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The Effect of Deferoxamine on the Preneoplastic Lesions in the Chemically Induced Hepatocarcinogenesis

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Iron is essential for the growth of all living cells. One of the most important intracellular roles of iron is the activation of ribonucleotide reductase, which is indispensible to the production of deoxyribonucleotide necessary for DNA synthesis. Deferoxamine (DFO) is an iron chelating agent and has been known to have an antiproliferative effect in various malignant cells including hepatocellular carcinoma and the effect seems to be related to depletion of iron. This study was undertaken to investigate the effect of DFO on preneoplastic lesions in chemically induced hepatocarcinogenesis. The resistant hepatocyte model was used and Sprague Dawley rats were divided into the following groups ; I: normal control, II: carcinogen administered group, III: carcinogen and DFO administered group. Rats were sacrificed at 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks and 8 weeks after partial hepatectomy (PH). DFO (50 mg/kg/day, I.P.) was daily injected from 3 weeks before administration of carcinogen to the time when rats were sacrificed. Hepatic iron content was higher in group II than in group III, especially at 3 days and 1 week after PH. Hyperplastic lesions of resistant hepatocytes were less well developed in group III than in group II. Bromodeoxyuridine labelling indices of oval cells and hyperplastic lesions of resistant hepatocytes were higher in group II than in group III except for rats examined at 3 days after PH. The results suggest that DFO has an antiproliferative effect on preneoplastic lesions in hepatocarcinogenesis and it might be related to reduction of the hepatic iron.

Key Words: Iron, deferoxamine, hepatic carcinogenesis, preneoplastic lesion, resistant hepatocyte

Hepatocellular carcinoma (HCC) is very common in Korea and the prognosis of HCC is poor in spite of the introduction of new therapeutic modalities. Recently the concern about the preneoplastic lesions of HCC is increasing. The preneoplastic lesions in chemically induced hepatocarcinogenesis include oval cell proliferation, hyperplastic foci of hepatocyte, hyperplastic nodules and cholangiofibrosis (Sell and Leffert, 1982; Sell and Dunsford, 1989).

Iron is essential for the growth of all living cells. Even though the precise nature of iron requirement for cell growth is not known, iron appears to be an essential component of ribonucleotide reductase, a critical enzyme required for DNA synthesis (Thelander and Reichard, 1979).

Deferoxamine mesylate (DFO) is an iron-chelating compound and has been used to treat patients with acute and chronic iron toxicity. DFO binds equimolar amount of fer-

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ric iron to form a DFO-Fe^{3+} complex, ferrioxamine with high stability ($Ka=10^{31}$) (Kerberle, 1964). DFO can chelate iron from ferritin and hemosiderin but not from cytochromes and hemoglobin. Furthermore, DFO has been shown to have an antiproliferative or a cytotoxic effect in a variety of malignant cells (Blatt and Stitely, 1987; Estrov *et al.* 1987; Becton and Bryles, 1988; Donfrancesco *et al.* 1990) and to cause cell death in HCC and hepatoblastoma cell lines, but not to have cytotoxic effect on human diploid cells of embryonic lung fibroblast (Hann *et al.* 1990).

The resistant hepatocyte model (Solt and Farber method), a rat model for hepatocarcinogenesis, has a major advantage in the homogeneity with respect to their stages in hepatocarcinogenic sequence. Initiation is made by a necrotic dose of diethylnitrosamine (DEN) and this procedure induces resistant hepatocytes which can be selectively stimulated to proliferate by a mitogenic stimulus such as partial hepatectomy (PH), in the presence of dietary N-acetyl-aminofluorene (AAF) on the level sufficient to inhibit proliferation of the uninitiated non-resistant hepatocyte (Solt *et al.* 1977; Farber, 1980; Solt *et al.* 1983).

The present study was undertaken to evaluate the effect of DFO on the preneoplastic lesions in hepatic carcinogenesis.

