 hr, but the incorporating activity of $^{35}$S-sulfate was significantly increased.

**Fractionation of gastric macromolecular glycoproteins (peak I) by DEAE-cellulose column chromatography**: Figure 5 shows the elution patterns of the stress control and AAHA treated sample on the DEAE-cellulose column (DE-52). When the macromolecular glycoprotein fraction (peak I) obtained from gel filtration was chromatographed on a DEAE-cellulose column (DE-52) of the macromolecular glycoprotein fraction (peak I) obtained by gel filtration. Rats were killed at 6 hr and 12 hr after the onset of stress. AAHA was given orally 30 min before stress at a dose of 1,000 mg/kg body weight. Peak I obtained by the Bio Gel A-1.5 m column from 3 rats were pooled and applied to the DEAE-cellulose column and eluted with a linear gradient of 0–0.4 M NaCl. Fractions of 2.3 ml were collected. Hexose was detected by the phenol/H$_2$SO$_4$ method (absorbance at 490 nm, stress control ○○○○, AAHA treated △△△△), and radioactivity was measured by a liquid scintillation counter (stress control ●●●●, AAHA treated ▲▲▲▲).

**Table 1.** The radioactivity of each fraction chromatographed on a DEAE-cellulose column (DE-52)

<table>
<thead>
<tr>
<th>Radioactivity ($\times 10^3$ dpm/g tissue)</th>
<th>Control</th>
<th>AAHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>l-a</td>
<td>l-b</td>
</tr>
<tr>
<td>Non-stress (0 hr)</td>
<td>1.61</td>
<td>3.28</td>
</tr>
<tr>
<td>2 hr-stress</td>
<td>1.54</td>
<td>6.30</td>
</tr>
<tr>
<td>6 hr-stress</td>
<td>1.34</td>
<td>3.54</td>
</tr>
<tr>
<td>12 hr-stress</td>
<td>6.44</td>
<td>13.0</td>
</tr>
<tr>
<td>24 hr-stress</td>
<td>2.60</td>
<td>2.64</td>
</tr>
</tbody>
</table>

Peak I obtained by the Bio Gel A-1.5 m column from 3 rats were pooled and applied to the DEAE-cellulose column (DE-52) and eluted with a linear gradient of 0–0.4 M NaCl. Their elution patterns at 6 hr and 12 hr are shown in Fig. 5. AAHA (1,000 mg/kg) was given orally 30 min before the stress load.
graphed on the DEAE-cellulose column using a linear gradient of NaCl in 50 mM Tris-HCl buffer, it was found that AAHA increased $^{35}$S-labeled glycoproteins at 12 hr. The radioactivity of each fraction on the DEAE-cellulose column is shown in Table 1. The radioactivity of peak 1-b (SGP fraction) increased from 13.0×10$^3$ dpm/g tissue to 22.6×10$^3$ dpm/g tissue by pretreatment with AAHA at 12 hr. Furthermore, highly sulfated glycoproteins in the AAHA treated sample were eluted at higher molarity of NaCl compared with that in the stress control. However no effect was observed at 6 hr after the onset of stress.

**Changes in gastric glycosaminoglycans in restrained and water immersed rats:** The uronic acid content in the rat gastric muscular layer did not change for all the periods of stress. (The uronic acid content: 0 hr, 30.2±3.6 μg/g tissue; 2 hr, 32.2±2.0; 6 hr, 31.5±1.7; 12 hr, 34.2±4.4; 24 hr, 32.9±4.4). The effect of AAHA on the uronic acid content was not observed at 6 hr and at 12 hr after the onset of stress. The incorporation of $^{35}$S-sulfate into the glycosaminoglycans was several percent of the total incorporating activity.

