Journal of Korean Medical Science
Vol. 10, No. 3, 183-188 June 1995

Transferrin Receptor Expression of the Hyperplastic Lesions of Hepatocyte in Experimental Hepatocarcinogenesis

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Transferrin receptor(TR) performs the major function of binding and internalizing its specific iron-loaded ligand, transferrin, and its expression is closely linked to the proliferation status of the cell. This study was undertaken to elucidate TR expression in the hyperplastic lesion of hepatocyte in chemically induced hepatic carcinogenesis. The resistant hepatocyte model was chosen for a rat model of carcinogenesis and Sprague-Dawley rats were divided into the following groups : the control groups of normal diet and iron-rich diet with or without hydroxyquinoline and the groups of carcinogen alone and carcinogen plus iron-rich diet with or without administration of hydroxyquinoline. Microscopic changes in the liver, expression of transferrin receptor and glucose-6-phosphatase were studied. The hepatocyte of the control group showed both cytoplasmic and membranous expression of TR. The liver of rats fed on high iron diet accumulated iron and the expression of TR was down regulated by intrahepatic iron accumulation. In the carcinogen administered group the resistant hepatocyte of hyperplastic lesion revealed strong membranous expression of TR and failed to accumulate iron in spite of high iron diet but in contrast the surrounding non-resistant hepatocyte expressed TR in both the membrane and cytoplasm and stored iron when fed on high iron diet. The strong membranous expression of TR is one of the characteristics of the resistant hepatocyte of hyperplastic lesion and it seems to be related to the inability to accumulate iron in spite of a high iron diet.

Key Words : *Transferrin receptor, Hepatocyte, Hyperplastic lesion, Hepatic carcinogenesis, Iron*

INTRODUCTION

Iron is essential for the growth of all living cells including tumor cells and transferrin receptor(TR) is a transmembrane homodimetric glycoprotein and performs an important role in intracellular delivery of iron(Hann et al., 1988 ; Bonkovsky, 1991). TR is present in higher levels in most rapidly proliferating normal and transformed cells and its expression is closely linked to the proliferation status of the cell (Bomford and Munro, 1985 ; May and Cuatrecasas, 1985).

Hyperplastic nodule is one of the preneoplastic lesions in hepatocarcinogenesis and is known to have increased numbers of TR with the same affinity to transferrin as that of normal hepatocyte(Eriksson et al.,

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This work was supported by the Research Grant for Basic Medical Sciences of the Ministry of Education, 1994, Korea.

1986). However, characteristically hepatocellular hyperplastic foci is unable to accumulate iron in spite of iron rich diet(Williams et al., 1976; Williams and Watanabe, 1978).

In vivo, iron is transported by the serum transferrin and is mainly delivered to cells after binding of transferrin to TR. It is suggested that the expression of TR, proliferation status of the cell, and intracellular iron delivery may be closely related and TR expression of normal hepatocyte may be different from that of hepatocellular hyperplastic lesion in hepatic carcinogenesis.

The present study was undertaken to evaluate the TR expression in hepatocytes of normal and iron loaded liver and in the hyperplastic lesion of hepatocytes in hepatocarcinogenesis.

MATERIALS AND METHODS

1. Animals and experimental design

Male Sprague Dawley rats initially weighing 140 to 160 gm were used. All animals received humane care in compliance with the guidelines as outlined in the "Guide for the Care and Use of Laboratory Animals"(National Institutes of Health publication no. 86-23, revised 1985).

The experimental animals were divided into two major groups : the control group(I) and the carcinogen administered group(II). The control group was divided into the following groups : I-A : normal control, I-B : ferrous sulfate administered group, I-C : ferrous sulfate and 8-hydroxyquinoline(HQ) administered group. The rats of normal control(I-A) were maintained on basal diet(Fe 65 mg/kg diet). The rats of I-B were fed ferrous sulfate(2.5% in the basal diet) (Sigma Chemical Co, St. Louis, MO, USA) and those of I-C were fed ferrous sulfate(2.5% in the basal diet) with HQ(0.8% in the basal diet) (Sigma Chemical Co, St. Louis, MO, USA) for 16 weeks, 18 weeks and 23 weeks. And then, five rats of each subgroup were sacrificed after a brief period of anesthetization with ether. The carcinogen administered group was divided into the following groups : II-A : only carcinogen administered group, II-B : carcinogen and ferrous sulfate administered group, II-C : carcinogen, ferrous sulfate and HQ administered group. The experimental design for the carcinogen administered group was based on the model described by Solt and Farber(Solt et al., 1977). Rats were given a single dose of diethylnitrosamine(DEN) (200 mg/kg I.P.) (Sigma Chemical Co, St. Louis, MO, USA). Two weeks later, the rats fed N-acetyl-aminofluorene(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(PH) after a week on AAF. The rats of II-A were kept on the basal diet. The rats of II-B were fed ferrous sulfate(2.5% in the basal diet) and those of II-C were fed ferrous sulfate(2.5% in the basal diet) and HQ(0.8% in the basal diet) from 12 weeks prior to injection of DEN to induce hepatic siderosis(Williams and Yamamoto, 1972), which was continued to a day before sacrifice. Five rats of each subgroup were sacrificed at 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks and 8 weeks respectively after PH under anesthetization with ether (Fig. 1).

