Effect of a cancer vaccine prepared by fusions of hepatocarcinoma cells with dendritic cells

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INTRODUCTION

T lymphocyte-mediated immune response plays an important role in the antitumor immune response. The sensibilization, activation and proliferation of T lymphocytes depend on antigen presenting cells (APC) which was able to present corresponding antigen peptides and to provide costimulatory signals[1]. However, many tumor cells have weak immunogenicity which expressed low levels or no MHC and costimulatory molecules, so that tumor antigen can not be effectively presented. Therefore, they cannot induce effective antitumor immune response in host, and can not effectively activate specific killing mechanism. APC cancer vaccines are expected to enhance the immunogenicity of tumor cells and increase the presenting ability of antigen presenting cells as well as induce effective specific T lymphocyte mediated antitumor immune response. Dendritic cells (DC) are a kind of the most potential antigen presenting cells (APC) which was able to present corresponding antigen peptides and to provide costimulatory molecules. As the immunogen, H₂₂-Dc was able to divide and proliferate in vitro, but its activity of proliferation was significantly decreased as compared with H₂₂ cells and its growth curve was flatter than H₂₂ cells. After subcutaneous inoculation over 60 days, H₂₂-DC showed no tumorigenecity in mice, which was significantly different from control groups (P<0.01). The spleen CTL activity against H₂₂ cells in mice implanted with fresh H₂₂-DC was significantly higher than control groups (P<0.01).

CONCLUSION

H₂₂-DC could significantly stimulate the specific CTL activity of murine spleen, which suggests that the fusion cells have already obtained the function of antigen presenting of parental DC and could present H₂₂ specific antigen which has not been identified yet, and H₂₂-DC could induce antitumor immune response; although simply mixed H₂₂ cells with DC could stimulate the specific CTL activity which could inhibit the growth of tumor in some degree, it could not prevent the generation of tumor. It shows that the DC vaccine is likely to become a helpful approach in immunotherapy of hepatocarcinoma.

Subject headings: cancer vaccine; dendritic cells; hepatocarcinoma cells; cell fusion; spleen; mouse

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Amresco Co. and PEG was from Sigma Co. Mouse hepatocarcinoma cell line (H22) was obtained from the Cancer Research Institute of Dalian Medical University.

**Methods**

**Isolation of DC** According to the previous METHODS[20-24] with minor modifications, DCs were isolated from murine spleen by metrizamide (145 g·L⁻¹) density gradient centrifugation, purified based on its characteristics of semi-adhesion to culture plates and FcR, and cultured in the medium containing GM-CSF and IL-4 (500 ng·L⁻¹), and a large number of DC were harvested.

**Cell fusion and selecting[25,26]** DCs were fused with H22 cells by PEG and the fusion cells were marked with CD11c MicroBeads. The H22-DCs were sorted with Mini MACS sorter. Fused cells were cultured in RPMI 1640 medium containing 20 mL·L⁻¹ fetal bovine serum, rmGM-CSF and rmIL-4 (500 ng·L⁻¹) for 2-3 wks.

**Cellular morphological analysis** Light microscopy and phase contrast microscopy were used to identify the morphological characteristics of H22-DC, H22 and DC.

**Immunocytochemical staining for CD80, CD86 and CD54** Cells were incubated with antibodies against CD80, CD86 and CD54. Membrane proteins were detected by ABC reagen and DAB staining, and photomicrographs were taken with an Olympus microphoto-microscope.

**Cell proliferation analysis in vitro** DCs were added into 24-well plates at 1.25×10⁴ cells per well and three wells were randomly selected to be counted every 24 h. Then, growth curve of H22-DC was drawn according to their average value, using H22 as control group at the same time.

**Tumorigenicity assays** This experiment was conducted in 3 groups. Each group included four experimental subgroups (H22-DC, H22+DC, H22, and PBS). Immunogen (0.1 mL 1×10¹⁰-2×10¹⁰·L⁻¹) of H22-DC was inoculated subcutaneously into the right armpit of H22-DC subgroup mice of each group and the same amount of H22, H22+DC and PBS were inoculated into the mice in each corresponding subgroup in the same way. The growth of tumors was observed every day and the survival time of mice was calculated. Meanwhile, mice in the second group were killed on the 14th day after implantation, and tumors were isolated and tumor weight was compared.