MATERIALS AND METHODS

Animals and experimental design

Male Sprague Dawley rats initially weighing 140 to 160 g were used. The rats were maintained on basal diet (Fe 65 mg/kg diet).

The animals were divided into the following groups; I: normal control, II: carcinogen administered group, III: carcinogen and DFO administered group. The experimental design of groups II and III was based on the model described by Solt and Farber (Solt *et al.* 1977). Rats were given a single dose of DEN (200 mg /kg, I.P.) (Sigma Chemical Co, St. Louis, MO, USA). Two weeks later, the rats were fed AAF (0.02% in the basal diet) (Sigma Chemical Co, St. Louis, MO, USA) for 2 weeks. The rats were subjected to a two-thirds partial hepatectomy after a week on AAF. Then the rats were kept on the basal diet until the termination of the experiment. A preliminary study had been performed to decide the relevant dose of DFO, and then in group III, DFO was injected in a daily dose of 50 mg/kg intraperitoneally (Sigma Chemical Co, St. Louis, MO, USA), starting 3 weeks before injection of DEN and continuing to the termination of rats. Rats of groups II and III were sacrificed at 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks and 8 weeks after PH (Fig. 1) respectively. At 2 hours before the sacrifice, each animal was given an injection of bromodeoxyuridine (BrdU) (200 mg/m^2 surface area) (Sigma Chemical Co, St. Louis, MO, USA) through the artery in tail portion.

Tissue preparation

A small piece of tissue obtained from each liver was fixed in 10% buffered formalin solution and embedded in paraffin. The remaining

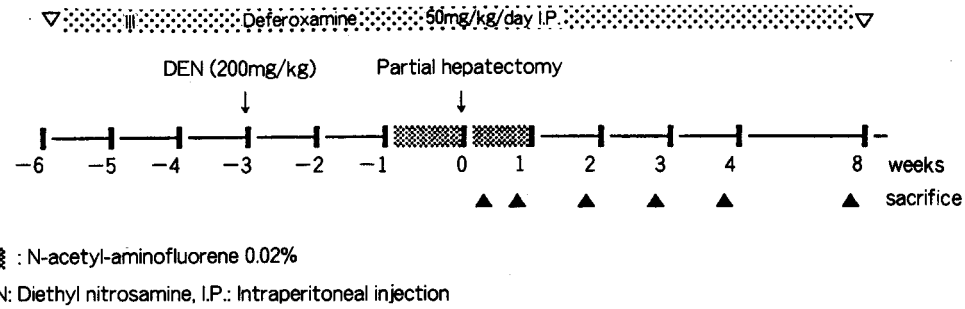

Fig. 1. *Schedule for drug administration in carcinogen administered group.*

part of the liver was processed for measuring the hepatic iron content.

Measurement of hepatic iron content

A piece of liver tissue, 1g in weight, was taken and dried by heater. It was burned at 400℃ by burner and then cooled at room temperature. It was dissolved in 15 ml of 5% HCl and passed through the filter (No. 42) and then diluted by adding 5% HCl making a volume of 25ml. The content of iron was estimated by atomic absorption spectrophotometer (Shidamazu AA-650, Tokyo, Japan). Fe standard solutions of 5, 10, 20 ppm were used for standardization.

Histologic and immunohistochemical examination

For histological examination, 4 μm thick paraffin sections were stained with hematoxylin and eosin and Perl's Prussian blue stain for iron.

The degree of oval cell proliferation and hepatocellular hyperplastic lesions were assessed in group II and group III according to the following criteria. Degree of oval cell proliferation was divided into 5 grades as follows: focal proliferation at periportal area (grade 1), focal proliferation in the lobule (grade 2), sheet-like proliferation at periportal area (grade 3), incomplete (grade 4) and complete (grade 5) encircling around hepatocellular hyperplastic lesions. The grades of hepatocellular hyperplastic lesions were divided according to the longest dimension of the lesion: below 250 μ (grade 1), 250 μ~500 μ (grade 2), 500 μ~1 mm (grade 3), 1 mm~2 mm (grade 4), above 2 mm (grade 5). Those hyperplastic lesions of the grade 4 or 5 (>1 mm in diameter) were defined as hyperplastic nodule.

