

3rd Yahya Cohen Lecture: The Role of the Myofibroblast-Like Cell in Hepatocellular Carcinoma-Host Defence?

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Abstract

Hepatocellular carcinoma is a common cancer in Asia with a poor prognosis. Tumour encapsulation has been shown to be a good, independent prognostic factor. The main cell in the liver responsible for extracellular matrix formation is the hepatic stellate cell, and this was investigated as the cell type responsible for encapsulation. This lecture discusses the role of hepatic stellate cells in hepatocellular carcinoma, in particular with tumour encapsulation as a host defence mechanism. The study involved four phases: (1) comparing encapsulated tumours with non-encapsulated tumours, (2) comparing with metastatic tumours to the liver, (3) creation of an animal model, and (4) investigating known cytokines with hepatic stellate cell stimulating activity. The results showed that the hepatic stellate cells were responsible for tumour encapsulation and that this was impaired in non-encapsulated tumours and in metastatic tumours. A successful animal model was created which allowed further work. However, the known cytokines that normally stimulate hepatic stellate cells were not shown to be involved in tumour encapsulation, suggesting that an unknown factor may be involved.

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Mr Chairman, Distinguished Guests, Fellow Academicians, Friends and Colleagues, Ladies and Gentlemen.

It is indeed a great honour for me to be named the third Yahya Cohen lecturer and I would like to express my deepest thanks and appreciation to the Academy of Medicine Singapore for having selected me for this award. At this lecture, I would like to share with you my work over the last three years, on the myofibroblast-like cell and its association with hepatocellular carcinoma or HCC as it is more commonly known. Over this period of time, I have been fortunate to have had great help in my research from many good friends and colleagues, both in Australia and in Singapore. Too many to name but listed here (see acknowledgements) and duly acknowledged and thanked for their contributions.

Introduction

HCC is a common tumour in Asia. In Singapore, it is the fourth commonest cancer in males, with an estimated incidence of 20 per 100,000 per annum, and is among the top 10 cancers in females. The prognosis is generally dismal with few surviving 2 years and beyond.

Numerous clinicopathological studies have shown

that local invasion of HCC is influenced by the presence of a capsule, and that this capsule confers some degree of survival advantage, as well as a longer disease-free interval. However, at this point of time, the cell of origin for this capsule formation is unclear. Various studies, primarily from Japan, have shown that tumour cells of HCC can produce various components of the extracellular matrix, like collagen types I, III, and IV, fibronectin and laminin. These findings have led some investigators to believe that the tumour cells of HCC may form the capsule, although this has never been demonstrated or proven.

Looking at it from a different angle, the host fibroblasts are the body's main source of extracellular matrix. In tumours like breast and colorectal cancers, these fibroblasts have been shown to limit local tumour invasion, and confer some measure of increased survival and longer disease-free interval. The equivalent of the host fibroblast in the liver is the myofibroblast-like cell. This cell is so named because of the presence of smooth muscle filaments within it, as can be seen on electron microscopy (Fig. 1). The myofibroblast-like cell has also been known by a variety of other names, although the original name of Ito cell, named after the person who first described it, is the most well known. These names

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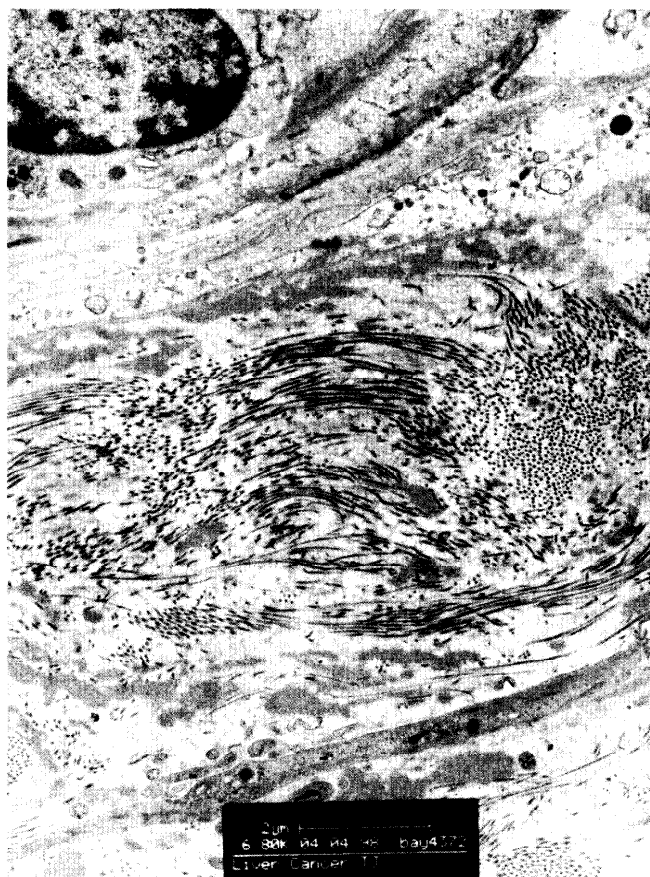


