行政院國家科學委員會專題研究計畫　成果報告

肝纖維化對肝浸潤 T 細胞活化的影響

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（一）研究計劃中文摘要：

關鍵詞：肝細胞癌，免疫治療，肝浸潤 T 細胞，CD25，纖維化

過去的研究指出，肝癌組織的腫瘤浸潤淋巴細胞，與肝癌的預後可能有關。既然腫瘤浸潤淋巴細胞可以跑到肝癌組織，可是肝癌卻仍可以不斷生長，此表示腫瘤浸潤淋巴細胞型態上或功能上的缺陷，因此我們去年研究肝癌病人腫瘤浸潤細胞其表現型及分子型態。我們發現大約 70%-90%的腫瘤及非癌肝組織浸潤淋巴細胞，有 CD45RA- / CD45RO+ / CD62L-記憶 T 細胞表現型。大約 60%-70%的腫瘤及非癌肝組織浸潤淋巴細胞，會表現 CD69 與 HLA-DR T 細胞活化標記。可是另一個 T 細胞活化標記 CD25，不論是在腫瘤或非癌肝組織浸潤淋巴細胞的表現都是被調降的。我們的結果意味著，「逃避免疫系統」的現象，並不只是發生在肝癌組織，可能在慢性肝病肝組織中就已經產生了。

CD25 的調降表現可能與腫瘤會分泌 matrix metalloproteinase 有關，然而在非癌肝組織浸潤淋巴細胞的 CD25 調降的原因仍不是十分清楚。由於 65%-80%的肝癌病人的非腫瘤肝組織，若不是肝硬化就是纖維化，再加上 matrix metalloproteinase 的活化與表現，與肝硬化及纖維化很有關係。因此，我們推論在非腫瘤肝組織的肝組織浸潤淋巴細胞，有可能也會經由 matrix metalloproteinase 或其它物質的活化與表現導致調降 CD25 的表現。

在本研究中，我們成功的建立小鼠的肝纖維化模式，然後再將小鼠的肝癌細胞移植到鼠的肝臟。在固定的時間下，從不同程度的纖維化的肝，以及從有或沒有肝腫瘤的肝，分離出肝組織浸潤淋巴細胞。然後再以流式細胞分析儀來分析其表現型及活化情況，並與纖維化的程度及 matrix metalloproteinase 的表現量做關聯。我們發現，MMP2 的表現與活化，在纖維化的肝是顯著比正常的肝來得高。可是，肝組織浸潤淋巴細胞 CD25 的表現量，在纖維化的肝與正常的肝之間並沒有顯著的差別。所以我們並無法證實，纖維化的肝會經由 matrix metalloproteinase 或其它物質的活化與表現導致調降 CD25 的表現。

（二）研究計劃英文摘要：

Keywords：Hepatocellular carcinoma, tumor-infiltrating lymphocyte, liver-infiltrating lymphocyte, phenotypes, CD25, fibrosis

In our previous study, we investigated the phenotypic analysis of tumor-infiltrating lymphocytes (TILs) in HCC tissues and the corresponding non-tumor liver tissues infiltrating lymphocytes (NILs). We found that Most of infiltrating lymphocytes from the HCC tissues and the corresponding non-tumor liver tissues were activated and expressed antigen-experienced phenotypes. Around 70%-90% NILs or TILs expressed the antigen-experienced or memory phenotypes of CD45RA- / CD45RO+ / CD62L-. Around 60%-70% CD4+ or CD8+ NILs and TILs expressed the activation markers CD69 and HLA-DR. However, we found that CD25 is under-expressed in both the NILs and TILs. Only 1.0% of the CD8+ NILs and 7.7% of CD8+ TILs expressed CD25 and 11.2% of CD4+ NILs and 28.2% CD4+ TILs expressed CD25. The downregulation of CD25 in NILs might suggest “immune escape” happens not only in the tumors, but also in the diseased liver.

It has been shown that CD25 downregulation in TILs is probably caused by matrix metalloproteinases (MMPs). Around 60%-85% of HCC patients have underlying liver cirrhosis. In the 15%-40% HCC patients with non-cirrhotic liver, various degrees of fibrosis can also be detected in the non-tumor liver portions. Accumuating data demonstrated that the MMPs play important

roles in the hepatic fibrogenesis. Thereore, if the MMPs secreted by the tumor cells can degrade CD25 expressed on the TILs, we would wonder whether the MMPs activated in the hepatic fibrosis can also degrade CD25 expressed on the liver-infiltrating lymphocytes of fibrotic livers.

Thus, our hypothesis generated from our previous study is: the liver-infiltrating lymphocytes are chronically activated. The liver-infiltrating lymphocytes will express the actiavation markers of HLA-DR, CD25, and CD69. In the process of hepatic fibrosis, the CD25 is downregulated because of something activated, such as MMPs, can downregulate the CD25. The existence of hepatic tumors will have further influences on the expressions of CD25.