BrdU was demonstrated by ABC method using 4 μm thick paraffin sections. After deparaffinization, the sections were incubated with 1N HCl for 20 min at room temperature, and then trypsin and 1% periodic acid were applied for 20 min and 15 min repectively. After a rinse in distilled water the sections were incubated overnight with monoclonal mouse anti-BrdU (Dako, Santa Babara, CA, USA, dilution 1:50). Biotinylated anti-mouse IgG (Vector, Burlingame, CA, USA) ser-ved as the second step and avidin-biotin peroxidase complex (Vector, Burlingame, CA, USA) served as the third step. Peroxidase activity was developed using 3-amino-9-ethylcarbazole (Biomeda, Fostar, CA, USA). After visualization of reaction product, sections were counterstained with Carazzi's hematoxyline, dehydrated and mounted.

Negative control for BrdU was made by omission of the primary anti-serum. Basal cells of epidermis were used as positive control for BrdU. BrdU labelling index was calculated as percent number of positively stained nuclei with anti-BrdU per 500 cells of each preneoplastic lesion.

Statistical analysis

Mann-Whitney test was used for statistical analysis. A difference was regarded as statistically significant, if p value was less than 0.05.

RESULTS

Hepatic iron content

The hepatic iron content of each group was presented in table 1. Hepatic iron content of normal control was 73.3±8.40 μg/g (50.0~96.5 μg/g wet liver weight), which was much higher than that of DFO only treated animals (51.2± 6.68). Hepatic iron content of group II was higher than those of normal control at 3 days and 1 week after PH ($p<0.05$). But there was no significant difference in hepatic iron content between group II and normal control at 2 weeks, 3 weeks, 4 weeks and 8 weeks after PH. Hepatic iron content of group III was not significantly different from those of normal control in all subgroups except those sacrified 8 weeks after PH. Hepatic iron content of group III was much lower than those of group II at 3 days, 1 week, 4 weeks and 8 weeks after PH ($p<0.05$), particularly at 3 days and 1 week.

Histologic findings

The administration of DFO did not alter the

Table 1. Hepatic iron content (μg/g wet liver weight)

Group	Time after partial hepatectomy					
	3 days	1 wk	2 wks	3 wks	4 wks	8 wks
I			73.3±8.40(7)			
II	162.8±24.62(6)*	104.6±6.37(5)*	68.3±7.19(5)	71.5±3.91(7)	75.9±5.40(9)*	68.8±8.08(8)*
III	64.3±3.68(5)	83.6±2.25(4)	94.9±30.82(5)	70.9±20.75(6)	54.0±4.06(5)	48.8±5.50(4)

( ): Number of animals
Values are mean±standard deviation.
*: Significant difference when compared with respective values of group III ($P<0.05$, Mann-Whitney test)

Table 2. Degree of oval cell proiferation and hyperplastic lesion of hepatocyte in the carcinogen administered group

Time after PH	Gruop	No. of animals	Oval cell proliferation					HL of hepatocyte				
			1	2	3	4	5	1	2	3	4	5
3 days	II	6	6	0	0	0	0	6	0	0	0	0
	III	5	5	0	0	0	0	5	0	0	0	0
1wk	II	5	2	0	3	0	0	3	1	1	0	0
	III	4	1	1	2	0	0	3	1	0	0	0
2wks	II	5	0	2	1	2	0	0	2	3	0	0
	III	5	0	3	1	1	0	4	1	0	0	0
3wks	II	7	0	7	0	0	0	0	6	1	0	0
	III	6	0	6	0	0	0	3	3	0	0	0
4wks	II	9	0	8	0	1	0	0	6	3	0	0
	III	5	0	5	0	0	0	2	3	0	0	0
8wks	II	8	0	6	0	1	1	0	4	3	0	1
	III	4	0	4	0	0	0	0	2	2	0	0

HL: hyperplastic lesion, PH: partial hepatectomy

hepatic morphology. There was no stainable iron in the liver of normal control on Prussian blue stain.