Fig. 1. Scanning electron micrograph of a hepatic stellate cell showing the presence of myofilaments within the cytoplasm. (Magnification x80,000)

include fat-storing cell, lipocyte, perisinusoidal cell, parasinusoidal cell, and pericyte. To avoid confusion among researchers and scientists, a worldwide consensus has been reached, and the cell is now known as the hepatic stellate cell. Henceforth in my lecture, this is the nomenclature I will use.

The hepatic stellate cell exists in two main forms. A quiescent form where it is lipid-droplet rich and stores vitamin A, and an activated form where it is primarily involved in extracellular matrix formation. An intermediate transitional form, between the quiescent and activated forms, has also been described, although its clinical significance is seldom a consideration.

The quiescent form of the hepatic stellate cell is best demonstrated by various special stains like Toluidine blue or Oil-Red-O that show up the lipid droplets, or by detection of vitamin A with gold chloride impregnation or immunofluorescence. Alternatively, electron microscopy may show the quiescent cell in its normal perisinusoidal position. In the activated form, immunohistochemical demonstration of alpha smooth muscle actin (α SMA) is the gold-standard, and can be seen in the activated hepatic stellate cell as a brown staining in the cytoplasm because of the chromogen 3,3'-diamino-

benzidine tetrahydrochloride (DAB). Other markers that have been described include desmin, glial fibrillary acid protein (GFAP) and vimentin.

The hepatic stellate cell has a variety of functions. In its resting form, it is primarily involved in the uptake, storage and release of retinoids, and is the body's main store for vitamin A. It is also a major body source of various cytokines. By virtue of its position in the perisinusoidal region, and the presence of myofilaments in its cytoplasm, the hepatic stellate cell has a unique function of controlling the pressure within the sinusoids by contraction or dilatation, although the actual clinical significance of this function is unclear.

In the activated form, the main function of the hepatic stellate cell is the fine control of the extracellular matrix in the liver. This it does by controlling three different aspects of extracellular matrix balance. First, the hepatic stellate cell synthesizes and secretes the various components of the matrix, including collagen, fibronectin and laminin. Second, it produces metalloproteinases, like collagenase, which are enzymes that break down the various matrix components. Thirdly, by a feedback mechanism, it secretes tissue inhibitors of metalloproteinases, or TIMP for short, which inhibits the activity of these metalloproteinases, and prevents the degradation of the extracellular matrix.

It should be evident to us all now that the hepatic stellate cell is an important cell in extracellular matrix formation in the normal liver. It is also the main cell source for matrix components in chronically diseased livers, especially in liver cirrhosis. In HCC, the hepatic stellate cell has been described around and within the tumour, although its role has not been determined in these circumstances. In some patients with HCC, a cytokine with known hepatic stellate cell activating function, transforming growth factor beta or TGF β , has been identified, both around the tumour, and within the capsule of the HCC. The presence of TIMP has also been detected in the serum of patients with HCC. These observations could suggest that the hepatic stellate cell may be involved with HCC.

The hypothesis put forth then, is that hepatic stellate cells are intimately related to HCC, and is the cell involved in capsule formation in these tumours. This capsule formation, could possibly be a form of host defence mechanism for HCC tumour containment by limiting tumour growth and preventing local invasion into the surrounding parenchyma.

Materials and Methods

In order to prove this hypothesis, I chose to proceed with a study design with four phases. Firstly, to compare encapsulated and non-encapsulated HCC to establish if hepatic stellate cells are indeed responsible for capsule

formation. Secondly, to compare the findings with non-primary liver cancers, on the presumption that these are more aggressive tumours. Thirdly, to establish an animal model that is similar to the human HCC and that would allow me to perform further *in vivo* studies that would not be possible in the human. And lastly, to investigate cytokines and growth factors with known hepatic stellate cell activating function for their effects on capsule formation.

Results

Let's look at the first phase. Tissue samples were obtained from resected specimens of patients with encapsulated HCC and a comparable number with non-encapsulated tumours.^{1,2} These samples were taken from various parts of the tumour and liver; the tumour centre, tumour-"normal" margin or interface where the capsule is expected to be, close "normal", and distant "normal". Technically speaking, some of these were not "normal" as some patients had cirrhosis, and should more correctly be termed non-tumour tissue, although "normal" is used for convenience here.

