RESEARCH ARTICLE

Kupffer Cells Suppress Hepatocarcinogenesis and Metastasis in Tumor Orthotopic Implanted Kunming Mice

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Abstract

In this research, we used $GdCl_3$ (gadolinium chloride) to restrain the function of Kupffer cells and assessed effects on hepatocarcinogenesis and metastasis in the Kunming mouse. A 0.25% $GdCl_3$ solution (10 mg/kg b.w.) was infused via the vena caudalis of each mouse 1 week before inoculation of H22 cells and was continued once per three days. Then we observed the follow indexes 3 weeks after injection of H22 cells: tumor weight, histologic characteristics of tumor tissue by light microscopy, ultramicrostructure of Kupffer cells under the electron microscope, distribution and number of Kupffer cells by histochemical staining, and TNF-α and IFN-γ levels in blood-serum and liver tissue by ELISA and RT-PCR. MMP-2 protein expression was tested by immunohistochemistry. The $GdCl_3$ pretreatment had no effect on the quantity of Kupffer cells, but clearly restrained their functions, with decrease of TNF-α and IFN-γ levels and elevation of MMP2. Tumor immunity functions were markedly suppressed and tumor growth was accelerated with appearance of metastasis. Furthermore, survival time of trial mice was shortened.

Keywords: Kupffer cells - hepatocellular carcinoma - gadolinium chloride - suppressive effects

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Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide, with the incidence on the rise globally (Jemal et al., 2011; Siegel et al., 2012). With the advancement of the modern medicine, for the late few years, some new methods were employed to HCC, such as biologic immunotherapy, liver transplantation, radiofrequency ablation, high focused ultrasound and etc. Some effects were gained; however, the 5-year survival rate was still less than 11 percent in these three decades (1975~2004), and improvement was not obvious (Ahmedin et al., 2009). Recently, biologic immunotherapy became the studying focus for its peculiar preponderance. And the mononuclear phagacytic system (MPS) was important ingredient of immune defence of organism. Activated macrophage through phagocytosis (Greten et al., 2006), CD 16 (FcγR III) and antibody dependent cell mediated cytotoxicity (ADCC) (Clynes et al., 2000), excreting TNF-α (Tumor Necrosis Factor-α) and IL-10 (interleukin-10) etc. cytokines (Li et al., 2002; Sturm et al., 2003), some enzymes and reactive oxygen species to dissolve and kill tumor cell or inhibit tumor growth (Smedsrod et al., 1994). The Carl von Kupffer described the macrophages in the liver first in 1886, and named them Kupffer cells (KCs). KCs were the dominated V-type macrophages, account for about 80% of the total number of macrophages in human body. In addition to the common function of phagocyte, other researchers recently reported that KCs undertook some unique functions, such as playing an important role in inhibiting growth and metastasis of liver tumor as a kind of natural killer cell. However, its specific role in hepatocarcinogenesis was still unclear. In addition, recent researches indicated that KCs played a promoting role in drug-induced hepatocarcinogenesis of animal model (Teufelhofer et al., 2005). In this paper, we used $GdCl_3$ to block the phagocytosis function of KCs, and observed the liver tumor growth, metastasis, immune response level and survival of tumor-bearing mice.

Materials and Methods

Material

Four weeks old male Kunming mice were bought from the Animal Center of Chongqing Medical University. The murine hepatocarcinoma cell line H22 was got from the Ultrasonic Imaging Research Institute of Chongqing Medical University. The sandwich method TNF-α ELISA kit, IFN-γ ELISA kit and goat anti-mouse CD68 monoclonal antibody were bought from Boshide Corporation, Wuhan, China. Goat anti-mouse MMP-2

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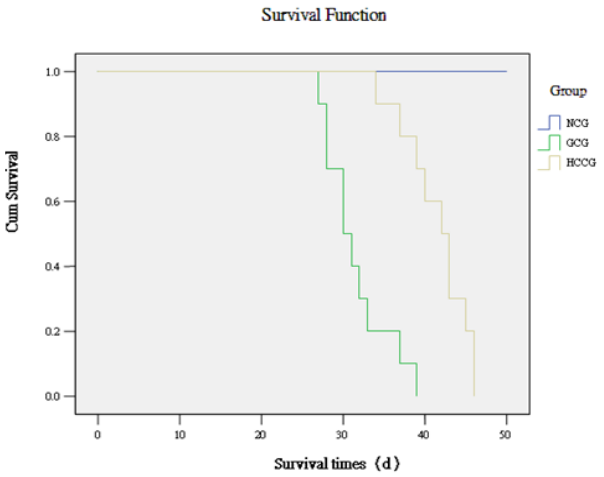