In groups II and III, oval cell proliferation and hyperplastic lesion of resistant hepatocytes were developed. At 3 days after PH, proliferation of oval cells in periportal area and occasional small aggregates of altered hepatocytes were evident. At 1 week after PH, oval cells showed sheet-like arrangement in the periportal area, some of which infiltrated deeply into the hepatic acini. Basophilic hyperplastic foci, composed of basophilic hepatocytes and intermediate cells of hepatocyte and oval cell, appeared at 1 week after PH. The basophilic hepatocytes had round vesicular nuclei. The number of oval cells and the area occupied by oval cells increased and the basophilic foci became enlarged at 2 weeks after PH. These findings were more pronounced in group II than in group III. At 3 weeks after PH, eosinophilic hyperplastic foci became to appear and they were composed of hepatocytes with abundant clear or eosinophilic cytoplasm. Focal proliferation of oval cells was found here and there in the lobule. By 3 and 4 weeks after PH, proliferation of hyperplastic foci was more pro-

Table 3. BrdU labelling indices of oval cell in the carcinogen administered group

Group	Time after partial hepatectomy					
	3 days	1 wk	2 wks	3 wks	4 wks	8 wks
II	7.8±1.25(6)*	11.5±2.69(5)	8.7±2.55(5)*	2.9±0.48(7)	4.4±2.00(9)	4.0±1.78(8)
III	15.4±3.15(5)	9.3±2.36(4)	5.0±2.04(5)	3.2±0.58(6)	2.6±1.08(5)	2.6±0.26(4)

Values are mean±standard deviation.
( ): Number of animals
*: Significant difference when compared with respective values of group III ($p<0.05$, Mann-Whitney test).

Table 4. BrdU labelling indices of resistant hepatocyte of hyperplastic lesion in the carcinogen administered group

Group	Time after partial hepatectomy					
	3 days	1 wk	2 wks	3 wks	4 wks	8 wks
II	4.9±0.90(6)*	4.8±1.88(5)	5.5±0.71(5)*	2.5±0.44(7)*	2.2±1.33(9)	1.3±0.21(8)
III	9.1±1.42(5)	4.7±0.15(4)	2.1±0.82(5)	1.3±0.23(6)	2.2±0.49(5)	1.4±0.17(4)

Values are mean±standard deviation.
In group I, where there is no hyperplastic lesion developed, the BrdU labelling index is 0.3±0.08.
( ): Number of animals
*: Significant difference when compared with respective values of group III ($p<0.05$, Mann-Whitney test).

nounced in group II than in group III and eosinophilic foci were larger in group II than in group III. Oval cells accompanying fibrosis began to encircle the large eosinophilic foci partly. In group II hyperplastic nodules were developed and oval cells accompanying slight fibrosis completely encircled the hyperplastic nodules at 8 weeks after PH. However, hyperplastic nodules were not developed in group III.

The hyperplastic lesion of resistant hepatocyte was less well developed in group III than in group II (Table 2). There was no significant difference in the proliferation of oval cell between group II and group III. The liver revealed no stainable iron in groups II and III on Perl's Prussian blue stain.

BrdU labelling indices

The mean value of BrdU labelling indices of normal hepatocytes in group I was 0.3± 0.08. The BrdU labelling indices of oval cells were higher than those of normal hepatocytes ($p<0.05$), as shown in table 3. In group II, the BrdU index of oval cells was highest at 1 week after PH and then decreased gradually. In Group III, it was highest at 3 days after PH and decreased rather abruptly. The BrdU labelling indices of oval cells were higher in group III than in group II at 3 days after PH ($p=0.01$) and were lower in group III than in group II at 2 weeks after PH ($p=0.02$). The other subgroup did not show any significant difference of BrdU labelling indices of oval cells between group II and group III.

