• LIVER CANCER •

# Antitumor immunopreventive and immunotherapeutic effect in mice induced by hybrid vaccine of dendritic cells and hepatocarcinoma *in vivo*

Jin-Kun Zhang, Jun Li, Juan Zhang, Hai-Bin Chen, Su-Biao Chen

**Jin-Kun Zhang, Jun Li, Juan Zhang, Hai-Bin Chen, Su-Biao Chen**, Cancer Pathology Laboratory, Shantou University Medical College, Shantou 515031, Guangdong Province, China

**Correspondence to:** Prof. Jin Kun Zhang, Tutor of Doctor, Cancer Pathology Laboratory, Shantou University Medical College, 22 Xinlinglu, Shantou 515031, Guangdong Province, China. jkzhang@stu.edu.cn
**Telephone:** +86-754-8900443    **Fax:** +86-754-8557562

## Abstract

**AIM:** To develop atumor vaccine by fusion of H22 hepatocarcinoma cells and DC, and to study its protective and therapeutical effect against H22 cell.

**METHODS:** H22-DC vaccine was produced by PEG fusion of H22 and DC induced by cytokine released from splenic mononuclear cells, sorted by CD11c magnetic microbead marker. It was injected through the tail vein of the mice and the $H_{22}$-DC oncogenesis was detected in the liver, spleen and lung. In order to study the therapeutical and protective effect of $H_{22}$-DC against tumor $H_{22}$, two groups were divided: immune group and therapeutic group. Immune group was further divided into P, D, HD and H subgroups, immunized by PBS, DC, $H_{22}$-DC and inactivated $H_{22}$, respectively, and attacked by $H_{22}$ cell. The tumor size, tumor weight, mice survival time and tumor latent period were recorded and statistically analyzed; Therapeutical group was divided into three subgroups of P, D and HD, and attacked by $H_{22}$, then treated with PBS, DC, and $H_{22}$-DC, respectively. Pathology and flow cytometry were also applied to study the mechanism how the $H_{22}$-DC vaccine attacked on the $H_{22}$ cell.

**RESULTS:** 1. No oncogenesis was found in spleen, lung and liver after H22-DC injection. 2. Hybrid vaccine immunized mice had strongest CTL activity. 3. In the immune group, latent period was longer in HD subgroup than that in P, H and D subgroup; and tumor size and weight were smaller in HD subgroup than that in P, H and D subgroup. 4. In therapeutic group, tumor size was smaller in HD subgroup than that in P, D subgroup.

**CONCLUSION:** 1. H22-DC tumor vaccine is safe without oncogenesis *in vivo*. 2. Hybrid vaccine can stimulate potent specific CTL activity against H22. 3. H22-DC vaccine has distinctive prophylatic effect on tumor H22 and can inhibit the tumor growth.

## INTRODUCTION

Tumor cells have low antigenicity and high antigen modulatory ability, by which tumor associated antigen or tumor specific antigen can't be efficiently presented to T cells and tumor specific killing T cell can't be efficiently activated[1-5]. Furthermore, as one of antigen presenting cells (APC), tumor cells are anergy to T cells because of their deficiency of costimulator. Dendritic cells (DC), as a potent professional APC, not only express MHC molecules and costimulators to present tumor antigen and activate tumor specific T cells, but also can directly activate NK cell to kill MHC molecule negative tumor cell in nonspecific immunity. So much attention was paid to DC for its potential application in antitumor immunity[6-10]. Hepatocarcinoma is a high malignant tumor, so it is an important assignment to find a suitable way to kill the hepatocarcinoma. Our previous work showed that hybrid vaccine could kill tumor cell *in vitro*[11-16]. In this report, we tried to find a way to apply the DC in the prevention and therapy of hepatocarcinoma.

## MATERIALS AND METHODS

### Materials

**Tumor cell line** $H_{22}$ cell line, constructed by Dalian medical university from the mouse ascite, was heterogeneous cells with higher declination to spread by lymph vessel.

