Immunohistochemical Demonstration of the Gap Junctional Protein Connexin 32 and Proliferating Cell Nuclear Antigen in Glutathione S-Transferase Placental Form–Negative Lesions of Rat Liver Induced by Diethylnitrosamine and Clofibrate*

SEIICHI ITO, CHISE TATENO,1 MINA TUDA, AND AKIRA YOSHITAKE

Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., 1-98-3 Kasugade-naka, Konohana-ku, Osaka, 554, Japan

* Address correspondence to: Seiichi Ito, Nippon Experimental Medical Research Institute Co., Ltd., 416 Nakasatomita Haruna-machi, Gunma-gun, Gunma 370-33, Japan.

1 Present address: Yoshizato MorphoMatrix Project, ERATO, JRDC, Hiroshima-Techno-Plaza, 13-26, Kagamiyama, 3-chome, Higashi-Hiroshima, Hiroshima 739, Japan.

ABSTRACT

The distributions of a gap junctional protein, connexin 32 (cx 32), and proliferating cell nuclear antigen (PCNA) were examined immunohistochemically in glutathione S-transferase placental form (GST-P)–negative foci, induced in rat liver by initiation with diethylnitrosamine (DEN, 200 mg/kg) followed by promotion with clofibrate (1% in diet) in an in vivo medium-term assay system for hepatocarcinogenesis. The results were compared to those in GST-P-positive foci induced by DEN alone. The treatment with clofibrate caused the appearance of GST-P-negative foci, increased in size as compared to GST-P-positive foci in the same liver or induced by the DEN alone. The proportion of PCNA-positive hepatocytes in GST-P-negative foci was significantly higher than in the surrounding parenchyma, indicating increased cell proliferation. The numbers of cx 32–positive spots per hepatocyte in GST-P-negative foci were clearly decreased, reaching 65.4% at week 20 and 51.8% at week 30 of values for surrounding normal hepatocytes. In GST-P-positive foci induced by DEN, only a slight decrease (80%) was observed at week 8. These findings show that a positive association between the sustained inhibition of gap junctional intercellular communication and increased cell proliferation of GST-P-negative foci in Fischer-344 male rats induced with DEN and promoted with clofibrate.

Keywords. Gap junction; peroxisome proliferator; cell proliferation; hepatocarcinogenesis

INTRODUCTION

Clofibrate, one of the groups of compounds known as peroxisome proliferators (PPs), is a hepatocarcinogen in rats and mice (13, 16). Because preneoplastic and neoplastic lesions in rat liver induced by PPs are negative for marker enzymes such as glutathione S-transferase (GST-P) and γ-glutamyltranspeptidase (12), it is rather difficult to detect the carcinogenic effects of PPs on rat liver by in vivo medium-term assay system (4, 5). It has also been indicated that the c-myc gene product is not a useful marker for GST-P-negative foci induced by diethylnitrosamine (DEN)–clofibrate (6). The mechanisms by which PPs lead to carcinogenesis are as yet unclear (1).

It has been postulated that modulation of gap junctional intercellular communication (GJIC) plays an important role in carcinogenesis, because many promoting agents or activated oncogenes have been known to inhibit GJIC (7, 8, 10, 17, 19, 20). For instance, Neveu et al (10) reported decreased expression of the major hepatocyte gap junction protein, connexin 32 (cx 32), to be a common observation in rat neoplastic lesions induced by initiation with ethylnitrosourea or DEN followed by promotion with several chemicals including PPs. Krutovskikh et al (7) also described GST-P-positive foci induced by DEN and 2-acetylaminofluorene to show marked reduction of GJIC as evidenced by a significant decrease in cx 32–positive spots.

In the present study, the distributions of cx 32 and proliferating cell nuclear antigen (PCNA) in GST-P-negative foci of rat liver induced by DEN–clofibrate were examined immunohistochemically in GST-P-negative foci of rat liver induced by DEN–clofibrate in an in vivo medium-term assay system were examined immunohistochemically and compared to those in GST-P-positive foci induced by DEN alone.

