Immunohistochemical and Ultrastructural Studies of Myofibroblasts in Hepatocellular Carcinoma

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Abstract : Sections from 13 hepatocellular carcinomas (HCCs) were examined to determine the myofibroblasts (MFBs) in tumour fibrous stroma and cirrhotic fibrous stroma, using monoclonal antibodies to specific actins HHF-35, 1A4 and sarcomeric actin. Histological grading of tumours with the expression of a labelling index using monoclonal antibodies to proliferative cell nuclear antigen (PC-10) were correlated with the amount of MFBs. The results showed a statistically significant increase in the amount of MFBs in tumour fibrous stroma. However, no significant correlation was found between the amount of MFBs with histological grading or the labelling index. However, a good correlation with statistically significant differences between the histological grading of HCC with the labelling index was shown. In conclusion, MFBs found in the tumour fibrous septa of HCC were a response by the stromal reaction or a desmoplastic reaction intended to limit the tumour size by contraction.

เรื่องย่อ : การตรวจหาเซลล์ไมโอไฟโบบลาสท์ในมะเร็งเซลล์ตับโดยวิธีทางอิมมูโนและจุลทรรศน์อิเลคตรอน

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ได้ศึกษาชิ้นเนื้อของมะเร็งเซลล์ตับจากผู้ป่วยจำนวน 13 รายเพื่อตรวจหาเซลล์ไมโอไฟโบบลาสท์ในบริเวณเนื้อเยื่อผังผืดหุ้มมะเร็งเซลล์ตับและบริเวณตับแข็ง การตรวจหาเซลล์ไมโอไฟโบบลาสท์ทำโดยใช้โมโนโคลนัลแอนติบอดีย์ชนิด HHF-35, 1A4 และ Sarcomeric actin ผลการตรวจพบว่า จำนวนเซลล์ไมโอไฟโบบลาสท์ในบริเวณ

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เนื้อเยื่อผังผืดหุ้มมะเร็งเซลล์ตับ มีจำนวนมากกว่าในบริเวณตับแข็งอย่างมีนัยสำคัญทางสถิติ และเชื่อว่าจำนวนเซลล์ไมโอไฟโบบลาสท์ที่เพิ่มขึ้นเป็นขบวนการของร่างกายในการที่จะจำกัดขอบเขตการงอกและแพร่กระจายของเซลล์มะเร็งโดยอาศัยคุณสมบัติเฉพาะตัวของเซลล์ไมโอไฟโบบลาสท์ในการบีบรัดก้อนเนื้อมะเร็งเซลล์ตับ นอกจากนี้ได้ทำการแบ่งเกรดของชนิดมะเร็งเซลล์ตับ เพื่อตรวจหาอัตราการเติบโตของเซลล์มะเร็งเซลล์ตับโดยใช้โมโนโคลนัลแอนติบอดีย์ชนิดแอนตีย์ PCNA ซึ่งค่าดัชนีการเติบโตของเซลล์มะเร็ง Labelling Index ที่ได้ใช้เปรียบเทียบหาความสัมพันธ์กับจำนวนเซลล์ไมโอไฟโบบลาสท์ในบริเวณเนื้อเยื่อผังผืดหุ้มมะเร็งเซลล์ตับ พบว่าไม่มีความสัมพันธ์อย่างมีนัยสำคัญทางสถิติ ระหว่างจำนวนเซลล์ไมโอไฟโบบลาสท์กับค่าดัชนีการเติบโตของเซลล์มะเร็ง หรือเกรดของชนิดมะเร็งเซลล์ตับ

