Antitumor effect of immunization with fusion of dendritic cells and hepatocellular carcinoma cells in mice

Hao Zhang, Shu-Sen Zheng, Guo-Ping Jiang, Li-Hua Wu, Fen Zhu and Zhen-Lin Yang

Hangzhou, China

BACKGROUND: **Important advances have made within the past few years in the treatment of hepatocellular carcinoma (HCC), however, the long-term prognosis after resection of HCC remains unsatisfactory as a result of a high incidence of recurrence. This study was to investigate immunization with fusions of DCs and HCC cells against HCC tumors transplanted to mice.**

METHODS: **Fusion cells of dendritic cells (DCs) and H22 cells were prepared with polyethylene glycol. Expression of MHC and costimulatory molecules by dendritomas were determined by FACs. To study the antitumor immune preventitive and therapeutic effects, fusions were subcutaneously injected into naive or tumor-bearing mice; the CTL activity was assumed by the LDH method, and the expression of tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in tumors were assayed by reverse transcription-polymerase chain reaction (RT-PCR).**

RESULTS: **The hybridomas of DCs and H22 cells acquired both DCs and H22 cells phenotypes. Immunization of BALB/C mice with DC/H22 fusions induced protective immunity against a high dose of H22 tumor challenge. After treatment with hybridomas, the survival time of tumor-bearing mice was extended. The expression level of TNF-α and IFN-γ mRNA was markedly increased.**

CONCLUSION: **The hybridomas of DCs and H22 cells could induce effective antitumor immune responses and may be potentially used in prevention and management of recurrence and metastasis of HCC.**

(*Hepatobiliary Pancreat Dis Int 2004; 3: 235-240*)

KEY WORDS: **dendritic cells; hepatocellular carcinoma; cell fusion**

Author Affiliations: Department of Hepatobiliary Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China (Zhang H, Zheng SS, Jiang GP, Wu LH, Zhu F and Yang ZL)

Corresponding Author: Hao Zhang, MD, Department of Hepatobiliary Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310003, China (Tel: 86-571-87236570; Fax: 86-571-8723 6570; Email: haozh@zju.edu.cn)

This work was supported by Zhejiang Academican Foundation (203201513).

© 2004, Hepatobiliary Pancreat Dis Int. All rights reserved.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Africa and Asia. The long-term prognosis after resection of HCC remains unsatisfactory as a result of a high incidence of recurrence.[1-3] Although prevention and effective management of recurrence are the most important strategies to prolong the long-term survival,[4,5] knowledge has indicated that relapsed HCC is refractory to either chemotherapy or radiotherapy, and the effects of immunotherapy with interferon-α or interleukin-2 have been marginal,[6] which is why an alternative treatment is required.

Dendritic cells (DCs) are recognized as the most powerful antigen-presenting cells (APCS) with an unique ability to stimulate naive resting T cells and to initiate primary immune responses in vitro and in vivo.[7-9] With the prodigious antigen-presenting capabilities of DCs and the development of techniques for the generation of sufficient DCs, multiple methods have been used to load tumor antigens onto DCs for use as vaccines.[10-12] As a novel strategy, fusion of DCs with cancer cells has been effective in treating metastatic cancers.[13] In the current study, we investigated immunization with fusions of DCs and HCC cells against HCC tumors transplanted to mice.

Methods

Animals and cell lines

Five- to six-week-old female BALB/C (H_2-K^d) mice obtained from the Animal Resources Center of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (CAS) were maintained in specific pathogen-free conditions and used at the age of 6-8 weeks. The experiments employing the mice were performed in accordance with the institutional guidelines. H22 cells, which had been established as a BALB/C mouse origin HCC cell line, were purchased from China Center for Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L of glutamine, 100

μ/ml of penicillin and 100 μg/ml of streptomycin.

Reagents

Recombinant mouse granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), tumour necrosis factor-α (TNF-α) and mouse lymphotactin ELISA kit were obtained from R&D Company, USA. Fluorescence-conjugated rat monoclonal antibodies against murine (CD80, CD86, CD40, CD54, $CD11_C$, DEC205, $I\text{-}A^d$, $H_2\text{-}K^d$) were purchased from BD Pharmingen, USA. PKH-26, PKH-2 and 50% PEG (polyethylene glycol, 50% PEG/10% DMSO in PBS) were purchased from Sigma, USA. LDH cytotoxicity assay kit was purchased from Roche Company, USA.