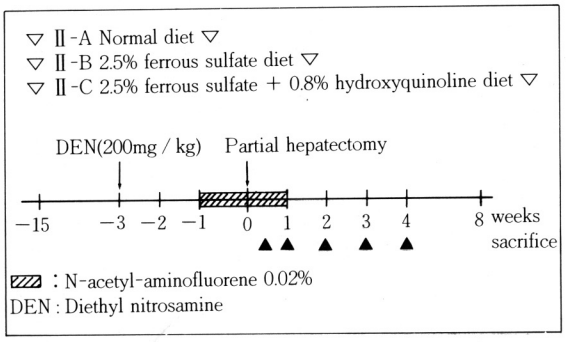

Fig. 1. Schedule of drug administration in the carcinogen administered group

2. Tissue preparation and (Immuno) histochemical method

A small piece from each liver was frozen in liquid nitrogen cooled isopentene at −6℃ for enzyme histochemistry and immuno-histochemistry. For glucose-6-phophatase(G6Pase) histochemistry, 20 μm thick cryostat sections were incubated in modified Wachstein and Meisel medium [0.25M tris-maleic acid buffer(pH 7.2), 5.0mM $MgSO_4 7H_2O$, 5.0mM $pb(NO_3)$ and 0.8mM glucose-6-phosphate] for 20min at 36℃. They were fixed in 10% neutral formalin and then washed with distilled water. After fixation in 1% ammonium sulfide they were washed, dehydrated and mounted.

For TR, cryostat sections(4 μm thick) were air dried on vecta bond(Dako, Santa Babara, CA, USA) coated slides and were fixed in cold acetone for 20 min. They were then treated with Tris buffered

saline(pH 7.6) for 10 min. The sections were incubated with a monoclonal anti-rat TR antibody(OX-26, Serotec Oxford, England, dilution 1 : 100) for an hour at room temperature. Rabbit anti-mouse antibody (Dako, Santa Babara, CA, USA) served as the second step and alkaline phosphatase anti-alkaline phosphatase(Dako, Santa Babara, CA, USA) served as the third step. The reaction product was visualized with fast red(Dako, Santa Babara, CA, USA). Then the sections were mounted. Negative control for TR was made by omission of the primary anti-serum, and the endothelium within the liver was used as positive control for TR.

The remaining part of each liver sample was prepared for histological examination with fixation in 10% buffered neutral formalin solution and then a small piece of it was embedded in paraffin and processed according to standard methods. Paraffin sections were stained with hematoxylin and eosin for general morphological assessment and iron staining with Perls' Prussian blue stain was carried out according to standard methods.

RESULTS

1. Histopathologic finding

The livers of I-A showed a normal appearance and those of I-B and I-C were normal except for hemosiderin-like golden yellow pigments in hepatocytes, Kupffer cells and portal tracts. The accumulation of pigments was more marked in I-C than in I-B.

In the carcinogen administered group the preneoplastic lesions developed in the order of the following sequences. At 3 days after PH proliferation of oval cells in the periportal area and occasional small aggregates of altered hepatocytes developed. Oval cells showed sheet like arrangement in the periportal area and infiltrated deeply into the hepatic acini at 1 week after PH. Basophilic hyperplastic foci, composed of intermediate cells of hepatocytes, oval cells and basophilic hepatocytes, appeared at 1 week after PH. The number of oval cells as well as the area occupied by oval cells increased and the basophilic foci became more prominent at 2 weeks after PH. At 3 weeks after PH eosinophilic hyperplastic foci composed of hepatocytes with clear and eosinophilic abundant cytoplasms and focal proliferation of oval cells were found in the lobule. Eosinophilic foci were enlarged and oval cells accompanying fibrosis began to encircle the eosinophilic foci partly. Hyperplastic nodules developed and oval cells with fibrosis encircled them completely at 8 weeks after PH. However, hepatocytes of hyperplastic nodules did not show cytologic atypia. There was no significant difference in the degree of proliferation of oval cells and the development of hepatocellular hyperplastic lesion among II-A, II-B and II-C and they appeared in regular sequence. Cholangiofibrosis accompanied stratification of ductular(? oval) cells with nuclear atypia and developed in 57%, 40%, and 0% of rats in II-B, II-C and II-A respectively at 8 weeks after PH.