INTRODUCTION

Hepatocellular carcinoma (HCC) is common in Thailand and has been found to be the third most common tumour in males.[1] Prognosis is poor with high incidence of vascular invasion.[2] Factors related to the prognosis of HCC include: stage, size, encapsulation, invasion and metastasis, number of tumours, histological grading and presence of cirrhosis.[3] However, patients with encapsulated HCC and fibrolamellar HCC are known to have a better prognosis.[4-6] In a recent study, the presence of myofibroblasts (MFBs) was observed in some HCC patients.[7] MFBs may have a role in limiting tumour expansion and preventing metastasis through their physical effect and anticollagenolytic properties.[8] Evidence exists of encapsulated HCCs having a better prognosis, and MFBs may have a role in limiting tumour expansion and metastasis. This study was therefore designed to define the detail expression of MFBs in the cirrhotic fibrous septa and tumour fibrous septa of HCCs. In addition, the correlation between the presence of MFBs, histological grading and rate of tumour growth are described.

MATERIALS AND METHODS

Patients

Thirteen consecutive cases of HCC were treated by hepatectomy. The median age of the patients was 50.7 years (range: 31 to 68 years), nine of whom were males and four were females. HBsAg was found in nine cases and cirrhosis in eight cases. The median size of the resected tumours was 7.6 cm (range: 2.5 to 18 cm). Eight tumours were in the right lobe and the other were in the left lobe.

Tumour histopathology

The resected liver specimens were immediately transferred to the Department of Pathology. The tissue blocks for study were sampled from edges of the tumour, solid tumour areas and also liver parenchyma, avoiding areas of necrosis and haemorrhage. The tissues were fixed in modified Millonig's phosphate buffer formalin and processed within 24 hours. For conventional section, the 3-micron paraffin sections were cut and stained with hematoxylin-eosin, Masson's trichrome, Gomori's methenamine silver and Periodic Acid Schiff stains. Classification and grading of HCCs were performed according to AFIP classification and Edmonson-Steiner's classification. The distribution of the tumours among the different histologic grades was: six cases of grade II and seven cases of grade III.

Immunohistochemistry

Staining for MFBs. To identify the MFBs, a commercial monoclonal antibody for specific actin was used: pan actin HHF-35 (DAKO, Dakopatts, Copenhagen, Denmark), alpha smooth muscle actin 1A4(DAKO) and sarcomeric actin (DAKO) at a dilution of 1:500. Bound monoclonal antibodies were located by the avidin-biotin peroxidase complex technique with 3-3'-diaminobenzidine as chromogen (Vectastain ABC Kit, Vector Laboratories, Burlingame, California). In negative control staining, non-immune mouse immunoglobulin was used instead of the specific antibody. The results of positive stains were spindle cells with dark brown granular cytoplasm. Positive cells in five representative high powerfields from ten sampling areas were counted. The amount of MFBs was scored as: low for negative cells or positive cells numbering less than five cells per high powerfield and high for positive

cells showing more than five cells per high powerfields.

Staining for PCNA. To detect of proliferative cell nuclear antigen (PCNA), a mouse monoclonal antibody to PCNA (PC10: NOVA Castra Lab) was used. The antigenic sites reacting with antibody were located by the avidin-biotin peroxidase complex procedure as described for specific actin above. The PC10 staining result was evaluated by counting the percentage of positive stained nuclei of 1,000 tumour cells, using a standard light microscope equipped with an index squared grid. In addition, positive cells in five representative high powerfields from 10 sampling areas were counted. The fraction of positive tumour cells was determined as the labelling index (LI %).

Tissue for ultrastructural examination

Tissue for ultrastructural examination were fixed in 2.5 per cent glutaraldehy in phosphate buffer, post-fixed in 2 per cent buffered osmium tetroxide and embedded in Epon-812 resin. One-micron thick sections were cut on a LKB ultramicrotome, stained with toluidine blue and used for orientation. Ultrathin sections were cut and mounted on copper grids, and double stained with uranyl acetate and lead citrate. Sections were viewed and photographed with a Jeol JEM-100 SX electron microscope at 80 kv.