Generation of dendritic cells from bone marrow

DCs were prepared according to the method described by Homma,[14,15] with modifications. Briefly, bone marrow cells were obtained from the femora and tibiae of female BALB/C mice. Red blood cells were lysed by treatment with 0.84% ammonium chloride solution. After being washed with PBS, the cells were plated in DMEM plus 10% FCS and 10 ng/ml of GM-CSF with conjunction of 10 ng/ml of IL-4. On day 3, nonadherent granulocytes, T and B cells were gently removed and fresh media were added. On day 5, loosely adherent proliferating DC aggregates were dislodged and re-plated in fresh media supplemented with 50 ng/ml of TNF-α. On day 7 of culture, released, nonadherent mature DCs were harvested.

Phenotypic analysis

After washing, DC and H22 cells were resuspended in PBS containing 1% of BSA, and stained with fluorescence-conjugated monoclonal antibody ($H_2\text{-}K^d$, $I\text{-}A^d$, CD80, CD86, CD40, CD54) or isotype control antibody for 30 minutes at 4 ℃. Stained cells were washed and analysed using a FACScan flow cytometer.

Cell fusion

DCs derived from bone marrow culture were fused with tumor cells at a 3:1 (DC: tumor) ratio using 50% PEG.[16] In brief, H22 cells were inactivated by 30 μg/ml of mitomycin, then washed and mixed with DC. After being washed with serum-free medium, 1 ml of PEG was added to the cell pellet for resuspending the cells by stirring for 2 minutes. An additional 10 ml of serum-free medium was added to the cell suspension over the next 3 minutes for continuous stirring. The cells were centrifuged at 400 × g for 5 minutes, and resuspended with RPMI-1640 medium supplemented with 20% FCS, 10 ng/ml of GM-CSF and 10 ng/ml of IL-4, cultured for 24 hours.

To determine the efficiency of cell fusion, H22 cells were stained with PKH-26 (red fluorescence) and DCs were stained with PKH-2 (green fluorescence). The cells stained with the fluorescence dyes were treated with PEG and cultured overnight as described above.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared by the Trizol method (Invitrogene) according to the manufacturer's instructions. cDNA was prepared from 1 μg of RNA by using a hexanucleotide random primer and M-MuLV reverse transcriptase (Fermentas) in a total volume of 20 μl. A volume of 2 μl was used for PCR amplification. The upstream primer for TNF-α was 5'-AGCCCACGT-AGCAAACCACCAA-3', and the downstream primer was 5'-ACACCCATTCCCTTCACAGAGCAAT-3', with an expected size of 446bp. The upstream primer for interferon-γ (IFN-γ) was 5'-TGGGGACTGAAG-TCCTAGAAG-3', and the downstream primer was 5'-TTACCCAGTCAGGGTTACTGCTGCTGTG-3', with a product size of 300bp. The upstream primer of mouse beta-actin was 5'-CTGGAGAAGAGCTATGAGC-3', and the downstream primer was 5'-TTCTGCATCC-TGTCAGCAATG-3', with a product size of 241bp. Amplification conditions for TNF-α were 2 minutes at 94 ℃ denaturation, followed by 35 cycles for 1 minute at 94 ℃ denaturation, annealing for 1 minute at 65 ℃, extention for 1 minute at 72 ℃ and a final extension step at 72 ℃ for 7 minutes. PCR conditions for IFN-γ were the same as for TNF-α except for annealing at 55 ℃ and 32 PCR cycles. PCR products were separated by 1.5% agarose containing ethidium bromide and the target bands were analyzed densitometrically using a Kodak science gel imaging system. Beta-actin was selected as internal control.