These tissue samples were formalin-fixed, paraffin-embedded and sectioned into 4 micron slices for analysis using immunohistochemistry for α SMA as the gold-standard marker for activated hepatic stellate cells. In addition, sections were also stained with haematoxylin and eosin for morphological assessment, and Masson's trichrome stain for collagen and reticulin. *In situ* hybridisation for procollagen $\alpha_1(I)$ messenger RNA was also performed to confirm synthesis of collagen by the hepatic stellate cells.

Looking at the stains for α SMA, there is intense hepatic stellate cell activity within the capsule around encapsulated tumours, and this corresponds to the intense staining of collagen obtained with Masson's trichrome. In the non-encapsulated tumour, there is hepatic stellate cell activity around the tumour but no evidence of capsule formation.¹

In situ hybridisation for procollagen $\alpha_1(I)$ mRNA, shown as black dots within hepatic stellate cells, proves that these cells are indeed laying down extracellular matrix in the capsule. What is more interesting, however, is that hepatic stellate cells around non-encapsulated tumours also seem to be demonstrating procollagen $\alpha_1(I)$ mRNA but without the end-result of matrix deposition and capsule formation. To me, this suggests some form of post-transcriptional inhibition of matrix deposition, possibly by a more aggressive tumour type.

To summarise, the colocalisation of α SMA, collagen and procollagen $\alpha_1(I)$ mRNA would strongly suggest that the hepatic stellate cell is the cell type responsible for capsule formation around HCC.

Another interesting phenomenon was observed in this

study. You have already seen the high numbers of activated hepatic stellate cells around the tumour. Further away from the tumour towards normal liver, the number of activated hepatic stellate cells reduces until it reaches negligible numbers in distant normal liver. This observation of a gradient would suggest that hepatic stellate cell activation was initiated or mediated by a diffusible agent from the tumour.

One final observation. In the section of an encapsulated HCC with satellite intrahepatic metastases, the presence of activated hepatic stellate cells in the capsule around the primary, and the contrasting absence around the metastases is glaring (Fig. 2). Would this then suggest that more aggressive tumour types could suppress hepatic stellate cell capsule formation?

To answer this question, we looked at various metastases to the liver. Colorectal metastases are by far the commonest that undergo resection, and we chose 10 of these. Resections for other forms of metastases are more difficult to obtain, but courtesy of Professor Russell Strong from Brisbane, we managed to obtain 10 other specimens. The histological types include carcinoid tumour (2), malignant melanoma (2), renal cell carcinoma, infiltrative ductal breast carcinoma, pancreatic islet cell carcinoma, granulosa cell carcinoma of the ovary, extrauterine endometrial sarcoma, and squamous cell carcinoma of the oesophagus.³

Looking at sections of a patient with colorectal metastasis to the liver, there is a noticeable absence of a capsule. However, there is marked peritumour activation of hepatic stellate cells as well as some collagen deposition, although not amounting to encapsulation.' The sections from the remaining 19 other metastatic tumour samples were similar in observations. It would seem then, that the hepatic stellate cell is activated in response to tumour growth, but is unable to mount an encapsulation response to these more aggressive tumour types. In addition, similar findings of a gradient of decreasing hepatic stellate cells away from the tumour towards normal liver would imply the effects of a soluble, diffusible agent from the tumour.^{1,3}

We move on now to phase three of the study. Here I attempted to establish an animal model that would allow work to be done on hepatic stellate cell interaction with HCC, and for this I would like to acknowledge with thanks the funding provided by the National Medical Research Council (NMRC) of Singapore. As with all animal work, strict ethics guidelines were followed to ensure the animals were treated humanely.

Buffalo rats of about 260 to 280 g weight were used, and these were inoculated with Morris 7777 hepatoma cells obtained from ATCC in the US, and grown in cell culture⁴ (Fig. 3). Injected intraparenchymally, these cells grew and became visible by the second week. Unlike