Figure 1. The Kaplan-Meier Time-survivor Curve of Experiment Mice in Every Group Demonstrated that Mice in GCG Suffered Worse Survival Times, Due to Inhibition of Tumor Immunity by Gdcl3 and its Suppression of Kcs

(Matrix Metallo Preteinases-2) monoclonal antibody was bought from Abcam Corporation, the U.S.A.. Goat-anti-mouse secondary antibody was bought from Maixin Biotechnology Corporation, Foochow, China. $GdCl_3$ was bought from Sigma Corporation of American.

H22 Cell Culture

H22 cells passaged in vivo of Kunming mouse were diluted with equal volume of normal sodium, and kept under 4°C. 4-week-old healthy male Kunming mice about 25±5 g were injected 0.5 ml H22 cell suspension (about 1×10^5) in abdominal cavity after sterilization. The abdomens of these mice appeared apparent apophysis after 2 weeks and the ascites was milky or partly bloody. 5~10ml ascites was drawn aseptically from these Kunming mice, and then centrifuge it for 3min under 500 rpm in room temperature. Supernatant fluid was abandoned, and remainder was washed with normal sodium repeatedly then diluted H22 cells with distilled water to 1×10^6/ml, stored in 4°C for use.

Tumor Model, Test and Analysis

Our study was approved by Institutional Ethics Committee of Chongqing Medical University. Sixty male Kunming of 4 weeks old were inoculated in the anteroright hepatic under liver capsule with 0.2 ml (2×10^5) H22 cell suspension and were randomly divided into two groups (each group 30 mice): $GdCl_3$ treated group (GCG) and hepatocellular carcinoma group (HCCG). The 0.25% $GdCl_3$ solution (10 mg/Kg) was infused via vena caudalis of mice in GCG 1week before inoculation and injection was continued for once per three days. The normal sodium was infused instead in HCCG. Simultaneously, normal control group (NCG) was set with 20 healthy Kunming mice with the same background conditions. Ten mice were taken out of each group for observing the survival. The remains in each group were killed to observe the follow indexes 3 weeks after injection of H22 cells: tumor weight, histologic characteristics of tumor tissue in light microscope, ultramicrostructure of KCs in electron microscope, distribution and number of KCs by histochemical stain, TNF-α and IFN-γ levels of blood-serum and liver tissue by ELISA and RT-PCR, and MMP-2 protein level of tumor tissues by IHC (Immunological Histological Chemistry).

Table 1. The Effect of $Gdcl_3$ Pretreatment on the Quality of Tumor (M±SD, g)

Group (n = 20)	quality of tumor (g)
HCCG	1.56±0.14
GCG	3.18±0.25*

*P<0.05, HCCG vs GCG

Statistical analysis

Data was assayed by SPSS 13.0 software. ANOVA and Student's t test were applied for analysis of measurement data. For the survival analysis of animals, Kaplan-Meier curves were established for each group, and the survivals were compared by the log-rank test. Differences between means or ranks as appropriate were considered significant when P<0.05. Results were presented as Means ± SD.

Results

Effect of $GdCl_3$ pretreatment for the survival of Kunming mice

No death occurred in the mice of NCG during 60days. Kaplan-Meier survive curve was analysed by SPSS13.0 displayed the median survival times of HCCG and GCG were 40.5±3.5d and 30.5±2.5d respectively. Log-rank test indicated that the survival time of NCG, HCCG and GCG decurtated gradually, the distinctions between every two groups all reached statistical significances (P<0.05) (Figure 1).

Effect of $GdCl_3$ pretreatment to tumorigenesis and tumor growth

The size of liver Clump was about 0.4 cm~0.8 cm of HCCG at 3W and there was not satellite focuses around it (Figure 2A). The size of liver Clump was 1.1 cm~1.3 cm companied with multitude satellite focuses (indicated by the arrows) of GCG at 3W (Figure 2B), which demonstrated significant tendency of metastasis. The tumor quality of this two groups was 1.56±0.14g and 3.18±0.25g respectively, the difference was significance (Table 1, P <0.05).