2. Expression of G6Pase

In the livers of the control group(I-A, I-B and I-C), hepatocytes showed G6Pase activity almost equally throughout the hepatic tissue, but within the liver lobule it was slightly weaker in the acinar zone 3.

In the carcinogen administered group(II-A, II-B and II-C), the hepatocellular hyperplastic lesion showed negative reaction to G6Pase. At first all basophilic hyperplastic foci showed a negative reaction to G6Pase and its activity recovered gradually. At 8 weeks after PH most hyperplastic nodules eventually restored G6Pase activity except for a few hepatocytes with consistent negative reaction. There was no difference in the pattern of G6Pase expression among II-A, II-B and II-C.

3. Expression of transferrin receptor and distribution of storage iron

1) The control group

The livers of the normal controls(I-A) showed no stainable iron on Prussian blue stain. TR was expressed equally throughout the hepatic lobule in terms of degree and zonality. The hepatocyte showed both cytoplasmic and membranous expression of TR although the membranous expression of TR was stronger than cytoplasmic expression.

The livers of I-B and I-C showed stainable iron in hepatocytes, Kupffer cells and portal tracts on Prussian blue stain. Accumulation of iron started from the acinar zone 1 and gradually extented to the acinar zone 3. The accumulation of iron increased in relation to the duration of iron administration and it was more pronounced in I-C than in I-B particularly after iron administration for 16 weeks and 18 weeks. The hepatocyte of I-B and I-C showed both cytoplasmic and membranous expression of TR and the hepato-

cytes of acinar zone 1 with marked iron accumulation showed weak expression of TR and those of acinar zone 3 with little iron accumulation showed strong expression of TR mainly in the cytoplasm. The expression of TR decreased as the accumulation of iron increased and TR of Kupffer cells and histiocytes continued to express strongly after TR expression of hepatocytes decreased. The pattern of TR expression showed no difference between I-B and I-C but the intensity was stronger in I-C than in I-B.

2) The carcinogen administered group

II-A showed no stainable iron on Prussian blue stain. In II-B and II-C hepatocellular hyperplastic lesion failed to accumulate iron in spite of the high iron diet whereas surrounding non-resistant hepatocytes showed accumulated iron. The inability of iron accumulation in hepatocellular hyperplastic lesion continued consistently and eventually hyperplastic nodules showed no stainable iron on Prussian blue stain at 8 weeks after PH. However, iron accumulation was found in Kupffer cells, histiocytes and portal tracts in both II-B and II-C.

The resistant hepatocyte of hyperplastic lesion showed an accentuated membranous expression of TR. In the groups fed the high iron diet(II-B and II-C) the membranous expression of TR of iron-free hepatocytes of hyperplastic lesion contrasted with both cytoplasmic and membranous TR expression of the surrounding iron stored hepatocytes(Fig. 2). The membranous TR expression of resistant hepatocyte increased gradually as basophilic hyperplastic foci, eosinophilic foci and hyperplastic nodules developed consecutively. The strongest membranous expression of TR without cytoplasmic expression was noted in the hyperplastic nodules(Fig. 3). The hepatocyte of hyperplastic lesion did not show any difference in pattern and intensity of TR expression regardless of iron administration, however the cytoplasmic TR expression of non-resistant hepatocytes was stronger in II-B and II-C than in II-A. Histiocytes and Kupffer cells also showed strong cytoplasmic expression of TR in both II-B and II-C.

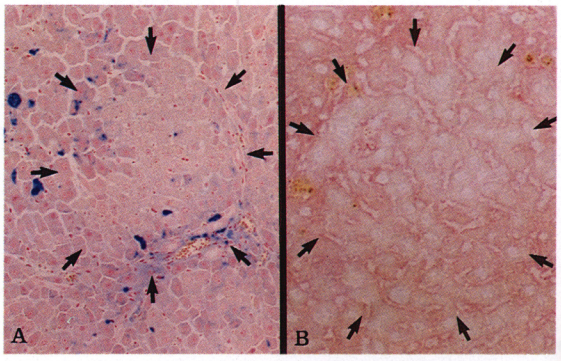

Fig. 2. Distribution of iron storage and transferrin receptor(TR) in the group with administration of carcinogen, ferrous sulfate and 8-hydroxyquinoline at 1-week after partial hepatectomy. A : Hepatocytes of hyperplastic lesion(arrow) showing no stainable iron in contrast to iron accumulation in surrounding hepatocytes(×200, Prussian blue). B : Hepatocytes of hyperplastic lesion(arrow) showing membranous expression of TR in contrast to cytoplasmic and membranous expression of TR in surrounding hepatocytes(×400, APAAP).