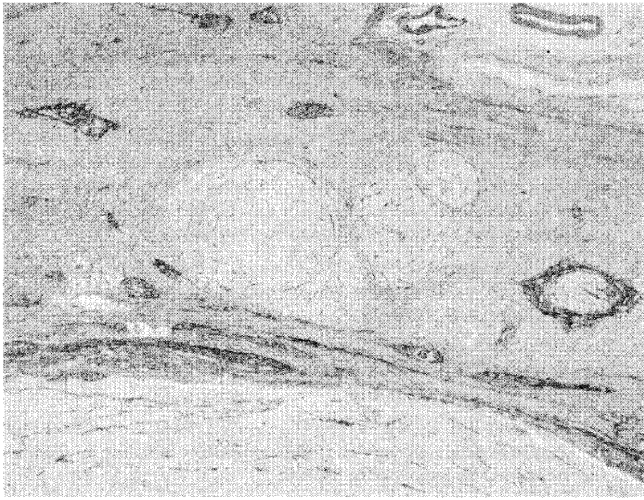


Fig. 2. Immunohistochemical staining for α -smooth muscle actin, showing increased expression around the primary encapsulated HCC, but obviously absent around the satellite intrahepatic metastases. (Magnification $\times 40$)

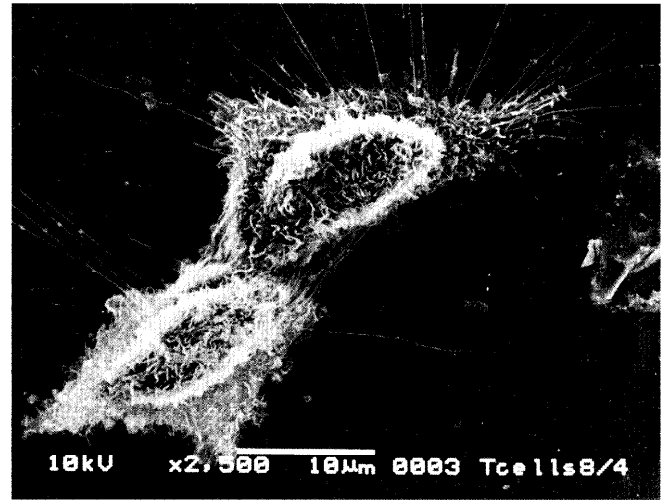


Fig. 3. Scanning electron micrograph of two Morris hepatoma cells in cell culture. (Magnification $\times 2500$)

subcapsular inoculations, which is the common technique used in animal HCC models, the intraparenchymal inoculations used here produced tumours that were within the liver and more like the human HCC.

Tissue sections were similarly subjected to immunohistochemistry for α SMA as the gold-standard marker for hepatic stellate cells, and Masson's trichrome stain for collagen. These showed an increase in hepatic stellate cells in the capsule, very similar to those seen in sections taken from human HCC that I showed earlier.

The Buffalo rat is a hardy animal, and we managed a 100% survival. Although tumour take was only 94.4%, this is much higher than with other tumour models. The consistent finding of a capsule and activated hepatic stellate cells within this capsule in all cases makes this an ideal model for studying hepatic stellate cell interaction with HCC. With the findings being so similar to those in humans, it is expected that observations made with this model may be extrapolated to the human. This is the first such model of hepatic stellate cell and HCC described, and will be published as an original article shortly."

I am presently at the final phase of my study. We know that various cytokines can influence hepatic stellate cell activity. Prime candidates with this activity include transforming growth factor beta (TGF- β), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and heparin-binding epidermal growth factor (Hb-EGF). It would thus be most logical to start looking at these cytokines and determining if these are indeed involved in hepatic stellate cell activation around tumours and in the capsule. Immunohistochemistry using monoclonal antibodies to these cytokines was used for this analysis.

With sections taken from human encapsulated HCC,

there was no demonstration of the cytokines TGF- β , bFGF, Hb-EGF and HGF in the capsule, although there was demonstration in the normal liver as shown by the brown stainings within the hepatocytes of the chromogen DAB. It would seem then that the major cytokines that affect hepatic stellate cell activity may not be involved in capsule formation in HCC.

Conclusion

Ladies and gentlemen, friends and colleagues, I have now come to the end of my presentation and I would like to highlight several points. From my earliest study, I have shown conclusively that the hepatic stellate cell is the cell type responsible for capsule formation in HCC. This encapsulation appears to be a form of host defence that is influenced by metastatic and more aggressive tumour types.

Based on a decreasing gradient of hepatic stellate cell activity away from the tumour, it would suggest that the agent responsible for activation of hepatic stellate cells around the capsule is a diffusible one. However, the presently known major cytokines do not appear to be this diffusible agent.

Several questions remain unanswered. What is the agent responsible for activating the hepatic stellate cell? And how do more aggressive tumours affect capsule formation and disrupt this host defence? At this point of time, there are no answers.

So what does the future hold? Is the hepatic stellate cell the key to controlling this dismal cancer? Can this form of host defence mechanism be enhanced and used as a novel way of limiting local cancer invasion? Can we really contain the crab in its cage? Only time will tell, and I hope I will be the one telling you.

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