**Animal** BALB/c mice were bought from laboratory animal center of antibiotic industrial institute in Sichuan china. All mice were male, specific pathogen free animals, with age of 6-8 weeks and weight of 15-20 g.

**Main reagents** Metrizamide was product of Amresco Company. Mini MACS cell sorter and CD11c MicroBeads marked antibody were products of Miltenyi Biotec GmbH Company. rmGM-CSF (granulocyte-macrophage colony-stimuting factor) was a product of R & D company. Polyethylene glycol (PEG) was purchased from Sigma Chemical Company. MTT solution was a product of Amresco Company.

### Methods

**Culture of tumor cells** $H_{22}$ cell was incubated in double-layer agarose culture medium (upper layer 0.3 %, base layer 0.6 %) with concentration of $5\times10^5$/L and cultured for 10 days. Single colony was selected and transferred to culture flask for expanding culture. Cells of the logarithmic stage were collected and conserved in frozen conditions for later use. 0.1 ml $1\times10^7$ $H_{22}$ cells were incubated subcutaneously at the right armpit, carcinogenesis *in vivo* was observed.

**Isolation of DC from spleen** Spleen of mice was grinded it into cell suspension, they were lysed by $NH_4Cl$, centrifugated by metrizamide gradient solution. Cells in the interface were collected, and cultured routinely by complete culture medium RPMI 1640 at 100 % humidity, 37 ℃, 50 mL· $L^{-1}$ $CO_2$ for 3 hours, then the suspended cells were removed and continued to be cultured for another 16hours, the non-adherent cells were collected, and adjusted to the concentration of $1\times10^9$/L, then

cultured in the complete medium with 200 mL· L$^{-1}$ NCS and rmGM-CSF (500 ng/L) for 5-7days[17].

**Fusion of DCs and H22** DCs and H$_{22}$ were washed by PBS for 3 times, then the mixture of these two kinds of cells (DCs: H$_{22}$=1:1) were centrifugated to remove the upper liquid gently and completely, the cell droplet was loosen by shaking, 500 mL· L$^{-1}$ PEG was added in the droplet in 37 ℃ water bath to fuse for 1 min, and simultaneously the tube was shook gently. D-hanks solution was added to terminate the fusion process for 5 min in 37 ℃ water bath, the upper liquid was removed by centrifugation, and the cell droplet was collected. the cell availability and number of fusion cells was detected by trypan blue staining.

**Screening and determination of hybrid cell** The fusion cells were marked by CD11c antibody, and sorted by Mini MACS to remove the CD11c− cells and the CD11c+ cells were collected and they were cultured in RPMI complete medium with rmGM-CSF (500 ng/L) and 200 mL· L$^{-1}$ NCS for 2-3 weeks[18].

**Carcinogenic effect of hybrid vacciney** *in vivo* Mice were divided into five groups of HD1group, HD2 group, D group, H group and P group. 0.1 ml hybrid vaccine was injected into tail veil of mice in the HD1group, mice in the other group were injected at right armpit subcutaneously with 0.1 ml 1-2×10$^7$/ml vaccine, H$_{22}$ cells+DC, H$_{22}$ cells and PBS, respectively. And, 14 days later, tissues of the injection site, spleen, liver and lung were isolated, the tumor weight was compared.

