Original

Close Relation of Transforming Growth Factor- α Positive Hepatic Foci to Hepatocellular Tumor Development in the Rat

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Abstract: The question of whether transforming growth factor- α (TGF- α) might be a useful marker of preneoplastic lesions during hepatocarcinogenesis was investigated using a medium-term liver carcinogenesis bioassay (Ito test) extended to 50 weeks. Male F344 rats, 6 weeks of age, received a single i.p. injection of 200 mg/kg diethylnitrosamine (DEN), then after 2 weeks, the animals were divided into four groups. Groups 1 and 2 were maintained on tap water supplemented with 10 ppm of N-nitrosomorpholine (NMOR), a genotoxic carcinogen, for 48 or 6 weeks, respectively. Animals in Group 3 were maintained on basal diet and tap water *ad libitum* as controls. Group 4 was included as the vehicle control and was maintained until experimental week 50. Two-thirds partial hepatectomy was performed on all rats at the end of week 3 of the experiment. Histopathology and immunohistochemical investigations of glutathione S-transferase placental form (GST-P) and TGF- α of the liver were performed sequentially at experimental weeks 8, 12, 20, 35 and 50. The numbers of TGF- α positive lesions were significantly lower than those of GST-P positive foci at all experimental time points. In contrast, the mean size values of TGF- α positive liver lesions in the continuous and 6-week treatments of DEN/NMOR, and the DEN alone protocol, were larger than those of their GST-P positive counterparts from week 35. Bromodeoxyuridine (BrdU) labeling indices for TGF- α positive foci and hepatocellular adenomas at week 20 were clearly higher than those for TGF- α negative lesions. In conclusion, the overexpression of TGF- α can be immunohistochemically detected in large hepatocellular lesions demonstrating increased levels of DNA synthesis. Therefore TGF- α may be a marker of a relatively late, progression stage of hepatocarcinogenesis. (J Toxicol Pathol 2006; 19: 99–106)

Key words: transforming growth factor- α (TGF- α), F344 rat, hepatocarcinogenesis, medium-term liver carcinogenesis bioassay

Introduction

Transforming growth factor- α (TGF- α), has been clearly shown to be involved in normal cell development, wound healing and the malignant transformation of cells^{1–3}. Synthesized as a 160-kDa membrane-spanning precursor, the more active 50-amino acid mature form is released from the cell surface by proteolytic cleavage^{4–7}. TGF- α positively regulates cell proliferation by means of paracrine or autocrine stimulation^{8,9} via binding to epidermal growth factor (EGF) receptors on the cell surface. In the liver, TGF- α gene expression is regulated during development. It is depressed in the adult liver, and enhanced in regenerating

liver and hepatocytes proliferating in culture^{8–10}. Rat liver cell lines obtained from experimentally induced hepatocellular carcinomas secrete substantial amounts of TGF- α ⁵, and urinary excretion of TGF- α is known to be elevated in some patients with hepatocellular carcinomas¹¹.

TGF- α transgenic mice, produced to clarify functions of TGF- α ^{12–18}, demonstrate enhanced development of hepatocellular carcinomas with diethylnitrosamine initiation and phenobarbital promotion, possibly through a mechanism of increased hepatocyte proliferation in precancerous lesions (alteration foci and adenomas)¹⁹. Overexpression of TGF- α has been linked with elevated hepatocyte proliferation and hepatocarcinogenesis in both humans and rats^{20,21}, especially in the progression stage^{22–25}, and there is evidence of its mechanistic role during hepatocarcinogenesis^{26,27}.

Initiated hepatocytes and early preneoplastic foci are well known to be characterized by alteration of the normal phenotype, and pronounced upregulation of the glutathione

Received: 24 December 2005, Accepted: 10 May 2006

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Fig. 1. Experimental Design.

S-transferase placental form (GST-P) which allows ready recognition of them in tissue sections²⁸. GST-P, which is an isozyme of GST contributing to the phase II detoxification enzyme, is induced in preneoplastic and neoplastic liver lesions. An epigenetic rather than a genetic mechanism accounts for the induction of GST-P in hepatocytes²⁹, and therefore, this enzyme has been extensively used for basic analysis of rat liver carcinogenesis³⁰.

In the present study, the relation between TGF- α expression and GST-P positive lesions was examined in rats treated with a representative genotoxic hepatocarcinogen, N-nitrosomorpholine (NMOR). The primary aim was to determine whether TGF- α might be a useful marker for the subset of preneoplastic lesions most likely to give rise to adenomas and carcinomas.

Materials and Methods

Chemicals

Diethylnitrosamine (DEN) and N-nitrosomorpholine (NMOR) were obtained from Tokyo Chemical Industry Co. Ltd., Tokyo, Japan.

