P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• LIVER CANCER •

Experimental study on therapeutic effect of *in vivo* expression of Cell I-Hep II recombinant polypeptide of fibronectin on murine H22 hepatocellular carcinoma

Gui-Mei Zhang, Yan Yang, Bo Huang, Hui Xiao, Dong Li, Zuo-Hua Feng

Gui-Mei Zhang, Yan Yang, Bo Huang, Hui Xiao, Dong Li, Zuo-Hua Feng, Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
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Correspondence to: Dr. Zuohua Feng, Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China. fengzhg@public.wh.hb.cn
Telephone: +86-27-83657574 **Fax:** +86-27-83657574
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Abstract

AIM: To investigate the inhibitory effect of *in vivo* expression of expressing plasmid pCH510 of recombinant fibronectin polypeptide (CH50) on hepatocellular carcinoma and the improved therapeutic effect of pCH510 in combination with chemotherapeutic agents and Hsp70-H22 hepatocarcinoma antigen peptide on tumor.

METHODS: Mice were inoculated with H22 hepatocarcinoma cells. The chemotactic effect of the expression of plasmid pCH510 on immunocytes was observed after *in vivo* transfection, tissue slicing and HE staining. Inhibitory effect of transfection with pCH510 on murine tumor originated from different inoculative doses was observed. The inhibitory effect of immediate transfection with pCH510 after chemotherapy on tumor was compared with that of transfection 5 days after chemotherapy. The change of function and amount of mouse peritoneal macrophages and the peripheral blood immunocytes resulted from administration of chemotherapeutic agents were detected. The peptides mixture was prepared from H22 hepatocarcinoma cells. pCH510 + Hsp70-H22 antigen peptides were injected into tumor-bearing mice with or without chemotherapy, to observe the inhibitory effects on tumor.

RESULTS: At the tumor tissue site injected with pCH510, there were a great number of immunocytes which mainly were macrophages, lymphocytes and neutrophils. Transfection of plasmid pCH510 inhibited significantly the murine tumor induced by different inoculative doses. The inhibitory effect was negatively correlated with the inoculative dose. The therapeutic effect was not improved by immediate transfection with pCH510 after chemotherapy, but was significantly improved by transfection with pCH510 5 days after chemotherapy. Chemotherapeutic agent decreased the number of immunocytes and suppressed their activation *in vivo*. After injection of drug, the amount of immunocytes was the lowest from d 1 to d 3 and returned to normal level on the 10^{th} day. Transfection with plasmid pCH510 alone could inhibit tumor induced by the inoculation with 10^4 H22 cells. The tumor originated from the inoculation with 10^5 H22 cells was inhibited by pCH510+Hsp70-H22 antigen peptides and that from the inoculation with 10^6 H22 cells was inhibited by pCH510+Hsp70-H22 antigen peptides in combination with chemotherapeutic agents.

CONCLUSION: *In vivo* expression of pCH510 recruits immune cells, inhibits tumor growth, and enhances the efficacy of chemotherapy. But the proper timing of combining chemotherapy with pCH510 must be taken into great account. The synergism of pCH510 and Hsp70-H22 peptides can improve the efficacy, which could be further enhanced if they are used following chemotherapy. Chemotherapeutic agent + pCH510 + Hsp70-H22 peptides is a promising therapeutic approach of combination treatment of tumor.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignant tumor with an increasing incidence, and remains a disease with a poor and dismal prognosis, and all forms of currently available conventional therapies are rarely beneficial[1]. Surgical resection is incapable of removing all HCC cells and so is chemotherapy[2]. However, in recent years, biotherapy has been reported with promising results as a new therapeutic approach of hepatocellular carcinoma[1-7]. On the other hand, the extraordinary versatility of gene therapy opens new possibilities for the treatment of hepatocellular carcinoma[8-10].

Fibronectin (FN) is an extracellular matrix glycoprotein which exists in extracellular matrix (cellular-type) and in blood (plasma-type). It is composed of multiple functional domains including cell-binding domain and heparin-binding domain, and additional regions such as EDA, EDB and V region that arise through alternative splicing. Recombinant peptides with different functional domains produce different effects on tumor cells. Hep II-V bifunctional-domain recombinant peptide can promote tumor invasion[11]. In contrast, Cell I-Hep II bifunctional-domain recombinant peptide has an inhibitory effect on adhesion, invasion, and spreading of tumor cells[12,13]. Fragments of fibronectin have chemotactic effect on immunocytes. David A.Norris[14] reported that fragments of fibronectin cleaved by enzymes were chemotactic to human peripheral blood monocytes, but intact FN was inactive. Doherty[15] also reported that FN fragments containing cell-binding domain could recruit macrophages. The chemotactic effect is mediated by the interaction between cell-binding domain and β1 integrin receptor on the cell surface[16,17]. These characteristics indicate the great potential of FN in tumor therapy. We have expressed in *E.coli* and purified recombinant