**Analysis of the CTL activity** 20 mice were classified to four groups of HD group, D group, H group and P group, each group had 5 mice. Mice were injected with 0.1 ml hybrid vaccine, DC, heat inactivated H$_{22}$ and PBS at the concentration of 1×10$^{10}$-2×10$^{10}$/L, twice every three days. All the mice were killed at 10 days after the last immunization and the spleen lymphocytes were separated and cultured under condition of complete medium with IL-2 (1×10$^5$u/L) and 100 mL· L$^{-1}$ FCS at saturated humidity, 37 ℃, 50 mL· L$^{-1}$CO$_2$ to induce the CTL. CTL and H$_{22}$ was mixed at 5:1 and 10:1 effector/target rate, respectively. In addition, there were a CTL control group, a H$_{22}$ control group and a culture medium control group. All specimens had 3 parallel wells on a 96-well culture plate. All were cultured under conditions of saturated humidity 37 ℃, 50 mL· L$^{-1}$CO$_2$ for 48 hours. MTT method was used to detect the CTL cytotoxity against H$_{22}$. Chief process was as below: adding 5 g/L MTT solution 20 ul in each well for 4 hour before the detection, then it was centrifugated to remove the free MTT; 150 ul DMSO was added to the cell droplet for 10 min to solve the MTT. Bio-Rad 350-uv automatic enzyme linker detector is used to detect the OD value at 570 wavelength[19]. All were repeated for 4 times.

**Protective effect of hybrid vaccine against H$_{22}$** 40 BALB/c mice were randomly divided into HD, D, H and P subgroups, ten mice in each subgroup, immunized by 0.1 mL hybrid vaccine, DC, heat inactivated H$_{22}$ and PBS, respectively, at concentration of 1×10$^9$/L, two times every three days by tail veil. Three days after the last immunization, all mice were injected by 1×10$^6$ H$_{22}$ at the right armpit subcutaneously. The mice weight, tumor formation, tumor size and day of mice death were recorded. 5 mice were randomly chose and killed on day 14, tumor tissue, lung tissue, liver tissue and lymph node of neck and armpit were sampled for routine paraffin embedded slice and HE staining. Other mice were kept observation for 50 days.

**Therapeutic effect of hybrid vaccine against H$_{22}$** 30 BALB/c mice were randomly divided into HD, D and P subgroups, ten mice in each subgroup. All mice was injected with H$_{22}$ tumor cell 1×10$^6$ subcutaneously at right armpit. 3 days later, tumor was formed in all mice which demonstrated the successful construction of tumor model. Mice in different groups were treated with 0.1 mL hybrid vaccine, DC, and PBS, respectively, at the concentration of 1×10$^9$/L, two times every three days, by the tail veil. The tumor weight and size were recorded each day. 5 mice were randomly chose and killed on day 14; pathological changes were observed in tumor tissue. The other mice were kept on feeding for 50 days.

### Statistical analysis
Data were analyzed by the method of ANOVA (SPSS 10.0 software).

## RESULTS

### Culture of tumor cell
H$_{22}$ grew *in vitro* in a suspended manner, single or integrated, its double proliferation time was 24 hours. H$_{22}$ cell can grow to form colony composed of hundreds of cells in 5-6 days, thousands cells in ten days on agarose cultured medium.

Incubation of 1×10$^6$ H$_{22}$ at right armpit subcutaneously *in vivo* can successfully induce tumor in all mice. Tumor grew aggressively to invade the surrounding tissue, tumor appeared necrosis when tissue bulk were big enough, even gangrenous impairment appeared on skin when tissue diameter was beyond 30 mm. Tumor tissue had integrated cell with few intercellular materials, had no funicular structure of normal liver. Tumor cells had distinctive cellular pleomorphism, nuclear pleomorphism, nuclear hyperchromatism and increased nuclear: cytoplasmic ratio (Figure 1).

### Morphology of spleen DC
DC was round and irregular with sharp and long, or blunt cell extension. Induced by rmGM-CSF for 5-7days, DC grew into a colony; outer cell of it had much extension. Sorted by Microbead marked CD11c, most of them were CD11c+.

### Carcinogenesis of Hybrid vaccine in vivo
No carcinogenesis appeared after hybrid vaccine was injected subcutaneously for 60 days, while H$_{22}$ was injected, tumor appeared in 100 % mice at site of injection. There was significant in tumor weight between the HD and H$_{22}$ subgroup (*P*<0.01). 1×10$^6$ hybrid vaccine was injected by tail veil, the mice' s spleen, lung and liver were sampled after 14 days, three slices were sectioned for each organ, no carcinogenesis appeared (Figure 2-3).