As shown in table 4, the BrdU labelling indices of the resistant hepatocytes of hyperplastic lesion were higher than those of normal hepatocytes but lower than those of oval cells ($P<0.05$). BrdU labelling indices of the hepatocellular hyperplastic lesions were higher in group III than in group II at 3 days after PH ($P=0.01$). Thereafter they began to decrease markedly in group III and became higher in group II than in group III at 2 and 3 weeks after PH ($p=0.01$, $p=0.00$) (Fig. 2).

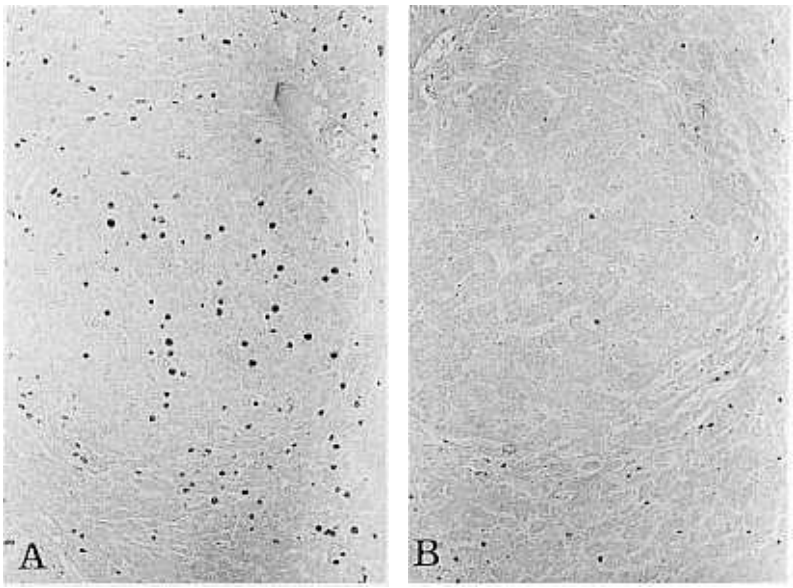

Fig. 2. BrdU labelled cells of hyperplastic nodule. The carcinogen only administered group (A) showing higher BrdU labelling index than the both carcinogen and deferoxamine administered group (B) (ABC, ×100).

DISCUSSION

This study shows that DFO has an anti-proliferative effect on preneoplastic lesions in hepatocarcinogenesis. Recently, antitumor activity of DFO had been demonstrated in neuroblastoma (Blatt and Stitely, 1987; Becton and Bryles, 1988; Donfrancesco *et al.* 1990), acute leukemia (Estrov *et al.* 1987), hepatoblastoma and HCC (Tabor and Kim, 1991; Hann *et al.* 1992), but was not proved in normal human diploid cells of embryonic lung fibroblast (Hann *et al.* 1990). Our study is the first demonstration of an in vivo effect of DFO on the preneoplastic lesions in hepatocarcinogenesis.

DFO crosses cell membrane, accumulates within the cell and chelates intracellular iron, ferritin and hemosiderin (Bottomley *et al.* 1985). Because iron is required for the activity of ribonucleotide reductase, which is important in the production of deoxyribonucleotides necessary for DNA synthesis (Thelander and Reichard, 1979), the iron chelating agent DFO appears to exert an inhibitory effect on proliferative activity of tumor probably by depleting iron (Hoffbrand *et al.* 1976). Without iron, cells are unable to proceed from the G1 phase to the S phase of the cell cycle (Larrik and Cresswell, 1979; Lederman *et al.* 1984).

The resistant hepatocyte model (Solt-Farber method) used in this study provides analyzable cell population that is homogeneous with respect to its stage in the hepatocarcinogenic sequence and the hyperplastic foci and nodules of resistant hepatocytes are considered as the early putative preneoplastic lesions (Solt *et al.* 1977; Farber, 1980; Solt *et al.*, 1983).