FN polypeptide containing Cell I-Hep II bifunctional-domain. The peptide has the activities of chemotaxis, macrophage activation, as well as inhibitory effect on tumor growth and metastasis[18]. On this basis, the eukaryotic expressing plasmid pCH510 of Cell I-Hep II bifunctional-domain recombinant polypeptide was constructed[19]. The main purpose of the study was to investigate the chemotactic effect on immunocytes and therapeutic effectiveness on mouse H22 hepatocellular carcinoma of *in vivo* expression of pCH510, and the role of *in vivo* expression of pCH510 in combined treatment of tumor, and to analyze the factors influencing the effect of combined treatment so as to provide experimental basis for potential use of pCH510 in tumor therapy.

MATERIALS AND METHODS

Reagents

Mouse H22 hepatocarcinoma cell line was purchased from China Center for Type Culture Collection (CCTCC, Wuhan). BALB/c mice were purchased from Medical Experimental Animal Center of Hubei Province. Eukaryotic expression plasmid pCH510 of Cell I-Hep II bifunctional-domain recombinant polypeptide CH50 of human fibronectin was constructed in our lab. Recombinant human heat shock protein 70 was expressed and purified in our laboratory. Mitomycin C (MMC) was the product of Zhejiang Hisun Pharmaceutical Corporation. IFN-γ and MTT were purchased from Sigma Co.

Inoculation of mouse with H22 cells

H22 cells were suspended in PBS at the concentrations of 1×10^8/ml, 1×10^7/ml, 1×10^6/ml, 1×10^5/ml respectively. 100 μl of the suspension was injected into the muscle of mouse at right hind limb. The inoculative doses were 1×10^7, 1×10^6, 1×10^5, 1×10^4, respectively.

Chemotaxis test for immunocytes of pCH510 transfection in hind limb muscle of tumor-bearing mice

After inoculation with 1×10^6 H22 cells, mice were randomly divided into 2 groups, 9 mice in each group, for the injection of 100 μg of plasmid pCH510, 100 μg of plasmid pcDNA3.1 respectively. The next day after inoculation, mice in each group were given an injection at the inoculation site. The tissue specimens from muscle tissue of injection site were prepared on day 1, 2, 3 after plasmid transfection, 3 mice from each group each day.

Inhibition test of tumor growth by pCH510 transfection

The mice in different groups were each inoculated with 1×10^7, 1×10^6, 1×10^5, 1×10^4 of H22 tumor cells respectively. Starting from d 2, the mice in each group were given an injection of 100 μg of plasmid pCH510 every day for 10 times at the inoculation site. The mice in control group were given an injection of control plasmid pcDNA3.1. The mice were dissected to measure the weight of tumor at different time after inoculation: on the 7th day for 1×10^7 inoculative dose, on the 10th day for 1×10^6, on the 14th day for 1×10^5, and on the 21st day for 1×10^4.

Inhibition test of pCH510 in combination with chemotherapy

The mice were inoculated with 1×10^6 H22 cells, and randomly divided into six groups designated as A, B, C, D, E, F (day 0). On day 1, the mice in group B were injected at inoculation site with 100 μg of plasmid pCH510, for 10 days. On day 2, the mice in Groups C, D, E, F were injected at inoculation site with 50 μg MMC. On day 3, the mice in group E were injected at inoculation site with 100 μg of plasmid pCH510, for 10 days. On day 6, the mice in group F were injected at inoculation site with 100 μg of plasmid pCH510, for 15 days. The mice in groups A, B, C were dissected to measure the weight of tumor on the 15th day, the mice in groups D, E, F were dissected to measure the weight of tumor on the 21st day.

Assay for function of mouse peritoneal macrophages after injection of MMC

Macrophages were collected from murine abdominal cavity 4 hours and 24 hours after ip injection of 50 μg MMC. The macrophages were cultured *in vitro* in the presence of IFN-γ and CH50. The activation and NO production of macrophages were determined after 48 hour cell culture by MTT.

Assay for amount of immunocytes in peripheral blood of mouse after injection of MMC

The mice were randomly divided into two groups for ip injection of 50 μg MMC and PBS respectively (designated as day 0). On days 1, 3, 5, 7, 9, 11, blood was collected from murine orbital vein, the cells were counted following the removal of red cells. At the same time, macrophages were collected from murine peritoneal cavity and counted.

Preparation of Hsp70-H22 antigen peptides

H22 tumor antigen peptides and Hsp70 were prepared according to the method described previously[20]. Hsp70 and peptides mixture from cells were mixed to bind each other[20]. Briefly, peptides and Hsp70, at the concentrations of 75 μg/ml and 250 μg/ml respectively, were mixed and incubated at 37 ℃ for 2 h in the presence of 1 mM of ADP and 1 mM of $MgCl_2$.

