Suppressive Effect of Irsogladine Maleate on Diethylnitrosamine-initiated and Phenobarbital-promoted Hepatocarcinogenesis in Male F344 Rats

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Modifying effects of irsogladine maleate (IRG) on diethylnitrosamine (DEN)-induced hepatocarcinogenesis were examined in male F344 rats. Six-week-old rats were divided into 8 groups. Groups 1 through 4 were given a single i.p. injection of DEN (200 mg/kg body weight) at the start of the experiment, whereas groups 5 through 8 received a single i.p. injection of saline as the vehicle treatment. Groups 1 and 8 were kept on the basal diet and distilled water throughout the experiment (36 weeks). Groups 2 and 7 were exposed to 500 ppm phenobarbital (PB) in the drinking water, starting one week after the carcinogen or vehicle treatment. Groups 3 and 5 were fed the diet mixed with 125 ppm IRG from one week after DEN or vehicle treatment. Groups 4 and 6 were given 125 ppm IRG-containing diet and drinking water with 500 ppm PB after the carcinogen or vehicle treatment. Liver neoplasms developed in groups 1 (1/15 rats, 7%) and 2 (14/14 rats, 100%). However, no liver tumors were found in rats of groups 3 through 8. Incidence and average number of liver neoplasms in group 4 (0/14 rats, 0%) were less than those in group 2 ($P<0.0001$). The number of glutathione S-transferase placental form (GST-P)-positive liver cell foci in group 3 or 4 was significantly smaller than that in the appropriate control ($P<0.01$, $P<0.001$, respectively). The average and unit areas of these foci in group 4 were also significantly smaller than those in group 2 ($P<0.001$, $P<0.05$, respectively). These results suggest that IRG could be a chemopreventive agent for rat liver carcinogenesis.

Key words: Irsogladine maleate — Hepatocarcinogenesis — Chemoprevention

Chemoprevention embraces the concept that non-carcinogenic synthetic chemicals or natural products can inhibit the process of carcinogenesis. A number of agents have proved effective against chemical carcinogenesis in different organs, and have been classified into two major categories, i.e., blocking and suppressing agents.

The mechanism of carcinogenesis is considered to involve multistep genetic alterations. Using animal models, the stages of initiation, promotion and progression have been well investigated, and in particular, studies on hepatocarcinogenesis have provided considerable information. Gap junctional intercellular communication (GJIC) has been reported to play an important role in the control of cell proliferation or differentiation, and the modulation of GJIC is suggested to be involved in hepatocarcinogenesis, especially in the promotion phase. The gap junctions mediate the transfer of signal-transducing factors such as calcium, cAMP, and inositol triphosphate. They are made up of oligomeric proteins composed of 6 subunits, called connexins (Cxs), and more than 10 connexins, are so far known in mammals. Among them, Cx 32 and Cx 26 are commonly expressed in hepatocytes. Furthermore, Cx 32 is known to decrease in preneoplastic and neoplastic lesions of the liver, while Cx 26 is reported to show a different expression pattern from that of Cx 32 in preneoplastic and neoplastic lesions. Previously, we have demonstrated that in vivo exposure to liver tumor promoters, such as phenobarbital (PB) or dichlorodiphenyltrichloroethane, decreased the size and altered the distribution of gap junctions in rat hepatocytes, suggesting inhibitory effects of these agents on GJIC. Agents that promote the function of gap junctions may have suppressing effects on carcinogenesis.

Recently, irsogladine maleate (IRG) (Fig. 1), an antiulcer agent, has been found to enhance GJIC, and combination chemotherapy with IRG and other agents was reported to be effective against metastatic tumors from gastric cancer. We have immunohistochemically examined the effect of IRG on gap junctions of the liver, and found that IRG inhibited the decrease of Cx 32 induced by PB (unpublished data).

In the present study, we examined the possible modifying effect of IRG in a rat hepatocarcinogenesis model using diethylnitrosamine (DEN) as an initiator. Dose
selection of IRG was based on maximum tolerated dose (MTD) values in the previous study. IRG was administered during the promotion phase, and glutathione S-transferase placental form (GST-P)-positive preneoplastic lesions were analyzed.

MATERIALS AND METHODS

Animals, diet, drinking water and carcinogen Weanling male F344 rats, purchased from Shizuoka SLC Co., Shizuoka, were used. Powdered CE-2 (Clea Japan Inc., Tokyo) was used as a basal diet. DEN and PB were purchased from Nacalai Tesque Inc., Kyoto and Maruishi Pharm. Co., Osaka, respectively. IRG was synthesized by Nippon Shinyaku Co., Inc., Kyoto.