Effect of $GdCl_3$ pretreatment to pathomorphology of tumor

HE stain show the morphosis of hepatic lobules of NCG was normal, the periportal and central vein structure was clear; cells arranged regularly (Figure 2C). The cells array was disorder and the atypia of organization was obvious in liver cancer tissue and the infiltration of inflammatory cells was evident in liver tissue around liver cancer in both HCCG and GCG (Figure 2D, Figure 2E). A satellite nodule was found in GCG (indicated by the arrows, Figure 2F), tumor cells presented stronger ability of metastasis in after suppression of KCs by $GdCl_3$. From the electron microscope, in normal mouse liver tissue, their phagocytic activity of the KCs did not strengthen

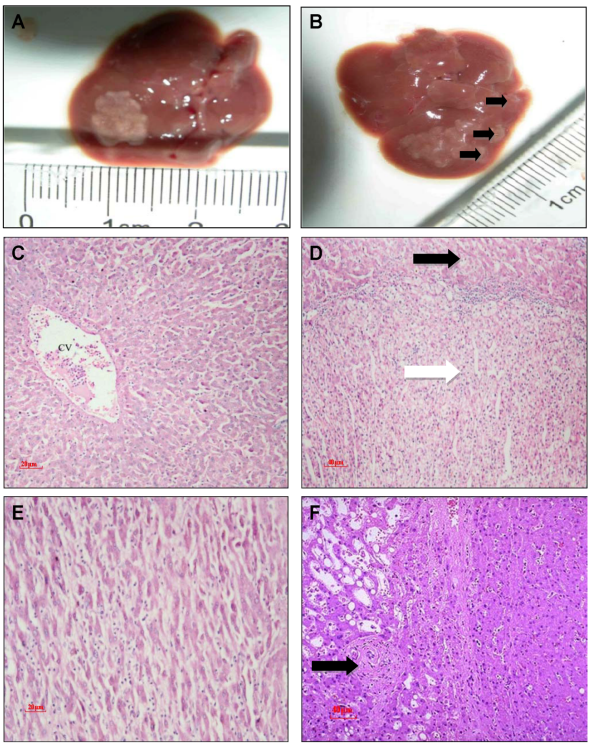

Figure 2. A. The size of tumor focus was about 0.8 cm of HCCG at 3W. B. The size of tumor primary focus was 1.1 cm which was accompanied with three satellite focuses of GCG at 3W (indicated by black arrows). Tumor in GCG showed stronger trend of growth and metastasis due to inhibition of tumor immunity by GdCl3 and its suppression of KCs. C. The histological structure of liver tissue around central vein in NCG was normal (HE×200). D. Significant pleomorphism and disorderly array of tumor cells were found in tumor tissue in HCCG at 3W (indicated by black arrow). However, in para-carcinoma tissue, normal histological structure was kept (indicated by white arrow, HE×100). E. The infiltration of inflammatory cells was evident in live tissue apart from tumor in HCCG at 3W (HE×200). F. Significant pleomorphism and disorderly array of tumor cells were found in tumor tissue in GCG at 3W, a round satellite focus was found (indicated by black arrow, HE×100)

obviously and phagolysosome in the intracytoplasm was few (Figure 3A). The KCs in HCCG was activated obviously in around cancer tissue with cellular swelling, densed chromatin, enlarged and irregular shaped nuclear and generous endoplasmic reticulum intracytoplasm whose membranes were close to cancer cells (Figure 3B). After $GdCl_3$ pretreatment for a week, a mass of phagocytized $GdCl_3$ particles were found in KCs of GCG (Figure 3C). But the function of KCs of GCG does not show to be activated obviously which was presented as nuclei condenses, less of phagosome and endoplasmic reticulum, but smaller pseudopodium was found (Figure 3C).

Effect of $GdCl_3$ pretreatment to the quantity of KCs

Immunohistochemical staining (CD68+, brownish red) confirm that the KCs is orbicular-ovate, asteroidal or fusiform (Figure 3D), but no KC was found in tumors in both HCCG and GCG (Figure 3E, Figure 3F). The quantity of KCs of the para-carcinoma tissue (<5 mm from tumor) and tissue far from cancer (>5 mm from tumor) in the