Inhibition test of pCH510 with Hsp70-H22 peptide on tumor growth

The mice were randomly divided into eight groups. Four groups were inoculated with 10^5 of H22 cells, the other four groups were inoculated with 10^6 of H22 cells (designated as day 0). On day 1, the mice in different groups were given injection of pCH510 + Hsp70-H22 antigen peptides, pCH510, Hsp70-H22 antigen peptide, and saline respectively, every other day for 9 times. The mice were dissected to measure the weight of tumor on the 21st day (for control group, on the 14th day or 10th day).

Inhibition test of chemotherapy + pCH510 +Hsp70-H22 antigen peptide on tumor growth

The mice were randomly divided into five groups and inoculated with 10^6 of H22 cells into the hind limb (designated as day 0). On day 1, the mice were injected with 50 μg of MMC at the inoculation site (the control group was given saline). On day 5, the mice injected with MMC were given injection of pCH510 + Hsp70-H22 antigen peptide, pCH510, Hsp70-H22 antigen peptide, and saline respectively, every other day for 14 times. The mice were dissected to measure the weight of tumor at different times: saline group on the 10th day, MMC group on the 15th day, MMC + pCH510 and MMC + Hsp70-H22 antigen peptide groups on the 30th day, and another MMC + pCH510 + Hsp70-H22 antigen peptide group on the 40th day. The size of tumor was observed.

Statistical analysis

The *t* test was used for statistical analysis. *P* value less than 0.05 was considered as significantly different.

RESULTS

Chemotaxis for immune cells of pCH510 expression in tumor tissue

Tumor cells in control group grew rapidly. On the 3rd day after inoculation, tumor nodes were observed. In the tissue section

of control group, there were a large amount of tumor cells which invaded muscle tissues. In the experimental group, tumor node was not observed. In the tissue section, tumor cells were distributed locally. There were a large amount of macrophages and lymphocytes in connective tissues and many neutrophils and lymphocytes on the edge of tumor and muscle tissues. Immune cells were seldom seen in normal muscle tissues which were not invaded by tumor cells (Figures 1 and 2).

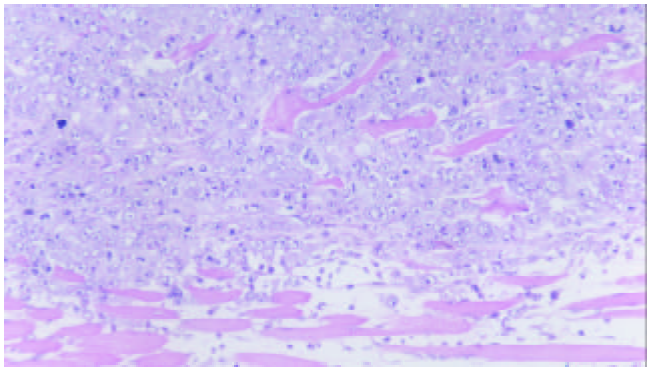

Figure 1 Control group: tumor cells grew rapidly and invaded the normal muscles (×200).

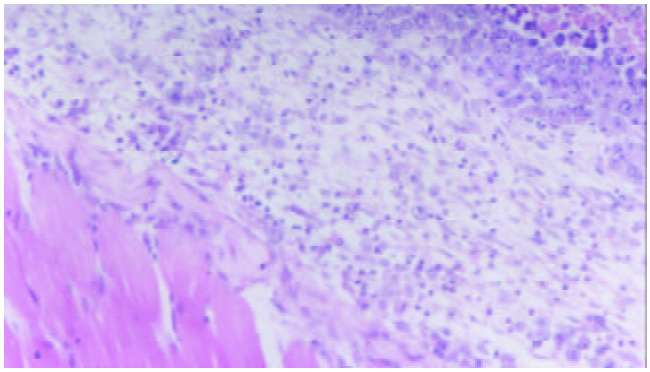

Figure 2 pCH510 group: transfection of pCH510 recruited macrophages, lymphocytes and neutrophils (×200).

Inhibitory effects of pCH510 on tumor growth

Plasmid pCH510 had inhibitory effects on murine tumor induced by different inoculative doses and the effect was negatively correlated with the inoculative dose. The lower the inoculative dose was, the more significant the effect. For 10^4 of tumor cells inoculation, pCH510 could completely inhibit the tumor growth in a period of 20 days (Table 1).

Table 1 Inhibitory effect of pCH510 on murine tumor inoculated with different doses of tumor cells

Inoculative doses	*n*	Dissecting time (d)	Tumor weight (g, $\bar{x}$±*s*)	
			Control	pCH510
10^4	8	21	2.19±0.35	0[a]
10^5	8	14	2.27±0.38	0.62±0.17[a]
10^6	8	10	2.32±0.42	1.35±0.24[a]
10^7	8	7	2.37±0.33	1.88±0.32[b]

[a]P<0.05 *vs* control; [b]P<0.01 *vs* control.