Statistical analysis

The correlation between the amount of MFBs, the labelling index and the histological grading were evaluated by the Mann-Whitney U test and chi-square test. The test calculation were made with the Stat View software (Abacus Concepts, Inc., Berkeley, California.).

RESULTS

The staining reaction of the monoclonal antibody to specific actins, HHF-35, 1A4, was confined to the cytoplasm of MFBs in the areas of the tumour fibrous septa and fibrous septa of cirrhotic liver as shown in figures IA, IB,IC. The monoclonal antibody to sarcomeric actin showed a negative result in all studied tissues. In table 1, the amount of MFBs shown by positive HHF-35 and 1A4 are correlated between cirrhotic fibrous septa and tumour fibrous septa, showing a statistically significant difference ($p<0.05$).

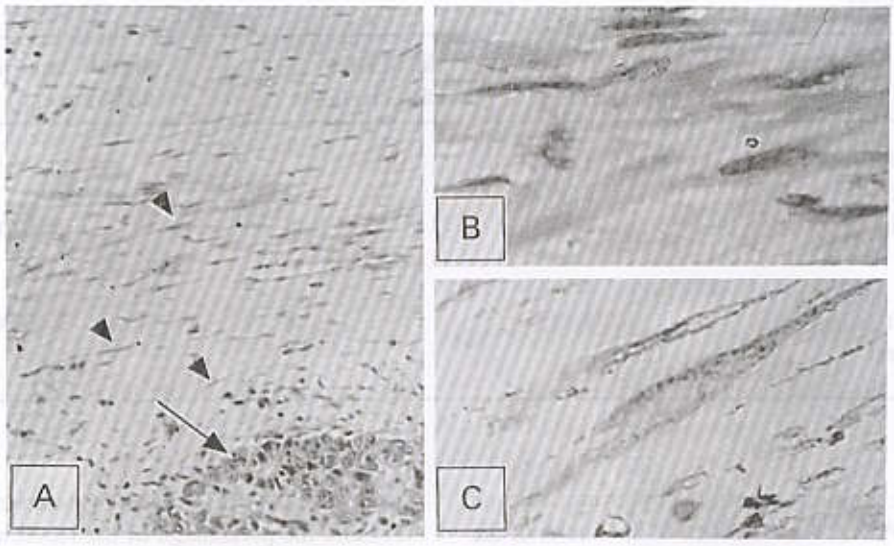


Figure I. Left (A). Presence of MFBs (arrow head) embedded in the tumour fibrous capsule of HCCs (arrow) (H&E stain x 200) .
Right upper (B). Histological characterisation of MFBs shows large, spindle-shaped, long cytoplasmic extensions with distinct acidophilic and fibrillar cytoplasm (H&Es stain x400).
Right lower (C). Immunoperoxidase staining for HHF-35 in the cytoplasm of MFBs shows dark brown granules (hematoxylin counterstain x 400).

Table 1. Correlation of the amount of MFBs between cirrhotic fibrous septa and tumour fibrous septa

	No. of cirrhotic fibrous septa	No. of tumour fibrous septa	Significance of difference, p
Amount of MFBs			
HHF-35 low	6	5	
HHF-35 high	0	8	p=0.0083,p<0.05
1A4 low	6	4	
1A4 high	0	9	p=0.0261,p<0.05
Chi-square test			

In table 2, the amount of MFBs in the areas of tumour fibrous septa were correlated between cases of HCC with cirrhosis and HCC without cirrhosis. Specific actin of 1A4 showed a statistically significant difference of $p<0.05$ while specific actin of HHF-35 showed no statistically significant difference.

Table 2. Amount of MFBs in the tumour fibrous septa: correlation between HCC with cirrhosis and HCC without cirrhosis.

