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Establishment of an *in vivo* Highly Metastatic Rat Hepatocellular Carcinoma Model

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We previously found by chance that N-nitrosomorpholine (NMOR) given after a multi-carcinogenic treatment induces liver carcinomas with 56% lung metastasis, and it was confirmed that hepatocellular carcinoma (HCC) with 100% lung metastasis was produced by 24-week treatment with NMOR and additional treatment with diethylnitrosamine (DEN). In the present study, we modified the duration of NMOR to establish an animal model with a simple experimental protocol and an appropriate experimental duration which would facilitate further study of the mechanisms of metastasis and antimetastatic agents. The results revealed DEN exposure followed by a 16-week treatment with NMOR to be a most efficient method for the induction of HCC metastasizing to the lung. Loss of cadherin, demonstrated immunohistochemically, occurred in an early stage of carcinogenesis, and this was reflected in malignant conversion of primary lesions. This model, with its essential similarities to malignant tumor behavior in man, should find application not only for elucidation of the mechanisms underlying metastasis, but also in the development of anti-metastatic agents.

Key words: Rat hepatocarcinogenesis model — Lung metastasis — N-Nitrosomorpholine — Cadherin

To form distant metastases, a sequence of many steps, including invasion, transport, arrest, adherence, extravasation, and tumor cell proliferation is required.[1)] To study the mechanisms underlying metastasis, many tools and models have been developed. Most of them use cancer cell lines or transplantable tumors, injected into blood vessels or the intraperitoneal cavity, or transplanted into the cecum, spleen or subcutis.[2–6)] These models have provided very useful tools for analysis of individual steps in the metastatic process. However, in order to analyze the natural course of metastasis, it is necessary to develop animal cancer models which feature frequent metastasis of primary tumors to distant organs. They are also required to assess the efficacy of therapeutic treatments for advanced cancers.

We previously found by chance that N-nitrosomorpholine (NMOR) given after a multi-carcinogenic treatment with N-diethylnitrosamine (DEN), N-methylnitrosourea (MNU), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), 1,2-dimethylhydrazine (DMH), and 2,2′-dihydroxy-di-N-propylnitrosamine (DHPN) induces liver carcinomas with frequent lung metastasis.[7)]

The purpose of the present study was to establish an animal model with a simple experimental protocol and an appropriate experimental duration which would facilitate further study of the mechanisms of metastasis and antimetastatic agents. We also examined cadherin expression in primary lesions by immunohistochemistry.

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MATERIALS AND METHODS

Animal experiment Five groups of 6-week-old male F344 rats (Charles River Japan Inc., Kanagawa) were used as shown in Fig.1. At the beginning of the experiment, groups 1, 2, 3 and 5 were given a single i.p. injection of DEN (Tokyo Kasei Kogyo Co., Tokyo) as an initiator of liver carcinogenesis at a dose of 100 mg/kg body weight. Then groups 1, 2 and 3 were given 120 ppm NMOR (Tokyo Kasei Kogyo Co.) in the drinking water for 8, 16 and 22 weeks, respectively. Animals in group 4 received NMOR for 22 weeks without the prior DEN treatment. Surviving animals were killed at the end of the experiment (week 22), and some were killed at weeks 8 and 16 in group 1 and at week 16 in group 2. A basal diet (BD), Oriental MF diet (Oriental Yeast Co., Tokyo) was given *ad libitum* throughout with free access to water.