Animals and maintenance

Male F344/DuCrj rats, 5 weeks of age, were purchased from Charles River Japan, Inc., Atsugi, Japan, and housed five to a polycarbonate cage with hardwood Beta chips (Northeastern Product Corp., Warrensburg, NY) for bedding. The animals were supplied with food (Oriental MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water *ad libitum* and the room temperature and relative humidity were controlled at $22 \pm 2^\circ\text{C}$ and $55 \pm 10\%$, respectively. Fluorescent lighting provided a 12-hr light/dark cycle. The animals were observed daily for abnormalities, and body weights and food and water consumptions were recorded

once a week until 14 weeks and then every 4 weeks until the end of the experiment.

After a one-week quarantine period, a total of 101 F344/DuCrj rats were randomly divided into 4 groups. The experimental design is illustrated in Fig. 1. Ninety-three rats (groups 1 to 3) were treated intraperitoneally with DEN at a dose of 200 mg/kg body weight and given basal diet for the first 2 weeks. Then the animals in groups 1 and 2 were maintained on tap water supplemented with 10 ppm NMOR for 48 or 6 weeks, respectively, while those in Group 3 were given basal diet and tap water *ad libitum*. The remaining 8 rats (Group 4) received a saline i.p. injection followed by unsupplemented water. Two-thirds partial hepatectomy was performed on all rats at the end of week 3 of the experiment. Five rats from groups 2 and 3 were sacrificed by exsanguination from the abdominal aorta under ether anesthesia and necropsied at the end of week 8, then 4 or 5 rats from each group, except Group 4, were similarly sacrificed at the ends of weeks 12, 20 and 35, and 8 to 11 rats of each group at the end of week 50. At necropsy, final body weights after fasting over night were recorded and the livers were removed and weighed. Each liver lobe was cut into 2- to 3-mm thick sections with a razor blade, fixed in 10% phosphate-buffered formalin and embedded in paraffin.

Histopathological and immunohistochemical procedures

Three serial sections through the liver from each animal were stained with hematoxylin and eosin (H&E), and then immunohistochemically stained for glutathione S-transferase placental form (GST-P) and TGF- α with anti-rat GST-P rabbit serum (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) at 1:2000 dilution, and anti-human TGF- α mouse serum (Oncogene Research Products, Boston, MA) at 1:500 dilution. The avidin-biotin peroxidase

Fig. 2. Numbers and areas of GST-P and TGF- α positive liver lesions in livers of DEN-initiated rats given NMOR. *, **: Significantly different from TGF- α positive lesions at $P < 0.05$, 0.01 , respectively.

Fig. 3. Numbers and areas of GST-P and TGF- α positive liver lesions in livers of DEN-initiated rats **: Significantly different from TGF- α positive lesions at $P < 0.01$.

complex (ABC) technique was employed using a rabbit Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and GST-P and TGF- α were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and new fuchsin, respectively. Representative paraffin liver sections were stained doubly for GST-P and TGF- α .

Image analysis

Liver lesions were classified according to criteria defined previously³¹. GST-P or TGF- α positive lesions were traced using a color image processor (SPICA-II; Nippon Avionics Co. Ltd., Tokyo, Japan), and their numbers and areas per cm² were measured.

Labeling indices for bromodeoxyuridine (BrdU)

During week 20, Alzet osmolar infusion pumps (Alza Corporation, Palo Alto, CA) filled with 200–300 μ g of BrdU were implanted intraperitoneally into 4 rats of Group 1

(continuous NMOR treatment, 3 days before sacrifice. Sections from the livers of these rats were also immunohistochemically stained for BrdU by the avidin-biotin peroxidase complex (ABC) technique, using a mouse Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). A mouse anti-bromodeoxyuridine monoclonal antibody at 1:2000 dilution (DakoCytomation, Denmark) was used as the antibody.

All experimental procedures were performed in accordance with the in-house guidelines for the Care and Use of Laboratory Animals at the DIMS Institute of Medical Science, Inc.

Statistical analysis

Mean values $\pm$ SD for body weights, liver-to-body weight ratios, BrdU labeling indices and liver lesions were assessed for significant differences between test and control groups by the two-tailed Student's *t*-test.