Effect of interval between injection of MMC and pCH510 on therapeutic efficacy

Chemotherapeutic agent alone or pCH510 alone could inhibit tumor growth. After chemotherapy, the effect was not improved by immediate 10-day pCH510 transfection. The tumor grew much slower if the mice were given 15-day pCH510 transfection 5 days after chemotherapy, suggesting that pCH510 in combination with chemotherapeutic agent in this way could significantly improve therapeutic effectiveness (Table 2).

Table 2 Inhibitory effect of pCH510 in combination with chemotherapy on murine tumor

Groups	*n*	Tumor weight (g, $\bar{x}$±*s*)	
		d 11	d 21
A: control	8	2.12±0.38	
B: pCH510 (d 2 to d11)	8	0.82±0.21[a]	
C: chemo I	8	0[a]	
D: chemo II	8		1.58±0.31
E: MMC+pCH510 (d 2 to d 11)	8		1.49±0.28
F: MMC+pCH510 (d 6 to d 20)	8		0.42±0.12[b]

[a]P<0.01 *vs* control; [b]P<0.01 *vs* chemo II.

Effect of MMC on the function and amount of macrophages in vivo

The metabolic activity of macrophages from murine abdominal cavity was inhibited by injection of MMC after 4 hours and 24 hours *in vivo*. The level of NO released by macrophage decreased significantly (Table 3). After injection with MMC intraperitoneally, the number of macrophage from murine peritoneal cavity and mononuclear cells from peripheral blood decreased dramatically. The number of immune cells reached the lowest from d 1 to d 3, and started recovery from d 5 and returned to normal level on the 11th day (Figure 3).

Table 3 Effects of MMC on activity of macrophages

Groups	*n*	4 h		24 h	
		Metabolism OD_{570},($\bar{x}$±*s*)	NO mol/L, ($\bar{x}$±*s*)	Metabolism OD_{570},($\bar{x}$±*s*)	NO mol/L, ($\bar{x}$±*s*)
Control	6	0.35±0.06	29.9±5.1	0.32±0.04	28.8±6.3
MMC	6	0.15±0.03[a]	16.5±4.3[a]	0.16±0.04[a]	13.9±5.4[a]

[a]P<0.01 *vs* control.

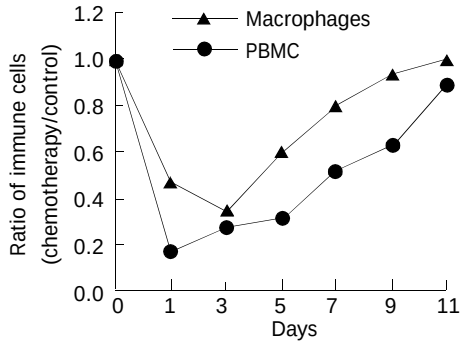

Figure 3 Numerical change of mouse celiac macrophages and peripheral blood mononuclear cells (PBMC) after injection of MMC.

Synergic effects of pCH510 with Hsp70-H22 peptides on inhibition of H22 tumor growth

pCH510 in combination with Hsp70-H22 peptides could produce a much stronger inhibitory effect on H22 tumor in mice. Although the tumor from 10^6 of H22 cells inoculation was not completely inhibited, the tumor from 10^5 of H22 cells inoculation was completely inhibited (Table 4).

Table 4 Inhibitory effect of pCH510+Hsp70-H22 antigen peptide complex on murine tumor

Group	*n*	10^5 H22 cells inoculation		10^6 H22 cells inoculation	
		Dissecting time (d)	Tumor weight (g, $\bar{x}\pm s$)	Dissecting time (d)	Tumor weight (g, $\bar{x}\pm s$)
Control	8	14	3.24±1.22	10	3.45±1.31
pCH510 plasmid	8	21	2.38±0.84	21	2.72±1.28
Hsp70-H22 peptide	8	21	1.44±0.45	21	2.58±0.93
Hsp70-H22 peptide+pCH510	8	21	0[b]	21	1.76±0.66[a]

[a]$P<0.05$ *vs* control; [b]$P<0.01$ *vs* control.

Table 5 Inhibitory effect of chemotherapy+pCH510+Hsp70-H22 peptide on murine tumor

Group	*n*	H22 cell inoculating	Dissecting time (d)	Tumor weight (g, $\bar{x}\pm s$)
Control	8	10^6	10	3.28±1.12
MMC	8	10^6	15	2.82±0.94
MMC+pCH510	8	10^6	30	1.51±0.53
MMC+Hsp70-H22 peptide	8	10^6	30	0.62±0.22[b]
MMC+pCH510+Hsp70-H22 peptide	8	10^6	40	0[a]

[a]$P<0.01$ *vs* control; [b]$P<0.05$ *vs* (MMC+pCH510).