### Activity of spleen CTL
CTL activity of mice in HD subgroup was significantly higher than that of D, H and P subgroup (Figure 4).

### The protective effect in vivo hybrid vaccine against H$_{22}$ in different subgroup
**Observation of carcinogenesis** Mice of different subgroups in protective group were injected with H$_{22}$ cells, the tumor latent time ranked as P<H<D<HD subgroup (*P*<0.05). This demonstrated that the HD subgroup had delayed tumor latent time (Figure 5A); tumor size and tumor weight ranked as HD<D<H<P subgroup (*P*<0.05) (Figure 5B, C), which showed that mice in the HD subgroup had the lowest tumor size and tumor weight, and the D subgroup had secondly lowest tumor size and tumor weight. Survival time among different subgroups had no significant difference (*P*>0.05) (Figure 5D).

**Pathology of different subgroups** Macroscopic structure showed the tumor tissue was hard and adherenced to the surrounding tissue. Cutting face of tumor tissue was gray and diffused dot-like brown. Microscopic structure showed that tumor had dot like or sheet like necrosis (Figure 6A, B, C, D).
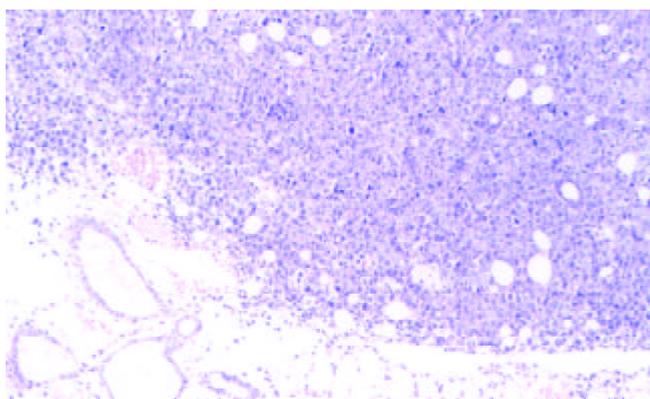
**Figure 1** Tumor tissue after 14 d subcutaneous incubation. HE, 3.3×10.
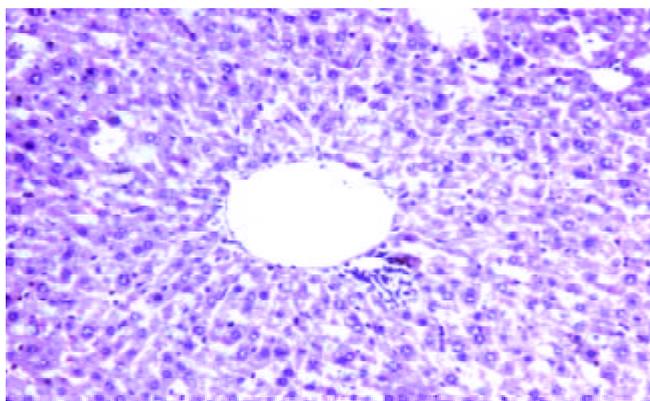


**Figure 2** Liver tissue of 14 d after hybrid vaccine injection by tail veil HE, 3.3×20.
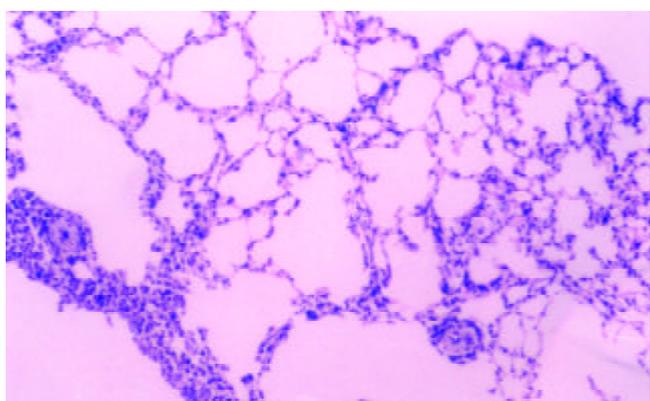