Animals were maintained three to a plastic cage under specific pathogen free (SPF) conditions. Although the total experimental period was originally designed up to week 24, the experiment was terminated at week 22 due to the poor survival rate in group 3. All animals were killed under ether anesthesia. After weighing the major organs, parts of the liver tumors were excised and frozen in liquid nitrogen. Ten percent neutral buffered formalin was injected into the lungs through the trachea. One liver slice

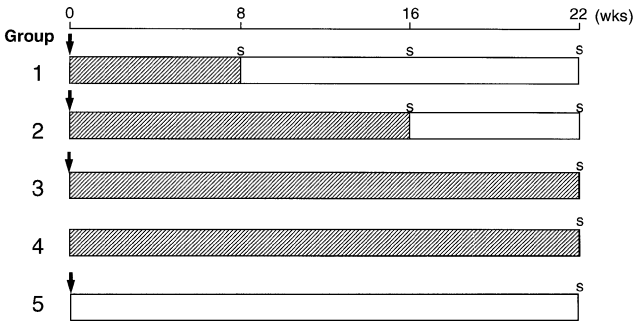

Fig. 1. The experimental protocol. ↓, DEN, i.p. injection of 100 mg/kg body weight; ▨, NMOR, 120 ppm in the drinking water; S, animals killed.

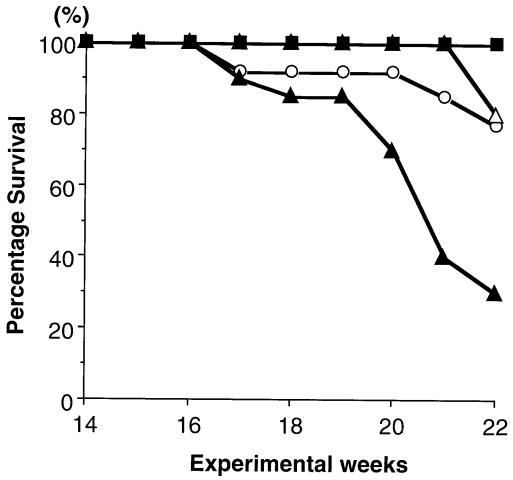

Fig. 2. Survival curves. ●, DEN+NMOR 8 weeks; ○, DEN+NMOR 16 weeks; ▲, DEN+NMOR 22 weeks; △, NMOR 22 weeks; ■, DEN.

was fixed in cold acetone, and remaining liver tissue and other organs were fixed in 10% buffered formalin. The acetone-fixed liver sections were immunohistochemically stained for binding of monoclonal pan-cadherin antibody (Sigma Immuno Chemicals, St. Louis, MO) using the avidin-biotin-peroxidase complex method (Vectastain ABC kit; Vector Lab. Inc., Burlingamee, CA). Step sections of livers were processed routinely for hematoxylin and eosin staining for identification of liver lesions.

Quantitative analysis

Lung metastatic nodules: Lung metastatic lesions were counted under a light microscope and the total areas of lung tissues per animal were measured with the assistance of an image analyzer (VIP-21C; Olympus-Ikegami Tsushin Co., Tokyo).

Cadherin staining: Pan-cadherin immunohistochemical evaluation was conducted for HCCs, adenomas and surrounding normal tissue. For quantitative analysis, immunostained sections were examined under a light microscope connected to an image analysis system, Image Processor for Analytical Pathology (IPAP; Sumika Technos Corp., Osaka).[8] Binary digitized images of liver lesions were obtained automatically by the programmed segmentation procedure. The length of positive cell surface for pan-cadherin staining was measured at a magnification of 600 (at least 10 fields) for each lesion. Here we use the term "staining index" to refer to the parameters expressed as the average positive length per unit area for each immunostained section.

RESULTS

Increase of body weight from week 8 to 22 was observed in group 1, but not in group 2, and the final val-