Table 1. Mean Sizes of GST-P and TGF- α Positive Liver Lesions

Group	Treatment	NMOR Treatment period (weeks)	Observation time (weeks)	Mean size of liver lesions (mm ²)	
				GST-P positive	TGF- α positive
1	DEN→NMOR+PH (Continuous treatment)	10	12	0.07 ± 0.01 ^b (5)	0.11 ± 0.12 (4)
		18	20	0.12 ± 0.07 (4)	0.57 ± 0.66 (4)
		33	35	0.57 ± 0.41 (5)	3.46 ± 3.39 (5)
		48	50	4.65 ± 5.71 (13) ^a	15.96 ± 12.89 (13) ^{a*}
2	DEN→NMOR+PH (Short treatment)	6	8	0.07 ± 0.01 (5)	0.06 ± 0.04 (5)
		6	12	0.08 ± 0.02 (5)	0.06 ± 0.04 (3)
		6	20	0.10 ± 0.01 (5)	0.06 ± 0.04 (5)
		6	35	0.21 ± 0.11 (5)	1.67 ± 1.97 (5)
		6	50	1.58 ± 1.70 (9)	15.97 ± 14.40 (9)**
3	DEN→PH		8	0.07 ± 0.02 (5)	0.06 ± 0.01 (2)
			12	0.06 ± 0.01 (5)	0.05 ± 0.01 (4)
			20	0.10 ± 0.03 (5)	0.06 ± 0.01 (3)
			35	0.35 ± 0.31 (5)	2.22 ± 2.52 (5)
			50	1.13 ± 1.27 (10)	23.88 ± 34.41 (9)
4	Saline→PH		50	0.04 ± 0.01 (5)	0 ± 0 (8)

*, **: Significantly different from GST-P positive liver lesions at P<0.05, 0.01, respectively.

a: Includes two moribund animals.

b: mean ± SD.

Numbers of rats examined are given in parentheses.

Table 2. BrdU Labeling Indices for Hepatic Lesions at Week 20 in Livers of Rats Treated with DEN/NMOR (Group 1)

Surrounding normal hepatocytes	Preneoplastic lesions		Hepatocellular adenoma
	GST-P(+)/TGF- α (-)	GST-P(+)/TGF- α (+)	GST-P(+)/TGF- α (+)
0.40 ± 0.08 (4) ^{a,c**}	0.69 ± 0.74 (13) ^b	3.70 ± 1.56 (2) ^b	14.25 ± 16.48 (2) ^b

**: Significantly different from the GST-P(+)/TGF- α (-) preneoplastic lesions at P<0.01.

a: Number of liver samples investigated are given in parenthesis.

b: Numbers of liver lesions investigated are given in parentheses.

c: mean ± SD.

Results

Body weights and liver weights

Mean body weights of rats treated with DEN/NMOR in both Groups 1 and 2 were lower than those of the control Group 3, at week 50 (data not shown).

Liver weights of rats treated with DEN/NMOR in Group 1 (continuous NMOR treatment) were greater than the values of Group 3, while those for rats treated with DEN/NMOR for only 6 weeks, Group 2, were increased at weeks 8 and 12 (data not shown).

Changes in GST-P and TGF- α positive liver lesions

Data for the numbers and areas of GST-P and TGF- α positive liver lesions are illustrated in Figs. 2 and 3. The numbers of GST-P positive lesions increased until week 35, but were in decline at week 50 in the groups receiving DEN/NMOR as well as DEN alone. Changes in the numbers of TGF- α positive lesions were similar to those of GST-P positive lesions but the absolute values were significantly lower than those of GST-P positive foci at all experimental

time points. The areas of both GST-P and TGF- α positive lesions increased throughout in a time-dependent manner. Significant differences in areas between GST-P and TGF- α positive lesions were not apparent in the groups receiving DEN/NMOR and DEN alone.

The mean sizes of GST-P and TGF- α positive liver lesions are given in Table 1. The mean size values for TGF- α positive liver lesions were larger than those for their GST-P positive counterparts from weeks 12 or 35 to the end of experiment in Groups 1 and 2, which were given DEN/NMOR continuously and for 6 weeks, respectively. They were also larger at all time points, with clear difference at weeks 35 and 50 in the group receiving DEN alone (Group 3).

BrdU labeling indices

BrdU labeling indices in hepatic lesions are given in Table 2. At week 20, values for TGF- α positive preneoplastic lesions and hepatocellular adenomas induced by the regime of DEN/NMOR (Group 1) were clearly higher than those for TGF- α negative lesions.

Fig. 4. Immunohistochemical findings for GST-P positive lesions(left) and TGF- α positive lesions (right) in the liver of a Group 1 rat at experimental week 12 ($\times 18$).

Fig. 5. Immunohistochemical findings for GST-P positive lesions (left) and TGF- α positive lesions (right) in the liver of a Group 1 rat at experimental week 35 ($\times 18$).

Fig. 6. Immunohistochemical findings of GST-P and TGF- α double staining of liver lesions (left) and histopathological findings (right, H&E staining) in the liver of a Group 2 rat at experimental week 50 ($\times 58$).

TGF- α expression in liver lesions

TGF- α was found to be expressed in centrilobular areas on the cell membranes of normal hepatocytes, as previously reported¹³ (Figs. 4 and 5). Percentage incidences of TGF- α overexpressing liver lesions are presented in Table 3. The

values were 2.2–13.2% of GST-P positive eosinophilic foci, overall. TGF- α overexpression in the adenomas and carcinomas was much higher with incidences of 69–100%.