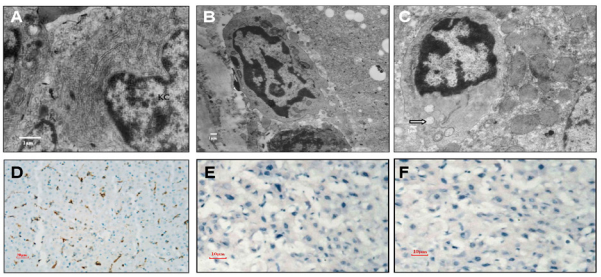

Figure 3. A. Phagocytic activity of KCs in NCG was not enhanced; few phagolysosomes in the cytoplasma were found (TEM×10000). B. A mass of phagocytized $GdCl_3$ particles were found in KCs in GCG whose KCs were pre-treated with $GdCl_3$ (indicated by white arrow). Nuclear condensation, less phagosomes and less endoplasmic reticulums were found in KCs which were pre-treated with $GdCl_3$ in GCG, however, small pseudopodium could be observed. (TEM×4200)C.The nucleus irregularly enlarged and generous endoplasmic reticulum would be found in KCs in para-carcinoma tissue in HCCG at 3W. Swelled KCs in para-carcinoma tissue in HCCG at 3W which were tightly contacted with malignant cell (TEM×4200). D.In NCG, many CD68 positive cells (KCs, brownish red) were found in liver tissue with stellate or spindly shapes. (×200) E.There was no CD68-expressing cell (KCs) was observed in cancer tissue in HCCG at 3W (×400). F.There was no CD68-expressing cell (KCs) was observed in cancer tissue in GCG at 3W (×400)

Table 2. The Effects of $Gdcl_3$ Pretreatment on the Quantity of KCs (M±SD)

Group (n=20)	The quantity of KCs (pc./PH)	
	Para-carcinoma tissue	Tissue apart from cancer
HCCG	9.40±2.37*	9.50±2.17*
GCG	9.20±1.87*	9.08±2.10*
NCG	4.80±1.31	

*P<0.01, all other groups compared with NCG

HCCG and GCG are 9.40±2.37 pc./PH, 9.50±2.17 pc./PH and 9.20±1.87 pc./PH, 9.00±2.10 pc./PH respectively, the quantity of KCs in NCG was 4.80±1.31 pc./PH. There was no KC in cancer tissue. The quantities of KCs in tissues para-carcinoma and far from cancer were more than in normal liver tissue (P<0.01), but between the both sides or both groups (HCCG and GCG) had not significance difference (Table 2, P>0.05).

Effect of $GdCl_3$ pretreatment to the levels of TNF-α, and IFN-γ

ELISA was employed to evaluate the level of blood serum. The TNF-α level of GCG and HCCG were higher than NCG (P<0.05), and TNF-α level of HCCG was higher than GCG also (Figure 4A, P<0.05). The IFN-γ level of HCCG was lower than that in NCG. However, the IFN-γ level further decreased in GCG when compared with that in HCCG (Figure 4B, P<0.05).

Effect of $GdCl_3$ pretreatment to TNF-α and MMP-2 of liver tissue

RT-PCR (Figure 4C) and semiquantitative analysis (Figure 4D) indicated that: the express levels of TNF-α mRNA of liver tissue or tumor tissue in NCG, GCG and HCCG were heightening gradually. Statistical differences