In vivo stimulation and CTL assay

Spleen cells were isolated from H22, DC, DC+H22 and DC/H22 vaccinated mice and enriched by nylon wool column. T lymphocytes from 4 mice in the same group were pooled and restimulated with irradiated H22 cells at a ratio of 20:1 for 5 days in RPMI-1640 medium containing 5 μ/ml of IL-2. After restimulation, the T lymphocytes were harvested and the activities of cytotoxic T lymphocytes were determined by measuring the specific cytotoxic activity against H22 cells in LDH assay at various effector/target (E/T) cell ratios. Shortly, after washing the effector and target cells with assay medium (RPMI-1640 with 1% BSA), the effector cells were co-cultured with target cells in a 96-well round bottom plate for 6 hours at 37 ℃, then the plate was centrifuged and the supernatant was transferred to another flat-bottom ELISA plate. One hundred μl of LDH detection mixture was added to each well and incubated in dark for 30 minutes at room temperature. Absorbance was measured by an ELISA reader at 490 nm. The spontaneous release of LDH by target cells or

effector cells was assayed by incubation of target cells in the absence of effector cells and vice versa, whereas the maximum release of LDH was determined by incubation of target cells in 1% Triton X-100 in assay medium. The percentage of cell mediated cytotoxicity was determined by the following equation: cytotoxicity (%) = [(effectors and targets mix-effectors control − spontaneous)/(maximum − spontaneous)] × 100.

Statistical analysis

The data were expressed as means ± standard deviation. Experimental results were analyzed using SPSS 10.0 statistical package. Differences among groups were assessed by Student's *t* test. A *P* value less than 0.05 was considered statistically significant.

Results

Preparation of DC/HCC fusion vaccine

The purity and phenotype of the bone marrow derived DCs were identified by labeling with a panel of Abs. The results showed that DCs derived from bone marrow culture were positive for H_2-K^d, I-A^d, CD80, CD86, CD40, CD54. The purity of DCs ($CD11_C^+$ cells) was greater than 80%; but the results confirmed that although H22 cells expressed moderate level of I-A^d, the expression levels of H_2-K^d, CD80, CD86, CD40 and CD54 were almost negative (Fig. 1). The hybrid DC/H22 cells acquired the phenotypes of both DC and H22 cells.

Fusion of DCs with syngeneic H22 cells by PEG was conducted at a DC to tumor ratio of 3:1; a fusion efficiency of 17%-26% was obtained reproducibly.

DC/HCC fusions up-regulating IFN-γ and TNF-α gene expression in tumors

To investigate the effect of DC/H22 fusions on IFN-γ and TNF-α gene transcription, tumors were harvested at day 14 after the first vaccine therapy. As shown in Fig. 2, the relative expression levels of IFN-γ and TNF-α mRNA in DC/HCC group were remarkably enhanced than in H22, DC and DC+H22 groups ($P < 0.05$), and IFN-γ and TNF-α mRNA expression levels in DC + H22 group were also higher than in H22 and DC groups ($P < 0.05$). This indicated that DC/H22 and DC+H22 vaccines could stimulate IFN-γ and TNF-α release from activated T cells (Table 1).