Inhibitory effects of chemotherapy+pCH510+Hsp70-H22 peptides on tumor growth

For tumor from 10^6 of H22 cells inoculation, the anti-tumor efficacy was significantly different if chemotherapeutic agent was used in combination with other agents. Compared with the protocols of chemotherapy in combination with Hsp70-H22 peptides and chemotherapy in combination with pCH510, the anti-tumor effect of chemotherapy in combination with both pCH510 and Hsp70-H22 peptides was the most powerful, which could completely inhibit the growth of residual tumor after chemotherapy (Table 5).

DISCUSSION

Tumor immuno-therapy comprises non-specific and specific immuno-therapy. The former includes some cytokines such as IL-12, IL-18, TNF-α, as well as some immune cells such as macrophages, natural killer cells and so on. The latter is cell-mediated immunity which is produced by the activation of $CD4^+$ and $CD8^+$ T cells stimulated by tumor specific antigen. Immuno-therapy holds great promise as an effective weapon against residual tumor cells after chemotherapy. Cell I-HepII double-domain polypeptide and CH50 can recruit macrophages and lymphocytes by chemotaxis, activate macrophages to inhibit the growth of tumor by non-specific immune response. It has a great potential in tumor therapy. It has been reported that fibronectin fragments containing Cell I-domain inhibited tumor invasion and metastasis[21,22]. Our results showed that recombinant FN polypeptide recruited immune cells into the local tumor tissue, increasing the local anti-tumor immunity. A large number of immune cells gathered around the tumor tissue which had been transfected with pCH510. Moreover, comparing the result of pCH510 transfection on the first day after inoculation with those of the other groups, the distribution of tumor cells was much more limited and the invasion of tumor cells to normal tissue was delayed, suggesting that transfection with pCH510 could inhibit tumor growth and invasion at the early stage of tumor growth.

Plasmid pCH510 had inhibitory effect on murine tumor from different inoculative doses and the effects was negatively correlated with the inoculative dose. The lower the inoculative dose was, the more significant the effect was. For 10^4 of tumor cells inoculation, pCH510 could completely inhibit the tumor growth in a period of 20 days. Chemotherapeutic agent kills tumor cells rapidly and powerfully. For 10^6 of tumor cells inoculation, the tumor did not grow within 11 days after chemotherapy. At the same time, the tumor weight in pCH510 transfection group was over 0.5 g, suggesting that chemotherapy should be chosen first to kill residual tumor cells after surgery.

It had an inhibitory effect on tumor growth no matter chemotherapeutic agent or pCH510 was used alone. But neither was powerful enough. When we tried to enhance the antitumor efficacy by combining anticancer drugs with immune modulator CH50, we found that the therapeutic effectiveness was not improved as we had expected by immediate 10-day transfection with pCH510 after chemotherapy. And there was no direct evidence to testify the inhibition of the expression of the plasmid by chemotherapy. CH50 plays an anti-tumor role by regulating host immune response. So we tried to find out the response pattern of host immune system to chemotherapy and the proper timing of transferring pCH510 after chemotherapy. Our results showed that chemotherapeutic agent not only decreased the number of immunocytes but also suppressed their activation. After injection of MMC, the amount of immunocytes was the lowest from d 1 to d 3 and returned to normal level on the 10^{th} day. Similar phenomenon was observed when dendritic cell (DC) counts and function were assayed in peripheral blood of lymphoma and solid tumor patients before and after chemotherapy[23]. The DC counts declined significantly within the first week from the start of chemotherapy, recovered in the second week, and exceeded the baseline values in the third week[23]. The count of immunocytes dropping to the lowest point means that the cytotoxicity of the drug is over. At this moment the host immune system is most severely damaged. If H22 cells were inoculated to the mice on the third day after chemotherapy, the tumor grew more fast than non-chemotherapy control (data not presented), suggesting that immuno-therapy was needed after chemotherapy. But immediate pCH510 transfection after chemotherapy recruited very few inactivated immune cells, as a result, the therapeutic effectiveness was not improved. In contrast, the therapeutic effectiveness was significantly improved by 15-day transfection with pCH510 5 days after chemotherapy. The proper timing of combining chemotherapy with pCH510 was on the 3^{rd} and 5^{th} day after chemotherapy.

Besides proper timing, another influential factor on

therapeutic effectiveness was the dose of chemotherapeutic drug which has also been reported recently[24-26]. One of the disadvantages of conventional chemotherapy is the cytotoxic side effect. How to make full use of the cytotoxicity to tumor cells and lower the side effects is the basis of evaluating the drug dose. In pilot experiments, four doses of MMC were set as following: 150 μg, 100 μg, 50 μg and 25 μg. Two of eight mice died from 150 μg MMC. None of the mice died from 100 μg MMC, but they were accompanied by obtuse reactions, light weight and lackluster hair. For 50 μg and 25 μg MMC, the mice were agile and no obvious side effects were found. Therefore, 50 μg of MMC was the suitable dose for mouse in our experiment. Our results were consistent with those of related reports[27, 28], which showed that combined treatment with low-dose chemotherapy could achieve better efficacy.

