Immunohistochemical Characterization of Hepatoblastomas in B6C3F1 Mice Treated with Diethylnitrosamine and Sodium Phenobarbital

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ABSTRACT. Hepatoblastomas (HBs) were induced in B6C3F1 male mice by diethylnitrosamine (DEN) and sodium phenobarbital (PB). Six-week-old mice received a single intraperitoneal dose of DEN followed by a continuous treatment with PB in diet at a concentration of 0 (group 1) or 500 (group 2) ppm for 50 weeks. HBs were observed in 13 of 21 (62%) group 2 mice, with typical histologic features as reported previously, while no such tumors were observed in group 1. Seven of 13 (54%) HBs were found in and/or adjacent to hepatocellular adenomas (HCAs) or hepatocellular carcinomas (HCCs). Immunohistochemically, all HBs were positive for S-100 protein but negative for keratin, α -fetoprotein (AFP), albumin (ALB) and vimentin, while HCC cells occasionally reacted positively for AFP with a mosaic pattern. HCC and HCA cells were occasionally positive for ALB. Non-neoplastic hepatocytes and normal bile ducts were positively stained for ALB and keratin/S-100 protein, respectively. S-100 protein is known to be expressed in many mesenchymal tissues and neoplasms including neuroectodermal elements but negative in cells of the hepatic lineage. Thus, the present immunohistochemical results suggested that mesenchymal differentiation occurs in mouse HB cells as observed in human HBs, one of the most frequent infant liver tumors in humans. Although the susceptibility of mouse HBs to PB-promotion suggests a hepatocytic histogenesis, the present immunohistochemical results support the hypothesis that the mouse HB is derived from pluripotent endodermal stem-like cells.

KEY WORDS: hepatoblastoma, immunohistochemistry, mouse, S-100 protein.

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Mouse hepatoblastomas (HBs) are rare, but occur spontaneously in aged animals or can be induced by certain chemicals in some strains [6, 7, 10, 19, 32]. Histologically, mouse HBs are well demarcated from surrounding normal tissues or hepatocellular neoplasms. In man, HBs are classified into several histological types and differentiation into various components is observed [1, 13, 26, 33]. Mouse HBs, however, lack a variety of subtypes and are histologically less differentiated. Previous immunohistochemical examination of the tumors revealed frequently positive for keratin, but no other specific immunoreactivity has been demonstrated so far [12, 19]. Although several pathways can be conceived in the development of mouse HBs, no clear answer has been available so far due to their poorly differentiation.

In the present study, we successfully produced HBs at a high rate in B6C3F1 mice which received a single dose of diethylnitrosamine (DEN) followed by a continuous dietary treatment with sodium phenobarbital (PB), a well known promoter of hepatocellular tumors in rodents [22], and made a comparative immunohistochemical examination of mouse HBs, hepatocellular adenomas (HCAs) and carcinomas (HCCs) in an attempt to cast light on the histogenesis of mouse HBs.

MATERIALS AND METHODS

Male 5-week-old B6C3F1 mice were purchased from Charles River Japan Inc. (Kanagawa, Japan) and acclimatized for 1 week in an air-conditioned animal room at 22°C with a 12 hr light/12 hr dark cycle. Mice were randomized

into 2 groups of 10 (group 1) and 24 (group 2) animals. They were fed basal powdered diet and tap water *ad libitum*. All mice were intraperitoneally administered a single dose of 80 mg/kg DEN (Tokyo Kasei Co., Ltd., Tokyo, Japan) at 6 weeks of age. Then, they were maintained on a diet containing PB (Tokyo Kasei Co., Ltd.) at a concentration of 0 (group 1) or 500 (group 2) ppm for 50 weeks. At termination, all surviving animals were on euthanasia under ether anesthesia.

Livers were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut in 3-4 μ m sections and stained with hematoxylin-eosin (HE). They were also examined immunohistochemically for keratin, S-100 protein, α -fetoprotein (AFP), albumin (ALB) and vimentin. The primary antibodies used were as follows: rabbit anti-keratin, predominantly of 56 and 64 kilodaltons, (ready to use; Dako Japan Co., Ltd., Kyoto, Japan), rabbit anti-S-100 protein (1:400 dilution; Dako Japan Co., Ltd.), rabbit anti-mouse AFP (1:1000 dilution; ICN Biomedicals Inc., Costa Mesa, CA, U.S.A.), goat anti-mouse ALB (1:1000 dilution; Bethyl Laboratories Inc., Montgomery, TX, U.S.A.), and goat anti-vimentin (1:250 dilution; ICN Biomedicals Inc.). Tissue sections were exposed to these primary antibodies, and then processed by avidin-biotin-peroxidase complex (ABC) methods using Vectastain(r) elite ABC kits (Vector Laboratories Inc., Burlingame, CA, U.S.A.). The sections for keratin or vimentin were incubated with 0.1% trypsin in phosphate buffered saline for 30 min before blocking endogenous peroxidase with 3% H₂O₂ in methanol. Visualization of binding was by the peroxidase-diaminobenzidine (DAB) reaction and the sections were counterstained with

hematoxylin to facilitate microscopic examination.