Sequential data for incidences of liver tumors in the rats are given in Table 4. Continuous treatment with DEN/

Table 3. Percentages of TGF- α Staining Liver Lesions Positive for GST-P

Group	Treatment	NMOR Treatment period (weeks)	Observation time (weeks)	Liver lesions		
				Eosinophilic foci	Hepatocellular adenomas ^a	Carcinomas
1	DEN→NMOR+PH (Continuous treatment)	10	12	6.4 ± 5.6 ^f (5) ^b	—	—
		18	20	4.3 ± 2.6 (4)	100 (2) ^c	—
		33	35	8.4 ± 2.7 (5)	82 ± 29 (5)	100 (5/5) ^d
		48	50	12.7 ± 6.2 (13) ^e	88 ± 23 (12)	89 (32/36)
2	DEN→NMOR+PH (Short treatment)	6	8	13.2 ± 7.2 (5)	—	—
		6	12	4.2 ± 4.5 (5)	—	—
		6	20	5.0 ± 2.5 (5)	—	—
		6	35	5.4 ± 2.2 (5)	75 ± 32 (4)	—
		6	50	5.4 ± 3.0 (9)	100 (8)	100 (7/7)
3	DEN→PH		8	4.8 ± 6.7 (5)	—	—
			12	10.8 ± 19.1 (5)	—	—
			20	7.6 ± 7.2 (5)	—	—
			35	10.8 ± 4.7 (5)	83 ± 29 (3)	—
			50	2.2 ± 2.1 (10)	69 ± 46 (8)	—
4	Saline→PH		50	0 (8)		

a: All of eosinophilic type.

b: Number of animals examined.

c: Number of adenoma-bearing animals.

d: Number of carcinomas.

e: Including two moribund animals.

f: mean ± SD.

Table 4. Numbers of Rats Bearing Liver Tumors (Percentages)

Group	Treatment	NMOR Treatment period (weeks)	Observation time (weeks)	No. of rats	Hepatocellular adenoma	Hepatocellular carcinoma ^a
1	DEN→NMOR+PH (Continuous treatment)	18	20	4	2 (50)	0
		33	35	5	5 (100)	3 (60)
		48	50	13 ^b	13 (100)	13 ^c (100)
2	DEN→NMOR+PH (Short treatment)	6	35	5	5 (100)	0
		6	50	9	8 (89)	5 (56)
3	DEN→PH	0	35	5	3 (60)	0
		0	50	10	8 (80)	0
4	Saline→PH	0	50	8	0	0

a: Including carcinoma within adenoma and hepatocholangiocellular carcinomas.

b: Includes two moribund animals.

c: Metastasis to the lung (6 rats).

NMOR induced hepatocellular adenomas at weeks 20 or more and hepatocellular carcinomas (HCCs) at weeks 35 or more (Group 1), while adenomas were noted at weeks 35 or more and HCCs at week 50 in the short treatment group (Group 2). Adenomas were observed in control rats treated with DEN alone, Group 3, at weeks 35 or more, but HCCs did not develop.

Discussion

In the present study on TGF- α overexpression in preneoplastic and neoplastic lesions induced by a two-stage hepatocarcinogenesis bioassay system with partial hepatectomy, an association with more advanced lesions was evident. This results agrees with the proposed stimulation

potential of TGF- α on the progression stage in rat hepatocarcinogenesis²⁵. The numbers of TGF- α positive lesions were significantly lower than those of GST-P positive counterparts, however, their areas were comparable because the large preneoplastic foci (eosinophilic), adenomas (eosinophilic) and carcinomas, and especially the latter two, tended to have strong TGF- α expression. In the early stage of liver carcinogenesis, in contrast, the rate for TGF- α positive foci was only 2.2–13.2% of that for GST-P positive foci, a result which is in agreement with a previous report^{32,33}.

The TGF- α overexpressing populations were also found to exhibit elevated cell proliferation activity, because BrdU labeling indices for TGF- α positive lesions were higher than those for the GST-P positive population. These

results strongly suggest that these lesions overexpressing TGF- α are more likely to progress to neoplasms. TGF- α induction is known to be mediated by an autocrine growth stimulatory loop and it is documented that TGF- α can act in concert with chemical carcinogens to promote hepatocarcinogenesis^{18,19}.

In conclusion, overexpression of TGF- α can be immunohistochemically detected in large preneoplastic and neoplastic hepatocellular lesions demonstrating increased levels of DNA synthesis. Therefore, it may be involved in a relatively late, progression stage of hepatocarcinogenesis, suggesting the potential use of TGF- α as a marker of hepatocarcinogenesis.

Acknowledgement: This study was supported by the Society for Promotion of Pathology of Nagoya, Japan.

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