**Figure 3** Lung tissue of 14 d after hybrid vaccine injection by tail veil  HE, 3.3×20.
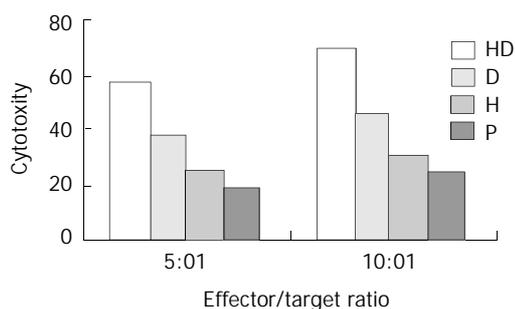


**Figure 4** CTL activity of different subgroup at different effector/target ratio.
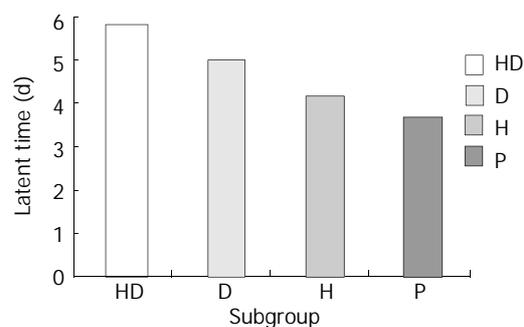


**Figure 5A** Comparison of latent time among different subgroups.
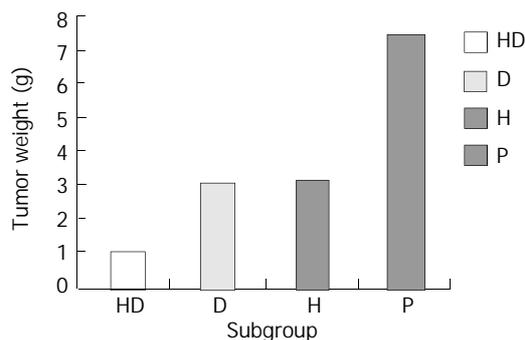


**Figure 5B** Comparison of tumor weight among different subgroups.
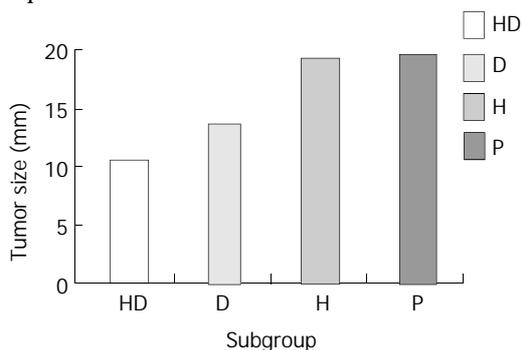


**Figure 5C** Comparison of tumor size among different subgroups.
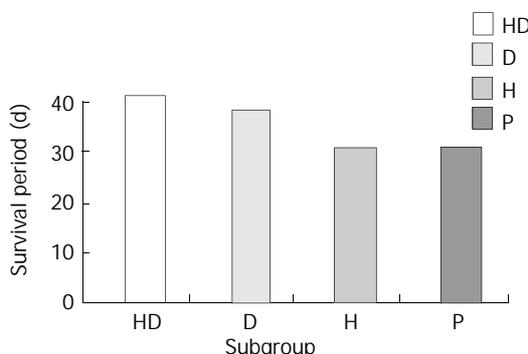


**Figure 5D** Comparison of survival period among different subgroups.

*Therapeutic effect of hybrid vaccine against H$_{22}$*

**Inhibitory effect of hybrid vaccine in carcinogenesis** Treated with hybrid vaccine, DC and PBS, tumor size in different subgroups ranked as HD<D<P ($P$<0.05) (Figure 7). Survival time and tumor weight had no significant difference among the different subgroups in therapeutic group.