Fusions of DC/HCC induce antitumor activity

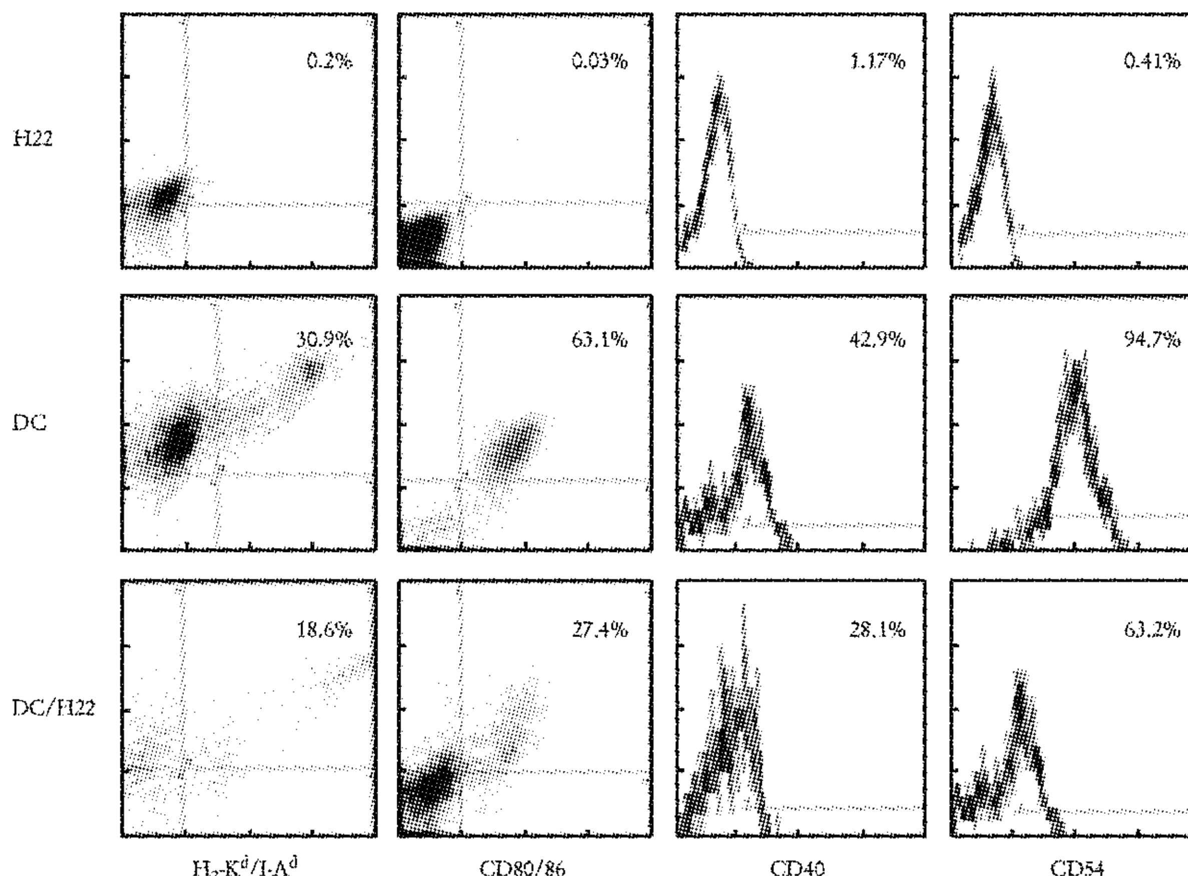

Fig. 1. FACS analysis of the phenotypes of DC, H22 and DC/H22 fusion cells.

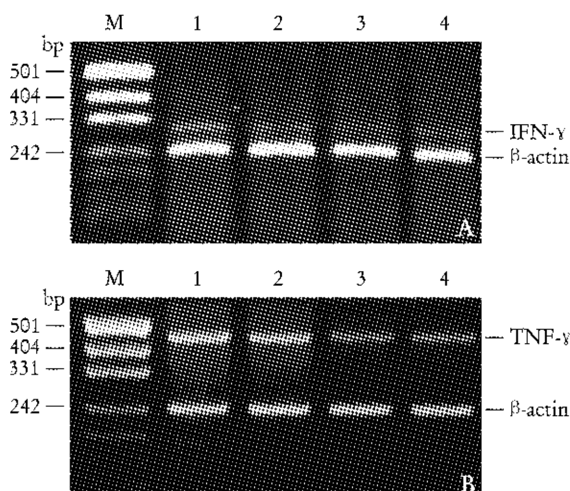

Fig. 2. Expression levels of IFN-γ (**A**) and TNF-α (**B**) mRNA. Line 1: DC/H22; Line 2: DC+H22; Line 3: DC; and Line 4: H22.

Table 1. Relative expression levels of IFN-γ and TNF-α mRNA in H22, DC, DC+H22 and DC/H22 groups (mean±SD)

Group	TNF-α/β-actin	IFN-γ/β-actin
H22	0.1403±0.0054	0.2053±0.0105
DC	0.1455±0.0051	0.2082±0.0075
DC+H22	0.2040±0.0066 *	0.2619±0.0192 *
DC/H22	0.2643±0.0167#	0.3741±0.0220#

*: $P<0.05$ vs H22 and DC groups; #: $P<0.01$ vs H22, DC and DC+H22 groups.

In order to assess the induction of antitumor immunity, mice were immunized with irradiated DCs, H22 cells, DC and H22 coculturing cells, and DC/H22 fusion cells for two times and then challenged with 1×10^5 viable H22 cells subcutaneously. All mice vaccinated with DC, H22, DC+H22 group cells rapidly succumed to their disease. In contrast, 2 mice immunized with DC/H22 fusion cells were protected against tumor challenge (Fig. 3A). In assessing treatment of established tumor, mice were first given 1×10^5 viable H22 cells on day 0 and then treated with H22, DC, DC+H22 and DC/H22 vaccines on day 2 and 8. Compared with H22, DC, DC+H22 groups, the DC/H22 group mice survived longer and half of them were tumor free (Fig. 3B). These results indicated that DC/H22 fusion vaccine could mediate marked antitumor effects.