All animals were housed in wire cages (3 rats/cage). They had free access to water and diet under controlled environmental conditions of humidity (50±10%), lighting (12 h light/dark cycle) and temperature (23±2°C). The experimental diet mixed with IRG and drinking water containing PB were prepared weekly and stored in a cold room (4°C).

Experimental procedure A total of 132 rats, 6 weeks of age, were divided into 8 groups (Fig. 2). Animals of groups 1 through 4 received a single i.p. injection of DEN (200 mg/kg body weight) at the start of the experiment. Groups 5 through 8 were given a single i.p. injection of saline (vehicle). Rats of groups 1 and 8 were fed the basal diet alone throughout the experiment (36 weeks). Groups 2 and 7 were given drinking water containing 500 ppm PB from one week after the carcinogen or vehicle treatment. Groups 3 and 5 were fed the diet mixed with IRG and drinking water containing PB were prepared weekly and stored in a cold room (4°C). The experimental diet mixed with IRG and drinking water containing PB were prepared weekly and stored in a cold room (4°C).

Fig. 1. Molecular structure of irsogladine maleate (C12H11Cl2N3O4: 372.17).

Fig. 2. Experimental design. : DEN 200 mg/kg body weight i.p. injection. : Saline. : PB 500 ppm in drinking water. : Basal diet. : Animals killed. : 125 ppm IRG.

Experimental procedure A total of 132 rats, 6 weeks of age, were divided into 8 groups (Fig. 2). Animals of groups 1 through 4 received a single i.p. injection of DEN (200 mg/kg body weight) at the start of the experiment. Groups 5 through 8 were given a single i.p. injection of saline (vehicle). Rats of groups 1 and 8 were fed the basal diet alone throughout the experiment (36 weeks). Groups 2 and 7 were given drinking water containing 500 ppm PB from one week after the carcinogen or vehicle treatment. Groups 3 and 5 were fed the diet mixed with 125 ppm IRG from one week after DEN or vehicle treatment. Groups 4 and 6 were given 125 ppm IRG-containing diet and drinking water with 500 ppm PB from one week after the carcinogen or vehicle treatment. At 11 and 21 weeks after the carcinogen treatment, 3 rats from each group were killed to determine the incidence of altered liver cell foci positive for GST-P. At the termination of the experiment (36 weeks), remaining animals were killed by exposure to ether. At autopsy, the location, number and size of liver tumors were recorded. Liver tissues were sliced into 2 pieces from each sublobe. One set of slices was fixed in cold acetone and another set was fixed in 10% buffered formalin, embedded in paraffin blocks, and processed for routine histological observation with the use of hematoxylin and eosin stain. The other organs were also subjected to routine histological processing for histopathological examination.

Immunohistochemical staining The liver sections from acetone-fixed tissues were stained for GST-P using the avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Lab. Inc., Burlingame, CA). Anti-GST-P antibody was provided by Dr. K. Satoh. The areas of GST-P-positive foci and the number of foci/cm² were measured by means of an image analyzer (IPAP) with a microscope (Sumitomo Chemical Co., Ltd., Osaka). GST-P-positive lesions composed of more than 11 cells were considered as altered liver cell foci.

Statistical analysis Differences of incidence or density of pathological lesions in the liver between groups were
foci were seen in all the DEN-treated animals. The den-
types of preneoplastic hepatocellular foci (clear, eosino-
philic and basophilic), which were also positive for GST-
P, were found in the groups exposed to DEN. The results
of quantitative analysis on the frequency of GST-P-posi-
tive foci is summarized in Table III. GST-P-positive
foci were seen in all the DEN-treated animals. The den-
sity and average area of GST-P-positive foci of group 2
were higher than those of group 1 or group 4 (Table III).
The density of GST-P-positive foci of group 3 was rather
lower than that of group 1, although the difference was
not significant. A significant decrease in the average area
of GST-P-positive foci was present in the animals fed the
IRG diet compared to the appropriate positive controls
(P<0.001 or P<0.0001). The area of GST-P-positive foci
was significantly decreased in the groups given IRG
(P<0.0001).

Table I. Body and Relative Liver Weights of F344 Rats Treated with DEN, PB and/or IRG