**Pathology of different subgroups** Macroscopic structure

showed the tumor tissue was hard and can't separate with surrounding tissue easily. Cutting face of tumor tissue is gray and diffuse dot-like brown. Microscopic structure showed tumor has dot like or sheet like necrosis (Figure 8A, B, C).
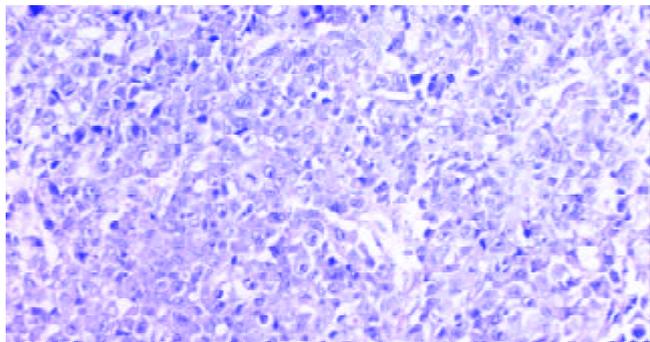


**Figure 6A**  There isn't obvious necrosis in tumor tissue of 14 d in pretective group P subgroup. HE, 3.3×20.
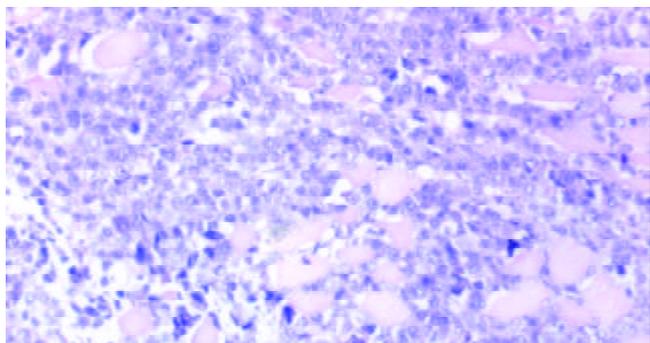


**Figure 6B**  There is dot like necrosis in tumor tissue of 14 d in pretective group H subgroup. HE, 3.3×20.
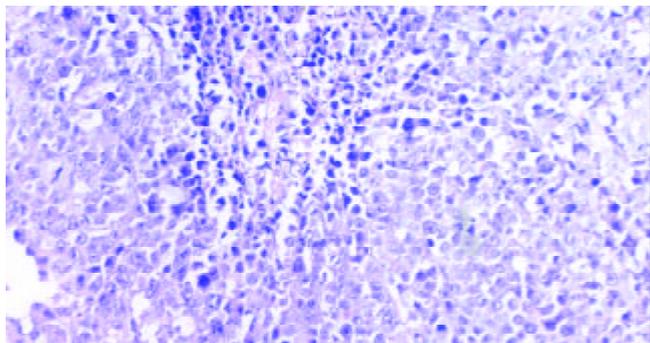


**Figure 6C**  There is sheet like necrosis in tumor tissue of 14 d in pretective group D subgroup. HE, 3.3×20.
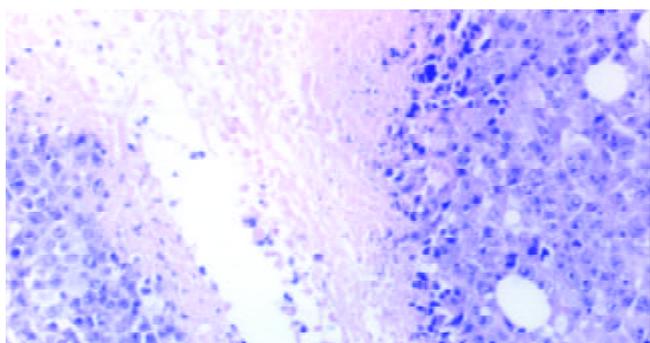


