Immunohistochemical investigation of hepatocellular pi class glutathione S-transferase in normal and regenerating rat liver

Masaaki Mori\textsuperscript{1)}, Masamichi Ishizaki\textsuperscript{2)} and Masahiko Onda\textsuperscript{1)}

\textsuperscript{1)}First Department of Surgery, Nippon Medical School
\textsuperscript{2)}First Department of Pathology, Nippon Medical School

Summary

We have immunohistochemically investigated the occurrence of pi class glutathione S-transferase (GST-pi) both in normal rat liver and in rat liver regenerating after hepatectomy. We made our observations by examining frozen liver sections rather than the paraffin sections used in previous studies. First we examined normal liver tissue. Light microscopic examination revealed weak, discontinuous immunofluorescence along the hepatocellular plasma membranes, indicating the presence of GST-pi. Then, using immunoelectron microscopy, we detected immuno-reaction products along the bile canalicular plasma membranes, indicating the location of the GST-pi more precisely. On the second and third days after hepatectomy, as the liver was regenerating, we observed that both the immunofluorescence and the reaction products were much more intense and that the bile canaliculi were dilated as well. These observations indicate that in regenerating liver, GST-pi which is a cytosolic enzyme, concentrates in the cytosol of the hepatocytes directly under the bile canalicular plasma membranes. These observations are the first reported morphological confirmation of induced GST-pi in regenerating liver.

Key words: pi class glutathione S-transferase, bile canaliculi, regenerating liver

Introduction

Glutathione S-transferases (GSTs) are a family of multifunctional proteins that enzymatically catalyze the conjugation of glutathione with a large number of compounds including electrophilic reactive drugs, carcinogens, and metabolities. GSTs also bind a variety of nonsubstrate ligands as binding proteins\textsuperscript{3,4). The cytosolic mammalian GSTs have been divided into three species-independent classes: alpha, mu, and pi. Immunocrossreactivity has not been observed among these classes, even for the GSTs of a single species\textsuperscript{5,6). Pi class glutathione S-transferase (GST-pi) of rat is identical with the placental form of glutathione S-transferase (GST-P) and consists of subunits 7-7, also called subunits Yp-Yp or Yf-Yf\textsuperscript{3,5,7).}

Recently, intense investigation has shown that GST-pi increases remarkably in the liver.

Correspondence to Masaaki Mori, First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113 Japan
with preneoplastic or neoplastic lesion induced by rat chemical hepatocarcinogenesis\textsuperscript{8,9} and that this increase is morphologically confirmed by applying immunohistochemical method\textsuperscript{5,10,11}. This usefulness of GST-pi as a reliable histological tumor marker has subsequently been reported not just for rat liver but also for other organs of other species including human, such as the uterine cervix, colon and brain\textsuperscript{12-16}. It is well known that the liver regenerates after partial hepatectomy. Hepatocytes proliferate vigorously in this condition, resembling in the neoplastic proliferation. A previous biochemical study revealed an increased level of GST-pi in those regenerating hepatocytes, although the level observed in the hepatocytes was not as high as that observed in preneoplastic and neoplastic lesion\textsuperscript{8}. The analysis of the mechanism of liver cell proliferation after hepatectomy is very important, because this phenomenon may relate to the neoplastic proliferation of hepatocytes. The comparative study of GST-pi in these proliferating states will give a clue to control the generation of hepatic tumor. While the significant morphological manifestation of GST-pi in preneoplastic and neoplastic condition has been extensively reported, no sufficient morphological study on this enzyme has been established either in normal or in regenerating liver. To elucidate the concerning role of GST-pi in hepatocyte proliferation, we examined intracellular occurrence of GST-pi both in normal and regenerating rat hepatocytes, using immunohistochemical techniques.