Fusions of DC and H22 cells induced marked immunity against H22 cells

To assess whether immunization with DC/H22 fusion cells induces an anti-H22 immune response, mice were vaccinated subcutaneously twice with 5×10^5 irradiated H22, DC, DC+H22 and DC/H22 cells. Immunization with DC/H22 induced more marked cytotoxic immune response than in H22, DC and DC+H22 groups ($P<0.05$, Table 2).

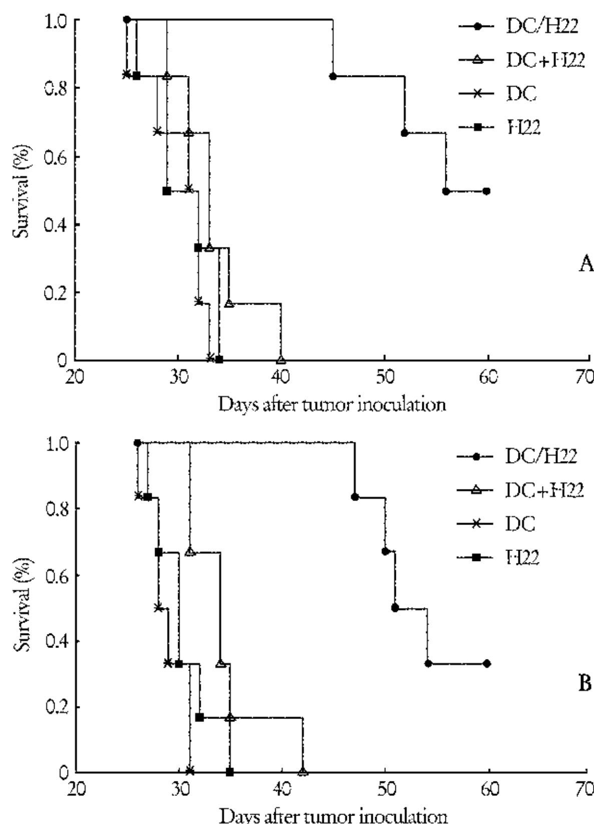

Fig. 3. Survival of mice after vaccination with H22, DC, DC+H22 and DC/H22 (**A**) or treatment of established tumor with above vaccine (**B**).

Table 2. CTL activity against H22 stimulated by different SC (E:T =50:1)

Group	CTL activity (mean±SD)
H22	0.246±0.017
DC	0.262±0.019
DC+H22	0.339±0.014 *
DC/H22	0.624±0.014#

*: $P<0.05$ vs H22 and DC groups; #: $P<0.01$ vs H22, DC and DC+H22 groups.

Discussion

The unique ability of DCs to take up, process, and present antigens and to activate naive $CD4^+$ and $CD8^+$ T cells makes them appropriate candidates for an experimental immune therapeutic approach. Several in vivo studies in mice as well as clinical phases I and phase II studies proved the marked efficacy of immunotherapy with monocyte-derived DCs.[17-20] Nevertheless, defined tumor specific antigens are rare or unknown in most of tumor entities, so clinical studies using DC vaccina-

tion protocols were mostly confined to melanoma and prostate cancer. In the current study, we demonstrated that fusions of DCs and HCCs not only increased the expression of TNF-α and IFN-γ in tumors, but more significantly, they mediated effective therapy of established tumors and induced detectable host protective immunity against HCC tumor challenge.

It has long been suspected that tumors have evolved multiple mechanisms to overcome the immune system in its role of immunosurveillance, which include down regulation of MHC and costimulatory molecules, alteration in the expression of tumor-associated antigen (TAA).[21,22] Our results also demonstrated that HCC cells express high levels of MHC I molecules, but there are rarely expression of MHC II, CD80, CD86, CD54, CD40 molecules, which may be the main mechanism of HCC immune escape. Furthermore, research has shown that HCC tumor could produce vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and IL-10 that can impair the maturation of DCs.[23] This is also an important mechanism of HCC resistance to the immune system.

Different strategies are available for animal experiments or clinical trials to deliver antigen to DC, such as pulsing synthetic or eluted peptides, transfection with cDNA or RNA encoding known as TAA, loading tumor lysates or tumor RNA.[24] But these approaches are currently limited for clinical application because few human tumor rejection antigens have been identified. The high polymorphism of the human HLA system has also made it difficult to identify tumor-associated peptides as a vaccine for cancer therapy. In addition, using tumor lysate or DNA/RNA loading method creats the risk of inducing immune responses against numerous self-antigens shared with normal cells.