**CTL activity assays** The third group mice were killed for examination at the 10th day after implantation, and the spleen was separated to prepare cell suspension, then the cells were cultured in 100 mL·L⁻¹ FCS-RPMI1640 medium containing the final concentration of 100 KU·L⁻¹ rhIL-2 by genetic recombination at 37°C in a saturated humidified 50 mL·L⁻¹ CO₂ atmosphere for 3 days. The anti-tumor experiment was conducted in four subgroups. Two ratios of effect (CTL) to target (H22) (5:1 and 10:1) were used in all groups. (1) Group A: CTL (H22-DC subgroup) + H22; (2) group B: CTL (H22+DC subgroup) + H22; (3) group C: CTL (H22 subgroup) + H22; (4) group D: CTL (PBS subgroup) + H22. In addition, T groups were only consisted of CTL as the corresponding control groups and group E was H22 control group. Culture medium of the control group only contained 100 mL·L⁻¹ FCS-RPMI1640. All of these groups were cultured in 96-well culture plates and each group had 3 wells at 37°C in a saturated humidified 50 mL·L⁻¹ CO₂ atmosphere for 48 h. Cytotoxicity was determined by MTT assay as previously described[27,28]. Briefly, freshly prepared and filtered 20 μL·MTT (5 g·L⁻¹ in PBS) were added to each well, and the cells were continuously cultured for 4 h. Then the supernatant was removed and 150 μL DMSO was added to each well and agitated for 10 min to fully liquify crystals, followed by reading on BIO-RAD 3550-UV type automatic ELSIA reader at 570 nm wavelength.

**Statistical analysis**

Statistical analysis was made using analysis of variance, if P <0.05, the result was considered statistically significant.

**RESULTS**

**DC morphology**

DCs are irregular shaped cells with many surface membrane processes, including spiky or spherical pseudopod-like processes. They have oval or irregular-shaped nucleus with wavy movement. The cytoplasm contains rich spherical mitochondrial adrias. Determined by immunocytochemical staining, DCs were CD80 and CD86 positive cells with irregular shape and brown-yellow fine granules in cytoplasm (Figures 5-7).

**Figure 1** H22-DC growth curve.

**Figure 2** Tumor mass of BALB/c mice on d14 after inoculation.

**Figure 3** Influence on CTL cells of kill activity in vitro on d10 after inoculation.
Characteristics of H22-DC and H22 and sorting of fusion cells
Marked with CD11c MicroBeads and sorted with Mini MACs, H22-DCs were CD11c+ cells (Figures 8-10), but H22 was CD11c- cells. By immunocytochemical staining, H22-DCs were CD80, CD86 and CD54 positive cells and H22 was negative cells. Cytokine of rmGM-CSF and rmIL-4 was able to induce proliferation of fusion cells and prolong their survival time. The fusion cells which were marked with CD11c MicroBeads and sorted with Mini MACs were mixed with unfused DC. If there were no rmGM-CSF and rmIL-4 in the medium, natural apoptosis would occur in DC after 10-14 days, but H22-DC would be still alive.

**Identification and analysis of characteristics of fusion cells**
H22-DCs have some characteristics of both of their parental cells such as suspended growth, oval, flat and irregular in shape, they also have irregular shape nucleus and rich mitochondrias (Figure 4). Proliferation of H22-DC in incubation without rmGM-CSF and rmIL-4 showed slow growth and low activity. However, H22-DC incubation with rmGM-CSF and rmIL-4 was able to divide and proliferate, but compared with H22, its activity of division and proliferation was significantly decreased and their growth curve was flatter. After subcutaneous implantation over 60 days, no tumorigenesis was induced in H22-DC of mice, but induced tumorigenesis (100%) was observed in control subgroups (iH22+DC and H22 subgroup) (Figure 1). The tumor weight of H22+DC subgroup mice implanted on day 14 was significantly different from that of H22 subgroup ($P<0.01$, Figures 2, 11-13).