**Materials and Methods**

(1) Treatment of animals

Forty young adult male Wistar rats weighting 160 g to 170 g (from Saitama Experimental Animal Supply, Saitama, Japan) were used in our experiments. We performed our experiments in compliance with "Nippon Medical School Resolution on The Use of Animal in Research". The rats were divided into three groups: normal, sham-operated, and hepatectomized. The rats in the sham-operated group were anesthetized with ether and with intraperitoneal injections of pentobarbital sodium (30 mg/kg), only laparotomized and then sutured. The rats in the hepatectomized group were subjected to about 70% partial hepatectomy as described by Higgins and Anderson\textsuperscript{17} using the same anesthesia as for the sham-operated group. The rats in the sham-operated group and the hepatectomized group were sacrificed successively between one and seven days after the operations at intervals of 24 h. We fed all three groups of rats a standard diet with water and maintained them in a 12 h-light-12 h-dark cycle. After aortic exsanguination, we sacrificed them under deep ether anesthesia and immediately removed the liver for analysis. The extracted liver was then divided into three portions. We used the first and second portions for the light microscopic examination and the third portion for the electron microscopic examination.

(2) Light microscopic examination

The first portion of the excised liver tissues was fixed with 5% neutralized formaldehyde and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin.

To observe immunofluorescence, we embedded the second portion of each liver in 7% carboxymethyl cellulose sodium salt (CMC), froze it in liquid n-hexane cooled with dry
ice/acetone, and stored it at −70°C. We fixed the 4 μm cryosections in acetone (−20°C) for 5 min, washed them in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) for 5 min, and incubated them with anti-GST Yp (compatible with rat GST-pi) rabbit serum (Biotrin International, Dublin, Ireland) (200 × diluted) overnight at 4°C. There are no crossreactivities of this antiserum to the other classes (alpha, mu)4. After washes in PBS for 5 min (3 times), we stained the sections with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG goat antibody (Zymed Laboratories, Inc., San Francisco, USA) for 60 min at room temperature. We then washed them again with PBS for 5 min (3 times). We mounted the stained sections using 50% glycerin in 0.5 M carbonate buffer, pH 9.5, and examined them with an Olympus BHF fluorescent microscope.

(3) Electron microscopic examination

We cut the third portion of the rat livers into small pieces and fixed the pieces in periodate-lysine-paraformaldehyde (PLP) fixative (10 mM sodium periodate, 75 mM lysine, 2% paraformaldehyde/37.5 mM sodium phosphate buffer, pH 6.2) for 6 h at 4°C. Then we washed the tissues in 0.01 M PBS with 10% sucrose, PBS with 15% sucrose, and PBS with 20% sucrose overnight at 4°C. Next we embedded the tissues in O.C.T. compound (Miles Inc., Elkhart, USA), quick-froze them in dry ice/acetone, and stored them at −70°C. Then we mounted the 6 μm cryosections on APS coated slides (Matsunami Glass Industries Ltd., Osaka, Japan), pretreated them with 10% normal swine serum for 10 min, and incubated them with the same anti-GST Yp serum as above at a 1:50 dilution overnight at 4°C. Next we washed the sections with PBS containing 10% sucrose for 10 min (5 times) at 4°C and incubated them with horseradish peroxidase-(HRPO)-labeled anti-rabbit immunoglobulin swine antibody (DAKO CO., LTD., Kyoto, Japan) (50 × diluted) overnight at 4°C. The sections were washed for 10 min (3 times) at 4°C with PBS containing 10% sucrose. Next we postfixated the sections in 1% glutaraldehyde for 5 min, treated them with 0.02% DAB/0.02% hydrogen peroxide for 10 min. Then we fixed them in 2% osmium tetroxide for 2 h and dehydrated them in a graded series of ethanol to 100%. Next we embedded the sections in Epok 812 and used heat to polymerize them. Then we used a Reichert-Nissei Ultracut N microtome to prepare ultrathin sections of the embedded liver material and examined those sections, with no additional staining, on a Hitachi H-7100 type electron microscope with a 75 kV accelerating voltage.

For control sections we used a preimmune rabbit serum instead of the anti-GST Yp rabbit serum, both for the immunofluorescence and electron microscopic examinations.

Results

1. Light microscopic findings

On the sections stained with hematoxylin and eosin, we found hepatocellular vacuolar change and many mitoses for a few days after hepatectomy (Photo 2-a), which were not present in the normal rat liver (Photo 1-a).