Table I. Body and Relative Liver Weights

Group	Treatment	Week 8		Week 16		Week 22	
		No. of rats	Body weight (g) Relative liver weight (%)	No. of rats	Body weight (g) Relative liver weight (%)	No. of rats	Body weight (g) Relative liver weight (%)
1	DEN+NMOR 8 wks	15	236.4±12.0 3.7± 0.2	15	313.3±15.2 3.8± 0.3	15	332.2±18.7 4.8± 0.7
2	DEN+NMOR 16 wks		ND	16	273.6±17.1 6.8± 0.7	13	267.2±19.6 9.0± 1.7
3	DEN+NMOR 22 wks		ND		ND	15	245.4±21.2 10.4± 1.6
4	NMOR 22 wks		ND		ND	15	285.8±26.0 9.0± 1.0
5	DEN		ND		ND	15	380.8±15.8 2.9± 0.1

ues for group 3 were the lowest in the groups receiving DEN and subsequent NMOR treatment (Table I). Decrease of body weight gain was observed for groups 2, 3 and 4 in which HCCs with lung metastasis were induced. Increase was observed in groups 1 and 5 without lung metastasis (Tables I and II). The relative liver weights in groups 2, 3

Table II. Incidences of HCCs and Lung Metastases

Group	Treatment	Week 8		Week 16		Week 22	
		No. of rats	HCCs Lung metastasis	No. of rats	HCCs Lung metastasis	No. of rats	HCCs Lung metastasis
1	DEN+NMOR 8 wks	15	0 (0)*** 0 (0)	15	3 (20) 0 (0)	15	9 (60) 0 (0)
2	DEN+NMOR 16 wks		ND	16	5 (31) 0 (0)	13	13 (100) 9 (69)***
3	DEN+NMOR 22 wks		ND		ND	18	17 (94)* 16 (89)***
4	NMOR 22 wks		ND		ND	13	12 (92) 1 (8)
5	DEN		ND		ND	15	0 (0)*** 0 (0)

Each number in parentheses represents percent of total animals.
*, *** : $P<0.05$, 0.001 compared to the values of each lesion in group 1 at week 22 (Fisher exact test).