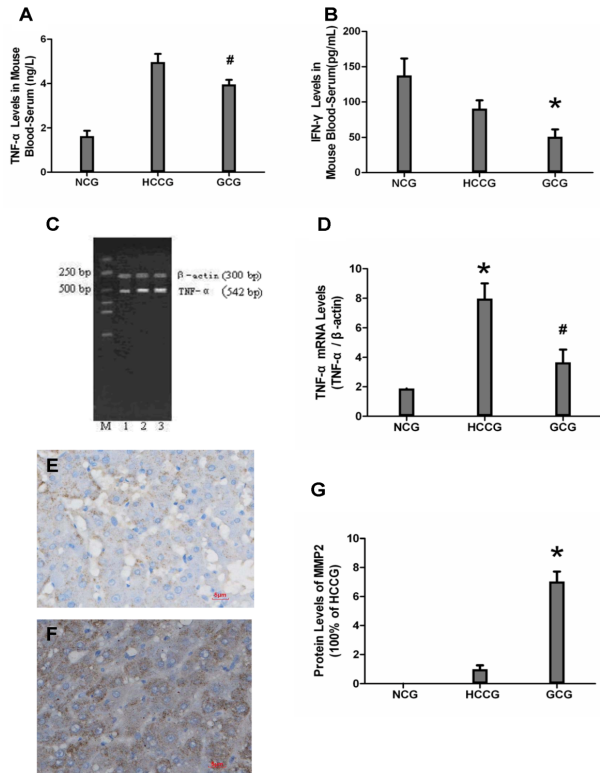

Figure 4. A & B. TNA-α and IFN-γ levels in mouse blood-serum were tested by ELISA. Significantly lower TNA-α (A) and IFN-γ (B) levels were found in GCG when compared with HCCG. $GdCl_3$ suppressed KCs and decreased the secretion and introduction of anti-tumor cytokines. **P*<0.01, HCCG vs NCG; #*P*<0.05, HCCG vs GCG. C. Express of TNF-α mRNA in liver tissue or tumor tissue in different groups, higher levels of TNF-α mRNA were found in HCCG and GCG. D. Semi-quantitative analysis of express levels of TNF-α mRNA in different groups, the highest level of TNF-α mRNA was found in HCCG, relative lower in GCG and the lowest in NCG. **P*<0.01, HCCG vs NCG; #*P*<0.05, HCCG vs GCG. M: Marker; 1: NCG; 2: GCG; 3: HCCG. E & F. IHC of MMP-2 indicated that higher MMP-2 protein level was found in GCG. G. Semi-quantitative analysis of express levels of MMP-2 protein levels by Image-Pro plus Software in HCCG and GCG (B) was accordance with IHC. **P*<0.01, HCCG vs NCG

existed between NCG and HCCG, NCG and GCG, GCG and HCCG (*P*<0.05). IHC was applied to test MMP-2 protein in tumor tissue, which indicated MMP-2 level in GCG group (Figure 4E) was much higher than that in HCCG (Figure 4F and Figure 4G, *P*<0.05), and no MMP-2 protein was found in normal liver tissue.

Discussion

Burnet introduced the Immune Surveillance Theory in the 60s of last century which presumed immune system could discriminate and kill the mutant cells specifically and eliminated the mutant cells before its malignant transformation, but the mutant cells would form tumor when the function of immunologic surveillance was weak. Fortunately, even if tumor was formed, immune system could figureht the tumor by certain immunological effects including cellular immunity and humoral immunity, especially cellular immunity. Tumor immunity depended on various kinds of effector cell, for instance, T cells, natural killer cells, natural killer T cells and macrophages. In addition, IgG could induce ADCC (antibody-dependent cell-mediated cytotoxicity) of many kinds of effector cells. For example, macrophages and NK (natural killer) cells could induce effective ADCC which led to cytolysis of tumor cells.

The macrophages not only acted as APC (antigen-presenting cell) for antigenic presentation, but also directly participated in killing tumor cell (Bertolino et al., 2002) including: 1. swallowing tumour cell directly (Greten et al., 2006), 2. killing neoplastic cell by ADCC through CD16 (FcγRIII) (Clynes et al., 2000), 3. secreting cytokine liked TNF-α and IL-1, directly or indirectly kill neoplastic cell (Karpoff et al., 1997), 4. producing some kind of enzymes and active oxygen molecule killed tumor cells directly or inhibited growth tumor cells. Injection of cytostatics to macrophages, for example silica or antimacrophage serum, in vivo, could accelerate the growth of tumor (Irie, et al., 2005). However, the Bacille Calmette Guerin or coryne bacterium parvum could restrain tumor growth and metastasis by activated macrophages (Yamada et al., 2000; Luo et al., 2004). Pathology biopsy showed that if there were macrophages around the tumor tissue, the incidence rate of the tumor metastasis was lower and the prognosis showed better also. On the contrary, the incidence rate of the tumor metastasis was higher and prognosis was worse (Satoh et al., 2003). KCs accounted 80% of sum total of macrophages not only played an important role in the mononuclear phagocytic system but also were of important significance to figureht against liver tumor. As the most important defense cells in liver, KCs induced cell swallow, production of cytokine, tumor immunity and mediated metabolism of cells by their surface receptors. Thus, they played an important role in defence mechanism and maintaining the balance of internal environment of the body. The quantity and function of KCs would alter obviously to react to the change of microenviroment when liver diseases occurred.