The growth of tumor from 10^6 of H22 inoculation was slowed down by chemotherapy in comparison with the tumor from 10^4 of H22 inoculation without treatment. Transfection with plasmid pCH510 alone did inhibit the tumor from 10^4 of H22 inoculation, but not the tumor from 10^6 of H22 inoculation with chemotherapy, suggesting that chemotherapy had a dual effect on tumor growth: on the one hand, they killed tumor cells, on the other hand, they promoted tumor growth by damaging host immune system. Furthermore, improvement of the therapeutic effectiveness by a single immune factor combined with chemotherapy was limited. The best way is the combination of chemotherapy with several kinds of synergic immune modulators. In tumor cells, there are a great amount of over-expressed proteins which can be cleaved into small peptides. These peptides can be used as tumor antigens to induce specific antitumor immune response. At present, only limited antigen peptides are identified, including MAGE-1, MAGE-3, HER-2/neu, and MUC-1[29]. On the other hand, the immunity of multi-valence CTLs induced by mixed antigen peptides from tumor cells is stronger than that of monovalence CTL induced by a given antigen peptide[30]. Heat shock protein 70, as a molecular chaperon, can bind antigen peptide to form Hsp70-peptide complex. The complex presents the antigen peptide to antigen presenting cells (APC) mediated by high affinity receptors on the surface of APC to induce CD_8^+ CTL response[31-35]. In addition to CD_8^+ CTL response, the Hsp70-peptides can also induce CD_4^+ T cell response and NK cell reaction[36]. Our experiment demonstrated that mixed antigen peptides obtained from H22 hepatocarcinoma cells, bound to Hsp70, could induce the production of $CD8^+$ CTLs which could kill specifically H22 cells and inhibit experimental tumor growth *in vivo*[20]. It is theoretically practical that immune cells recruited by pCH510 can be activated by Hsp70-H22 peptide to produce powerful anti-tumor immunity, which was verified by our experiment. The results in this paper showed that the combination of non-specific immune response with specific immune response could be a better strategy for the treatment of tumor. The Cell I-Hep II recombinant polypeptide expressed by plasmid pCH510 could recruit macrophages and other immune cells *in vivo*, activate macrophages and produce non-specific antitumor immunity. Hsp70-H22 peptides could specifically activate $CD4^+$, $CD8^+$ T cells to produce specific anti-tumor immunity. Immune cells were recruited into the tumor tissue by the expressed product of pCH510 and activated by Hsp70-H22 peptides, producing a much stronger anti-tumor immunity.

Nowadays tumor immuno-therapy is focused on specific immune response induced by antigens such as using tumor antigen peptides, tumor idiotype antibody[37]. Tumor antigen peptides can be obtained by some methods such as phage display[38], isolation from Hsp70, hsp90, and gp96-peptide complex[39, 40]. But it is difficult to get mixed antigen peptides, and the complex obtained by the latter method is very little. In this paper we provided a new method which is simple and effective, to obtain mixed antigen peptides from tumor cells by freezing and thawing, heating and acid precipitating.

Transfection with plasmid pCH510 alone could inhibit the tumor originated from 10^4 inoculative dose of H22 cells. The tumor originated from 10^5 inoculative dose of H22 was inhibited by pCH510+Hsp70-H22 antigen peptides. Following the treatment with MMC, the two factors could inhibit the tumor from 10^6 inoculative dose of H22. These results indicate that immuno-therapy is just effective on a small amount of tumor cells. The chemotherapeutic agent is the indispensable agent once the tumor load is high. Chemotherapeutic agents can kill tumor cells rapidly and powerfully, but not completely. Combination of anticancer drugs and immune modulators is necessary. And the combination of pCH510 and Hsp70-peptides has been proved to be one of the choices for the treatment of residual tumor after chemotherapy.

In summary, recombinant polypeptide CH50 is a promising non-specific immune modulator. It can be expressed *in vivo* to inhibit experimental tumor growth. The inhibitory effect can be further improved by combining pCH510 with Hsp70-tumor antigen peptides. Furthermore, the two immune modulators can synergize with chemotherapeutic agents to treat tumor. But chemotherapeutic agents damage the host immune sysytem, so that immuno-therapy has no effect within 3 or 5 days after chemotherapy. For different immuno-therapy agents, different chemotherapeutic drugs and different patients, the combining pattern is possibly different. Individualized treatment regimen should be adopted.