Immunization of BALB/C mice with DC/H22 cells produces significant therapeutic or protective effect on HCC cells. It is conceivable that DC/H22 fusions injected subcutaneously reach the peripheral lymph node and spleen, and then T lymphocytes are primed through the MHC I and MHC II pathway for the tumor antigens presented by DCs. The activated $CD8^+$ T cells represent the main CTL population that effectively eliminates autologous H22 cells. In addition, proliferated $CD4^+$ T cells increase production and secretion of Th1 associated cytokines (TNF-α, IFN-γ, IL-2), which act as growth factors for activated T and NK cells, and facilitates specific cytotoxic T lymphocyte responses. Fusion of DCs with tumor cells is a novel and promising method in delivering tumor antigens into DCs.[25-27] The hybrid cells retain important characteristics of both APC and tumor cells. These include the endogenous expression of multiple tumor antigens and their presentation in context with high level of MHC I, II, and costimulatory molecules of the DC parter. Secondly, the fusion of tumor cells with DCs expressing the entire repertoire of tumor antigens (Ags) should allow immunization of host with multiple tumor Ags without knowing the identity of the tumor Ags.

Although DC and H22 coculturing group has less T cell stimulating activity than fusions, it still has more potent effect in inducing antitumor immunity than H22 or DC group. This is possible that some tumor cells secrete tumor antigen in the form of exosomes, and DCs could capture secreated tumor antigen and present it to host T cells.[28]

Li[29] reported that purified hybrid cells from DCs and tumor cell fusions had superior effects of antitumor immunity. Owing to potential difficulties in the preparation of sufficient pure fusion cell fraction for clinical use, in the present study, we did not purify the hybrid cells from the fusions as immunogen and use all of the PEG treated mixtures administered to mice. The tumor suppression was satisfactory.

Moreover, DCs were stimulated with TNF-α to obtain fully mature DCs before fusion. Recent data support this approach. Because immature DC could induce tolerance to antigens used for vaccination, infections of peptide-pulsed immature DC but not mature DC into healthy persons can result in antigen-specific inhibition of effector T-cell functions in vivo.[30] In contrast, mature DCs with high expression of MHC and costimulatory molecules, are resistant to the inhibitory effects mediated by IL-10 and other immunosuppressive compounds, and are of critical importance for the induction of tumor specific cytotoxic T cell mediated immunity.

In conclusion, the vaccination of mice with fusions of DCs and HCC cells induces effective antitumor activity that is characterized by activation of $CD8^+$ and $CD4^+$ T cells, which indicates that using hybridoma vaccines may be a promising strategy for effective prevention and management of recurrent HCC.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References

1 Tung-Ping Poon R, Fan ST, Wong J. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. Ann Surg 2000;232:10-24.
2 Shen BY, Li HW, Regimbeau JM, Belghiti J. Recurrence after resection of hepatocellular carcinoma. Hepatobiliary Pancreat Dis Int 2002;1:401-405.
3 Tang ZY. Small hepatocellular carcinoma: current status and prospects. Hepatobiliary Pancreat Dis Int 2002;1:349-353.
4 Sun XY, Wu ZD, Hu JB. Suicide gene therapy of hepatocellular carcinoma and delivery procedure and route of therapeutic