RESULTS

Six of the 24 animals in group 2 died or were on euthanasia because of loss of condition. The cause of death or morbidity was ascribed to hepatic tumors because there were no major pathological findings other than the tumors. Three of these mice were excluded from evaluation due to severe autolysis.

Tumors were found only in group 2. The incidences of HCAs, HCCs and HBs were 17/21 (81%), 13/21 (62%) and 13/21 (62%), respectively (Table 1). All 13 HBs were complicated with HCAs or HCCs. In particular, 7 of them (54%) were found in and/or adjacent to HCAs or HCCs on the same slides. The others occurred independently. HBs showed typical histologic features as mentioned in previous reports. Namely, most tumor cells were elongated to spindle-shaped with irregular hyperchromatic nuclei and scant basophilic cytoplasm. Between these basophilic tumor cells, nests or rows of HB cells with moderately abundant eosinophilic cytoplasm were observed. They varied in size and were arranged radially or concentrically around blood vessels, being found as palisading arrangements or ribbons (Fig. 1). HCAs and HCCs also exhibited typical histologic features, the component cells having abundant eosinophilic cytoplasm and large nuclei. HCAs were relatively well demarcated from surrounding parenchyma but with some compression. HCA cells were similar to normal hepatocytes, but cellular atypia were present. HCCs showed highly cellular atypia with trabecular growth patterns (data not shown).

The immunohistochemical findings are summarized in Table 2. All HBs were positive for S-100 protein (Fig. 2)

but negative for keratin (Fig. 3), AFP (Fig. 4), ALB and vimentin. The expression of S-100 protein was heterogeneous between the HB cells and strongly positive cells were scattered in the tumor tissues. HB cells in palisading arrangements and ribbons tended to be particularly strongly positive for S-100 protein. On the other hand, all HCCs and HCAs were negative for S-100. HCCs occasionally stained positively for AFP (4/13, 31%, Fig. 4) with mosaic patterns, but all HCAs were negative. As for ALB, positive cells were sparsely observed in all HCCs and HCAs examined (data not shown). Non-neoplastic hepatocytes were positive for ALB and normal bile ducts were clearly immunoreactive for keratin. The former were negative for S-100 protein while weak staining was seen in the latter. HCAs, HCCs, non-neoplastic hepatocytes and normal bile ducts had no immunoreactivity for vimentin.

DISCUSSION

Mouse HBs can be induced by certain chemicals in some strains [10, 19]. For example, Diwan *et al.* [4, 5] reported a high incidence in D2B6F1 mice administered DEN and PB. In the present study, HBs were induced in 62% of B6C3F1 mice treated with a single dose of DEN and PB in diet for 50 weeks, and this result, including the data for HCAs or HCCs, generally corresponded with the literature.

Human HBs, distinct liver tumors which may occur in infants under 3 years old [11, 13], are classified into fetal, embryonal, microtrabecular, and 'small cell' or anaplastic types on the basis of their histological or immunohistochemical characteristics [1, 13, 26, 33]. Mouse HBs are histologically similar to the embryonal or 'small cell' types [4, 6]. In the present immunohistochemical examinations, mouse HBs were found not to react with antibodies against keratin,

Table 1. Incidence of liver tumors

Group	Treatment	Effective No. of Animals	Hepatoblastoma	Hepatocellular Carcinoma	Hepatocellular Adenoma
1	DEN	10	0(0)	0(0)	0(0)
2	DEN+PB	21	13(62)	13(62)	17(81)

Data shown are the numbers of mice with tumors; incidences as percentages are given in parentheses. DEN: Diethylnitrosamine, PB: Sodium Phenobarbital.

Table 2. Immunohistochemical findings for neoplasms and non-neoplastic liver cells

	S-100	Keratin	α -Fetoprotein	Albumin	Vimentin
Hepatoblastomas	++ ^{a)}	—	—	—	—
Hepatocellular Carcinomas	—	—	+(4/13,31%)	+ ^{b)}	—
Hepatocellular Adenomas	—	—	—	+ ^{b)}	—
Non-Neoplastic Hepatocytes	—	—	—	+	—
Normal Bile Ducts	+	++	—	—	—

(): Incidence of positive cases.

a) Positive cells were scattered mainly in areas arranged in ribbons or rows.

b) Positive cells were sparsely observed.