**Discussion**

It is well known that the gastric surface mucosal cells produce a large amount of glycoproteins with the concomitant death of the cells, and these gastric glycoproteins including sulfated glycoproteins (SGP) are important in protecting the underlying mucosa from peptic digestion. There are many reports about the relationship between gastric mucosal damage and the synthesis of gastric glycoproteins (1-6). It has been demonstrated that the mucosal SGP had apparent peptic inhibitory activity due to the binding of SGP to the protein substrate for pepsin and protected the gastric mucosal components from peptic digestion (24). It was found from the present study that the gastric tissues were able to synthesize SGP in vitro and newly synthesized SGP released into the medium was only 10 percent. Liau and Horowitz (14) reported in an in vitro experiment that the medium fraction contained a large amount of $^{35}$S-labeled glycosaminoglycan and glycoprotein. This may be attributed to the differences in the experimental system (incubation medium, tissue preparation, fractionation, etc.).

It has been observed in in vivo studies that the incorporation of $^{35}$S-sulfate into the gastric mucosal SGP significantly decreased in the restrained rats (17-19). Such a rapid decrease in the incorporating activity of $^{35}$S-sulfate can be best explained by assuming that the supply of $^{35}$S-sulfate into the locus of the gastric macromolecular glycoprotein synthesis was remarkably decreased, possibly through the reduction of the mucosal blood flow (19). However, the influence of the mucosal microcirculation on the glycoprotein synthesis can be eliminated in the in vitro study. The results obtained here indicate that the SGP synthetic activity does not decrease at least during the test period with stress load, but rather increases at 2 hr and at 12 hr after the onset of stress when a sufficient amount of $^{35}$S-sulfate is supplied. The increase in the SGP synthetic activity may represent the defensive reaction of the gastric tissue against stress.

It has been reported that highly sulfated glycoproteins showed stronger inhibitory activity to peptic digestion (24). Therefore, we carried out a further investigation on the DEAE-cellulose column. The elution patterns showed that peak I-a was less sulfated than peak I-b. Moreover, we demonstrated from the pulse-chase experiment that highly sulfated glycoproteins (peak I-b) could be synthesized from less sulfated glycoproteins (peak I-a) (Data not shown). A slight amount of hexose was detected in the SGP fraction (peak I-b). In contrast to hexose content, high radioactivities of $^3$H and $^{35}$S in this fraction were observed. This suggests that SGP has a high rate of turnover.

The major portion of radioactivity was incorporated into SGP in the gastric mucosa, and the incorporation into sulfated glycosaminoglycans in the muscular layer was very low. The uronic acid content in the muscular layer was unchanged with stress. Accordingly, it was considered that the contribution of glycosaminoglycans in stress-induced ulcer was very small.

It was reported that macromolecular...
glycoproteins with or without sulfate groups decreased before the macroscopic changes of the mucosa appeared in aspirin-induced ulcer (5). Thus, it was suggested that the decreased mucous glycoprotein in mucosa would have a harmful effect on the protection of mucosa from peptic digestion. In our present study on stress-induced ulcer, it was indicated that SGP had the important role of assisting the healing process of damaged mucosa since the SGP synthetic activity (highly sulfated glycoproteins) remarkably increased at 12 hr after the onset of stress when the gastric tissue was directed toward the healing process.

An anti-ulcer agent, AAHA, has been reported to have a potent inhibitory effect on the formation of various types of acute gastric ulcers or erosions in rats (25). It was proved from the present study that AAHA had a stimulatory effect on the SGP synthesis, particularly the synthesis of highly sulfated glycoproteins at 12 hr after the onset of stress. This suggests that AAHA exerts its effect on the healing process. One of the authors previously reported that an anti-ulcer agent, 2-carboxymethoxy-4,4'-bis(3-methyl-2-butenyloxy)chalcone (SU-88), which has a stimulatory effect on the gastric mucosal microcirculation (26–28), increased the incorporation of $^{35}$S-sulfate in vitro (19). In contrast to SU-88, AAHA has no effect on the improvement of the mucosal microcirculation. The effect observed here may be attributed to its direct action on the SGP synthesizing cells.

In conclusion, our in vitro study shows that the SGP synthetic activity does not decrease with stress load, but rather increases at 2 hr and at 12 hr after the onset of stress. This strongly suggests that SGP facilitates the restoration of the gastric mucosa damaged by stress.

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