**CTL activity assays**
MTT assays showed that CTL activity of spleen in H22-DC group was significantly higher than that in H22+DC, H22 or PBS group ($P<0.01$, Figure 3).
DISCUSSION

Steinman and Cohn first isolated DC from the spleen of mice in 1973[20,21]. Since then, scholars have successfully isolated DC from thymus, aggregated lymph follicle, tracheas of mice, livers of rats and human peripheral blood. In recent years, mature DC was considered able to effectively present tumor-peptide epitopes and induce cytotoxic T lymphocytes (CTL) to produce strong specific antitumor immune response[22-24]. Wu and Kufe et al prepared DC vaccine using activated B cells and DC fused with tumor cells by traditional fusion METHODS in 1994 and 1997, respectively. This experiment was based on the established METHODs of isolation and generation of DC[25-27] by chemical fusion with PEG and techniques of immunomagnetic beads, it not only apparently simplifies the complicated sorting process of traditional fusion methods, but also effectively increase the purity of cell sorting. It is simple and feasible. The CD11c monoclonal antibody N418 is specific for the integrin αx subunit of αxβ2 which was the leukocytic integrin expressed on mouse splenic DC[28,29]. The principle of MACS CD11c+ cell sorting is that the cells are labelled by MicroBeads coupled with CD11c antibodies and passed through a sorting column which is placed in the magnetic field of a MACS sorter. The magnetically labelled CD11c+ DC are retained in the column while the unlabeled CD11c− cells passed away. After getting the column from the magnetic field, the magnetically retained CD11c+ DC can be eluted as the fraction of positively sorting cells. The effect of MACS sorter is confirmed by fluoroimmunoassay, PCR, FISH and FACS. The advantages of MACS are: it can process numerous cells, its sorting purity is very high, and it can be operated easily.

Much data in recent years show that DC played a very important role in the tumor immune response[30-32], especially with the development of gene therapy against tumor specific antigen. But at present, T cells epitopes of tumor specific antigens in most of the human cancers besides melanoma, breast cancer and ovarian cancer are not very clear[33]. Thus, cancer vaccine directly fused DC with tumor cells has become an important way in active immunotherapy of tumors[34-38]. It is simple and reliable and of practical value. At the same time, a tumor immunotherapy approach of specifically distinguishing and killing tumor cells but normal cells of host in vivo has developed[39-45].

By sorting with Mini MACS marked with mouse CD11c MicroBeads, H2−DCs have some characteristics of two parental cells, being irregular in shape. Apoptosis occurred in DC-DC and DC mixed with H2−DC respectively after 7-10 d and 10-14 d in the medium with no rmGM-CSF and rmIL-4, but H2+DCs were still alive. H2−DC could divide and proliferate quickly in the initial stage but soon their growth slowed down and their activity of dividing and proliferating reduced. We failed to establish the cell line in vitro, possibly due to the growth nature of the parental cells in vitro and loss of chromosome with the time of incubation.

After subcutaneous implantation over 60 days, H2−DC showed no induced tumorigenesis in BALB/C mice, but did it in H2 control group (100%). It suggested that H2−DC has lost its tumorigenicity in vivo. The tumor weight in H2+DC control group was significantly different from that of H2 control group when it had been implanted for 14 days ($P < 0.01$). It shows that DC simply mixed with tumor cells could obviously inhibit the development of tumor in the early stage, but could not prevent the generation of tumor, which means that DC played a positive role in the course of presenting tumor antigen and inducing specific antitumor immune response in the early stage of tumorigenicity. By selecting the spleen of mice in H2−DC, H2+DC and H2 group on d10 after implantation spleen CTL activity in vitro was induced in our experiment, and the results showed that the spleen CTL activity of H2−DC inoculated group was significantly higher than H2 inoculated group ($P < 0.01$), which suggests that the active immunity of cancer vaccine can produce specific antitumorimmunoprotection in mice. DC and H2−DC could induce specific antitumorimmune response and stimulate production of effective T lymphocytes in mice, and H2−DC induced no tumorigenesis. It indicates that DC directly fused with hepatocarcinomar cells is likely to become a helpful approach in immunotherapy for hepatocarcinoma.

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