When we incubated the frozen rat liver sections of the normal and sham-operated groups with anti-GST Yp rabbit serum, we found noticeable immunofluorescence in the biliary
epithelial cells and weak, discontinuous immunofluorescence along the hepatocellular plasma membranes (Photo 1-b). All fluorescence levels in the normal and sham-operated groups remained the same thereafter. On the second and third days the fluorescence along the hepatocellular plasma membranes of the hepatectomized group was more intense than in the normal and sham-operated groups, and the strongest fluorescence was in the hepatectomized group on the second day (Photo 2-b). The fluorescence on the fourth day was markedly less than on the second day (Photo 3-b) and vacuolar change and mitoses can be hardly observed.
Photo 3-a  Rat liver on the fourth day after hepatectomy. Hepatocellular vacuolar change and mitoses can be hardly observed. (hematoxylin-eosin staining, ×200)

Photo 3-b  Immunofluorescent staining of GST Yp (rat GST-pi) in a section of regenerating rat liver on the fourth day after hepatectomy. Immunofluorescence can be seen in the biliary epithelial cells (arrow). The fluorescence along the hepatocellular plasma membrane is markedly less than in Photo 2-b. (×200)

Photo 4  Immunoelectron micrograph in a normal rat liver section, incubated with anti-GST Yp (rat GST-pi) serum. (Electron-dense) reaction products can be seen along the bile canalicular plasma membranes and the bile canaliculus can be seen to be filled with microvilli. BC; bile canaliculus. (×10,000)

Photo 5  Immunoelectron micrograph in a section of regenerating rat liver on the second day after hepatectomy, incubated with anti-GST Yp (rat GST-pi) serum. A few microvilli can be seen in the dilated bile canaliculus and strong reaction products can be seen along the bile canalicular plasma membranes. BC; bile canaliculus. (×10,000)

in paraffin embedded section with H-E staining (Photo 3-a). After the fourth day we observed no significant difference among the livers from the three groups. We did not see significant change in the fluorescence of the biliary epithelial cells at any time.
2. Electron microscopic findings

In the normal and sham-operated groups, we observed weak immuno-reaction products along the bile canalicular plasma membranes and found the bile canaliculi to be abundantly filled with microvilli (Photo 4). In the hepatectomized group, we observed many dilated bile canaliculi on the second and third days after the hepatectomy, with a few microvilli in the canaliculi and strong immuno-reaction products along the bile canalicular plasma membranes (Photo 5). On the fourth day after the hepatectomy we could not see dilation of the canaliculi and saw only weak immuno-reaction products along the membranes (Photo 6). Just as in our light microscopic examination, we found no significant differences among the three groups after the fourth day. These chronological data are summarized in Table 1.

In the control sections that we incubated with a preimmune rabbit serum instead of anti-GST Yp rabbit serum, we saw no specific immunofluorescence and immuno-reaction products at any time.

![Photo 6](Image)

Photo 6 Immunoelectron micrograph in a section of regenerating rat liver on the fourth day after hepatectomy, incubated with anti-GST Yp (rat GST-pi) serum. No dilation of the bile canaliculus is visible and weak reaction products along the bile canalicular plasma membranes can be seen. BC; bile canaliculus. (×10,000)

Table 1 Chronological evolution of morphological data on light and electron microscopy

<table>
<thead>
<tr>
<th></th>
<th>Normal group</th>
<th>Hepatectomized group</th>
<th>(POD)</th>
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<tr>
<td></td>
<td>Sham-operated group</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Intensity of IF along hepatocellular plasma membranes</td>
<td>±</td>
<td>±</td>
<td>+</td>
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<tr>
<td>Intensity of IF in biliary epithelial cells</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Dilation of bile canaliculi on electron microscopy</td>
<td>−</td>
<td>−</td>
<td>+</td>
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IF: immunofluorescence, POD: postoperative days,
−: negative, ±: weakly positive, +: positive, ++: strongly positive
Discussion

GST-pi appears to be a new marker enzyme for carcinogenesis of the liver. Various studies have indicated that GST-pi is also a good marker for detection of premalignant lesion in investigating the mechanism of the hepatocellular proliferation, and an analysis of the distribution of GST-pi in regenerating liver is very important in this context. However, previous studies have not clarified the morphological occurrence of GST-pi either in normal or in regenerating liver. In this study we have used frozen sections to demonstrate the occurrence of GST-pi in normal and regenerating rat liver, instead of using paraffin embedded sections for immunohistochemistry. Our results clearly indicated that GST-pi occurs in biliary epithelial cells, although the similar findings has been reported briefly and ambiguously by other investigators by using paraffin embedded sections. We have newly demonstrated that GST-pi occurs along the hepatocellular plasma membranes at immunofluorescence level and that the staining increases as the progress of the regeneration. Using immunoelectron microscopy, we definitely demonstrated reaction products along the bile canalicular site of plasma membranes of the hepatocytes, thus confirming the existence of the GST-pi there. This finding means that the positive immunofluorescence of hepatocytes in light microscopic level really represents the bile canalicular site of plasma membranes.