Gadolinium chloride hexahydrate ($GdCl_3$) is a kind of experiment medicine which molecular weight was 371.70. It is white fine powder; isotonic Na chloride could dissolve it easily. $GdCl_3$ can inhibit KCs specifically, but has no effect to the phagocytes in other organs, thus it was widely used in the studies of KCs (Lee et al., 2003). Research indicated that the level of TNF-α in circulating blood degraded obviously after the function of KCs was restrained by $GdCl_3$, which mechanism was that (Hustik et al., 1980) $GdCl_3$ hindered Ca^{2+} inflow which was essential for activation of KCs, and inhibited the contact of KCs and other cells, so that phagocytic function of KCs was suppressed. Then activity of NF-κB was inhibited, production and releasing of immune correlation factors for example TNF-α, etc. were decrease, phagocytosis and the function of antigen presentation of KCs were also restrained.

Our study showed that life span of the liver cancer mice which were pretreated with $GdCl_3$ shortened obviously; in addition, the growth and development of liver tumor were accelerated. Median survival times which were calculated through Kaplan-Meier survival curve indicated that liver cancer mice in HCCG enjoyed longer lives (40.5±3.5

days) than those in GCG (30.5±2.5 days), and Log-rank test indicated that the survival time of NCG, HCCG and GCG decurtated gradually, the distinctions reached Statistical significances (P<0.05). HE stain showed the histological morphology of hepatic lobules of mice in NCG was normal, the periportal and central vein structure was clear, the cells arrayed regularly. The cells array was disordered and atypia was obvious in liver cancer tissue, and the infiltration of inflammatory cells was evident in live tissue around liver cancer in both HCCG and GCG. In addition, satellite lesion was found in GCG, which indicated higher viability of tumor cells and ability to metastasize. From the electron microscope, KCs in HCCG were activated obviously in para-carcinoma tissue with cellular swelling, densed chromatin, enlarged and irregular shaped nuclear and generous endoplasmic reticulum intracytoplasmic. In addition, their membranes contacted with cancer cells, which indicated close interaction between these two kinds of cells. However, the function of KCs in GCG did not show to be activated obviously by the suppressive effect from $GdCl_3$. Those could confirm that the activation of KCs played a very important role in inhibition of growth and development of HCC.

CD68 was a glucoprotein with 110KD molecular weight which was concerned with the production of cytolysosome, and it was also the specific surface marker of histoleucocyte and macrophage. To detect CD68 was one of the methods for confirming KCs (Ikarashi et al., 2013). Our study adopted immunohistochemistry, used anti-CD68 monoclonal antibody to test the distribution of KCs in liver tissue, and the result indicated that the quantity of KCs in liver cancer was significant smaller than para-carcinoma tissue and tissue far from the cancer, which is accord to other literatures (Liu et al., 2003; Okino et al., 2005). This might be concerned with the destruction of nomal structure of hepatic lobule and the microenvironment of hepatic sinusoid which KCs lived relied on (Roland et al., 1994). Our study hinted the quantities of KCs in para-carcinoma tissue and tissue far from cancer tissue in both GCG and HCCG were larger than that in normal liver tissue. However, the quantities of these two groups and these two regions had no significance difference. In summary, with the appearance of tumor immunity in hepatocarcinogenesis, the quantity of KCs would increase obviously. And $GdCl_3$ pretreatment had no effect on the quantity of KCs but restrained the function, and further suppressed their immunologic function against tumor.

Besides direct phagocytosis, activated KCs could also produce or induced diverse effectors, including TNF-α, IFN-γ, etc. which could kill and inhibit tumor cells non-specifically. The mechanisms of TNF-α suppressed tumor cells in vitro were: dissolving directly and repressing proliferation, these could cause tumor necrosis; toxic action to capillary endothelium cell which could block regional blood supply then caused tumor necrosis; strengthening the activity of macrophage system and NK cells, played synergistic anti-tumor effect. Research had showed that plasma level of TNF-α was positively associated with the course of HCC (Wang et al., 2003). Our study demonstrated that the level of TNF-α in HCCG was highter than that in GCG, which hinted if the function of KCs had not been restrained, organism could exert normal immunologic surveillance for tumor, and KCs could secret TNF-α by stimulation of tumor. Thus survival time of mice in HCCG was longer than that in GCG, and growth rate of tumor was lower than the latter. Interestingly, we also found significantly higher protein level of MMP-2 which was a marker protein of ability for tumor metastasizing in GCG group. This found was accordance with gross of tumor tissues in our research. The relation between KCs and tumor metastasis was still unclear; researches indicated the polarization of KCs might be involved. However, more works on this mechanism were needed.

Acknowledgements

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