

An Animal Model for the Study of Hepatic Stellate Cell and Hepatocellular Carcinoma Interaction[†]

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Abstract

The recognition of hepatic stellate cells (HSC) around and within hepatocellular carcinoma (HCC) in human livers has generated interest in the interactions between HSC and HCC. We explored the possibility of creating an animal model to allow *in vivo* investigations of this interaction. Eighteen adult Buffalo rats were inoculated with 1×10^6 cells obtained from cultures of Morris 7777 hepatoma cell line (A TCC). The rats were sacrificed at 2-, 3-, and 4-week intervals. Identification of activated HSC was with immunohistochemistry for α -smooth muscle actin (α -SMA). There was 100% survival of all animals until sacrifice. Tumour formation occurred in 94.4% of rats, and was of a good size by two weeks. Expression of α -SMA was observed around and within all HCC, but absent from normal tissue, and this showed colocalisation with collagen deposition. These findings are consistent with those previously reported in resected HCC in humans. The high survival, good tumour yield, consistent generation of activated HSC around the tumours, and similarities in histological appearance to the human HSC-HCC distribution pattern, make this a reliable animal model for *in vivo* studies on HSC-HCC interaction.

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Introduction

Hepatic stellate cells (HSC) are mesenchymal cells that are prominent in various forms of liver injury, in particular with cirrhosis or carbon tetrachloride-induced fibrosis.¹ HSC are also being increasingly reported around and within hepatocellular cancers (HCC), and have been thought to be responsible for the capsule formation in these tumours.^{2,3} While much experimental work has been done regarding HSC in animal models of liver injury and fibrosis, little is known about the association between HSC and HCC. Also, it is impossible to perform studies on HSC-HCC interaction in humans, apart from static observations on histological and immuno-cytological assessment. In contrast, an animal model can create a consistent supply of tissue for study of HSC-HCC interaction, as well as allow for dynamic studies involving introduction of various agents or milieu changes that may influence this interaction. In this study, we attempted to create an animal model for HCC that

will generate a response from HSC to enable *in vivo* study of HSC-HCC interactions.

Materials and Method

Animal HCC Model

All research work was done in accordance with the international guiding principles for animal research.⁴ Three-month-old Buffalo rats (Laboratory Animal Centre, National University of Singapore) weighing approximately 280 g were used. Morris 7777 hepatoma cells (ATCC, USA) were cultured at a concentration of 1×10^6 cells per flask, in F12 medium with 10% fetal calf serum and an antibiotic combination of penicillin, streptomycin and amphotericin. Upon cell confluence, the cells were washed with Hank's Balanced Salt Solution (HBSS) before centrifugation to obtain a pellet. These were reconstituted with F12 medium to obtain 0.1 ml volumes of 1×10^6 cells each and inoculated intraparenchymally into the liver via a mini-laparotomy.

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From our pilot studies, tumour growth of approximately 0.5 cm^3 is expected one week after inoculation. Eighteen animals were inoculated, and these were humanely sacrificed (in accordance with the hospital ethics committee recommendations) in three batches of six animals each at intervals of 2, 3 and 4 weeks post-inoculation.

Tissue Preparation

At sacrifice, the tumour was measured using calipers at its widest diameter, and along a line perpendicular to this plane. Tissue samples were taken from three distinct areas of the liver, viz. the normal liver at a distance from the tumour, the centre of the tumour, and the interface between tumour and normal liver. The formalin-fixed, paraffin-embedded tissue samples were then sectioned at $4 \mu\text{m}$ thin cuts for analysis. Activated HSC were identified directly by the expression of $\alpha\text{-SMA}$ as the gold standard, and indirectly by the presence of collagen. Immunohistochemical staining for $\alpha\text{-SMA}$ was performed using a mouse monoclonal anti- $\alpha\text{-SMA}$ antibody (1:400, clone 1A4; Sigma Chemical Co, St Louis, MO, USA) as the primary antibody, and a DAKO LSAB2 kit (DAKO, USA). The chromogen substrate used was 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co, St Louis, MO, USA), and the counterstain was haematoxylin. Collagen deposition was demonstrated by Masson's trichrome stain. In addition, haematoxylin and eosin staining was used for morphological analysis of the tumour. A semi-quantitative scoring system, reported previously by the principal author,² was used for grading the expression of $\alpha\text{-SMA}$. This

scoring system is based on the number of cells expressing $\alpha\text{-SMA}$, as well as the pattern of layering of the HSC. Single HSC staining was labelled "1+", HSC in a monolayer is "2+", more than 1 but less than 10 cell layers thick is "3+", and more than 10 HSC cell layers thick is "4+".

Results

All animals remained healthy until the time of sacrifice. At sacrifice, tumour was seen in 94.4% (17/18) of the animals inoculated. One animal at four weeks did not show tumour formation. The mean tumour diameter was 6.2 mm (range 3.1 to 8.0 mm) for animals sacrificed at 2 weeks, 10.7 mm (range 8.3 to 14.8 mm) at 3 weeks, and 11.3 mm (range 3.6 to 18.3 mm) at 4 weeks. All the lesions were well-circumscribed but a complete tumour capsule was evident in only 58.8% (10/17) of the tumours, with the remainder having deficient or incomplete capsule formation. There were no satellite or multiple lesions in any of the animals. Haematoxylin and eosin stains confirmed the presence of HCC, and the histological features are almost identical to a human HCC.