Our light microscopic examination exhibited only weak fluorescence along the hepatocellular plasma membranes in the normal and sham-operated rats. On the other hand, intense fluorescence was there in regenerating rat liver on the second and third days after hepatectomy. Biochemically, significant induction of GST-pi has been observed after 70% partial hepatectomy, with the strongest expression seen 48 h after hepatectomy. This biochemical result corresponds with our morphological observation that the strongest fluorescence along the hepatocellular plasma membranes occurred on the second day after hepatectomy. Moreover, our electron microscopic examination showed frequent dilation of the bile canaliculi on the second and third days after hepatectomy, as previously reported. The amplification of the immunofluorescence in regenerating liver corresponds with induced hepatocellular GST-pi appearing along the dilated bile canaliculi.

It is reported that induced GST-pi has been constantly observed immunohistochemically in the cytosol of preneoplastic and neoplastic lesions during chemical hepatocarcinogenesis. However, previous studies have not provided morphological confirmation that regenerating liver also induces the production of GST-pi. That means less GST-pi is produced in regenerating rat liver after hepatectomy than in preneoplastic hepatic hyperplastic nodules or in hepatocellular carcinoma tissues. In addition, the manner of expression of GST-pi in neoplastic cells is diffuse cytosolic distribution. The difference of these characteristics in expression of this enzyme between tumor and regeneration suggests that the mechanism of cell proliferation in neoplastic state and regenerating state in the liver is essentially different.

It has been reported that activity of γ-glutamyl transpeptidase (γ-GTP) also increased in regenerating rat liver after 70% partial hepatectomy. Enzymatic steps in mercapturate
formation play an important role in detoxication. Previous studies have established that GSTs catalyze the first step in mercapturate formation, while \( \gamma \)-GTP catalyzes the second step\(^2,21\) and exists on the bile canalicular plasma membranes of the hepatocytes\(^22-24\). There is a system that transports glutathione S-conjugates into bile canaliculi through the bile canalicular plasma membranes\(^25\). GST-pi is a cytosolic enzyme and \( \gamma \)-GTP is a membrane-bound enzyme. Therefore, the proximity of these two enzymes indicates that the enzymatic processes are taking place in the cytosol of the hepatocytes directly under the bile canalicular plasma membranes through the action of GST-pi and on those membranes through the action of \( \gamma \)-GTP. The catalytic action of these two enzymes in mercapturate formation may play an active role especially in the region associated with the bile canalicular plasma membranes in liver regeneration.

Paraffin embedding of histological specimen has usually been used in immunohistochemical investigations of GSTs, but our results indicate that the studies with using paraffin section\(^10,11,18\) have not clarified the histological occurrence of hepatocellular GST-pi in normal rat liver and only equivocal results have been shown in regenerating rat liver. These are probably due to the reduced immunoreactivity. The hepatocellular GST-pi along the bile canaliculi of normal rat liver is barely detectable on paraffin embedded sections because of low concentration of this enzyme there. The reason why previous studies have not demonstrated GST-pi along the bile canaliculi is ascribed to that the embedding process exposed the sections to too much heat. The methods we used in our study never required the sections to be exposed to heat higher than room temperature before immunoreaction. Our results indicate that in order to detect such small amounts of GST-pi immunohistochemically, it is indispensable to use an embedding method that keeps the immunoreactivity level higher than paraffin embedding does.

In conclusion, GST-pi occurs in the cytosol of the hepatocytes directly under bile canalicular plasma membranes, indicating that this enzyme is active there in normal liver. In the process of rat liver regeneration the induction of GST-pi occurs along the dilated bile canaliculi, in contrast to neoplastic state in which the enzyme induction occurs throughout the cytosol. Our study provides the first morphological confirmation of the induction of GST-pi in the regenerated liver.

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