以雷射捕捉顯微分離法研究肝星狀細胞基因之表現
Study of gene expression of hepatic stellate cell using laser capture microdissection

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一、中文摘要

肝硬化的成因是由於膠質及其它細胞間質異常沉積，造成正常組織結構破壞，引起肝功能失償。這些間質蛋白是由肝星狀細胞所製造。間質異常沉積可以因製造增加或是代謝減少所引起。間質金屬蛋白酵素(MMP)可分解細胞間質所有成份，是間質代謝的主要酵素。間質金屬蛋白酵素可經由間質蛋白酵組織抑制因子(TIMP)加以調節。肝星狀細胞已知可製造 MMP-1、MMP-2、MMP-3、TIMP-1 及 TIMP-2。根據以往的研究，在肝硬化時可觀查到 MMP-2、TIMP-1 及 TIMP-2 的增加。但是我們的研究卻顯示 MMP-1 增加, 而無 MMP-2、TIMP-1 及 TIMP-2 的變化。肝星狀細胞基因的表現受到周圍細胞及間質的影響, 因此研究膠質, MMPs 及 TIMPs 的表現應直接從肝組織中擷取肝星狀細胞加以分析，而非從組織中分離細胞或培養後再分析。

本計畫利用雷射捕捉顯微分離法來擷取硬化肝及非硬化中之肝星狀細胞, 淬取其 RNA，反轉錄後作競爭性 PCR，以定量 MMP 及 TIMP 的表現。我們試用了冷凍組織及福馬林固定之組織切片做雷射捕捉顯微分離法(LCM)。LCM 每次約捕捉 5 個細胞。以 actin 為引子所作之 RTPCR 顯示只有在冷凍組織切片才能粹取出 mRNA。大約要 100 個細胞的 mRNA 才作的出 actin 之 RTPCR。我們用抗平滑肌 actin 及抗 desmin 抗體來分辨肝星狀細胞。只有在福馬林固定之切片染得出星狀細胞。由於其細長的形狀及緊靠在肝細胞旁，使得單獨分離不可能。因此以雷射捕捉顯微分離法來分析肝星狀細胞基因的表現並不可行。。

關鍵詞：肝硬化、星狀細胞、間質金屬蛋白分解酵素

Abstract

Liver cirrhosis is characterized by pathological deposit of collagen and other excellular matrix proteins resulting in disruption of normal liver architecture and a compromise in liver function. These matrix proteins are synthesized by activated hepatic stellate cells (HSC). The accumulation of matrix proteins may reflect increased matrix synthesis and/or decreased matrix degradation. Matrix metalloproteinases (MMP) are able to degrade all of the components of extracellular matrix and are the major enzymes in matix turnvoer. Specific inhibition of MMPs occurs by interaction with the tissue inhibitors of matrix metalloproteinases (TIMP). The activated hepatic stellate cells (HSC) is known to secrete procollagen, three MMPs and two TIMPs: interstitial collagenase (MMP-1), gelatinase A (MMP-2) and stromelysin (MMP-3), TIMP-1 and TIMP-2. In this project we tried to isolate HSC using laser capture microdissection

(LCM) to study the expression of MMPs and TIMPs. We tried both frozen liver tissue and formalin-fixed tissue. PixCell Laser Capture Microdissection System captured about 5 cells in each laser excitation. RTPCR with actin primer only succeeded in frozen tissue. The detection limit is about 100 cells. We used anti-smooth muscle actin and anti-desmin antibody to indentify HSC. HSC can only be stained in formalin-fixed tissue. The filamentous-shaped HSC made the capture of pure cell impossible. We concluded that at present time using LCM to isolate HSC for study expression of MMP and TIMP is not feasible.

Keywords: Liver cirrhosis, Stellate cell, Matrix metaloproteinase

二、緣由與目的

Cirrhosis of liver is characterized by pathological deposit of collagen and other excellular matrix proteins resulting in disruption of normal liver architecture and a compromise in liver function. These matrix proteins are synthesized by activated hepatic stellate cells (HSC). The accumulation of matrix proteins may reflect increased matrix synthesis and/or decreased matrix degradation.

matrix metalloproteinases (MMP) belong to a family of zinc- and calcium-dependent endopeptidases. They are able to degrade all of the components of extracellular matrix and are considered to be major candidates in matix turnvoer. Interstitial collagenase (MMP-1) can cleave collagen I, II and III and is secreted as a latent proenzyme which can be activated in vivo by plasmin and stromyelin (MMP-3). Gelatinase A (MMP-2) and gelatinase B (MMP-9) can cleave collagen IV, V, VII, X and XI, fibronectin, elastin and proteoglycan. MMP-2 and is secreted as latent proenzymes, proMMP-2 and, and is activated by membrane type MMP (MT-MMP) and.

MMPs can be inhibited by general protease scavenger, such as a2-macroglobulin. Specific inhibition of MMPs occurs by interaction with the tissue inhibitors of matrix metalloproteinases (TIMP).