Increased $\alpha\text{-SMA}$ expression was noted in the interface around all tumours (Figs. 1a, b & c) and this colocalised to the areas of collagen deposition seen on Masson's trichrome staining (Figs. 1d, e & f). No expression of $\alpha\text{-SMA}$ was seen in normal liver tissue at a distance from the tumour, and minimal or absent expression was seen in the normal liver tissue just beyond the tumour-liver interface (Table I). This gradient of decreasing $\alpha\text{-SMA}$ expression from the tumour towards

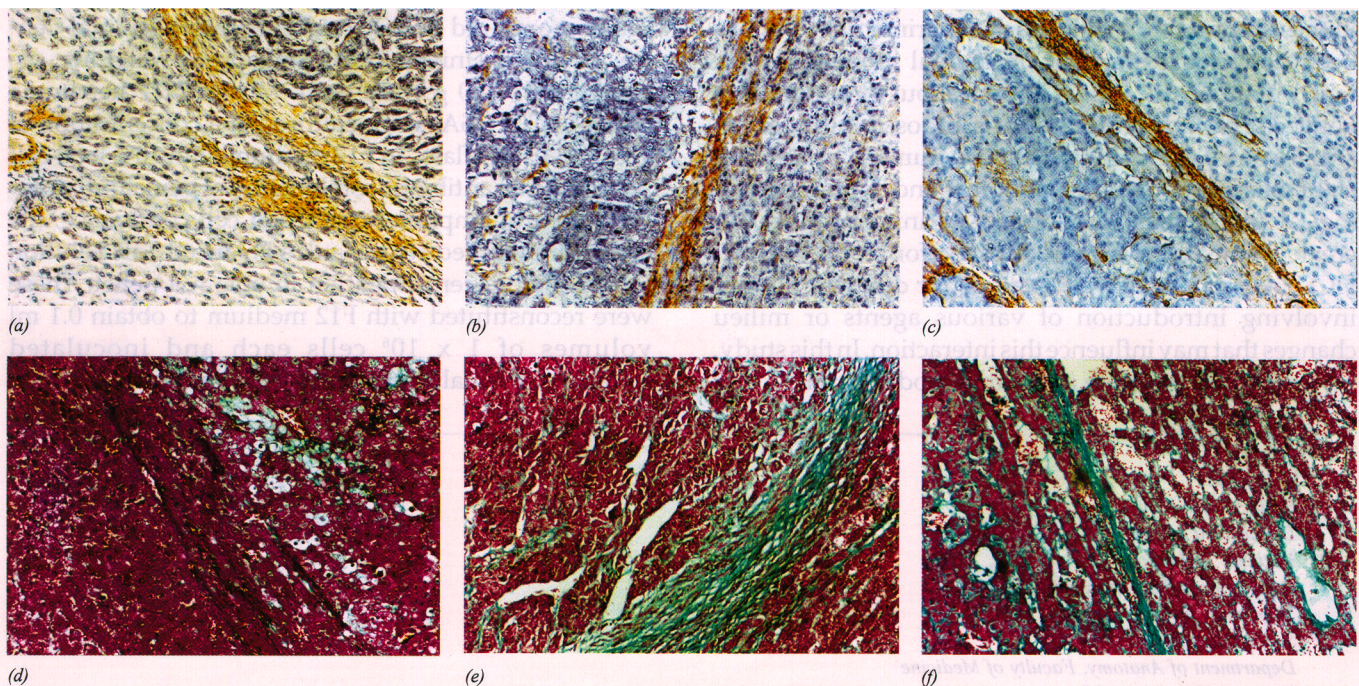


Fig. 1. The morphological appearance of the tumour, the expression of $\alpha\text{-SMA}$, and the collagen deposition at 2 (a, d), 3 (b, e) and 4 (c, f) weeks of sacrifice are identical (a-c, immunohistochemical stain for $\alpha\text{-SMA}$; d-f, Masson's trichrome stain) (Magnification $\times 100$).

normal tissue observed is similar to that previously reported in human specimens.² In the single animal that showed no tumour growth, there was no evidence of a-SMA expression or collagen deposition in the sections obtained from the site of inoculation.