MATERIALS AND METHODS

Animals and Treatment. Fischer-344 male rats weighing approximately 130 g each at the commencement were used in this study. They were divided into 3 groups. Rats in Group 1 were given a single intraperitoneal injection of DEN (200 mg/kg), dissolved in 0.9% NaCl, to initiate hepatocarcinogenesis. After 2 wk on basal diet, they were fed 1% clofibrate in the same basal diet for 28 wk. The animals were subjected to a two-thirds hepatectomy at week 3 and then sacrificed at week 8, 20, or 30. Rats in Group 2 were given 0.9% NaCl by intraperitoneal injection instead of DEN solution and treated in the same manner as Group 1. Rats in Group 3 were treated in the same manner as Group 1 but without the clofibrate in the
TABLE I.—Numbers and areas of GST-P-positive and -negative foci in liver of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Term week</th>
<th>GST-P-positive foci</th>
<th>GST-P-negative foci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No./cm²</td>
<td>Area (mm²/cm²)</td>
</tr>
<tr>
<td>1</td>
<td>DEN—clofibrate</td>
<td>8</td>
<td>8</td>
<td>1.74 ± 1.13</td>
<td>0.10 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20</td>
<td>2.29 ± 0.92</td>
<td>0.17 ± 1.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>30</td>
<td>2.09 ± 0.91</td>
<td>0.28 ± 0.13</td>
</tr>
<tr>
<td>2</td>
<td>NaCl—clofibrate</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>DEN</td>
<td>10</td>
<td>8</td>
<td>4.49 ± 2.52</td>
<td>0.26 ± 0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20</td>
<td>13.80 ± 3.57</td>
<td>1.36 ± 1.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>30</td>
<td>9.31 ± 2.76</td>
<td>1.06 ± 0.34</td>
</tr>
</tbody>
</table>

* The foci counted were larger than 0.2 mm in diameter.

Fig. 1.—Average areas (mm²) of GST-P-positive and -negative foci in rat livers of Groups 1 and 3 at weeks 8, 20, and 30. GST-P-negative foci were not detected in Group 3. Bar = SE.

Immunohistochemistry. For histopathological analysis, portions of the liver were fixed in 10% buffered formalin, and 3–5-μm paraffin sections were stained with hematoxylin and eosin. Serial sections were prepared for immunohistochemical staining with anti-GST-P rabbit antibody (15) (1/1,000 dilution) and anti-PCNA mouse monoclonal antibody (1/200 dilution; PC10, Novocastra Laboratories Ltd.) using the avidin–biotin–peroxidase complex (ABC) method. Nuclei were counterstained with Mayer’s hematoxylin. The numbers and areas of GST-P-positive and -negative foci/nodules were measured using a color video image processor (VIP-21CH, Olympus-Ikegami Tsushin, Tokyo). The numbers of PCNA-positive nuclei per >1,000 cells were counted under a microscope.

Further liver slices were immediately frozen on dry ice acetone. Frozen sections (6 μm) were fixed with acetone for 5 min at −20°C, dried, and incubated with an anti-cx 32 rabbit polyclonal antibody (9) (1/1,000 dilution) for 30 min at room temperature. After washing in phosphate-buffered saline, the sections were reacted with anti-rabbit immunoglobulin (1/200, Vecta stain ABC kit, Vector Laboratory, Inc., USA) for 30 min and then incubated with fluorescein-conjugated streptavidin (1/30 dilution) for 30 min at room temperature. The sections were coverslipped with 90% glycerin including 0.1% phenylenediamine and viewed and photographed under a fluorescence microscope. Some sections were double-stained for cx 32 and GST-P by utilizing rhodamine-conjugated anti-rabbit immunoglobulin. The number of fluorescent spots on hepatocyte plasma membranes was counted using an image analysis system with photographs (LA-555, Pias, Inc., Osaka).

RESULTS

Table I summarizes data for numbers and areas of GST-P-positive and -negative foci in livers of rats treat-

Table II.—Proportion (%) of PCNA-positive nuclei in GST-P-negative and -positive foci and surrounding hepatocytes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Proportion (%) of PCNA-positive nuclei</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>20 Wk</td>
</tr>
<tr>
<td>Group 1 (DEN-clofibrate)</td>
<td></td>
</tr>
<tr>
<td>GST-P-negative foci</td>
<td>0.47</td>
</tr>
<tr>
<td>GST-P-positive foci</td>
<td>0.41</td>
</tr>
<tr>
<td>Surrounding hepatocytes</td>
<td>0.06</td>
</tr>
<tr>
<td>Group 3 (DEN)</td>
<td></td>
</tr>
<tr>
<td>GST-P-positive foci</td>
<td>0.70</td>
</tr>
<tr>
<td>Surrounding hepatocytes</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Fig. 2.—DEN–clofibrate induced GST-P-negative lesion (A, circumscribed by arrows) at week 30. The number of PCNA-positive nuclei is increased in this lesion (B). Serial sections.

ed with the 3 different protocols. In Group 3 (DEN only), GST-P-positive foci were induced at week 8 and markedly increased in number at weeks 20 and 30, but no GST-P-negative foci were found. Group 1 (DEN–clofibrate) demonstrated significantly smaller numbers and areas of GST-P-positive foci than Group 3 throughout the study period, whereas GST-P-negative foci, first detected at week 20, were clearly increased in terms of number and size at week 30. In Group 2 (NaCl–clofibrate), neither GST-P-positive nor -negative foci were found.