REFERENCES

1 **Kountouras J**, Boura P, Kouklakis G. Locoregional immunochemotherapy in hepatocellular carcinoma. *Hepatogastroenterology* 2002; **49**: 1109-1112

2 **Tang ZY**. Hepatocellular carcinoma-cause, treatment and metastasis. *World J Gastroenterol* 2001; **7**: 445-454

3 **Fan J**, Wu ZQ, Tang ZY, Zhou J, Qiu SJ, Ma ZC, Zhou XD, Ye SL. Multimodality treatment in hepatocellular carcinoma patients with tumor thrombi in portal vein. *World J Gastroenterol* 2001; **7**: 28-32

4 **Shi BM**, Wang XY, Mu QL, Wu TH, Liu HJ, Yang Z. Angiogenesis effect on rat liver after administration of expression vector encoding vascular endothelial growth factor D. *World J Gastroenterol* 2003; **9**: 312-315

5 **Shi M**, Wang FS, Wu ZZ. Synergetic anticancer effect of combined quercetin and recombinant adenoviral vector expressing human wild-type p53, GM-CSF and B7-1 genes on hepatocellular carcinoma cells *in vitro*. *World J Gastroenterol* 2003; **9**: 73-78

6 **Liu JW**, Tang Y, Shen Y, Zhong XY. Synergistic effect of cell differential agent-II and arsenic trioxide on induction of cell cycle arrest and apoptosis in hepatoma cells. *World J Gastroenterol* 2003; **9**: 65-68

7 **Wang X**, Liu FK, Li X, Li JS, Xu GX. Retrovirus-mediated gene transfer of human endostatin inhibits growth of human liver carcinoma cells SMMC7721 in nude mice. *World J Gastroenterol* 2002; **8**: 1045-1049

8 **Mohr L**, Geissler M, Blum HE. Gene therapy for malignant liver disease. *Expert Opin Biol Ther* 2002; **2**: 163-175

9 **Ruiz J**, Mazzolini G, Sangro B, Qian C, Prieto J. Gene therapy of hepatocellular carcinoma. *Dig Dis* 2001; **19**: 324-332

10 **Sangro B**, Qian C, Schmitz V, Prieto J. Gene therapy of hepatocellular carcinoma and gastrointestinal tumors. *Ann N Y Acad Sci* 2002; **963**: 6-12

11 **Kapila YL**, Niu J, Johnson PW. The high affinity heparin-binding domain and the V region of fibronectin mediate invasion of human oral squamous cell carcinoma cells *in vitro*. *J Biol Chem* 1997; **272**: 18932-18938

12 **Saiki I**, Yoneda J, Kobayashi H, Igarashi Y, Komazawa H, Ishizaki Y, Kato I, Azuma I. Antimetastatic effect by anti-adhesion therapy with cell-adhesive peptide of fibronectin in

combination with anticancer drugs. *Jpn J Cancer Res* 1993; **84**: 326-335

13 **Yoneda J**, Saiki I, Kobayashi H, Fujii H, Ishizaki Y, Kato I, Kiso M, Hasegawa A, Azuma I. Inhibitory effect of recombinant fibronectin polypeptides on the adhesion of liver-metastatic lymphoma cells to hepatic sinusoidal endothelial cells and tumor invasion. *Jpn J Cancer Res* 1994; **85**: 723-734

14 **Norris DA**, Clark RA, Swigart LM, Huff JC, Weston WL, Howell SE. Fibronectin fragment (s) are chemotactic for human peripheral blood monocytes. *J Immunol* 1982; **129**: 1612-1618

15 **Doherty DE**, Henson PM, Clark RA. Fibronectin fragments containing the RGDS cell-binding domain mediate monocyte migration into the rabbit lung. A potential mechanism for C5 fragment-induced monocyte lung accumulation. *J Clin Invest* 1990; **86**: 1065-1075

16 **Hauzenberger D**, Klominek J, Sundqvist KG. Functional specialization of fibronectin- binding β1-integrins in T lymphocyte migration. *J Immunol* 1994; **153**: 960-971

17 **Harler MB**, Wakshull E, Filardo EJ, Albina JE, Reichner JS. Promotion of neutrophil chemotaxis through differential regulation of β1 and β2 integrins. *J Immunol* 1999; **162**: 6792-6799

18 **Zhang G**, Feng Z, Zhang H, Fan Q, Li D. Augmentation of recombinant fibronectin polypeptide CH50 on the antitumor function of macrophages. *J Tongji Med Univ* 1998; **18**: 5-9

19 **Li D**, Feng Z, Ye S, Zhang G, Zhang H, Huang B, Xiao H. Construction and expression of eukaryotic expressing vector pCH510 of polypeptide CH50 and its chemotaxis and antitumor function by *in vivo* transfection. *J Tongji Med Univ* 2001; **21**: 1-5

20 **Feng ZH**, Huang B, Zhang GM, Li D, Wang HT. Investigation on the effect of peptides mixture from tumor cells inducing anti-tumor specific immune response. *Science in China* 2002; **45**: 361-369

21 **Van Golen KL**, Bao L, Brewer GJ, Pienta KJ, Kamradt JM, Livant DL, Merajver SD. Suppression of tumor recurrence and metastasis by a combination of the PHSCN sequence and the antiangiogenic compound tetrathiomolybdate in prostate carcinoma. *Neoplasia* 2002; **4**: 373-379