gene in vivo. Hepatobiliary Pancreat Dis Int 2002;1:373-377.
5 Blum HE. Molecular therapy and prevention of hepatocellular carcinoma. Hepatobiliary Pancreat Dis Int 2003;2:11-22.
6 Palmieri G, Montella L, Milo M, Fiore R, Biondi E, Bianco AR, et al. Ultra-low-dose interleukin-2 in unresectable hepatocellular carcinoma. Am J Clin Oncol 2002;25:224-226.
7 Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767-811.
8 Yi Q. Dendritic cell-based immunotherapy in multiple myeloma. Leuk Lymphoma 2003;44:2031-2038.
9 Turtle CJ, Hart DN. Dendritic cells in tumor immunology and immunotherapy. Curr Drug Targets 2004;5:17-39.
10 Dallal RM, Lotze MT. The dendritic cell and human cancer vaccines. Curr Opin Immunol 2000;12:583-588.
11 Ueno H, Tcherepanova I, Reygrobellet O, Laughner E, Ventura C, Palucka AK, et al. Dendritic cell subsets generated from CD34(+) hematopoietic progenitors can be transfected with mRNA and induce antigen-specific cytotoxic T cell responses. J Immunol Methods 2004;285:171-180.
12 Strome SE, Voss S, Wilcox R, Wakefield TL, Tamada K, Flies D, et al. Strategies for antigen loading of dendritic cells to enhance the antitumor immune response. Cancer Res 2002;15;62:1884-1889.
13 Kugler A, Stuhler G, Walden P, Zoller G, Zobywalski A, Brossart P, et al. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med 2000;6:332-336.
14 Homma S, Toda G, Gong J, Kufe D, Ohno T. Preventive antitumor activity against hepatocellular carcinoma (HCC) induced by immunization with fusions of dendritic cells and HCC cells in mice. J Gastroenterol 2001;36:764-771.
15 Siders WM, Vergilis KL, Johnson C, Shields J, Kaplan JM. Induction of specific antitumor immunity in the mouse with the electrofusion product of tumor cells and dendritic cells. Mol Ther 2003;7:498-505.
16 Soruri A, Fayyazi A, Neumann C, Schlott T, Jung T, Matthes C, et al. Ex vivo generation of human anti-melanoma autologous cytolytic T cells by dentritic cell/melanoma cell hybridomas. Cancer Immunol Immunother 2001;50:307-314.
17 Azuma T, Horie S, Tomita K, Takahashi T, Tanaka Y, Kashiwase K, et al. Dendritic cell immunotherapy for patients with metastatic renal cell carcinoma: University of Tokyo experience. Int J Urol 2002;9:340-346.
18 Ehtesham M, Kabos P, Gutierrez MA, Samoto K, Black KL, Yu JS. Intratumoral dendritic cell vaccination elicits potent tumoricidal immunity against malignant glioma in rats. J Immunother 2003;26:107-116.
19 Kaminski ER, Goddard RV, Prentice AG. Dendritic cells and their potential therapeutic role in haematological malignancy. Leuk Lymphoma 2003;44:1657-1666.
20 Ridolfi R, Ridolfi L, Petrini M, Fiammenghi L, Riccobon A. Dendritic cell vaccination and immunostimulation in advanced melanoma. Expert Rev Vaccines 2003;2:825-833.
21 Bremers AJ, Parmiani G. Immunology and immunotherapy of human cancer: present concepts and clinical developments. Crit Rev Oncol Hematol 2000;34:1-25.
22 Piemonti L, Zerbi A, Di Carlo V. Strategies for tumor immune escape. Drugs Today 2003;39:701-724.
23 Kakumu S, Ito S, Ishikawa T, Mita Y, Tagaya T, Fukuzawa Y, et al. Decreased function of peripheral blood dendritic cells in patients with hepatocellular carcinoma with hepatitis B and C virus infection. J Gastroenterol Hepatol 2000;15:431-436.
24 Moingeon P. Cancer vaccines. Vaccine 2001;19:1305-1326.
25 Gong J, Koido S, Chen D, Tanaka Y, Huang L, Avigan D, et al. Immunization against murine multiple myeloma with fusions of dendritic and plasmacytoma cells is potentiated by interleukin 12. Blood 2002;99:2512-2517.
26 Kjaergaard J, Shimizu K, Shu S. Electrofusion of syngeneic dendritic cells and tumor generates potent therapeutic vaccine. Cell Immunol 2003;225:65-74.
27 Sloan AE, Parajuli P. Human autologous dendritic cell-glioma fusions: feasibility and capacity to stimulate T cells with proliferative and cytolytic activity. J Neurooncol 2003;64:177-183.
28 Celluzzi CM, Falo LD Jr. Physical interaction between dendritic cells and tumor cells results in an immunogen that induces protective and therapeutic tumor rejection. J Immunol 1998;160:3081-3085.
29 Li J, Holmes LM, Franek KJ, Burgin KE, Wagner TE, Wei Y. Purified hybrid cells from dendritic cell and tumor cell fusions are superior activators of antitumor immunity. Cancer Immunol Immunother 2001;50:456-462.
30 Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 2001;193:233-238.

Received January 6, 2004
Accepted after revision March 24, 2004