| Treatment       | No. of rats (initial) | 11 wks     | 21 wks     | 36 wks     |
|-----------------|-----------------------|------------|------------|------------|
|                 | Body weight (g)       | Relative liver weight(%) | Body weight (g) | Relative liver weight(%) | Body weight (g) | Relative liver weight(%) |
| DEN alone       | 21                    | 292±2*     | 3.43±0.03  | 330±11     | 3.33±0.20  | 378±44     | 3.42±0.20  |
| DEN+PB          | 21                    | 317±5*     | 4.53±0.13  | 337±13     | 4.25±0.12* | 342±36*   | 4.80±0.25* |
| DEN+IRG         | 21                    | 274±7*     | 3.53±0.10  | 310±28     | 3.22±0.18  | 288±29*   | 3.14±0.28* |
| DEN+PB+IRG      | 21                    | 289±35*    | 3.92±0.61  | 281±13*    | 4.08±0.33  | 288±40*   | 4.25±0.23* |
| IRG alone       | 12                    | 292±41     | 3.23±0.37  | 315±15     | 3.27±0.49  | 332±15    | 3.04±0.15  |
| PB + IRG        | 12                    | 286±24     | 4.91±0.13  | 298±11     | 4.82±0.34  | 303±12    | 4.28±0.18  |
| PB alone        | 12                    | 311±9      | 4.72±0.08  | 327±19     | 4.68±0.11  | 342±24    | 4.57±0.34  |
| Vehicle control | 12                    | 278±10     | 3.11±0.13  | 325±18     | 3.15±0.15  | 397±27    | 3.22±0.24  |

*Mean±SD.

RESULTS

General observation There was no clear evidence of
toxicity in the animals exposed to the IRG diet (Table I).
Two rats in group 1 and one rat in group 2 died of pneu-
monia before the termination of the experiment, and no
neoplasms were found in them. Mean body weights of the
animals given the basal diet alone (group 1), IRG diet
alone (group 5), PB alone (group 7), and PB and IRG
(group 6) were respectively 397±27g, 333±15g, 342±24g
and 303±12g at the termination. Mean relative liver
weights of the groups treated with PB were larger than
those of the groups without PB treatment, though the
differences were not statistically significant. In other organs,
no apparent toxic effects were found in IRG-treated
groups.

Tumor incidence Liver tumors were recognized only in
groups 1 and 2. They were all of hepatocellular origin
(Table II). Hepatocellular adenomas were found in both
groups, but liver cell carcinomas were seen only in group
2. Interestingly, no hepatocellular neoplasms were present
in any group exposed to IRG. The incidence of hepatocel-
lular carcinoma or adenoma, and the incidence of total
liver neoplasms of group 2 were significantly higher than
those of the other groups (P<0.01).

Incidence of hepatocellular foci In this study, three
types of preneoplastic hepatocellular foci (clear, eosino-
philic and basophilic), which were also positive for GST-
P, were found in the groups exposed to DEN. The results
of quantitative analysis on the frequency of GST-P-posi-
tive foci are summarized in Table III. GST-P-positive
foci were seen in all the DEN-treated animals. The den-

DISCUSSION

The results of the present study clearly indicate the
inhibitory effect of IRG on DEN-induced rat hepatocarci-
ogenesis in rats when given during the promotion
phase. In this study, IRG showed a prominent suppressive
effect on hepatocarcinogenesis promoted by PB, suggest-
ing that IRG basically acted as an antipromoter. Quantita-
tive analysis of the altered liver cell foci using the
phenotypic marker GST-P was consistent with the inci-
dences of liver neoplasms. These liver cell foci are gener-
ally recognized as preneoplastic lesions in the lineage of
hepatocellular carcinoma development and are considered
to reflect the carcinogenic potential, due to the consistent
manner in which liver cell foci appear during the post-ini-
tiation stage of hepatocarcinogenesis.24)

IRG has been reported to enhance GJIC.16–18) An
inverse correlation between the expression of Cx 32 and
BrdU labeling index has been reported in partially hepatec-
tomized liver.8,9) Reduction of GJIC is suggested to be
associated with the process of mitosis.25) Thus, modulation
of carcinogenesis by GJIC is considered to be important,
especially in the promotion phase.35) Tsuda et al. reported a
progressive decrease of Cx 32 expression in liver carci-
Table II. Incidence of Hepatocellular Neoplasms in Each Group

| Treatment          | No. of rats (terminal) | Incidence(%) | Multiplicity |       |
|--------------------|------------------------|--------------|--------------|-------|
|                    |                        | Hepatocellular neoplasm | Adenoma | Carcinoma | Hepatocellular neoplasm | Adenoma | Carcinoma |
| DEN alone          | 13                     | 1(7)         | 1(7)         | 0     | 0.07±0.25* |
| DEN+PB             | 14                     | 14(100)      | 9(64)        | 7(50) | 0.82±1.11* |
| DEN+IRG            | 15                     | 0            | 0            | 0     | 0.07±0.25 |
| DEN+PB+IRG         | 15                     | 0*           | 0            | 0     | 0.07±0.25 |
| IRG alone          | 6                      | 0            | 0            | 0     | 0         |
| PB +IRG            | 6                      | 0            | 0            | 0     | 0         |
| PB alone           | 6                      | 0            | 0            | 0     | 0         |
| Vehicle control    | 6                      | 0            | 0            | 0     | 0         |

a) Mean±SD.
b-d) Significantly different from the rats treated with DEN alone by Fisher’s exact probability test (b) P<0.0001, (c) P<0.01, (d) P<0.005.

c-g) Significantly different from the rats treated with DEN alone by Student’s t test (e) P<0.0005, (f) P<0.005, (g) P<0.05.
h-j) Significantly different from the rats treated with DEN alone by Fisher’s exact probability test (h) P<0.0001, (i) P<0.005, (j) P<0.005.