	No. of HCC without cirrhosis	No. of HCC with cirrhosis	Significance of difference, p
Amount of MFBs			
HHF-35 low	3	2	
HHF-35 high	2	8	p=0.2070,p>0.05
1A4 low	4	0	
1A4 high	1	8	p=0.0024,p<0.05
Chi-square test			

The staining reaction of the monoclonal antibody to PCNA was confined to the nuclei of the tumour cells (figure II). The mean percentages of the labelling index and the SD were 55.15 and18.85 respectively; the median was 59 and the range was 27 to 81. Table 3 shows that in the correlation between the amount of MFBs shown by positive HHF-35 and 1A4 and the percentage of the labelling index there was no statistically significant difference. However, the correlation between the histological grading and percentage of the labelling index shows a significant difference ($p<0.05$).

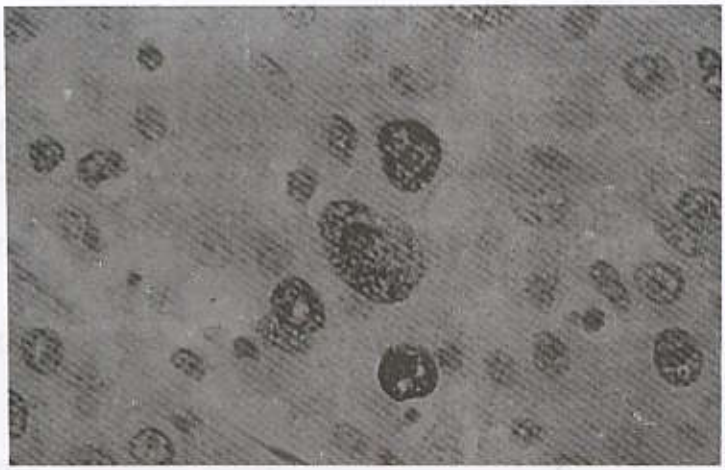


Figure II. Immunoperoxidase staining for PC10 (monoclonal antibody to proliferative cell nuclear antigen) in the nuclei of HCCs shows intranuclear dark brown grunules (methyl green counterstain x400).

Table 3. The labelling index LI%: correlation between amount of MFBs and histological grading of tumours in 13 HCC patients.

	No. of tumours	LI% (mean ± SD)	Significance of difference, p
Amount of MFBs			
HHF-35 low	5	62.8 ±1.9	p=0.31, p> 0.05
HHF-35 high	8	50.4 ±18.0	
1A4 low	4	65.5 ± 23.1	p=0.12, p>0.05
1A4 high	9	50.6 ± 15.9	
Histological grade			
grade II	6	41.5 ± 13.7	p=0.022, p<0.05
grade III	7	66.9 ± 14.4	

Mann Whitney U test

Using the labelling index cut-off point of 49 per cent, two subgroups (LI%<49 and LI%>49) were correlated with the amount of MFBs and histological grading. (Table 4) The results also showed a statistically significant difference in correlation between histological grading and subgroups ($p<0.05$), but was not significantly different in the correlation between the amount of MFBs and subgroups.

Table 4. Subgroup of the labelling index (LI%<49 and LI%>49), the amount of MFBs and the histological grading of tumour in 13 HCC patients.

	No. of tumours LI%<49	No. of tumours LI%>49	Significance of difference, p
Amount of MFBs			
HHF-35 low	1	4	p=0.14,p> 0.05
HHF-35 high	5	3	
1A4 low	1	3	p=0.31,p>0.05
1A4 high	5	4	
Histological grade			
grade II	5	1	p=0.013,p<0.05
grade III	1	6	
Chi-square test			

At electron microscopic level, MFBs were easily observed between tumour cells and in the perisinusoidal spaces. MFBs shared the morphologic features of fibroblasts, the presence of rough endoplasmic reticulum (RER) with occasionally dilated cisternae and the features of smooth muscle cells, and the presence of microfilaments. MFBs were either spindle or stellate-shaped cells. Packed longitudinally oriented microfilaments with dense bodies were aggregated at the periphery of the cells. Stromal MFBs were commonly found in the fibrous capsule of the tumour cells, sometimes showing the feature of tapering cell processes (figure IIIA, IIIB).