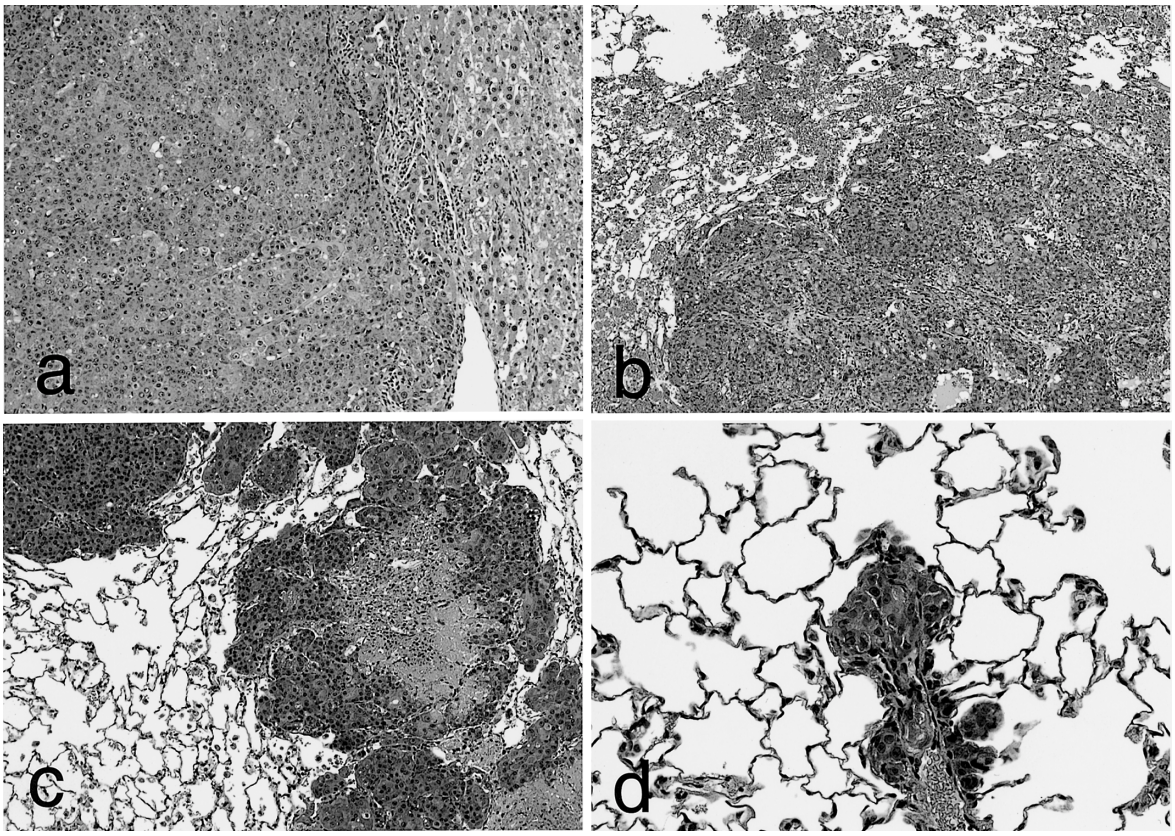

Fig. 3. Most HCCs found in groups 1–4 were moderately differentiated (a). Lung metastases with bleeding (b), or central necrosis (c), or extravasation from vessels (d) were observed in groups 2–4.