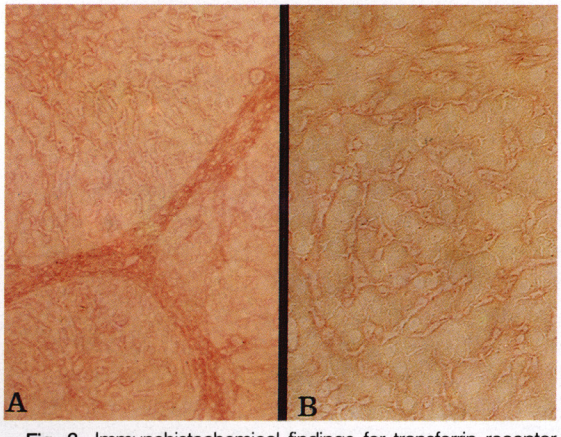

Fig. 3. Immunohistochemical findings for transferrin receptor in the hyperplastic nodule. Hyperplastic nodule showing strong membranous expression of transferrin receptor(A : ×100, B : ×400, ABC).

DISCUSSION

TR plays a major role in binding and internalization of specific iron-loaded ligand, transferrin. The intrahepatic iron regulates the number of TR on the cell surface slowly by alteration in the synthesis or removal of TR, or rapidly by a shift in the proportion of the total TR population on the cell surface(Bomford and Munro, 1985). In the iron administered control groups of this study, expression of TR was stronger in hepatocytes of acinar zone 3 than in those of acinar zone 1, which was contrary to the pattern of iron accumulation. The expression of TR decreased as the accumulation of iron increased, which suggests that the expression of TR is to be down regulated by in-

trahepatic iron accumulation.

The resistant hepatocyte model (Solt-Farber method) used in this study provides analyzable cell populations that are homogeneous with respect to their stage in the hepatocarcinogenic sequence and the hyperplastic foci and nodules of resistant hepatocyte are considered as early putative preneoplastic lesion(Williams and Yamamoto, 1972 ; Solt et al., 1977 ; Farber, 1984). In this study the resistant hepatocytes of hyperplastic lesion failed to accumulate iron regardless of the high iron diet. This agrees with previous reports(Williams and Yamamoto, 1972 ; Williams et al., 1976 ; Williams and Watanabe, 1978) and the inability to accumulate iron in spite of high iron supply is considered to be a characteristic feature of hepatocellular hyperplastic lesion.

Expression of TR is also closely linked to the proliferation status of the cells and the number of TR was reported to be increased in hepatocyte nodule(Eriksson et al., 1986). In this study the resistant hepatocyte of hyperplastic lesion showed strong accentuated membranous expression of TR without cytoplasmic expression and this pattern is similar to the previous report of increased numbers of surface TR in regenerating rat liver after partial hepatectomy (Akiko and Nobu, 1989). In cellular incorporation of iron, the binding of iron-containing transferrin or ferrotransferrin to cell surface receptor(TR) is the initial step and then ferrotransferrin-receptor complex is internalized via clathrin coated pits. During endocytosis, iron is dissociated from transferrin, probably in edosomes with acidic pH(pH 5). The apotransferrin, still bound to TR, is then recycled to the cell surface, where apotransferrin is released(Bomford and Munro, 1985 ; May and Cuatrecasas, 1985). Therefore, any disturbance dysfunction of these steps in intracellular delivery of iron causes inability of iron accumulation in spite of a high iron diet. In this study the resistant hepatocyte of hyperplastic lesion revealed a strong membranous expression, whereas the surrounding iron stored non-resistant hepatocyte showed stronger expression of TR in the cytoplasm than in the membrane. TR of hepatocellular hyperplastic lesion was reported to have the same affinity to transferrin as that of normal hepatocyte(Eriksson et al., 1986) and the increased membranous expression of TR in resistant hepatocyte may suggest the existence of a rate-limiting step in transferrin endocytosis. Though the nature of the signal to induce internalization of TR is not known precisely, phosphorylation of TR is suggested to be operative(Johnstone et al., 1984 ; May et al., 1984 ; Testa et al., 1984 ; Hebbert and Morgan., 1985).

Thus the strong membranous expression of TR is one of the characteristics of resistant hepatocyte of hyperplastic lesion and it seems to be related to the inability to accumulate iron in spite of a high iron diet.

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