Fig. 1. Hepatoblastoma. Left: Hepatoblastoma tumor cells are radially arranged around blood vessels. HE, $\times 55$. Right: Tumor cells are elongated to spindle-shaped with scant basophilic cytoplasm. They are often arranged in ribbons or rows palisading around vascular space. HE, $\times 210$.

Fig. 2. Immunohistochemical staining for S-100 protein. S-100 positive tumor cells were scattered, mainly in an area of palisading arrangement. $\times 220$.

Fig. 3. Immunohistochemical staining for keratin. Hepatoblastoma cells (T) are negative for keratin while a normal bile duct (arrow) stains positively. $\times 110$.

Fig. 4. Immunohistochemical staining for α -fetoprotein (AFP). Hepatoblastoma cells are negative for AFP (left) while hepatocellular carcinoma cells are positive, demonstrating a mosaic pattern (right). $\times 220$.

AFP, ALB or vimentin. Nonoyama *et al.* [19] earlier reported 7 of 16 mouse HBs with squamous metaplasia to show positive reactions for keratin. Differentiation into ductal structures has also been described [12, 20]. In man, it is considered that the 'small cell' type HB, positive for low molecular-weight keratin, differentiates into the embryonal or fetal type. Some of them become to express high molecular-weight keratin [1]. The reason for the discrepancy with the present results remains unclear, but the negativity of our mouse HBs for keratin, predominantly of higher molecular-weight, might point to their being less-differentiated.

Unlike to human HBs which are observed only in infancy or childhood, mouse HBs are usually observed at the late-stage of chemical hepatocarcinogenesis studies and seem to grow later than HCAs or HCCs [3, 5, 19, 32]. In this study, nearly half of HBs were observed in and/or adjacent to HCAs or HCCs, in line with the previous reports suggesting an origin from hepatocellular lineage. In this context, it is important that HCAs, HCCs and HBs were all observed only in PB-treated mice in this study. It is reported that PB is a mitoinhibitor of DNA synthesis in normal hepatocytes, but promotes growth of initiated hepatocytes or neoplastic cells [18, 22, 29, 34], again suggesting that the origin or precursor of HBs might be identical to that of hepatocellular tumors, susceptible to the promoting effects of PB.

However, one of the most important findings in this study was the immunoreactivity of HB cells for S-100 protein, a mesenchymal marker, in clear contrast to the negative results for HCCs, HCAs, and normal hepatocytes. To our knowledge, this is the first report describing positive reactions of mouse HB cells for S-100 protein. It is, however, in line with the previous observations in human for HCCs with sarcomatoid differentiation [16, 21] and HBs [17, 25, 36] which were positive for neuron-specific enolase (NSE) and S-100 protein. Shiga *et al.* [27] reported a HB in a dog to be positive for NSE, but no neuroendocrine granules were observed on electron microscopic examination. S-100 protein is known to be expressed in many neuroectodermal tissues or neoplasms, as well as various other mesenchymal tissues/tumors [2, 9, 15, 35]. Since, in the present study, mouse HBs were negative for vimentin and there are no studies with immunoreactivity for NSE and electron microscopic features, the significance of the S-100 protein expression remains unclear.

Nonoyama *et al.* [19] previously reported that mouse HBs were morphologically composed of epithelial and mesenchymal components suggesting an origin from bipotential liver blastema cells. Likewise, multidirectional differentiation of HB cells has been extensively characterized in human cases [17, 25, 36]. Furthermore, we recently reported that a cell line established from one of the mouse HBs obtained in the present study required hepatocyte growth factor for optimal growth and expressed *c-met*, a protooncogene encoding the hepatocyte growth factor receptor, suggesting mouse HB can possibly originated from an early stage of hepatocellular tumor because of the lack of autonomous growth [14]. Though the existence of

hepatic stem cells is still controversial [8, 23, 28, 30, 31], the present results illustrate a possibility of multidirectional differentiating potential in mouse HB cells. As recently speculated for human HBs [26], furthermore, mouse HB cells may provide a potential of endodermal cells or play roles, less committed, as even stem cells [24].

In conclusion, although it is not possible to define exactly the histogenesis of mouse HBs, the present study suggests two possible origins — conversion from de-differentiated hepatocellular tumors and *de novo* generation from undifferentiated stem cells.

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