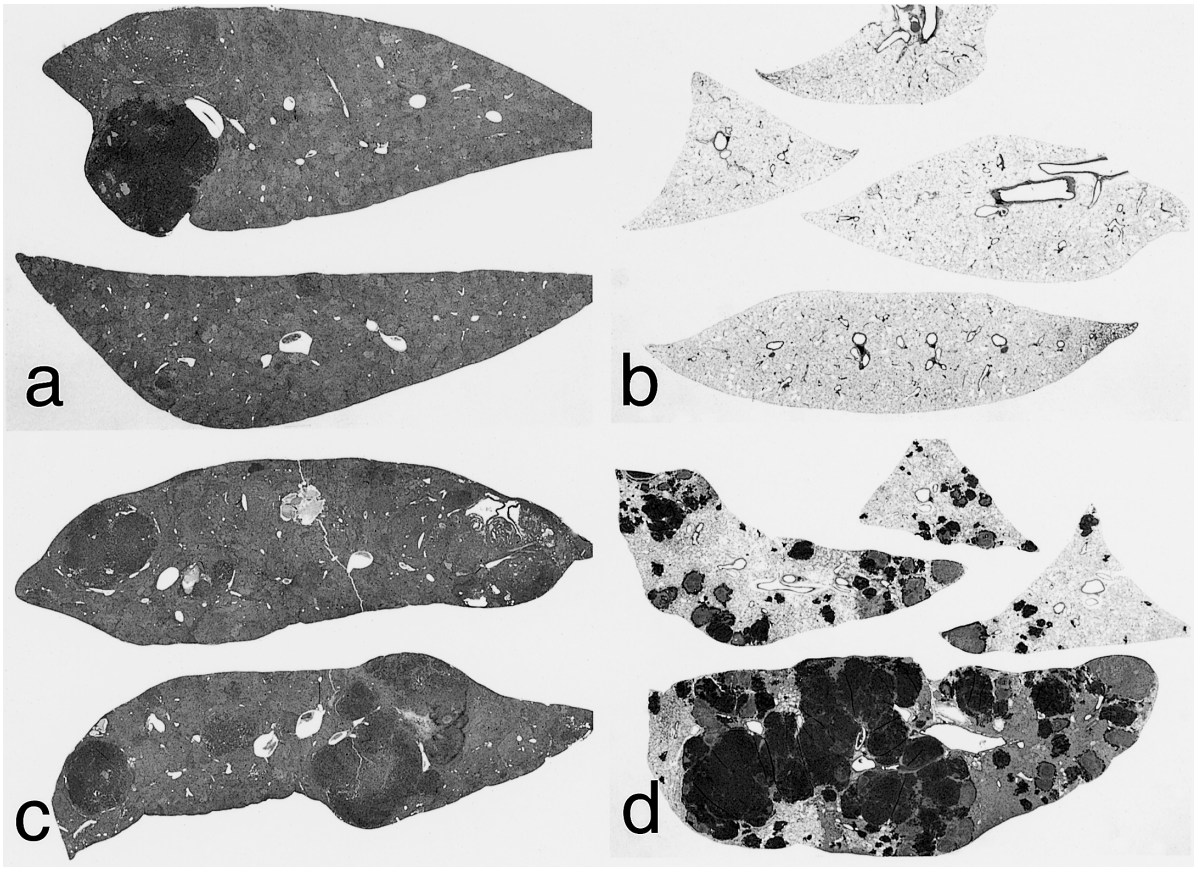

Fig. 4. Macroscopic sections of liver (a and c) and lung (b and d). Treatment with NMOR for 16 weeks after DEN exposure produced a high rate of lung metastasis at week 22 (d), but not at week 16 (b), in spite of a high incidence of HCCs (a).

and 4 were larger than in groups 1 and 5. Long-term NMOR treatment to induce HCC with lung metastasis is necessary to cause loss of body weight gain.

The first mortalities were observed in week 16 for both groups 2 and 3 (Fig. 2). The reason for the deaths was massive bleeding from either primary HCCs in the liver or metastatic nodules in the lung. The survival rate in group 3 decreased in week 18 and finally only 30% of the animals survived. The final survival rates in groups 2, 4 and 5 were 80, 80 and 100%, respectively, in line with the frequencies of metastatic HCC development.

Metastases in the lungs of groups 2–4 were moderately differentiated HCC and many demonstrated bleeding (Fig. 3b) or central necrosis (Fig. 3c). Small metastases with extravasation from a vessel were also found in the lungs of groups 2–4 (Fig. 3d). Most HCCs found in groups 1–4 were moderately differentiated (Fig. 3a), and the rest of them were well or poorly differentiated. No histological difference was observed between HCCs with and without lung metastasis.

Twenty to thirty percent incidences of HCCs were induced in groups 1 and 2 by week 16 (Table II, Fig. 4a). At week 22, the incidences in groups 1–4 were 60–100%. We found no HCC in group 5 given a single i.p. DEN treatment, and there was no histological difference in HCC chronologically.

We observed lung metastasis in the groups given NMOR for 16 weeks (group 2, Fig. 4d) or 22 weeks (group 3), at incidences of 69% and 84%, respectively. The average numbers of metastatic lesions per cm^2 of lung tissue were 8.5 and 9.7. Administration of NMOR alone without DEN induced lung metastasis in only one of 15 rats treated (Table III). The areas of metastatic nodules in groups 2 and 3 were 2.70 and 0.99 (mm^2/cm^2), respectively. Thus, the longer treatment with NMOR resulted in a greater number of metastatic lesions, but did not affect their size.

Quantitative data for pan-cadherin immunohistochemical staining are summarized in Table IV. Although the staining pattern was rather homogeneous in normal-look-

Table III. Quantitative Analysis of Lung Metastases at Week 22

Group	Treatment	No. of rats	Lung metastasis			
			Incidence (%)	No.	No. (No./cm^2)	Area (mm^2/cm^2)
1	DEN+NMOR 8 wks	15	0 (0)	0	0.00± 0.00*	0.00±0.00
2	DEN+NMOR 16 wks	13	9 (69)	207	8.50±11.73	2.70±5.06
3	DEN+NMOR 22 wks	19	16 (84)	330	9.66± 7.47	0.99±1.40
4	NMOR 22 wks	15	1 (7)	7	0.17± 0.67*	0.02±0.07
5	DEN alone	15	0 (0)	0	0.00± 0.00*	0.00±0.00

∗ : $P<0.05$ compared to the values in group 2 (Student's *t* test).