To date, Four TIMPs have been described: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. TIMP-1 and TIMP-2 have been studied in detail, while TIMP-3 and TIMP-4 are less well charaterized. TIMP-1 and TIMP-2 bind to active MMPs in a non-covalent but irreversible manner. Binding is stoichiometeric and blocks the active sites of MMPs. Both TIMP-1 and TIMP-2 also bind to specific pro-gelatinase species preventing their activation by MMP-3. In addition TIMP-1 prevents formation of gelatinase B homodimer and gelatinase-MMP1 heterodimer. Thus TIMPs play an importnt role in regulation of activity of MMPs. HSC

are known to produce procollagen, MMP-1, MMP-2, MMP-3, TIMP-1 and TIMP-2. MMP-1 activity has been studied in various liver diseases. The results of these studies are inconsistent and must be interpreted with caution because of assay conditions probably incorrectly ascribe gelatinase activity to collagenase activity. In general there is tendency of decreasing collagenase activity during progressive fibrosis. This pattern has been demonstrated in human and primate alcoholic liver injury and CCl4-induced rat liver injury(). In one study of human liver cirrhosis, a reduction of about 60% of MMP-1 was noted by western blot analysis () . Increased expression of MMP-2 was observed in experimental liver fibrosis as well as in cirrhotic human liver. In rat model of liver injury induced by CCl4 or bile duct ligation, TIMP-1 mRNA increased within 6 h, and remained elevated throughout the course of study, while rat interstitial collagenase mRNA showed no parallel change. In another CCl4 model, after 4 weeks of injury the fibrotic liver is allowed to recover. Both TIMP-1, TIMP-2 and procollagen I mRNA levels return to control value with collagenase mRNA expression remained at levels comparable to peak fibrosis, whereas collagenase activity in liver homogenates increased through recovery. TIMP-1 and TIMP-2 mRNA was found to be increased in fibortic human liver with no significant change in MMP-1 expression (). TIMP-1 protein expression was shown to be increased in liver cirrhosis by ELISA (). These observations imply that TIMPs plays an important role in liver fibrosis. HSC thus play complex roles in accumulation of matrix protein, degradation of matrix protein and protection of matrix degradation. The present data about gene expression of HSC were from purified HSC or cultured HSC. They are criticized by contamination of other cell types. Furthermore, expression of HSC is affected by surrounding cells and matrix. Task of analyzing gene expression patterns of procollagen, MMPs and TIMPs is better performed by extraction of specific HSCs from their complex tissue milieu. In situ hybridization or histochemical studies are qualitative and cannot fulfill this aim. Recently a new technique called laser capture microdissection is developed, with which a transfer surface is placed onto the tissue section and then focally bonded to the targeted cell by laser excitation, allowing targeted cell to be selectively removed for

molecular analysis.
The technique is able to extract single cell from its surrounding tissue, and is an ideal tool for studying HSC gene expression. In this project laser capture microdissection method and competitive PCR
were attempted to study expression of MMP and TIMP of HSC in situ.

三、結果與討論

We tried to perform laser capture microdissection on both frozen and formaline-fixed liver tissues. There was limitation of PixCell Laser Capture Microdissection System (Arcturus Engineering, Inc. CA, USA)
in isolating HSCs from liver tissue. About 5 cells were captured by one laser excitation.
On frozen tissue section, hepatocytes were captured and messenger RNA was extracted and RTPCR was done with actin primers. The lower limit of detection was about 100 cells. For formaline-fixed tissue, the extraction of messenger RNA was rather difficult and RTPCR failed to detect actin. To identify activated HSC, immunohistochemical stain was done with anti-smooth muscle actin and anti-desmin antibody. Activated HSCs failed to show up on frozen section, but did showed up in formaline-fixed tissue section. On tissue with minimal change or mild chronic hepatitis, the numbers of activated HSC was very low, about 5 to 10 cells per section. In liver cirrhosis, the activated HSCs distributed along fibrous septa and were abundant in numbers. The activated HSCs were elongated and thin, and abutted very closely with hepatocytes. This made it impossible to capture HSCs separately from hepatocytes. In conclusion, we met two difficulties to analyze HSC by LCM: 1. Current LCM equipment is not accurate enough to isolate single cell or cell with thin elongated shape. 2. Activated HSC can only be identified in formaline-fixed tissue, which made extraction of messanger RNA for RTPCR very difficult. We found that primer sets for MMP-2 (5-GTGCTGAAGGACACACTAAAGAAGA-3, 5-TTGCCATCCTTCTCAAAGTTGTAGG-3) and MMP-3 (5-GAACAATGGACAAAGGATACAACA-3, 5-TTCTTCAAAAACAGCATCAATCTT-3) are suitable for RTPCR and competitive PCR.

四、計畫成果自評

Although we did not succeed in isolation of HSC, we did learn a lot of technical tips in doing LCM and its limitations that are helpful for implementation of LCM for further studies. We also had found out suitable primer sets for RTPCR for MMP-2 and MMP-3 that also be useful for

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