**Figure 6D**  There is extensive necrosis in tumor tissue of 14 d in pretective group HD subgroup. HE, 3.3×20.
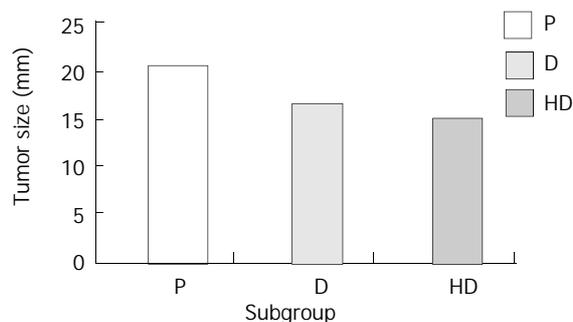


**Figure 7**  Comparison of tumor size on day 14 among different subgroups of therapeutic group.
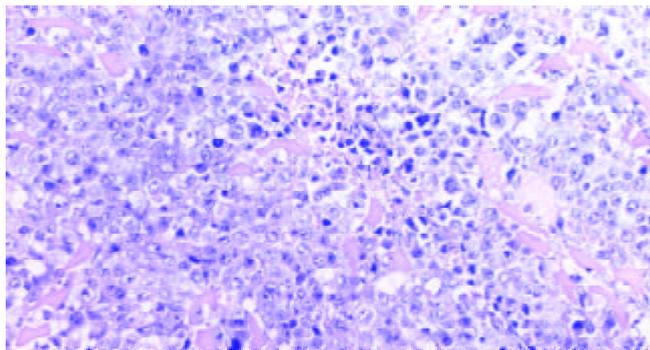


**Figure 8A**  There is dot or sheet like necrosis in tumor tissue of 14d in therapeutic group P subgroup. HE, 20×3.3.
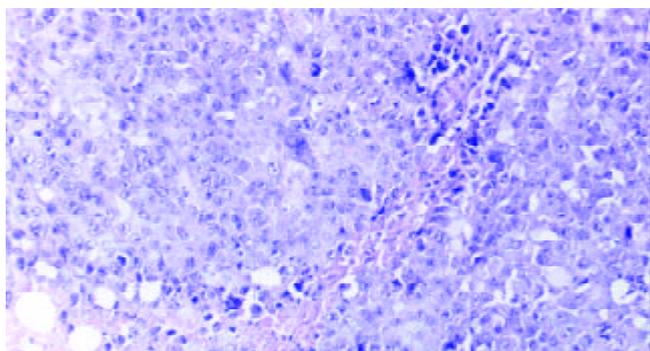


**Figure 8B**  There is sheet like necrosis in tumor tissue of 14 d in therapeutic group D subgroup. HE, 20×3.3.
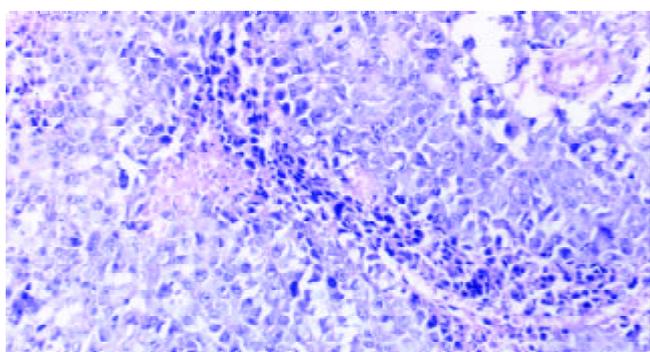


**Figure 8C**  There is sheet like necrosis in tumor tissue of 14 d in therapeutic group HD subgroup. HE, 20×3.3.

## DISCUSSION

$H_{22}$ ascite hepatocarcinoma has strong invasion and spreading declination by lymph vessel. In this report, $H_{22}$ cells had 100 %

carcinogenesis effect on BALB/c mice, which was consistent with other report[19]. H$_{22}$ cells grow quickly *in vitro*, which ccan be easily used to construct the tumor animal model, and express low level of MHC molecule. So H$_{22}$ cells are suitable for DC antitumor research.