In this current project, we used the murine model of hepatic fibrosis. We successfully grew the HCC tumors in the fibrotic livers in the murine models. We found that the MMP-2 expressions were upregulated and activated in the fibrotic livers. However, there were no significant differences in the CD25 expressions on the liver-infiltrating lymphocytes between the fibrotic livers and control livers. Thus, it was possible that the low expression of CD25 on the liver-infiltrating lymphocytes was not directly related the MMPs in the fibrotic livers.

（三）成果內容:

Introduction

In our previous study, we investigated the phenotypic analysis of TILs in HCC tissues and the corresponding non-tumor liver tissues infiltrating lymphocytes (NILs). We found that around 70%-90% NILs or TILs expressed the antigen-experienced or memory phenotypes of CD45RA- / CD45RO+ / CD62L-. Around 60%-70% CD4+ or CD8+ NILs and TILs expressed the activation markers CD69 and HLA-DR. However, we found that CD25 is under-expressed in both the NILs and TILs. Only 1.0% of the CD8+ NILs and 7.7% of CD8+ TILs expressed CD25 and 11.2% of CD4+ NILs and 28.2% CD4+ TILs expressed CD25. Most of infiltrating lymphocytes from the HCC tissues and the corresponding non-tumor liver tissues were activated and expressed antigen-experienced phenotypes. The downregulation of CD25 in NILs might suggest “immune escape” happens not only in the tumors, but also in the diseased liver.

The exact mechanisms leading to CD25 downregulation on activated TILs are unknown. However, one of the causes for CD25 downregulation in TILs is that tumor-derived matrix metalloproteinases (MMPs) induce the proteolytic cleavage of IL-2Rα on activated T cells (1). However, the interaction between MMPs and immune system is very complicated and not quite well understood (2). This observation that CD25 downregulation is probably caused by MMPs is quite interesting. One of the most important finding in our previous study was that the CD25 is also downregulated in the NILs in addition to the TILs. And the downregulation of CD25 is even more evident in the NILs than in the TILs. There is no straightforward explanation for this phenomenon based on our previous study.

Accumuating data demonstrated that the MMPs play important roles in the hepatic fibrogenesis (3-6). Thereore, if the MMPs secreted by the tumor cells can degrade CD25 expressed on the TILs, we would wonder whether the MMPs activated in the hepatic fibrosis can also degrade CD25 expressed on the liver-infiltrating lymphocytes of fibrotic livers.

Thus, our hypothesis generated from our previous study is: the liver-infiltrating lymphocytes

are chronically activated. The liver-infiltrating lymphocytes will express the actiavation markers of HLA-DR, CD25, and CD69. In the process of hepatic fibrosis, the CD25 is downregulated because of something activated, such as MMPs, can downregulate the CD25. The existence of hepatic tumors will have further influences on the expressions of CD25.

Materials and Methods

We used the mice models of liver fibrosis to investigate the phenotypic characterization of liver-infiltrating lymphocytes in the fibrotic liver and the correlation of CD25 expression with the expression level of intrahepatic MMPs.

The BALB/c mice were purchased from NTUH and used at 6 weeks of age. Procedures were performed according to the NTUH guideline and recommendations for the proper care and use of laboratory animals. . One set of mice will receive intraperitoneal injection of 1 μL/g body weight of CCl4 in a 1:5 ratio with corn oil twice weekly for a total of 8 weeks. The other set of mice will receive corn oil injection only. Five mice in each set were sacrificed every two weeks.

Normal and cirrhotic mice were anesthetized by i.p. administration of 2.5% Avertin (0.012 ml/g body weight) for laparotomy. A small midline laparotomy incision was made and the left lobe of the liver was exteriorized. HCC cells (BNC) ($5x10^5$) in 10 μl Hanks buffered salt solution were injected with a 30-gauge needle directly into the liver parenchyma.

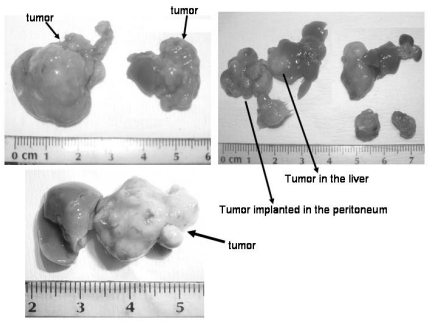
The mice were sacrificed the pre-determined time. The liver-infiltrating lymphocytes were isolated and subjected to three-color flow cytometry using anti-CD4, anti-CD8 and anti-CD25 antibodies. The proteins and mRNA were extracted from the liver tissues. The extracted proteins were subjected to gelatin zymography will be done as described (4, 5). The mRNA were used to MMP-2 RT-PCR.

Results

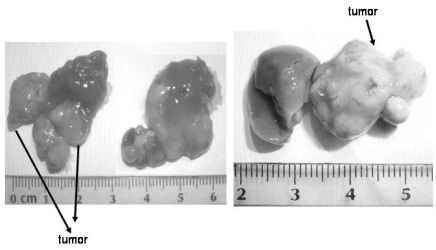
HCC in the fibrotic livers

As listed in the figure 1, the tumor sizes in the CCL4-treated mice were similar to those in the controlled mice. However, in the CCL4-treated mice, some mice died within one day after tumor challenge or even before tumor challenge. However, because only 1-3 mice were still alive during the time of sacrifice, this observation should be confirmed in the future. The reasons are not exact known. However, autopsy on these mice revealed massive ascites. Thus, these mice might died of CCL4-related hepatoxicity.