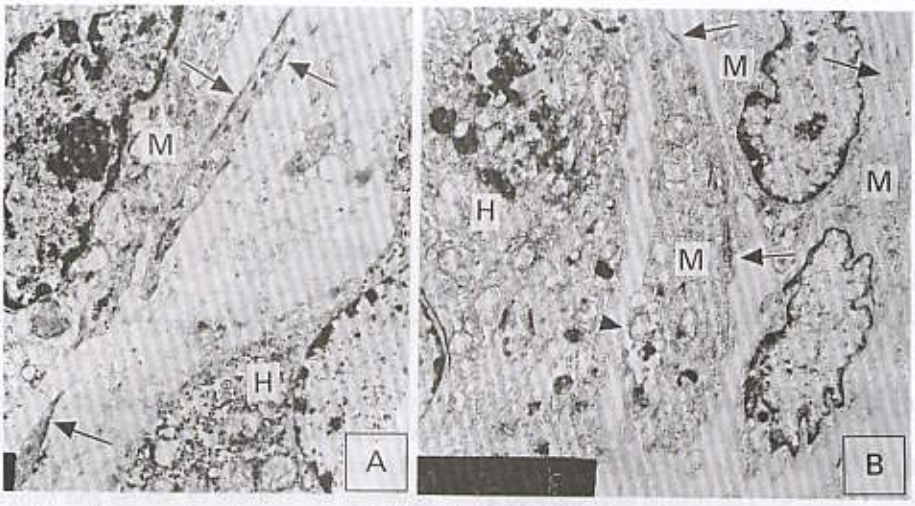


Figure III. Electron micrographs demonstrate MFBs. A. MFBs(M) show a long spindle-shaped cell with tapering processes between hepatic tumour cells (H). The longitudinally oriented microfilaments with associated dense bodies are concentrated in the periphery or the subplasmalemmal regions (arrows). B. MFBs share morphologic features of fibroblasts, the presence of rough endoplasmic reticulum (RER) with occasionallly dilated cisternae (arrow heads) and the features of smooth muscle cells, and the presence of microfilaments at the periphery of cells (arrows).

DISCUSSION

MFBs are a cell type with ultrastructural and biological features of fibroblasts and the contractile properties of smooth muscle cells.[9] MFBs have been observed for distribution in normal, reactive and neoplastic human tissue. By immunocytochemical criteria, Gabbiani's group supported the concept that MFBs were derived from fibroblasts rather than smooth muscle cells,[9] In contrast, Tsukada's group demonstrated the smooth muscle-like nature of MFBs but did not directly address the issue of their derivation.[10] In our study, the distribution of monoclonal antibody to smooth muscle actin(1A4) was prominent in MFBs, which may indicate evidence of origin in smooth muscle.[11] However, the electron micrographs also revealed structures compatible with both smooth muscle and fibroblast in the component of MFBs.[7]

MFBs have been found in three pathologic conditions: (a) the conditions related to the inflammation, wound healing and tissue remodelling such as liver cirrhosis; (b) fibromatoses and (c) the stromal reaction to neoplasia or the desmoplastic reaction.[12,13]

Studies of MFBs in cirrhotic liver show the presence of those cells in the fibrotic septa of active cirrhosis. The mechanism is the response to injury and the healing process or tissue remodelling.[14-16] In active cirrhosis, MFBs were numerous and produce mostly collagen type III (which is found only in the early phase of wound healing); when the granulation tissue reaction was resorbed, MFBs disappeared.[17]