Table III. Results of the Quantitative Analysis of GST-P-positive Foci in Rats of Each Group

| Treatment          | Density (/cm²) | Average area (×10² µm²) | Density (/cm²) | Average area (×10² µm²) | Density (/cm²) | Average area (×10² µm²) |
|--------------------|---------------|-------------------------|---------------|-------------------------|---------------|-------------------------|
|                    | 11 wks        | 21 wks                  | 36 wks        |                         |               |                         |
|                   | 0.89±0.70*    | 2.28±1.33               | 1.13±0.75     | 3.21±2.34               | 3.79±0.63     | 10.95±7.76              |
| DEN+PB             | 7.15±2.75     | 2.68±4.68               | 14.22±5.49    | 5.29±6.24               | 19.30±1.68    | 21.87±9.77              |
| DEN+IRG            | 0.55±0.26     | 1.89±1.57               | 0.92±0.40     | 3.71±4.05               | 3.40±1.34     | 7.80±7.71               |
| DEN+PB+IRG         | 2.79±1.61     | 2.52±2.95               | 5.20±1.36     | 2.43±2.64               | 8.15±3.89    | 8.92±6.32               |
| IRG alone          | 0             | 0                       | 0             | 0                       | 0             | 0                       |
| PB +IRG            | 0             | 0                       | 0             | 0                       | 0.27±0.30     | 6.72±5.57               |
| PB alone           | 0             | 0                       | 0.12±0.08     | —                       | 0.60±0.59     | 8.45±4.10               |
| Vehicle control    | 0             | 0                       | 0             | 0                       | 0.23±0.41     | 4.02±0.26               |

a) Mean±SD.
b,c) Significantly different from the rats treated with DEN alone by Student’s t test (b) P<0.0001, (c) P<0.001.
d) Significantly different from the rats treated with DEN+IRG by Student’s t test (P<0.001).
c) Significantly different from the rats treated with DEN+PB by Student’s t test (P<0.001).

genesis, together with an inverse correlation of the expression with hepatocellular proliferation, suggesting that Cx 32 expression has an important role in the hepatocarcinogenesis. The results of this study are consistent with such a hypothesis.

Eghbali et al., injected transfected and non-transfected human hepatoma cell lines into nude mice to examine the effect of the Cx 32 gene, and found that the tumor derived from Cx 32-transfected cells was significantly smaller than that derived from non-transfected cells. Negative growth control in vivo and in vitro was also observed in other tumorigenic cell lines transfected by gap junction genes. It is speculated that connexin gene expression basically increases gap junctional protein, enhances intercellular communication and influences cell proliferation. This may be one mechanism the suppressing effect of IRG on hepatocarcinogenesis.

Recently, IRG has been reported to inhibit the induction of tissue-type plasminogen activator synthesis in endothelial cells, and the angiogenesis induced by epidermal growth factor. The induction of tissue-type plasminogen activator is considered to be indispensable for growth factor-dependent angiogenesis. Larson and Haudenschild have reported that junctional coupling is slightly reduced during the repair of wounded aortic endothelial cells. On the other hand, Pepper and Meda indicated that migrating endothelial cells express plasminogen activator activity, while GJIC itself increases during the migration
of endothelial cells. These findings suggest that IRG may suppress angiogenesis, which may also play an important role in tumor growth, through abrogation of the induction of plasminogen activator by modulating GJIC of endothelial cells. This could be another mechanism by which IRG suppresses hepatocarcinogenesis. In addition, IRG has been shown to activate GJIC through the M₁ muscarinic acetylcholine receptor. Activation of the M₁ receptor inhibits Raf activation induced by growth factors. Therefore, the suppressive effect of IRG on hepatocarcinogenesis found in the present study may be due to signaling through the M₁ muscarinic acetylcholine receptor.

In this study, body weight gain tended to be decreased by the IRG treatment, though no particular toxic effects caused by IRG were found in histopathological examination. The dose of IRG in this study was the MTD value based on the previous study. Further work is needed to confirm the chemopreventive effects at lower doses.

Although more studies are necessary to confirm dose-dependency, and to establish the precise mode of action of IRG and the underlying mechanisms during carcinogenesis, the results of the present investigation strongly suggest that IRG, currently being used as a therapeutic agent, could also be a promising chemopreventive agent for human liver neoplasia.

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