Figure 1 shows average sizes (area, mm²) of GST-P-positive and -negative foci in Groups 1 and 3. Clofibrate did not alter the sizes of GST-P-positive foci as compared

Fig. 3.—Numbers of cx 32–positive spots per hepatocyte in GST-P-negative or -positive foci and surrounding parenchyma in rat liver at weeks 8, 20, and 30. **p < 0.01; *p < 0.05, significantly different from surrounding hepatocytes by Student’s t-test. Bar = SE.
to those in Group 3 (DEN only). However, the sizes of GST-P-negative foci in Group 1 were clearly increased after 30 as compared to 20 wk.

Table II shows proportions (%) of PCNA-positive nuclei in GST-P-positive or -negative foci and surrounding hepatocytes at weeks 20 and 30. The ratios of PCNA-positive nuclei in both GST-P-positive and -negative foci were significantly elevated at weeks 20 and 30 (Fig. 2A, B).

Figure 3 summarizes data for the numbers of cx 32-
positive spots per hepatocyte in GST-P-negative and -positive foci of Groups 1 and 3 as compared to those in surrounding hepatocytes. In the GST-P-negative foci of Group 1, numbers of cx 32-positive spots were significantly smaller than those in the surrounding hepatocytes at weeks 20 and 30 (see Fig. 4A, B). Relative numbers of cx 32-positive spots in GST-P-negative foci were 65.8 ± 6.0% and 51.9 ± 12.5% of those in the surrounding hepatocytes at weeks 20 and 30, respectively. In the GST-P-positive foci of Group 3, numbers of cx 32-positive spots were slightly decreased to 80.1 ± 9.9% at week 8 but recovered thereafter (Fig. 4C, D).

**DISCUSSION**

Recent evidence (2, 3) suggests that the mechanism of tumor promotion in the liver of rodents by clofibrate may be a selection process that allows proliferation to selectively occur in so-called spontaneously initiated hepatocytes, which arise during aging.

As shown in Table I, GST-P-positive foci were induced by a single administration with DEN (Group 3), but subsequent administration of clofibrate (Group 1) decreased development of such GST-P-positive lesions while causing large GST-P-negative foci to arise. Clofibrate itself did not induce any type of focus during the 28-wk administration period (Group 2). These results suggest that clofibrate promotes the DEN-initiated cells giving rise to GST-P-negative foci.

Clofibrate enlarged the size of GST-P-negative foci at weeks 20 and 30 but showed negative effects on the size of DEN-induced GST-P-positive foci. PCNA immunohistographs showed that the cell proliferation in both GST-P-negative and -positive foci was considerably elevated as compared to that in surrounding hepatocytes. The results indicate that clofibrate selectively promotes cell proliferation and growth of GST-P-negative foci. It is noteworthy that the proportion of PCNA-positive nuclei in surrounding parenchyma was smaller in Group 1 than in Group 3, suggesting that clofibrate reduces cell proliferation of normal hepatocytes from rats initiated with DEN. Tanaka et al (18) similarly reported that clofibrate treatment of rats for 13 wk inhibited DNA synthesis of hepatocytes, dose-dependently.

It has been described that decreased expressions of a gap junction protein, cx 32, were consistently observed in rat altered hepatic foci induced by either ethynitrosourea or DEN, followed by promotion with phenobarbital (PB), dioxin, chloroendic acid, C.J. Solvent Yellow, tamoxifen, Wy-14, 643, or ciprofibrate. And the decrease of cx 32 in altered hepatic foci was rapidly reversible after withdrawal of PB (10). The decreased expression of cx 32 spots in GST-P-negative foci induced by DEN–clofibrate observed in the present study seems to coincide with the preceding results. The fact that no changes in the distribution of cx 32 spots were found in GST-P-positive foci induced by DEN at weeks 20 and 30 suggests that the decreased expression of cx 32 spots found in GST-P-positive foci at week 8 recovered without any dosing with PPs such as clofibrate.

A single injection of DEN (200 mg/kg body weight) causes hepatocellular carcinomas to develop in 35% of Fischer-344 rats maintained for their life span (11). The carcinomas are considered to develop from GST-P-positive foci through neoplastic nodules, although the majority of foci/nodules disappear during this process. The present finding that dosing with clofibrate after initiation by DEN is associated with a clear and constant reduction of GJIC within GST-P-negative foci, linked to the fact that 0.5% dietary supplementation with these agents causes hepatocellular carcinomas in 91% of Fischer-344 rats (14), suggests a direct link between GST-P-negative foci (GJIC-impaired), increased mitosis, and tumor growth. The interrelationship between mitosis and altered GJIC cannot be further defined based on this study. In conclusion, the mechanism of development of GST-P-negative foci induced by DEN–clofibrate may be considerably different from that of GST-P-positive foci.

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