In HCCs, the influence of MFBs on tumour growth and invasion are topics of interest. The desmoplastic reaction inducing the fibrous capsule and intratumoral fibrous septa are the mechanism for preventing tumour growth and invasion and for localising the tumour. Encapsulated HCCs and fibrolamellar type HCCs are good examples and are known to have good prognosis.[4-6] MFBs found in the fibrous capsule and fibrous stroma generate the forces responsible for tumour contraction throught the actin bundles and they also synthesize collagen type III for strengthening the stroma.[17-19] Our study yielded a good correlation with a statistically significant difference in the amount of MFBs in the cirrhotic fibrous septa and tumour fibrous septa (table 1); this difference suggested that in the cirrhotic fibrous septa, MFBs were scanty as a result of the late stage of healing, while in the tumour fibrous septa, MFBs were in early stage and acted as a preventive mechanism in localising and contracting the tumour. Table 2 also showed a statistically significant difference in the amount of MFBs in the tumour fibrous septa shown by 1A4 in the correlation between HCCs without cirrhosis and HCC with cirrhosis. In HCC with cirrhosis, the amount of MFBs found in the tumour fibrous septa are higher than the number of MFBs found in HCCs without cirrhosis. However, the result did not provided a clear conclusion that stromal response to tumours in cases with cirrhosis is higher than in cases without cirrhosis.

Vascular invasion is a biologic hallmark of hepatocellular carcinoma and the major cause of cancer- related morbidity and mortality. In penetrating the blood vessel walls, tumour cells must first adhere to the matrix component. Degradation is caused by the tumor cells either secreting the proteolytic enzyme by themselves or by inducing host cells to elaborate the proteases.[20] Type IV collagenase is suggested as the agent used to break down the type IV collagen of the epithelial and vascular basement membranes, a view that is supported by the studies of Liotta and colleagues.[21] The role of MFBs in preventing vascular invasion was proposed by Seemayer who suggested that the myofibroblastic stromal response acted as a defence against neoplasia by impeding the motility and accessibility of tumour cells to blood vessels.[12] In addition, the study by Barsky suggested that MFBs have a role in preventing metastasis by their anticollagenolytic properties.[8]

Using the growth rate of HCCs correlated between the level of histological grading and growth fraction to calculate the labelling index has been mentioned in many studies.[22,23] Our previous study of unpublished data, an analysis of 51 cases of HCC using monoclonal antibody to anti PCNA, revealed that grade II and clear cell types of HCC had a slower rate of proliferation than grade III and grade IV HCCs.[24] Briefly, grade II and clear cell types of HCC had a labelling index below 49 per cent, while in grade III and grade IV HCC the percentage of the labelling index was above 49 per cent. Thus, the cut-off point of the labelling index was proposed as 49 per cent. In this study, the correlation between histo-

logical grading and the labelling index showed the same result as the previous study [24] with a statistically significant difference ($p<0.05$) in grade II and grade III HCCs (tables 3 and 4). However, the results of the correlation between the amount of MFBs in the tumour fibrous septa and the percentage of the labelling index showed no statistically significant difference (tables 3 and 4). Those findings may suggest that the number of MFBs found in the tumour fibrous septa did not relate to the rate of growth of the tumour or histological grading, but was in fact the response of stromal reaction to neoplasm. This mechanism refers to the repairing process or tissue remodelling and thus needs to be explored.