22 **Saito N**, Mitsuhashi M, Hayashi T, Narumo C, Nagata H, Soyama K, Kameoka S, Harumiya S, Fujimoto D. Inhibition of hepatic metastasis in mice treated with cell-binding domain of human fibronectin and angiogenesis inhibitor TNP-470. *Int J Clin Oncol* 2001; **6**: 215-220

23 **Markowicz S**, Skurzak HM, Walewski J. A method for directly determining the number of dendritic cells and for evaluation of their function in small amounts of human peripheral blood. *Arch Immunol Ther Exp* 2001; **49**: 51-57

24 **Hanahan D**, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. *J Clin Invest* 2000; **105**: 1045-1047

25 **Klement G**, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Kerbel RS. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* 2000; **105**: R15-24

26 **Vacca A**, Iurlaro M, Ribatti D, Minischetti M, Nico B, Ria R, Pellegrino A, Dammacco F. Anti angiogenesis is produced by nontoxic doses of vinblastine. *Blood* 1999; **94**: 4143-4155

27 **Bello L**, Carrabba G, Giussani C, Lucini V, Cerutti F, Scaglione F, Landre J, Pluderi M, Tomei G, Villani R, Carroll RS, Black PM, Bikfalvi A. Low-dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth *in vivo*. *Can cer Res* 2001; **61**: 7501-7506

28 **Ng CP**, Bonavida B. A new challenge for successful immunotherapy by tumors that are resistant to apoptosis: two complementary signals to overcome cross-resistance. *Adv Cancer Res* 2002; **85**: 145-174

29 **Wang RF**, Rosenberg SA. Human tumor antigens for cancer vaccine development. *Immunol Rev* 1999; **170**: 85-100

30 **Heiser A**, Maurice MA, Yancey DR, Wu NZ, Dahm P, Pruitt SK, Boczkowski D, Nair SK, Ballo MS, Gilboa E, Vieweg J. Induction of polyclonal prostate cancer-specific CTL using dendritic cells transfected with amplified tumor RNA. *J Immunol* 2001; **166**: 2953-2960

31 **Arnold-Schild D**, Hanau D, Spehner D, Schmid C, Rammensee HG, de la Salle H, Schild H. Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J Immunol* 1999; **162**: 3757-3760

32 **Castellino F**, Boucher PE, Eichelberg K, Mayhew M, Rothman JE, Houghton AN, Germain RN. Receptor-mediated uptake of antigen / heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways. *J Exp Med* 2000; **191**: 1957-1964

33 **Basu S**, Binder RJ, Ramalingam T, Srivastava PK. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70,and calreticulin. *Immunity* 2001; **14**: 303-313

34 **Blachere NE**, Li Z, Chandawarkar RY, Suto R, Jaikaria NS, Basu S, Udono H, Srivastava PK. Heat shock protein-peptide complexes, reconstituted *in vitro*, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J Exp Med* 1997; **186**: 1315-1322

35 **Castelli C**, Ciupitu AM, Rini F, Rivoltini L, Mazzocchi A, Kiessling R, Parmiani G. Human heat shock protein 70 peptide complexes specifically activate antimelanoma T cells. *Cancer Res* 2001; **61**: 222-227

36 **Tamura Y**, Peng P, Liu K, Daou M, Srivastava PK. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* 1997; **278**: 117-120

37 **Timmerman JM**, Singh G, Hermanson G, Hobart P, Czerwinski DK, Taidi B, Rajapaksa R, Caspar CB, Van Beckhoven A, Levy R. Immunogenicity of a plasmid DNA vaccine encoding chimeric idiotype in patients with B-cell lymphoma. *Cancer Res* 2002; **62**: 5845-5852

38 **Wu Y**, Wan Y, Bian J, Zhao J, Jia Z, Zhou L, Zhou W, Tan Y. Phage display particles expressing tumor-specific antigens induce preventive and therapeutic anti-tumor immunity in murine p815 model. *Int J Cancer* 2002; **98**: 748-753

39 **Ishii T**, Udono H, Yamano T, Ohta H, Uenaka A, Ono T, Hizuta A, Tanaka N, Srivastava PK, Nakayama E. Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. *J Immunol* 1999; **162**: 1303-1309

40 **Belli F**, Testori A, Rivoltini L, Maio M, Andreola G, Sertoli MR, Gallino G, Piris A, Cattelan A, Lazzari I, Carrabba M, Scita G, Santantonio C, Pilla L, Tragni G, Lombardo C, Arienti F, Marchiano A, Queirolo P, Bertolini F, Cova A, Lamaj E, Ascani L, Camerini R, Corsi M, Cascinelli N, Lewis JJ, Srivastava P, Parmiani G. Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96-peptide complexes: clinical and immunologic findings. *J Clin Oncol* 2002; **20**: 4169-4180

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