In this study, the hepatic iron content of group III was significantly elevated at 3 days and 1 week after PH, followed by returning to the normal value, indicating that hepatic recruitment of iron is an early event in experimental hepatic carcinogenesis.

It had been shown that rat hepatoma cells take up ^{59}Fe from transferrin more extensively than adult rat hepatocytes in culture. However, the hepatoma cells incorporate iron into ferritin with lesser degree, presumably because more iron is used for the synthesis of

iron-containing enzymes necessary for cell growth (Linder *et al.* 1970; Bomford and Munro, 1985). In this study the hepatic iron content was estimated by atomic absorption spectrophotometer, which represented the amount of iron contained in enzymes as well as in the storage form. Livers of group II and group III showed no stainable iron on Prussian blue stain. These results suggest that most of hepatic iron was used for iron-containing enzymes at 3 days and 1 week after PH. The low level of hepatic iron in group III is then considered to represent insufficiency of iron store which is necessary to activate the essential enzyme for growth, such as ribonucleotide reductase.

In group III, the hepatic iron content was relatively lower and the development of preneoplastic lesions was less prominent than in group II. Hyperplastic nodule was not developed in group III. Then it is suggested that DFO has an antiproliferative effect on preneoplastic lesions in hepatocarcinogenesis, probably through lowering the hepatic iron content. The antiproliferative effect of DFO was more marked on hyperplastic lesion of the resistant hepatocytes than the oval cells.

BrdU is incorporated into DNA in the place of thymidine and detection of BrdU in the cellular DNA enables a fraction of cells in the S phase of their cycle to be enumerated and it is one of useful methods to evaluate the proliferative activity (Morstyn *et al.* 1983). The cell cycle of the resistant hepatocyte is lengthened mostly due to prolongation of the S phase and the mean cycle length is 38.6 hours in the rat (Rotstein *et al.* 1984). BrdU labelling indices of preneoplastic lesions were lower in group III than in group II in accordance with the lower hepatic iron content of group III, supporting the antiproliferative effect of DFO.

The antiproliferative effect of DFO was reported in HCC and hepatoblastoma cell lines, but whether the addition of iron could overcome the effect of DFO remains controversial. In addition, the administration of calcium disodium versenate, a chelating agent with similar affinity for iron, showed no inhibitory effect on the cultured HCC cells and hepatoblastoma cells (Tabor and Kim, 1991; Hann *et al.* 1992). So for the antiproliferative effect of DFO, there might be other mechanisms in addition to iron chelation .

Furthermore, in spite of much less hepatic iron content, the BrdU labelling indices of oval cells and resistant hepatocytes at 3 days after PH were higher in group III. The reason is not yet known. In this context, the relationship between cellular proliferation and the transferrin receptor (TR) is interesting. Cells acquire iron from transferrin by TR mediated endocytosis (Karin and Mintz, 1981). TR is present in high levels in most rapidly proliferating normal cells and transformed cells. Its expression is closely linked to the proliferation status of the cell (Bomford and Munro, 1985; May and Cuatrecasas, 1985). Although TR expression is not entirely cell cycle dependent, the number of TR expression is greater in S and G2 phases than in G1 phase (Musgrove *et al.* 1984), and the regulation of TR expression in proliferating cells is assumed to be closely related to DNA synthesis (Hedley *et al.* 1985). The number of TR was reported to be increased in the hyperplastic nodule of resistant hepatocytes (Eriksson *et al.* 1986). DFO was reported to increase the number of TR probably by chelating the regulatory pool of iron within the cell and by arresting the cell cycle in S phase when TR was maximally expressed (Bomford *et al.* 1986). This prolongation of S-phase by DFO might be the mechanism by which high BrdU labelling indices of oval cells and resistant hepatocytes at the early proliferating stage result in.

In conculsion, the results suggest that DFO has an antiproliferative effect on the preneoplastic lesions in hepatocarcinogenesis and it might be related to the reduction of hepatic iron content.

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