Figure 1. Hepatic tumors in the CCL4 and control groups



(A). Tumor in the CCL4 group

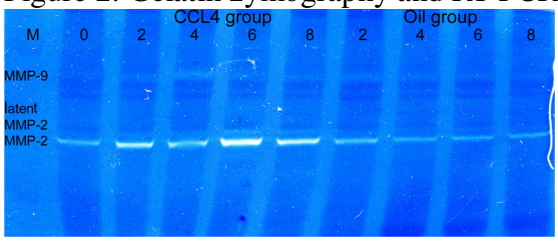


(B). Tumor in the control group

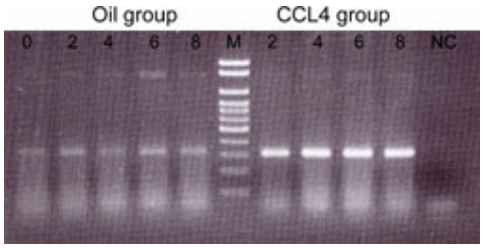
CD25 expression and MMP2

As shown in Figure 2A, the MMP2 were activated in the CCL4-treated livers, esp in week 6 and week 8. The MMP-2 expressions were upregulated in the CCL4-treated livers.

Figure 2. Gelatin zymography and RT-PCR of MMP-2 in CCL4-treated and control mice



(A) Gelatin zymography



(B) RT-PCR of MMP-2

CD25 expression

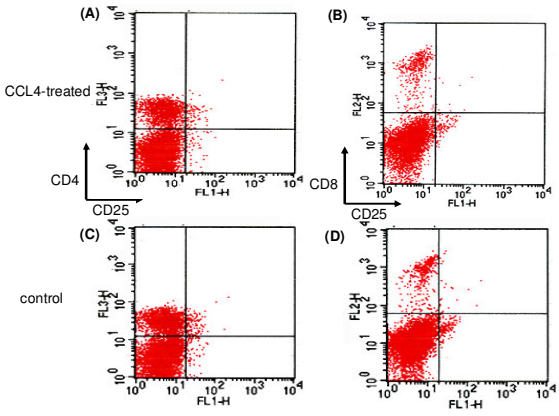


Figure 3. As shown in the figure 3, the CD25 expressions were low in the liver-infiltrating lymphocytes of both the control and CCL4-treated mice. There were significantly difference in the CD25 expressions on either CD4+ or CD8+ T cells. Thus, the CD25 expression was probably not correlated the MMP-2 expression.

Discussion

Our study demonstrated that it was feasible to grow hepatic tumors in the fibrotic livers. However, the unexpected is the high death rate of CCL4-treated mice in our first two sets of EXPs. The reasons are not exact known. Initially, we thought that this might be caused by some toxins contaminated in the tumor cell lines. However, the situation was not improved after we used another vial of stock cell lines. We also checked the cell lines and did not find any bacterial contamination. Another reason is that the mice with fibrotic livers induced by CCL4 were more vulnerable to avertin. To rule out this possibility, we ordered another set of mice and used the same protocol of CCl4 induction. We gave avertin without tumor challenge. The mice did not expire. Thus, the early mortality of mice after tumor challenge is probably due to tumor challenge per se. The cause of

death was not due to internal bleeding because we autopsy these mice and did not find large amount of bloody ascites. Another factor to be resolved is the tumor volume challenged. In the past 4 round of experiments, the tumor cells were resuspended in 30-40 ul volume. This volume might be too large for the mice, especially in the CCl4 treated group. Thus, we ordered another set of mice and repeat the experiment with the same tumor dose but less volume (20 ul). The immediate mice death decrease. Thus, tumor volume might be one of the factors. We are currently repeating another new EXPs.

Because of few mice were available in the tumor challenge groups, the flow cytometry and gelatin zymography were available only in the mice without tumor challenge.

Our results showed that the MMP-2 were activated and the expressions of MMP-2 were upregulated in the CCL4-treated livers. These findings were consistent to the previous ones. However, the CD25 expressions were low in the liver-infiltrating lymphocytes of both the control and CCL4 treated livers. Therefore, we failed to demonstrated that the CD25 expressions on the liver-infiltrating lymphocytes were directly related to the expressions of MMP-2.

(四) 參考文獻

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(五) 計劃成果自評

We demonstrated that the HCC tumor can grew in the fibrotic livers in the murine models. The significance of this model is that it is more similar to the clinical situations. However, we also encountered an unexpected difficulty: the high death in the initial two set experiments during tumor challenge. We resolved this problem by reducing the tumor volumes injected to the murine livers. In addition, we failed to demonstrate that the expressions of CD25 on the liver-infiltrating lymphocytes of fibrotic livers were correlated to the expressions of MMPs. Further studies were needed to investigate this problem.