In this report, DC was separated through centrifugation by metrizamide; the maturation of DC was induced by cytokine of rmGM-CSF; DC and H$_{22}$ were fused through PEG, they were marked by CD11c, and sorted by Mini MACS. By above process, the hybrid vaccine was successfully constructed[15,17,19,20]. The methods were simple and easily operated. Hybrid Vaccine, injected subcutaneously, has no tumor formation at local tissue, and injected by tail veil, it also has no carcinogenesis in the liver, lung and spleen, so it loses the carcinogenic effect of H$_{22}$ cells, and is safe to develop tumor vaccine *in vivo*.

In the protective group, different subgroups had different tumor latent time, and different interval between the H$_{22}$ incubation and tumor formation. The HD subgroup had the longest tumor latent time; the D subgroup had the second longest tumor latent time. The results showed that the hybrid vaccine and DC could delay the tumor carcinogenesis. The HD subgroup had the lowest tumor size and tumor weight, the D subgroup had the second lowest tumor size and tumor weight. The results showed that hybrid vaccine had the strongest anti-tumor effect. Hybrid vaccine could express some DC characteristics such as membrane molecule MHC-I, II molecule and costimulator, secrete some cell factors which can stimulate the T cell expansion, capture and present endogenous tumor associated antigens derived form parents H$_{22}$ cells, so it can efficiently stimulate the immune response[21-25]. There were similar results in other research reports on hybrid vaccines of DC with MC38, NS1, B16 melanoma, RMA-s lymphpma and renal carcinoma[26-35].

Mice immunized by hybrid vaccine could induce the tumor specific memory T cells, which could quickly be activated and expanded when they contact with tumor antigen again. When CD40 which was expressed by memory T cell integrated with CD40L of DC, it could stimulate DC to secrete high level of IL-12, and to enhance the expression of ICAM-1, CD80 and CD86. The activated DC could stimulate the proliferation of T cells and secretion of IFN-γ of T cells[36-39]. It was reported that IFN-γ gene modified H$_{22}$ cells could enhance the antigenicity and the expresseion of MHC-II molecule from 10 % to 19 %; TNF gene modified H$_{22}$ cell also could enhance the MHC-I molecule expression in different clones with different transfection methods which was enhanced to 28.39 % and 35.78 % in two clones which had the highest expression of MHC-I ; GM-CSF gene modified H$_{22}$ had lower oncogenicity *in vivo*, the possible mechanism attributed to the enhanced MHC expression[40-44].

Hybrid vaccine also takes part in nonspecific immunity; it can directly activate NK cells to kill MHC-tumor cells. Some reports demonstrated DC could produce large number of INF-γ, which can inhibit the virus replication and stimulate the NK and macrophages[45-47]. In this report, the hybrid vaccine antitumor function perhaps attributed to both adaptive and inborn immunity.

In the therapeutic group, tumor size in the HD subgroup was significantly lower than that in other subgroups after treated for 14 days, which showed that H$_{22}$-DC could inhibit the tumor growth. Some reported the similar effect. For example, after breast cancer cells hybridised with CD14$^+$ derived DC, $^3$H incorporation assay showed that hybrid cell can stimulate the proliferation T cells, while only tumor cell, DC and mixture of DC and tumor cell have no such effect[48-55].

From above, it is concluded that hybrid vaccine acquired the function of parents cell such as antigen presenting function of DC and T stimulating ability and capture the tumor derived antigen. Hybrid vaccine has the protective and therapeutic effect against H$_{22}$. This research can provide some evidence for the clinical application of tumor vaccine.

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