TABLE I: α -SMA DISTRIBUTION IN VARIOUS REGIONS OF THE SECTIONS IN THE 18 RATS. THE SCORING USED IS AS REPORTED PREVIOUSLY.²

No.	Tumour size (mm)	Capsule	Within tumour	Junction	Close normal	Distant normal
1	3.1 x 3.0	Complete	1+/2+	3+	1+	0
2	6.5 x 6.1	Complete	1+/2+	4+	1+	0
3	4.6 x 2.5	Incomplete	1+/2+	3+	1+	0
4	7.7 x 6.4	Incomplete	1+/2+	2+/3+	1+	0
5	8.0 x 4.9	Complete	1+/2+	3+	0/1+	0
6	7.4x5.8	None	1+/2+	0/1+	0/1+	0
7	8.3 x 5.7	Complete	1+/2+	3+/4+	0/1+	0
8	8.3 x 6.9	None	2+	2+/3+	1+	0
9	11.1 x 11.1	None	2+/3+	2+/3+	0/1+	0
10	8.1 x 6.6	Complete	2+/3+	2+/3+	1+	0
11	13.4 x 9.4	Incomplete	2+	2+/3+	1+	0
12	10.0 x 14.8	Incomplete	2+	2+/3+	0/1+	0
13	16.6 x 15.9	Complete	1+/2+	2+/3+	0/1+	0
14	18.3 x 12.7	Complete	1+/2+	3+	0/1+	0
15	11.6 x 10.5	Complete	1+/2+	2+/3+	0/1+	0
16	3.3 x 3.6	Complete	1+/2+	2+/3+	0/1+	0
17	None	None	0	0	0	0
18	6.6x3.1	Complete	0/1+	3+	0/1+	0

Discussion

The role of HSC in fibrogenesis in various forms of diseased liver states has been well studied in both human livers and animal models. However, interest in the interactions between HSC and HCC have only been recent following observations of increased numbers of activated HSC around, as well as within liver tumours.^{2,5,6} Reasons for this pattern of distribution have not been fully elucidated. The increase in HSC around HCC has been postulated to be a form of host defence mechanism causing encapsulation of the HCC, as HSC were demonstrated to contribute to capsule formation in these tumours.² In addition, HSC is also found within HCC as well as in other tumours to the liver,⁷ and could possibly be responsible for the extracellular matrix deposition required for scaffolding and development of neovascularisation in tumour growth. The actual role of HSC in these regions will require more investigations, both in human livers and in animal models.

Numerous *in vivo* models have been described for the study of HSC, although these have been primarily with regards to liver cirrhosis. Common techniques involved creating a diffuse liver parenchymal damage that would generate a diffuse HSC activation. The rat and mouse models have been extensively used and techniques

include the administration of carbon tetrachloride (CCl₄),⁸⁻¹⁰ or involved ligation of the common bile duct.^{10,11} These models however did not produce a site-selective activation of HSC, as is seen in the HSC distribution from human HCC specimens. Furthermore, observations from our previous work noted that the HSC distribution around HCC was not influenced by the presence of adjacent, concomitant cirrhosis, suggesting that different mechanisms may be involved in activating HSC in tumours and in cirrhosis, making the diffuse models useless for studying HSC-HCC interactions.

Likewise with HCC animal models, there have been numerous techniques reported for tumour induction, also with rats and nude mice. *De novo* tumour forming models can occur in Long-Evans cinnamon rats that undergo spontaneous progression from acute to chronic hepatitis and subsequently to HCC.¹² Deficiency of dietary choline is also a potent stimulus for HCC induction.¹³ The common techniques of generating HCC revolve around chemical tumour induction or direct tumour inoculation into the liver. Chemical induction has the disadvantages of a long period of model preparation time required to allow carcinogenesis. Also, the development of the tumour may be sporadic, unreliable, and not well-circumscribed or diffuse. Various hepatocarcinogens have been described and include phenobarbitone,¹⁴ diethylnitrosamine,¹⁵ 3'-methyl-4-dimethylamino-azobenzene¹⁶ and oral contraceptives.¹⁷ Insulin, from transplanted pancreatic islet cells, has also been used to induce HCC in a rat model, although after 15 to 22 months, only 19% of the animals showed a hepatocellular carcinoma.¹⁸ We chose the tumour inoculation method as the one most suitable to explore, because our prior experiences with the technique had yielded consistently good results of well-circumscribed lesions. The previously described methods advocated a subcapsular technique that is thought to contain the inoculum under the capsule, and prevent diffusion into the parenchyma, thereby increasing the chances of solid tumour formation. In most human HCC, however, the tumour lies within the parenchyma, and the intraparenchymal route was chosen in our model as being closest to the actual situation. In our model, there was one rat at 4 weeks that did not form a solid tumour and is presumed to be due to diffusion of the cells away from the inoculation site.

Buffalo rats, compared to nude mice, are hardy laboratory animals, and have been used for developing HCC models.^{19,20} The 100% survival of all animals until sacrifice is not unusual, with none of the rats showing evidence of being moribund or near death. In addition, there was a high reliability of 94.4% tumour formation, with tumours being a good size by two weeks.

The expression of a-SMA has been established as a reliable, gold-standard indicator of HSC. In our model,

we observed a 100% presence of a-SMA-expressing activated HSC around all the tumours generated. The distant normal tissue served as a control, and did not express any a-SMA except at the portal triads, which is the expected normal. In addition, the expression of a-SMA was colocalised to the deposition of collagen at the tumour-normal liver interface. The tumours morphologically resembled human liver tumours, and the pattern of HSC distribution throughout the liver was similar to that observed in previously published work on human liver tumours by the principal author.*

We therefore conclude that our animal model is a simple, reliable one that closely resembles the pattern of HSC-HCC interaction in humans, and should prove to be a useful model for the *in vivo* study of HSC-HCC interactions.

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