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Expression of tenascin in bile duct cancer of hamster liver by combined treatment of dimethylnitrosamine with *Opisthorchis viverrini* infections

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Abstract

Tenascin is an extracellular matrix glycoprotein known to be an essential factor for the modulation of reciprocal interactions between the epithelium and mesenchyme during embryogenesis and tumourigenesis. The interactions between the expression of tenascin in the liver of Syrian golden hamster and the development of bile duct cancer in an *Opisthorchis viverrini*-associated cholangiocarcinoma model were investigated. The tenascin was expressed in connective tissues surrounding the dilated ducts, ductal rims and the stroma of cancers, and strongly in the stroma flame of necrotic cancer nodules. The mRNA signal for tenascin was also recognized in the stroma cells. The potential roles of tenascin as prognostic tumour markers are discussed.

Introduction

In Thailand, liver cancer is the most common fatal neoplasm (Srivatanakul *et al.*, 1988). There are two main types of primary cancers in the liver, namely, hepatoma derived from hepatocytes and cholangiocarcinoma derived from bile duct epithelia. Hepatoma is a frequent cancer in many countries, whereas cholangiocarcinoma is much less frequent in most parts of the world except in certain parts where liver flukes, *Opisthorchis viverrini*, are endemic. It is well documented that *O. viverrini* infection is highly prevalent in northeast Thailand (Preuksaraj, 1984; Jongsuksuntigul & Imsomboon, 1997). Our recent studies on parasitic diseases in northeast Thailand as part of the International Scientific Research Program, demonstrated that *O. viverrini* infection of stool samples in residents was about 30% of the population (unpublished). A positive correlation of liver flukes to cholangiocarcinoma development has been demonstrated by the geographical relationship between distributions of *O. viverrini* infection and cancer incidence (Vatanasapt *et al.*, 1990; Haswell-Elkins *et al.*, 1992). In addition, laboratory studies showed that a combined administration of chemical carcinogens such as dimethylnitrosamine (DMN)(Sigma) and *O. viverrini* infection induced a high occurrence of cholangiocarcinoma in Syrian golden hamsters (Thamavit *et al.*, 1978, 1987).

Tenascin is an extracellular matrix glycoprotein known to be an essential factor for the modulation of reciprocal interactions between the epithelium and mesenchyme during embryogenesis (Chiquet-Ehrismann *et al.*, 1986; Ekblom & Aufderheide, 1989; Erickson & Bourdon, 1989) and tumourigenesis (Mackie *et al.*, 1987; Sakakura, 1995). Several studies suggested that tenascin expression

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and/or its serum level are closely related with tumour progression, burden and metastasis of cancers such as heptocellular and colorectal carcinoma (Yamada *et al.*, 1992; Riedl *et al.*, 1995). The present study examined the pattern and distribution of tenascin expression in the liver of hamsters with chronic *O. viverrini*-associated cholangiocarcinoma together with a histological analysis. In addition, the consequence of pre-immunization of hamsters with *O. viverrini* antigen on the occurrence and histopathological features of bile duct cancer was investigated.

Materials and methods

Preparation of O. viverrini *metacercariae*

Metacercariae were prepared from naturally infected cyprinid fish acquired from endemic areas in northeast Thailand. Fish were minced, homogenized and digested with artificial gastric juice. The entire digest was filtered, washed and resedimented several times with normal saline and the *O. viverrini* metacercariae were identified and collected under a dissecting microscope.

Experimental groups

A total of 203 Syrian golden hamsters (male) aged 6–8 weeks were used and these were divided into seven groups: group 1 was maintained as an untreated control (normal control group); group 2 was infected with 100 infective metacercariae of *O. viverrini* via a stomach tube at 10 weeks of age (Ov group); group 3 was given 12.5 ppm DMN in drinking water for 8 weeks starting from 14 weeks of age (DMN group); group 4 was immunized with a single injection of 50 mg crude phosphate buffer saline extract of *O. viverrini* adult in an equal volume of complete Freund's adjuvant (Im group); group 5 was infected with *O. viverrini* metacercariae followed by DMN administration (Ov + DMN group); group 6 was immunized, infected and administered DMN (Im + Ov + DMN group); group 7 was immunized and given DMN in drinking water (Im + DMN group). Hamsters were kept in the animal unit of the Faculty of Medicine, Khon Kaen University where they were maintained under a 12 h dark–light photoperiod at a temperature of 24–28°C. Food and water were given *ad libitum* and the animals were checked daily. Animal welfare was maintained under guidelines issued by the National Research Council of Thailand. Hamsters were sacrificed at 12, 26, and 35 weeks after *O. viverrini* infection (0, 14 and 23 weeks after the termination of DMN treatment) using evaporation of ether in a glass chamber. The abdomen of each hamster was opened up and blood was drawn from the heart and the hepatobiliary system was then carefully removed. Livers were fixed in 10% buffered formalin, serially sliced to a thickness of 2 mm, embedded in paraffin, cut at 5 μm and stained with haematoxylin and eosin. For investigation of liver cirrhosis, sections were stained with Azan-Mallory. Experimental procedures are outlined in table 1.

Immunohistochemistry

Sections from all specimens were examined by immunostaining for tenascin by methods described previously (Kalembeyi *et al.*, 1997). Briefly, after deparaffinization with xylene and hydration with downgraded ethanol, sections were treated with a 0.4% pepsin solution for 20 min at 37°C, incubated in 0.6% H_2O_2 in methanol for 30 min to block the endogenous peroxidase activity, and treated in PBS solution supplemented with 5% normal goat serum for 30 min to block the non-specific binding of rabbit immunoglobulins. Rabbit anti-human tenascin polyclonal antibody (Kalembeyi *et al.*, 1997) (diluted 1:500) was applied to the sections for 1 h at room temperature. Sections were then treated with a goat anti-rabbit peroxidase-labelled antibody (MBL) for 30 min. Colour development was done with a 3,3′-diaminobenzin tetrahydrochloride–H_2O_2 solution.

In situ hybridization

Freshly dissected liver tissues were trimmed and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer at 4°C. The methodology for *in situ* hybridization and colour development was as described previously (Ishihara *et al.*, 1995). Tissue sections were treated with protease K for 10 min. Sense and antisense strands of tenascin cRNA probes were prepared by *in vitro* transcription of mouse tenascin cDNA (Saga *et al.*, 1991) using a digoxigenin RNA labelling kit (SPR/T7, Boeringer Mannheim, Mannheim Germany) as previously reported (Tsukamoto *et al.*, 1991).

Table 1. Experimental procedures for the induction of cholangiocell carcinomas in Syrian hamsters infected or immunized with *Opisthorchis viverrini* and/or given dimethylnitrosamine.

	Im with Ov antigen	+	Ov infection	+	DMN in drinking water	Time of sacrifice
Birth	6		10		14–22	22, 36, 45 wks (after birth)
			0		4–12	12, 26, 35 wks (after Ov infection)
					0	0, 14, 23 wks (after treatment)

Ov, *Opisthorchis viverrini* metacercariae infection; DMN, 12.5 ppm dimethylnitrosamine (Sigma) given in drinking water for 8 weeks after 4 weeks of Ov infection; Im, immunization by subcutaneous injection of *Opisthorchis viverrini* extracts before 4 weeks of Ov infection.

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Table 2. Development of cholangiocarcinomas in Syrian hamsters infected or immunized with *Opisthorchis viverrini* and/or given dimethylnitrosamine.

Groups	Weeks after Ov infection	No. hamsters	With cancer		
			No.	macro	micro
Control	12	9	0		
	26	5	0		
	35	4	0		
Ov	12	5	0		
	26	10	0		
	35	11	0		
DMN	12	5	0		
	26	10	0		
	35	10	3	1	2
Im	12	5	0		
	26	8	0		
	35	4	0		
Ov+DMN	12	5	0		
	26	17	17	2	15
	35	16	16	7	9
Im+Ov+DMN	12	5	0		
	26	8	6	3	3
Im+DMN	12	5	0		
	26	5	0		
	35	5	1	1	

Abbreviations as in table 1.

Results

Survival of hamsters

Survival of hamsters from each experimental group was checked every week after *O. viverrini* infection. Hamster survival in the control group, DMN group and Im group was 100% at 35 weeks after *O. viverrini* infection. Survival of the Ov group and Im + DMN group was greater than 85% at 35 weeks after *O. viverrini* infection. In contrast, survival of the Ov + DMN group and Im + Ov + DMN group was lower, 62% and 20% at 26 weeks after *O. viverrini* infection, respectively. As all hamsters from the Im + Ov + DMN group were sacrificed at 26 weeks after *O. viverrini* infection, there are no data available at 35 weeks. Survival of the Ov + DMN group at 35 weeks was 22%. Eggs of *O. viverrini* were found in the faeces of hamsters infected with *O. viverrini* metacercariae.

Development of cancer

As shown in table 2, almost all hamsters infected with *O. viverrini* metacercariae, followed by chemical carcinogen treatment, developed cholangiocancer more than 14 weeks after termination of DMN administration. Tumours were multiple including micro- and macroscopic foci. Histological types of cancers are summarized in table 3. Cholangiocell carcinoma (CCC) was commonly observed in all cases (fig. 1a), associated with cystoadenocarcinoma (CAC) in many cases (fig. 1b). Hepatocholangiocell carcinoma (HCCC) was also found in some cases, particularly in larger sized tumours (fig. 1c). Besides cancers, a variety of pathological changes such as lymphocyte infiltration around the bile ducts and small blood vessels, bile ductule proliferation, dilated bile ducts, cholangiofibrosis, and cirrhosis (fig. 2) were observed (table 4). A comparison of Ov + DMN with and without *O. viverrini* extract pre-immunization

Table 3. Histological types of cancers in Syrian hamsters infected or immunized with *Opisthorchis viverrini* and/or given dimethylnitrosamine.

Groups	Weeks after Ov infection	No. hamsters	No. with cancers	Histological type		
				CCC	CAC	HCCC
DMN	35	10	3	3(100%)	0	0
Ov+DMN	26	17	17	17(100%)	4(23.5%)	0
	35	16	16	16(100%)	7(43.8%)	3(18.8%)
Im+Ov+DMN	26	8	6	6(100%)	4(66.7%)	1(16.7%)

Percent indicates the rate of histological types in each cancer.
CCC, cholangiocell carcinoma; CAC, cystoadenocarcinoma; HCCC, hepatocholangiocell carcinoma; other abbreviations as in table 1.

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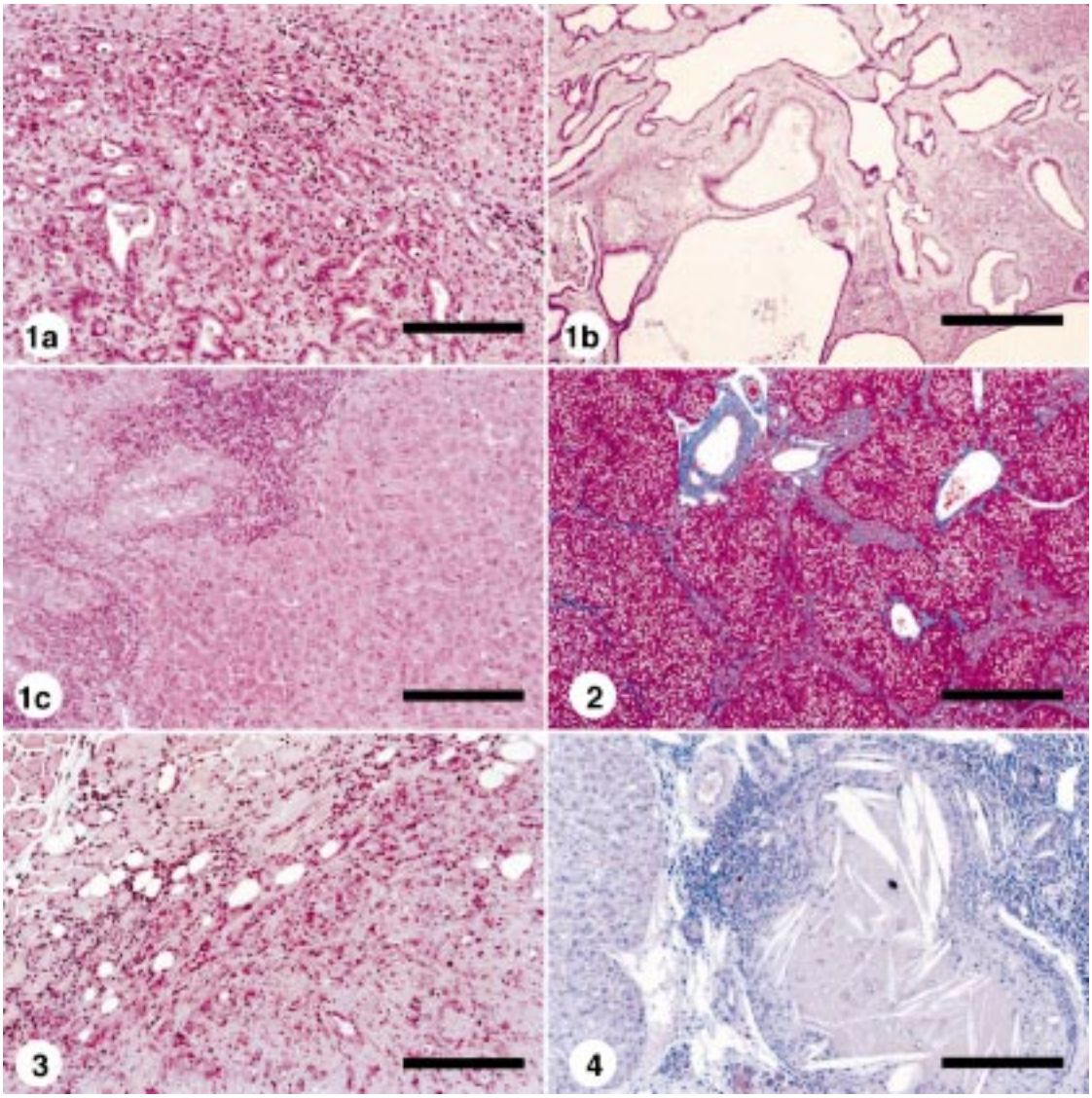

Fig. 1. Section of liver cancer in hamsters treated with a combination of *Opisthorchis viverrini* infection and dimethylnitrosamine administration. (a) Cholangiocell carcinoma (bar = 200 μm), (b) cystoadenocarcinoma (bar = 500 μm), (c) hepatocholangiocell carcinoma. Stained with haematoxylin and eosin (bar = 200 μm). Fig. 2. Section of liver cirrhosis stained with Azan-Mallory (bar = 500 μm). Fig. 3. Cholangiocell carcinoma invading the diaphragm (bar = 200 μm). Fig. 4. *Opisthorchis viverrini*-induced granulomatous tissue in the liver parenchyma (bar = 200 μm).

revealed an obvious difference in the degree of lymphocyte infiltration. Ten of 17 hamsters showed a massive infiltration with lymphocytes around the bile ducts, while less infiltration occurred in the pre-immunized group. In the group Im + Ov + DMN, the development of CCC seemed to be delayed or decreased. Two of eight hamsters had not developed cancers by 26 weeks. However, one of the cancers of the remaining six hamsters was highly malignant with invasion occurring into the diaphragm (fig. 3). Hamsters treated only with DMN developed cancer in 30% and cirrhosis in 50% of cases during the later experimental stages. In the group pre-immunized with *O. viverrini* extracts, both cancer and cirrhosis were also observed. With regard to liver cirrhosis, Ov + DMN treatment induced cirrhosis in 100% of hamsters treated. In a few cases of *O. viverrini* infection, eggs were seen in the granulomatous tissue (fig. 4) and in giant cells near the dilated bile duct filled with liver flukes.

Immunohistochemistry

The results of immunostaining for tenascin are summarized in table 5. Positive staining was found in

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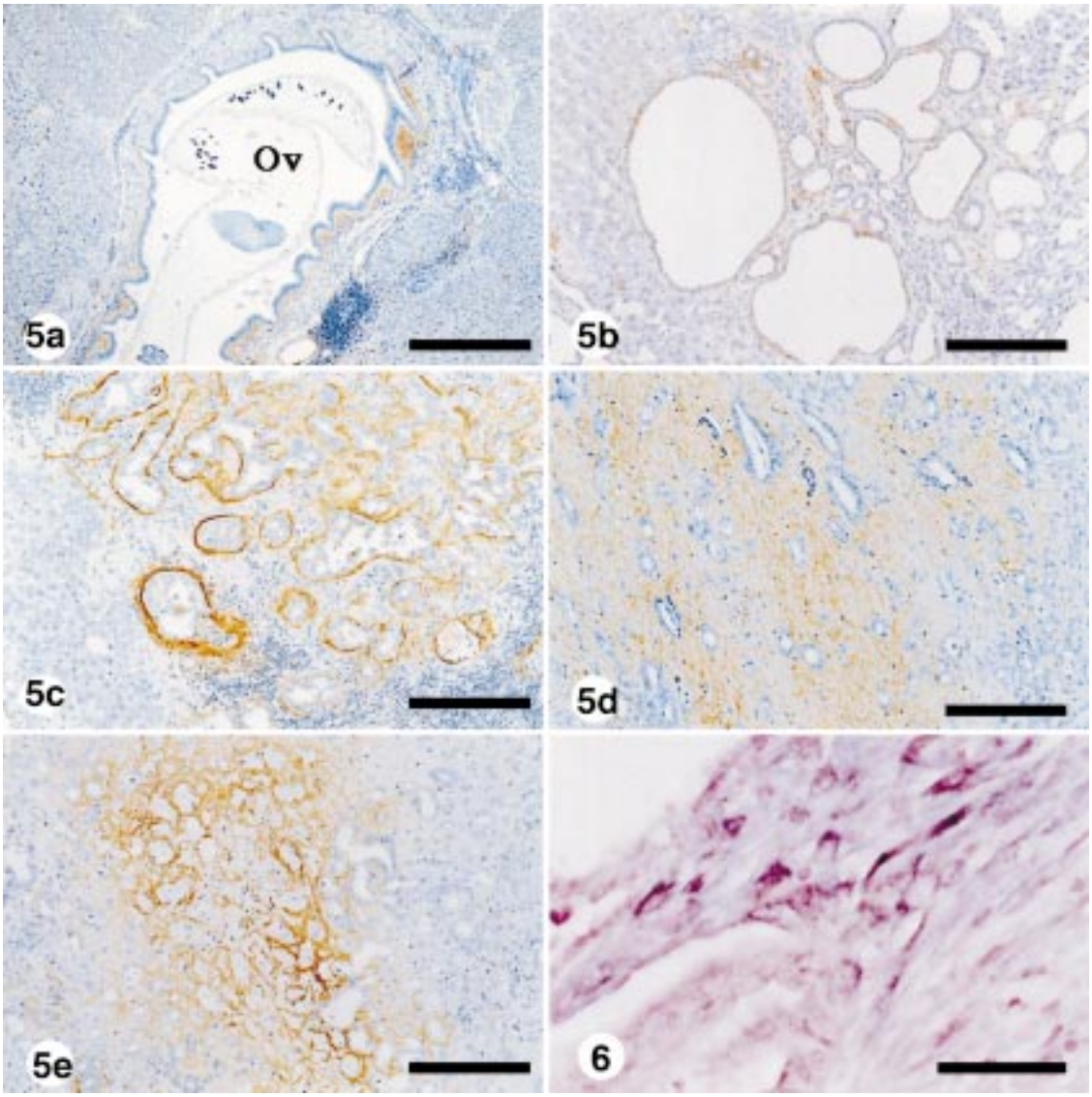

Fig. 5. Tenascin immunohistochemistry. Tenacin is positively stained (brown colour) in the connective tissue beneath the epithelium of a dilated bile duct with *Opisthorchis viverrini* (Ov) inside (a) (bar = 500 μm), periductal rims around the hyperplastic bile ducts (b) (bar = 200 μm), periductal rims and the stroma of cholangiocell carcinoma (c) (bar = 200 μm), the stroma of invading cancers (d) (bar = 200 μm), and the stroma of necrotic cancers (e) (bar = 20 μm). Fig. 6. *In situ* hybridization of tenascin mRNA expression in cholangiocell carcinoma. Some cancer cells and many stroma cells are positive (bar = 50 μm).

the connective tissue surrounding dilated bile ducts infected with *O. viverrini* (fig. 5a), in periductal rims around the bile ducts and also in hyperplastic ductules in experimental groups treated with DMN (fig. 5b). In cancer tissue, tenascin staining appeared as rings surrounding the tumour duct with diffuse staining in the interstitial matrix of tumours (fig. 5c,d). Distribution of tenascin is limited to the periductal rims at the initial stages of cancer development with diffusion of tenascin occurring as malignancy advances. The strongest staining is noticed in large tumours at the apoptotic cancer cell masses (fig. 5e).

In situ hybridization

To investigate which cells produce tenascin, liver sections from fresh samples were examined for tenascin mRNA expression by *in situ* hybridization. The mRNA signals were recognized in stroma cells and also in a few cancer cells (fig. 6).

Discussion

As shown in table 2, liver cancer can be induced in Syrian golden hamsters by DMN administration alone

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Table 4. Incidence of cholangiocellular lesions in Syrian hamsters infected or immunized with *Opisthorchis viverrini* and/or given dimethylnitrosamine.

Groups	Weeks after Ov infection	No. hamsters	Lymphocyte infiltration			Bile ductule proliferation	Dilated bile duct and cholangiofibrosis	Cancer	Cirrhosis
			−	+	+ +				
Control	12	9	9	0	0	0	0	0	0
	26	5	5	0	0	0	0	0	0
	35	4	4	0	0	0	0	0	0
Ov	12	5	0	2	3	4	5	0	0
	26	10	0	6	4	5	10	0	0
	35	11	0	7	4	3	11	0	3
DMN	12	5	2	3	0	1	0	0	0
	26	10	5	5	0	1	0	0	3
	35	10	2	8	0	3	0	3	5
Im	12	5	5	0	0	0	0	0	0
	26	8	8	0	0	0	0	0	0
	35	4	4	0	0	0	0	0	0
Ov + DMN	12	5	0	3	2	2	5	0	4
	26	17	0	7	10	17	17	17	17
	35	16	0	13	3	16	16	16	16
Im + Ov + DMN	12	5	0	3	2	1	5	0	5
	26	8	1	7	0	8	8	6*	7
Im + DMN	12	5	1	4	0	3	2	0	0
	26	5	0	5	0	3	0	0	2
	35	5	0	5	0	3	0	1	4

Abbreviations as in table 1.
*One liver shows invasion of cancer to diaphragm.

after long latent periods. At 23 weeks, followi#ng termination of DMN in drinking water for 8 weeks, cancers developed in three of ten hamsters and the cancers were all CCC (table 3) as also observed under the electron microscope (Tesana *et al.*, 2000). The development of bile duct cancer increased to 100% when combined with *O. viverrini* infection, which does not induce neoplastic changes by itself (table 2). These results are consistent with those reported by Thamavit *et al.* (1978, 1987).

Possible mechanisms of *O. viverrini* action are: (i) mechanical obstruction and damage of the bile duct epithelium; (ii) chemical induction of hyperplasia in the bile ducts; and (iii) immunological injury of the epithelium leading to cell death. Of these three possibilities, mechanical (Bhamarapravati *et al.*, 1978; Thamavit *et al.*, 1993) and chemical (Isseroff *et al.*, 1977) influences of *O. viverrini* on cholangiocancer development have so far been demonstrated. Immunological influences, however, have not been investigated experimentally. Therefore, although it was appropriate to examine the effect of pre-immunization with *O. viverrini* extracts, the results of these experiments are inconclusive. Two of eight hamsters did not develop cancers at 14 weeks after termination of DMN administration (table 2), suggesting that the pre-immunization was effective in reducing carcinogenesis. However, one of six hamsters showed aggressive behaviour as the cancer invaded into the diaphragm. Comparing pre- and non-immunized livers, lymphocyte infiltration around the bile duct filled with liver flukes was much stronger in non-immunized animals. However, these results indicate that the effect of pre-immunization or the influence of *O. viverrini* on host immunity remains unclear. DMN-induced liver cirrhosis was enhanced by a combined infection with *O. viverrini* (table 4). At 14 weeks after termination of the DMN treatment, cirrhotic changes occurred in all animals.

Tenascin is an extracellular matrix glycoprotein which is expressed at embryogenesis (Chiquet-Ehrismann *et al.*, 1986; Ekblom & Aufderheide, 1989, Erickson & Bourdon, 1989) and tumourigenesis (Mackie *et al.*, 1987; Sakakura, 1995). Besides the oncofetal expression of tenascin, it has also been found in many tissues with inflammation (Mackie *et al.*, 1988). These findings generally acknowledge that tenascin is induced in the stroma as a result of interactions with either the embryonic or neoplastic epithelium and probably functions as a homeostatic factor in the repair of tissue perturbations.

In the present study, positive staining of tenascin was found in the connective tissue surrounding the dilated bile ducts infected with *O. viverrini*, together with basement membranes of hyperplastic ductules, the periductal rims and the stroma of cancers (table 5, figs 5 and 6). Inside the large tumours, necrotic foci were found to be strongly stained. When cancer cells invaded the diaphragm, tenascin appeared in the stroma tissue. Tenascin staining became stronger with the advancement in the malignancy of the liver cancer and this has previously been demonstrated by many researchers (Sakakura, 1995). Speculated roles of tenascin in cancer development are various, including the stimulation of cancer cell growth, promotion of cancer cell migration and invasion, and a role as a mechanical barrier against

Table 5. Tenascin immunohistochemistry in the liver of Syrian hamsters infected or immunized with *Opisthorchis viverrini* and/or given dimethylnitrosamine.

Groups	Weeks after Ov infection	No. hamsters	Tenascin staining				
			−	+			
				Periduct dilated with Ov	Surrounding duct and proliferated ductule	Periduct of tumour	Interstitial matrix of tumour
Control	12	9	9	0	0	0	0
	26	5	5	0	0	0	0
	35	10	10	0	0	0	0
Ov	12	5	0	5	0	0	0
	35	21	6	15	0	0	0
DMN	12	5	5	0	0	0	0
	26	10	10	0	0	0	0
	35	8	3	0	5	5	1
Im	26	8	8	0	0	0	0
	35	4	4	0	0	0	0
Ov+DMN	12	5	3	2	0	0	0
	26	17	0	17	17	17	2
	35	23	0	23	23	23	12
Im+Ov+DMN	12	3	0	3	3	0	0
	26	8	0	8	6	6	3*
Im+DMN	12	5	4	0	1	0	0
	26	5	1	0	4	0	0
	35	5	4	0	1	0	0

Abbreviations as in table 1.
*One liver shows invasion of cancer to diaphragm. Positive staining of tenascin is also seen in the invading stroma.

cancer cell outgrowth. Further studies are now required to confirm the precise role of tenascin in cancer development.

The present study is the first report of tenascin expression in bile duct cancer induced by a combined parasitic infection and carcinogen treatment.

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