Table IV. Quantitative Analysis of Pan-cadherin Staining in the Liver

Group	Treatment	Staining index (mm/mm^2)					
		Week 8		Week 16		Week 22	
1	DEN+	Normal	3.8±1.0	Normal	2.9±1.3	Normal	3.3±0.9
	NMOR 8 wks	Nodule	2.0±0.7***	Nodule	1.4±0.6*	Nodule	1.0±0.3
		HCC	ND	HCC	0.9±0.7	HCC	0.7±0.3
2	DEN+			Normal	3.5±0.9	Normal	ND
	NMOR 16 wks			Nodule	1.3±0.6	Nodule	0.9±0.3
				HCC	0.4±0.2	HCC	0.3±0.3*
3	DEN+					Normal	ND
	NMOR 22 wks					Nodule	0.8±0.4
						HCC	0.4±0.4
4	NMOR 22 wks					Normal	ND
						Nodule	1.7±0.5**
						HCC	0.8±0.5
5	DEN alone					Normal	3.3±0.9
						Nodule	ND
						HCC	ND

∗, ∗∗, ∗∗∗ : $P<0.05$, 0.01, 0.001 compared to the values of each lesion in group 1 at week 22 (Student's *t* test).

ing tissue (Fig. 5b), it was heterogeneous in adenomas and HCCs (Fig. 5, b and d), with a reduction in the staining index from adenomas to HCCs. No time course changes were evident. In addition, the index of HCCs in the groups with many metastatic nodules (groups 2 and 3) was much lower than in groups with little or no lung metastasis (groups 1, 4 and 5).

DISCUSSION

NMOR and DEN have been widely used as hepatocarcinogens in animal models, and the induced preneoplastic lesions and malignant tumors have been well characterized.[9–13] Lung metastasis by induced HCC has been reported in rats given either DEN or NMOR by Lijinsky *et al.*[10, 11] who described the relationship between the chemical structure of the carcinogen, the tumors induced and the proportion of tumors that formed metastases. However, in the present study, treatment with NMOR alone or with DEN+8-week NMOR resulted in only a few lung metastases. In contrast, DEN+16 or 22-week NMOR treatment was associated with high frequencies, with a dependence on NMOR treatment time. The very high metastasis rate (up to 80%) means that treatment of rats with DEN and NMOR provides a good animal model for induction of metastatic HCCs.

While the 16-week treatment with NMOR after DEN produced a high rate of lung metastasis at week 22, metastatic lesions were lacking at week 16 in spite of the development of HCCs (Table II). Histologically, we observed not only large metastatic nodules, but also extravasation in the lung at week 22 (Fig. 3d). This findings suggests that a multi step process of metastasis (including invasion, transport, arrest, adherence, extravasation, and tumor cell proliferation) proceeded between weeks 16 and 22. Therefore, using this model, chemical substances could be applied in the intervening period to investigate modifying factors, particularly those leading to inhibition of lung metastasis formation.

Change in the expression of cadherin, a major adhesion molecule of epithelia,[14–16] has been implicated in carcinogenesis, because loss is frequent in human and murine

