Pathol. Oncol. Res. (2014) 20:357–365
DOI 10.1007/s12253-013-9704-3

RESEARCH

Autologous Dendritic Cell Based Adoptive Immunotherapy of Patients with Colorectal Cancer—A Phase I-II Study

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Received: 28 November 2012 / Accepted: 2 October 2013 / