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Full Length Research Paper

Vaccination with apoptosis colorectal cancer cell pulsed autologous dendritic cells in advanced colorectal cancer patients: Report from a clinical observation

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To investigate vaccination with apoptosis colorectal cancer (CRC) cell pulsed autologous dendritic cells (DCs) in advanced CRC, 14 patients with advanced colorectal cancer (CRC) were enrolled and treated with DCs vaccine to assess toxicity, tolerability, immune and clinical responses to the vaccine. No severe toxicity was observed and the vaccine was well tolerated. The levels of interleukin-2 (IL-2), interleukin-12 (IL-12) and interferon-γ (IFN-γ) significantly increased after DCs vaccination ($P < 0.05$). Mean of carcinoembryonic antigen (CEA) lever decreased from 99.5 to 71.4 ng/ml. Five patients showed a positive skin response to the apoptotic cells loaded DC vaccines in the delayed type hypersensitivity (DTH). In the five DTH positive patients, we found a patient with a lymphoglandulae coeliacae metastasis; temporary seroperitoneum regression was demonstrated by CT scan after full immunizations during 12 weeks. However, a rescan after 6 weeks showed new lesions on the abdomen as well as re-growth of the primary lesion. During the two years of clinical observation, all patients survived the first year, while the survival rate decreased from 80 to 20% during the second year owing to the advancement of CRC. In conclusion, the strategy for vaccination of apoptosis colorectal cancer cell pulsed-DCs may raise the clinical response of CRC patients by activating Th1 responses. Furthermore, indications of both immunologic and clinical effect were found in heavily pretreated patients with advanced colorectal cancer.

Key words: Dendritic cells, immunotherapy, colorectal cancer.

INTRODUCTION

Immunotherapeutic approaches to the treatment of cancer have evolved during the last decades. Since patients with colorectal cancer (CRC) have shown natural T-cell responses against their tumor, it is assumed that CRC might be targeted by immunotherapy. Such therapy has evolved from nonspecific immunotherapy to highly specific passive and active therapies (Burgdorf et al., 2009a). Of the different approaches proposed, immunotherapy based on dendritic cells (DCs) loaded with tumor anti-gens has shown to be promising. Numerous vaccine trials based on DCs have been carried out in CRC (Nagorsen and Thiel, 2006) as well as in a variety of other cancers (Cranmer et al., 2004). Encouraging results have been shown, primarily in malignant melanoma and non-Hodgkin's lymphoma. Melcher et al. (1998) have shown that necrotic cell death provides immunologic signals that recruit and activate DCs, whereas, apoptotic cell death requires additional signals to become immunogenic. These authors and others have suggested that, this may be due

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in part to the high expression of inducible heat shock proteins (HSP) found in necrotic, but not apoptotic cells. HSP have been described to participate in the immunologic defense against infectious diseases and cancers (Udono and Srivastava, 1993). Major roles of HSP in immunogenicity include chaperoning antigenic peptides6 and participating in antigen presentation and cross-presentation. HSP are often observed in fresh tumor cells, and HSP are reportedly released only from tumor lysates but not from apoptotic cells, (Somersan et al., 2001) though the authors admitted that secondary necrosis originating from apoptosis could release HSP. Various apoptosis-inducing agents, including ultraviolet B (UVB), influenza virus, ceramide, anti-Fas, semisynthetic dimerizing ligand AP2018713 and betulinic acid, have been exploited to produce apoptotic cells for experimental therapy. To date, no consensus has been reached on the efficacy of apoptotic cells in antitumor treatment. However, interesting studies have correlated HSP70 expression in apoptotic cells after HS (Feng et al., 2003) with therapeutic efficiency. Nevertheless, none of them has evaluated the concomitant effect of other expressed molecules following apoptosis induction. We thus suggest that, the different proapoptotic treatments may induce different levels of HSP expression and may therefore, alter the immune response. Hence, we investigated whether particular apoptosis-inducing agents could modulate HSP expression and increase the immunogenicity of cancer cells used as antitumor vaccines.

CRC is potentially curable by surgery alone, while oncological treatment modalities appear to prolong life for majority of the patients. Unfortunately, 20 to 60% of the patients with stage II-III CRC that have undergone intended curative surgery will subsequently relapse (Mosolits et al., 2005; Tebbutt et al., 2002). The further investigation is demanded to improve and combine with therapeutic options. Immunotherapy may play an important role in this setting and cancer vaccines based on DCs may have the potential as one of the modalities in future treatment options. In order to optimize immunotherapy, in this case, vaccines are based on DCs and in order to select the right treatment for the specific patient, it is necessary with an extensive registration and immune monitoring during investigational treatment. Hitherto, carcinoembryonic antigen (CEA) is the only recommended soluble biological marker in CRC (Duffy et al., 2007; Locker et al., 2006). Meanwhile, the CT scan and survival situation of patients were observed to explore the clinical response of DCs. The patients are evaluated in a classical phase I/II designed for immunologic and clinical response to explore the initial effect of vaccination with apoptosis colorectal cancer cell pulsed-DCs.

MATERIALS AND METHODS

Patients and eligibility criteria

The main criteria for patient inclusion in the phase 1 and 2 trial were: (1) Histologically proven metastatic or advanced colorectal cancer; (2) progressive disease and no standard systemic treatment indicated; (3) at least one measurable lesion or osteolytic bone metastasis; (4) high expression of the CEA; (5) World Health Organisation (WHO) performance status 0 to 2; (6) life expectancy of more than 3 months.

The main exclusion criteria included: (1) Evidence of brain metastasis; (2) use of immunosuppressive drugs such as steroid; (3) radiation therapy or chemotherapy within the prior 4 weeks; (4) significantly increased blood liver-enzyme level (> 2.5 x upper normal limit); (5) other malignancies. The study protocol as been approved by the Institutional Ethical Committees (310800024), China County and the Chinese Medicines Agency. Written consent was obtained from all patients at study entry.

Generation of DCs

All procedures were performed according to good laboratory practice standards approved by the Chinese Medicines Agency. The patients underwent unmobilized leukapheresis using a continuous flow blood cell separator for isolation of large scale mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated over the Ficoll, washed, suspended in culture medium (CM) (X-Vivo 15, 200 mM 2% L-glutamine,1% autologous heat-inactivated plasma) at 7×10^6 cells/ml and separated by 90 min adherence to plastic Corning dishes (Corning USA). Non-adherent cells were removed and adherent cells were subsequently cultured for 7 days with 250 U/ml rh-IL-4 (R&D, USA) and 1,000 U/ml GM-CSF (R&D, USA). Cells were harvested at the fifth day and loaded with heat shocked apoptosis tumor cells. The cells were added with 50 U/ml TNF-α for the maturation on day 6 for 24 h. Cells were washed twice, resuspended in 2 ml NS and transferred to a 3 ml insulin syringe for injection on day 7. Aliquots of 1.2-2.0×107 cells were frozen in 90% autologous serum, 10% dimethyl sulfoxide (DMSO) (Sigma USA). Sterility controls of DCs were negative at all times.

Generation of apoptotic cells

Colon cell lines HCT-116 were plated at 3 x 105 ml^{-1} and after 24 h culture at 37°C, cultured at 42°C for 2 or 4 h, followed by exposure to 10 µg/ml BA (Betulinic acid) for additional 24 h at 37°C. Annexin V and prodidium iodide (PI) staining were used to measure the death and apoptosis of tumor cells. Then, the cells were frozen at -80°C in complete medium containing 10% DMSO, as required.

DC phenotypic analysis

Aliquots of the cultured cells were subjected to phenotypic analysis at time of cryopreservation. The expressions of the cell surface antigens HLA-DR, CD11c, CD83, CD80 and CD86 were analyzed. Antibodies against HLA-DR, CD11c, CD83, CD80 and CD86 (BD, USA) were used together with relevant iso-type controls. For four-color analyses of DCs, cells were labeled simultaneously with lineage cocktail and four of the relevant DC cell surface markers. Flow cytometric analysis was performed on a FACS Calibur flow cytometer (BD Bioscience). Data were analyzed by using CellQuest software (BD Bioscience).

Treatment

Eligible patients were to receive a total of four immunizations with at least 1.6×10^7 cell shocked tumor cell pulsed autologous DCs. The first and second vaccinations were given weekly and the third and

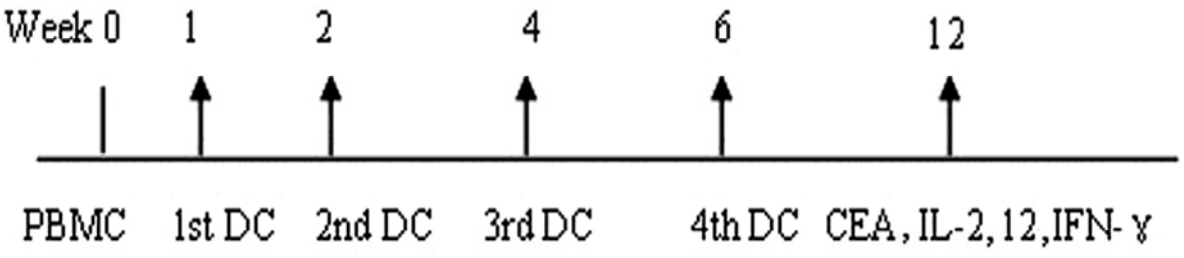

Figure 1. Detection of blood biomarker tested at 12 weeks.

fourth were given biweekly. The vaccine was administered subcutaneously (s.c.) near the inguinal region on the same thigh every time. The detection of blood biomarker were tested at 12 weeks (Burgdorf et al., 2009b, Dupont et al., 2005) (Figure 1).

Cytokine analyses

The cytokines IL-2, IL-12 and IFN-γ were analyzed in plasma using a multiplex platform (Luminex 100TM). Human extracellular protein buffer reagent kits (Invitrogen Corp, USA) were used and set up according to instructions from manufacturer. All samples were analyzed in triplicates.

Delayed-type hypersensitivity (DTH) response

To test the cell-mediated cytotoxicity response, 10 mg apoptosis tumor cells were administered intradermally into the forearm before and after treatment. A positive DTH skin-test reaction was defined as >5 mm diameter induration after 48 h.

Clinical monitoring

Patients receiving one vaccination or more were evaluated for toxicity and adverse events and patients receiving four vaccinations or more were evaluated for tumor response. With the exception of patients with osteolytic bone metastasis, all patients underwent response evaluation according to the RECIST criteria (James et al., 1999) at baseline. After the fourth DCs vaccination, CT scan was observed at 12 weeks. The survival situation was observed during the two years after DCs treatment.

Carcinoembryonic antigen (CEA) analyses

All the enrolled patients had a high expression of CEA. The level of CEA was tested before and after the DC vaccines. Levels of CEA were determined in serum using a commercially available enzyme linked immunosorbent assay (ELISA) platform (IBL, Immuno Biological Laboratories, Minneapolis, MN, USA).

Statistical analysis

Continuous variables were reported as medians (range). Changes in cytokine/protein levels of interleukin (IL)-2, IL-12, IFN-γ, CEA, were tested with T test. Graphs made for all analyses showed significant changes for a subgroup during treatment. P-values less than 0.05 were considered significant. All calculations were performed using the Statistical Package for the Social Sciences (SPSS) 11.0.

RESULTS

Patient characteristics

A total of 14 patients were enrolled; however, three quit prior to the second treatment and one after the third vaccination, either due to rapid disease progression or due to demonstration of extensive metastases. Prior treatment included operation and chemotherapy. The remaining 10 patients completed the full immunizations (Table 1).

DC phenotypic analysis

The cell percentage of $CD11c^+CD14^-$, $CD11c^+HLA^+DR^+$, $CD11c^+CD80^+$, $CD11c^+CD83^+$, $D11c^+CD86^+$ were (93.45 ± 1.25)%, (89.79 ± 1.35)%, (87.85 ± 1.62)%, (70.74 ± 6.45)% and (95.54 ± 2.18)%, respectively (Figure 2).

Generation of apoptotic cells

Colon cell lines HCT-116 were plated at 3 x 10^5/ml and after 24 h culture at 37°C, cultured at 42°C for 2 or 4 h, followed by exposure to 10 µg/ml BA (Betulinic acid) for additional 24 h and 48h at 37°C. Annexin V and PI staining revealed that, 65.8% of cells treated with Betulinic acid (BA) were apoptotic at 24 h post-inducing and 86.9% were apoptotic if induced for 48 h (Figure 3). The apoptotic cells of the control were less than 4%.

Immunological response

The levels of different cytokines were tested before and after the treatment. The levels of IL-2, IL-12 and IFN-γ increased significantly after the vaccination. (P values < 0.05) (Figure 4 a to c).

DTH response

The DTH was performed before and after treatment. Five of fourteen patients showed reactivity to the apoptosis

Table 1. Characteristics of the 14 patients enrolled for treatment.

Patient no.	Patient characteristics				
	Age	Gender	Primary lesion	Metastatic site	Prior treatment
1	52	Male	Rectum	Lung	OP+chemo
2	46	Male	Ascending colon	None	OP+chemo
3	71	Female	Descending colon	abdominal	OP+chemo
4	60	Female	Descending colon	Cavity liver	OP+chemo
5	53	Male	Ascending colon	Lung	OP+chemo
6	43	Female	Ascending colon	Liver	OP+chemo
7	70	Male	Descending colon	Bone	OP+chemo
8	64	Male	Descending colon	Liver	OP+chemo
9	52	Female	Ascending colon	None	OP+chemo
10	55	Female	Ascending colon	Nodi lymphaticicoeeliaci	OP+chemo
11	55	Male	Rectum	None	OP+chemo
12	51	Female	Ascending colon	None	OP+chemo
13	52	Male	Descending colon	Liver	OP+chemo
14	58	Male	Rectum	Lung	OP+chemo

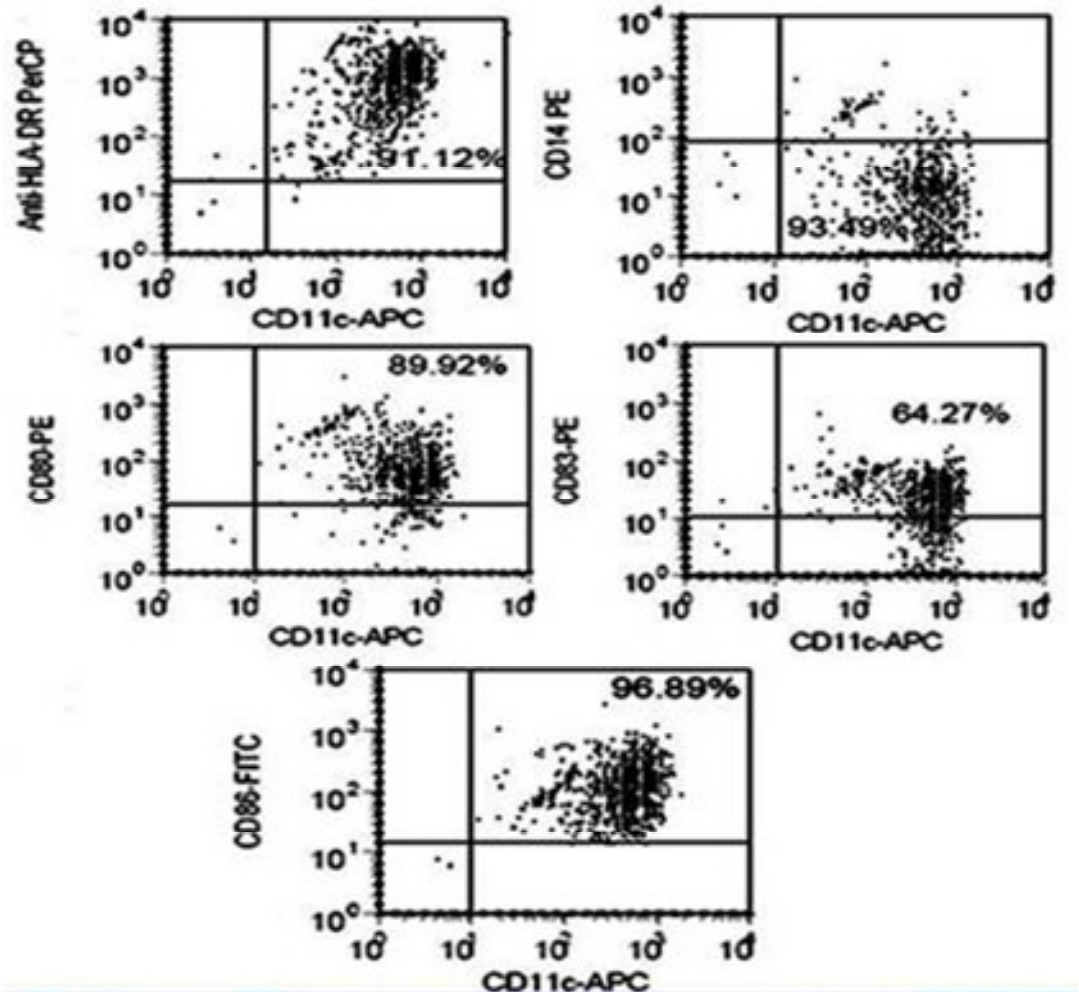

Figure 2. The cell percentage of CD11c+CD14-, CD11c+HLA+DR+, CD11c+CD80+, CD11c+CD83+, D11c+CD86+ were (93.45 ± 1.25)%, (89.79 ± 1.35)%, (87.85 ± 1.62)%, (70.74 ± 6.45)%, (95.54±2.18)%, respectively.

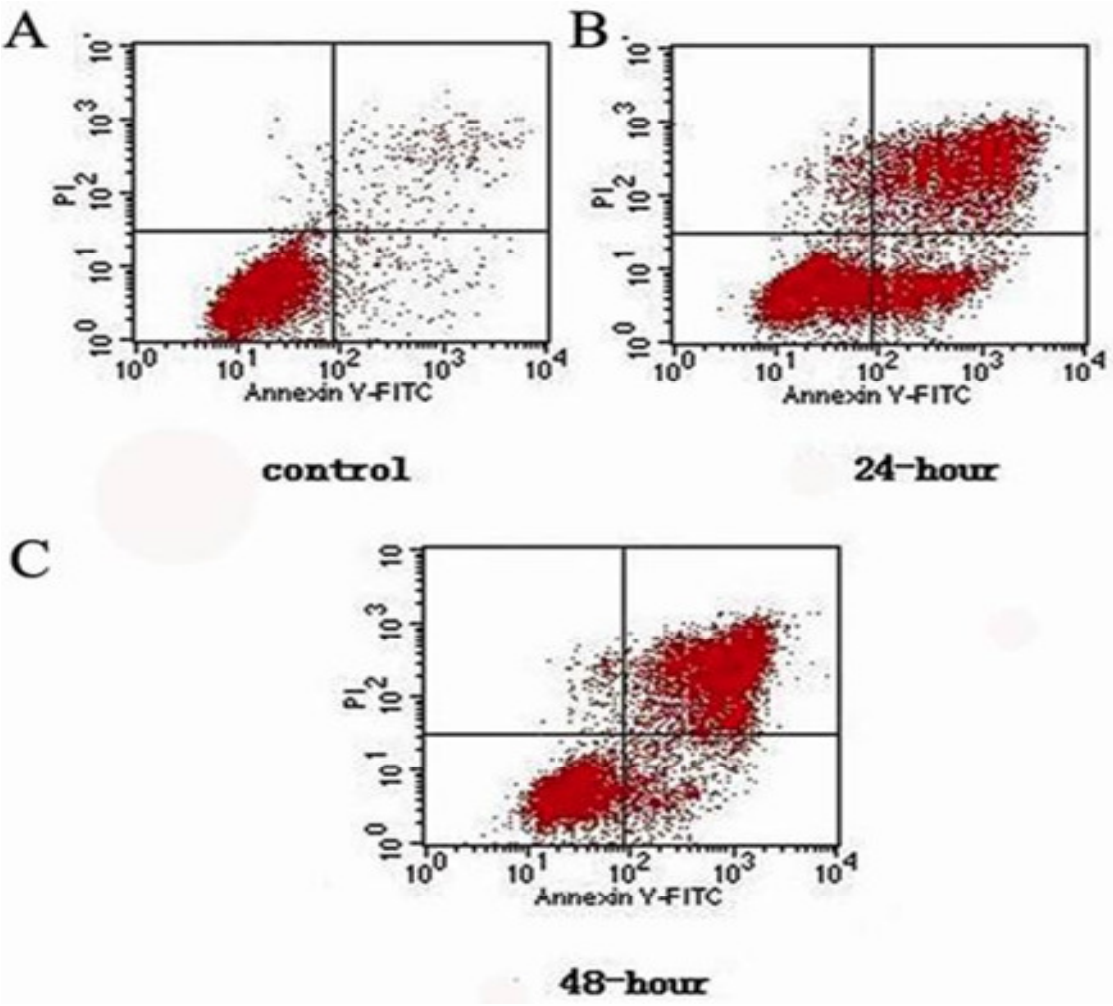

Figure 3. Annexin V and PI staining revealed that, 65.8% of cells treated with BA (Betulinic acid) were apoptotic at 24 h post-inducing, and 86.9% were apoptotic if inducing for 48 h. The apoptotic cells of the control were less than 4%.

tumor cells before and after vaccination (Figure 5). One of the five patients had a reaction on CT.

CEA analyses

The CEA lever was tested before the treatment and after the treatment at the 12th weeks. For patients with DCs treatment, there were significant differences in CEA levels ($p < 0.05$) (Figure 6)

Clinical response

During clinical observation, we found a patient with a lymphoglandulae coeliacae metastasis; temporary sero-peritoneum regression was demonstrated by CT scan after full immunizations at 12 weeks. However, a rescan after 6 weeks showed new lesions on the abdomen as well as re-growth of the primary lesion (Figure 7).

Survival rate

During the two years of clinical observation, all patients survived before the first year, while the survival rate was decreased from 80 to 20% during the second year owing to the advancement of CRC (Figure 8).

DISCUSSION

In this clinical study, 14 patients with CRC were treated with apoptosis tumor cells loaded DCs. No severe toxicity was observed during the whole treatment and the vaccine was well tolerated. Immune monitoring and key cytokines is an important step in the conduction of trials dealing with the effect of immunotherapy, including trials

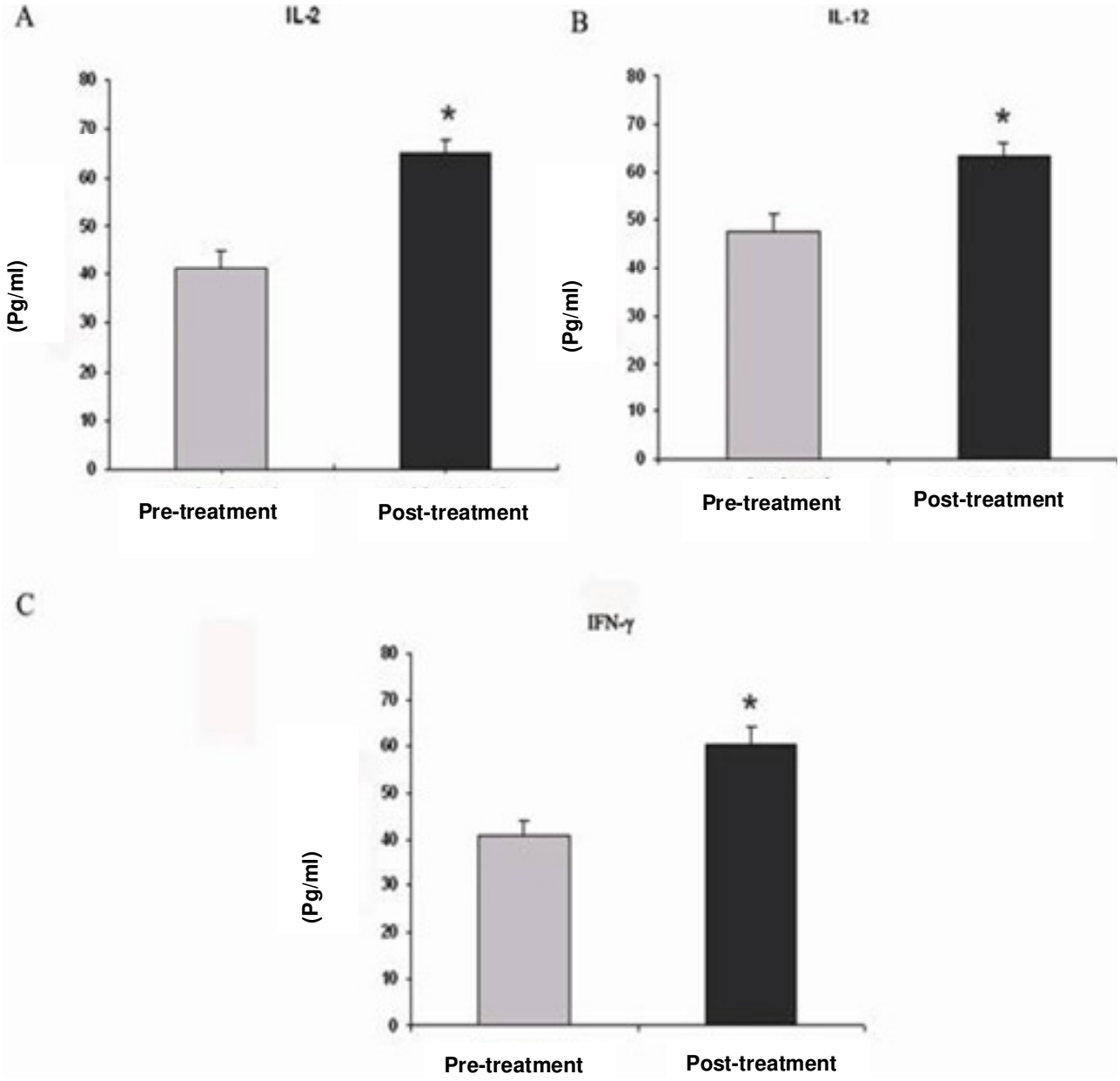

Figure 4. A to C, the levels of IL-2, IL-12 and IFN-γ increased significantly after the vaccination. (P values < 0.05).

in patients with malignant diseases. In the present study, we examined responses in blood cytokines during treatment with a cancer vaccine based on DCs loaded with apoptosis cell lines. We found the levels of IL-2, IL-12 and IFN-γ in the DCs vaccine group significantly increased and life time was delayed significantly ($P < 0.05$) after the vaccination. In the immune homeostasis of healthy individuals, both cellular and humoral immunological responses are tightly balanced during Th1 responses (Huang et al., 2007; Dranoff, 2004; Gutcher and Becher, 2007; Nembrini et al., 2006). In cancer immunotherapy, the general concept is that a Th1-dominant response directed against the tumor is favorable (Nishimura et al., 2000). The Th1 response may lead to activation of tumor specific CD8+ cytotoxic T-lymphocytes (CTL) capable of killing or impairing proliferation of tumor cells. The Th1 polarization of T-cells is driven by cytokines, primarily IFN, TNF-alpha, IL-2 and IL-12 (Gutcher and Becher, 2007). The polarization of T-cells induced by antigen presenting cells, of which the DCs is the most potent, requires three distinctive signals. The first signal is represented by the presentation of antigen on MHC molecules to T-cell receptors. The second signal is the co-stimulatory interaction between the antigen presenting cell and the T-cell. The third signal is the secretion of cytokines that directs the polarization of T-cells (Takagawa et al.,

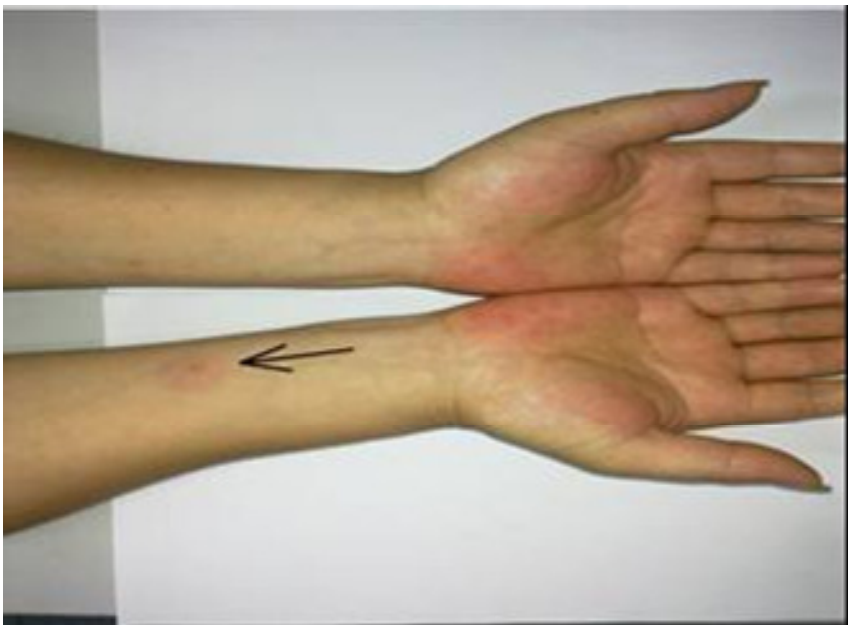

Figure 5. Five of fourteen patients showed reactivity to the apoptosis tumor cells before and after vaccination. One of the five patients had a reaction on CT.

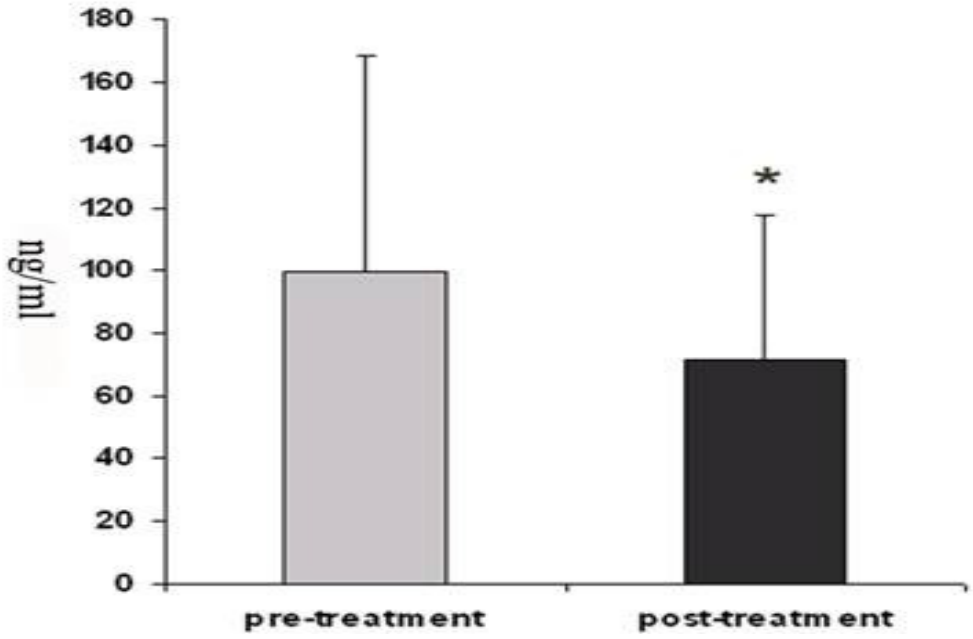

Figure 6. Mean of carcinoembryonic antigen (CEA) lever decreased from 99.5 to 71.4 ng/ml.

2008). The initial increases in IFN and IL-2, IL-12 could indicate a therapeutic potential of the vaccine because they promote a Th1-dominant immune response with cytotoxic activity. Five patients had a DTH positive response and one of the five patients had a regression of the lymphoglandulae coeliacae metastasis, the other four patients remain stable for more than 15 months.

CEA is a known tumor marker in colorectal cancer (Takagawa et al., 2008). It is widely used in daily clinical practice. For patients with DCs treatment in this study, mean of CEA lever decreased from 99.5 to 71.4ng/ml ($P < 0.05$). This observation supports that CEA might be used in the immunomonitoring during treatment with dendritic cell based vaccines. During clinical observation, we

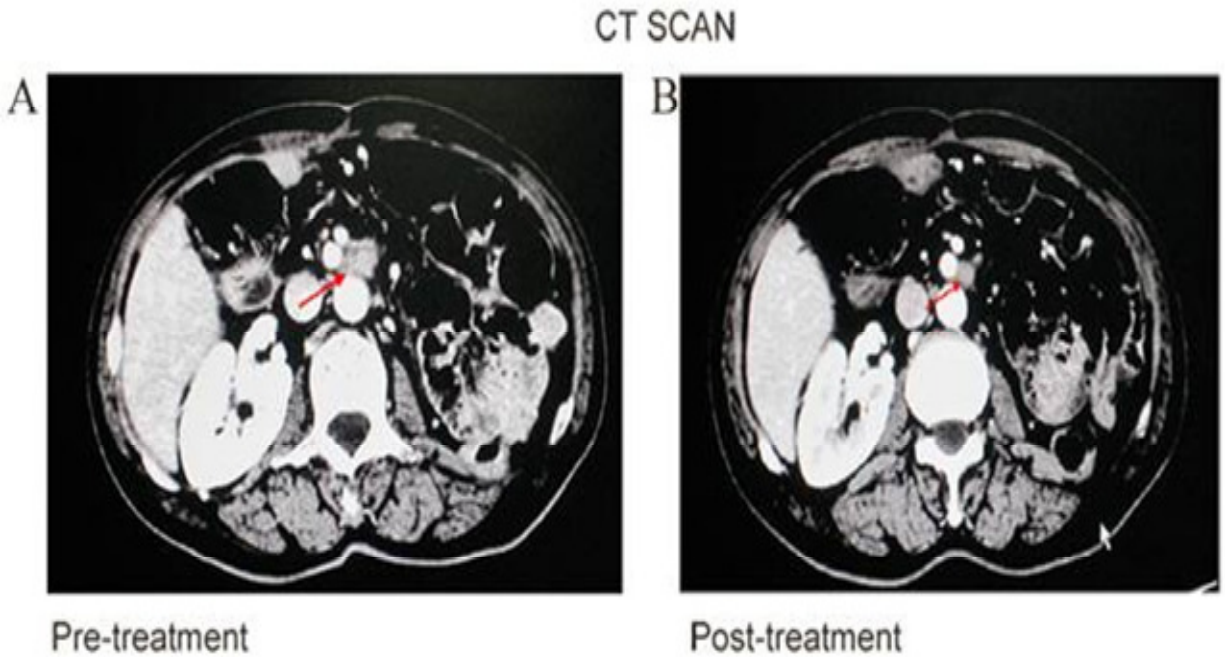

Figure 7. A patient with a lymphoglandulae coeliacae metastasis; temporary seroperitoneum regression was demonstrated by CT scan after full immunizations at 12 weeks.

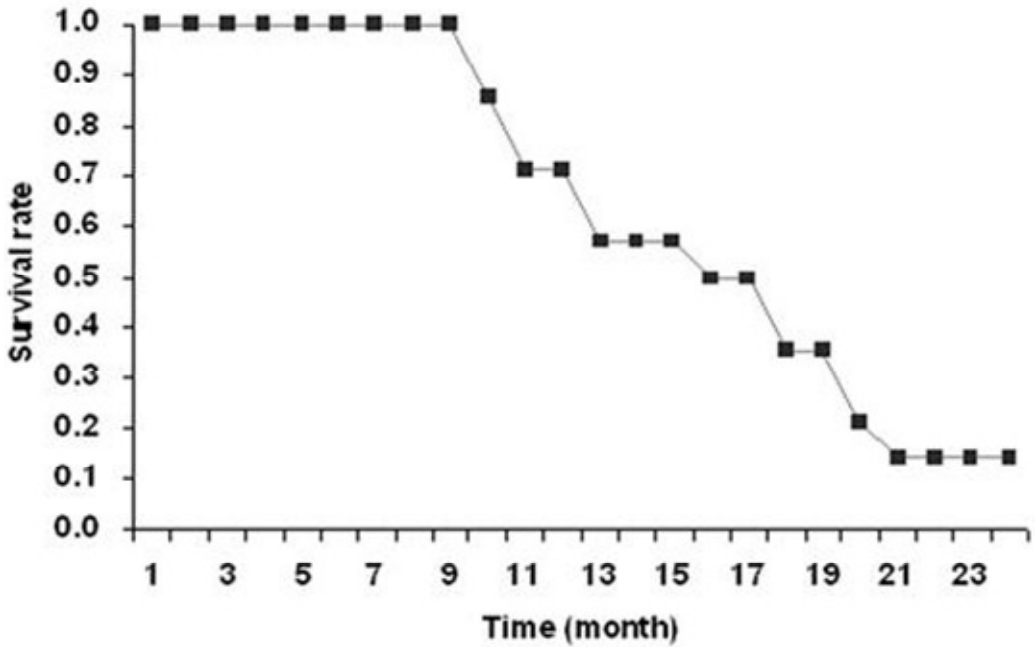

Figure 8. During the two years of clinical observation, all patients survived the first year, while the survival rate decreased from 80 to 20% during the second year owing to the advancement of CRC.

found a patient with a lymphoglandulae coeliacae metastasis; temporary seroperitoneum regression was demonstrated by CT scan after full immunizations at 12 weeks. All patients survived before the first year, while the survival rate was decreased from 80 to 20% during the second year owing to the advancement of CRC. Patients included in the trial had metastatic colorectal cancer often with a high tumor burden and were all heavily pretreated; as a consequence, significant tumor responses may not be obtainable despite activation of the Th1 immune system.

However, the clinical results obtained here encourage further clinical studies at an earlier stage of the disease. The fresh tumor specimen obtained from the surgery instead of the tumor cell line could be prepared for the antigen loading in the coming research, which could make more specific immunologic responses and stronger clinical responses.

ACKNOWLEDGEMENT

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Abbreviations

DCs, Dendritic cells; **CRC,** colorectal cancer; **IL,** interleukin-2; **IFN-γ,** interferon-γ ; CEA, carcinoembryonic antigen ; **DTH,** delayed type hypersensitivity; **PBMCs,** peripheral blood mononuclear cells; **CM**, culture medium; **BA**, betulinic acid; **PI,** prodidium Iodide; **HSP,** heat shock proteins; **WHO,** World Health Organisation; **DMSO,** dimethyl sulfoxide; **ELISA,** enzyme linked immunosorbent assay; **CTL,** cytotoxic T-lymphocytes.

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