REFERENCES

1. Tepmongkol P. Tumour registry. Siriraj Cancer Center. Statistical Report 1996. Faculty of Medicine Siriraj Hospital, Mahidol University.
2. Fujimoto J, Okamoto E, Yamanaka N, et al. Flow cytometric analysis of hepatocellular carcinoma. Cancer 1991; **68:** 2566-70.
3. Craig JR, Peter RL, Edmonson HA. Tumors of the liver and intrahepatic bile ducts. Atlas of Tumor Pathology. Second Series, Fascicle 26, Armed Forces Institute of Pathology, Washington, DC, 1988.
4. Lai CL, Wu PC, Lam KC, Todd D. Histologic prognostic indicators in hepatocellular carcinoma. Cancer 1979; **44**: 1677-83.
5. Craig JR, Peter R, Edmonson HA, Omata M. Fibrolamellar carcinoma of the liver: a tumour of adolescents and young adults with distinctive clinicopathologic features. Cancer 1980; **46:** 372-79.
6. Hsu HC, Wu TT, Wu MZ. Tumor invasiveness and prognosis in resected hepatocellular carcinoma; clinical and pathogenetic implications. Cancer 1988; **61:** 2095-99.
7. Chau KY, Lily MA, Wu PC, Yau WI. Myofibroblasts in hepatitis B related cirrhosis and hepatocellular carcinoma. J Clin Pathol 1992; **45:** 446-48.
8. Barsky SH, Nelson LL, Levy VA. Tumour desmoplasia inhibits angiogenesis. Lancet 1987; **ii:** 1333-37.
9. Gabbiani G, Ryan GB, Majno G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. Experientia 1971; **27:** 549-50.
10. Tsukada T, Tippens D, Gordon D, Ross R, Gown AM. HHF-35, a muscle actin specific monoclonal antibody. I. Immunocytochemical and biochemical characterization. Am J Pathol 1987; **126:** 51-60.
11. Darby I, Skalli O, Gabbiani G. Alpha-smooth muscle actin is temporarily expressed by myofibroblasts during experimental wound healing. Lab Invest. 1990; **63:** 42-6.
12. Seemayer TA, Lagace R, Schurch W, Thelmo WL. The myofibroblast: biologic, pathologic, and the theoretical considerations. Pathology Annual 1980; **15:** 443-70.
13. Skalli O, Gabbiani G. The biology of the myofibroblast. Relationship to wound contraction and fibrocontrative diseases. In: Clark RAF, Henson PM,eds. The molecular and cellular biology of wound repair. Plenum Publishing Corporation, New York, 1988: 373-402.
14. Bhatal PS. Presence of modified fibroblasts in cirrhotic livers in man. Pathology 1972; **4:** 139-44.
15. Irle C, Kocher O, Gabbiani G. Contractility of myofibroblasts during experimental liver cirrhosis. J Submicrosc Cytol 1980; **12:** 209-17.
16. Rudolph R, McLure WJ, Woodward M. Contractile fibroblasts in chronic alcoholic liver cirrhosis. Gastroenterology 1979; **76:** 704-9.
17. Rudolph R, Guber S, Suzuki M, Woodward M. The life cycle of myofibroblast. Surg Gynecol Obstet 1977; **145:** 389-94.
18. Gabbiani G, Lelous M, Bailey AJ, Bazin S, Delareney A. Collagen and myofibroblasts of granulation tissue. Virchows Arch(Cell Pathol) 1976; **21:** 133-45.
19. Barskey SH, Rao CN, Grotendorst GR, Liotta LA. Increased content of type V collagen in desmoplasia of human breast carcinoma. Am J Pathol 1982; **108:** 276-83.
20. Pauli BV, Knudson W. Tumor invasion : a consequence of destructive and compositional matrix alteration. Hum Pathol 1988; **19:** 628-9.
21. Liotta LA, Barskey SH, Rao CN. Biochemical mechanisms of tumor invasion and metastases Prog. Clin. Biol. Res. 1988; **256:** 3-14.
22. Silvestrini R, Costa A, Gennari L, Doci R, Bombardier E, Bombelli L. Cell kinetics of hepatic metastases as a prognostic marker in patients with advanced colorectal carcinoma. HPB Surg 1990; **2:** 135-44.
23. Tarao K, Shimizu A, Harada M, et al. Invitro uptake of bromodeo-xyuridine by human hepatocellular carcinoma and its relation to histopathologic findings and biologic behavior. Cancer 1991; **68:** 1789-94.
24. Atisook K, Dechsukhum C, Staphanachai C, et al. Hepatocellular carcinoma: a correlation of histologic grading with immunohistochemical identification of proliferative hepatocytes with PC-10. (unpublished datum).