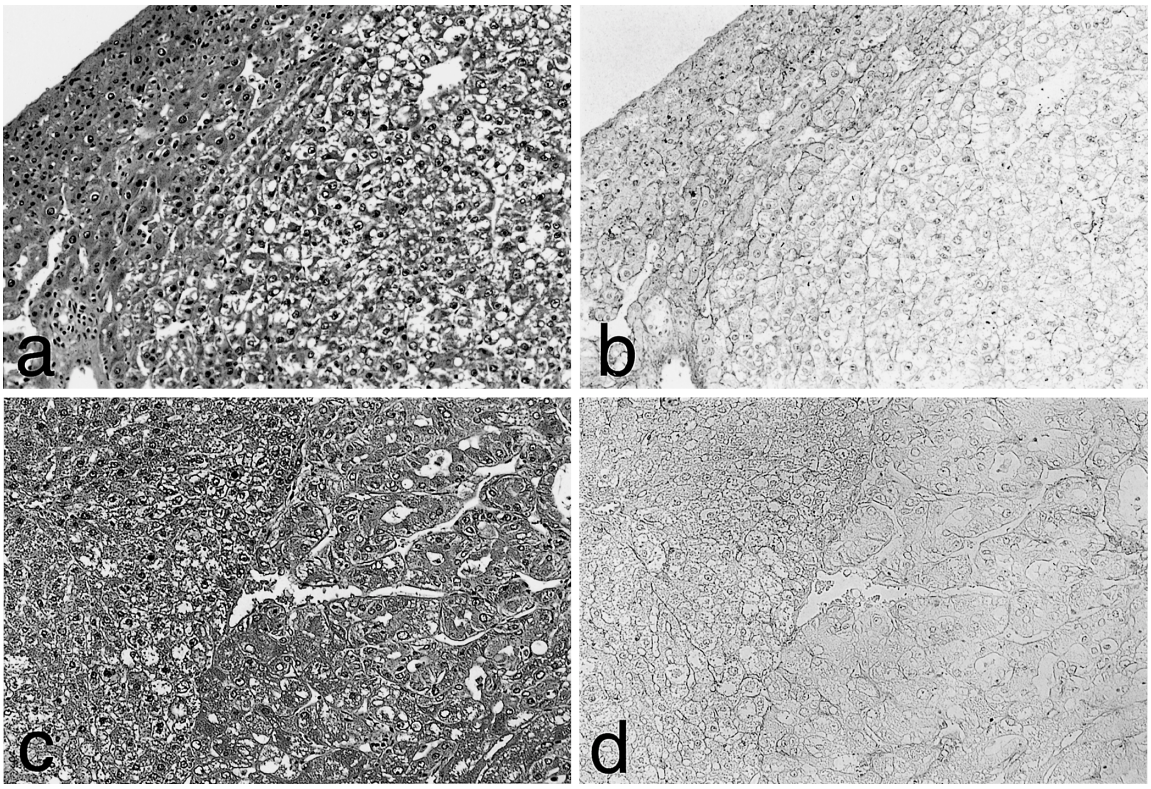

Fig. 5. Liver sections from acetone-fixed tissues immunohistochemically stained for monoclonal pan-cadherin antibody (b and d). Serial sections of the livers were processed routinely for hematoxylin and eosin staining for identification of liver lesions (a, adenoma and surrounding normal tissue; c, HCC and adenoma).

high-grade epithelial cancers.[17–19] Re-establishing functional cadherin complexes in tumor cell lines results in reversion from an invasive to a benign epithelial phenotype.[20, 21] Pan-cadherin immunohistochemical evaluation in the present study showed expression to be decreased in the order of adenoma, HCC and advanced HCC. Our data are in accordance with the report of Sundfeldt *et al*. that reduction of E-cadherin expression is observed in early progression to the malignant phenotype with further loss in benign and borderline-type ovary tumors, and high-grade and moderately to poorly differentiated lesions.[22] In contrast, studies of a transgenic mouse that expresses the SV40 T antigen under the control of the insulin promoter in the β-cells of pancreatic islets of Langerhans (Rip1Tag2) demonstrated that loss of E-cadherin-mediated cell adhesion is one rate-limiting step in the progression from adenoma to carcinoma.[23] Taken together the results suggest that down-regulation of cadherin expression may occur as an early event of carcinogenesis with a progressive decrease through normal-looking tissue, adenoma and HCC. However, a quantitative difference of cadherin expression was observed between HCC with metastasis and that without metastasis, indicating that further study is needed to clarify what part this difference plays in the metastasis formation.

In conclusion, the rat model presented here provides a good tool for rapid induction of metastatic HCC. To our knowledge, this is the first model to reflect the natural course of malignant tumors which metastasize to lung, and this model should be applicable not only for the elucidation of mechanisms underlying metastasis, but also to test anti-metastatic agents.

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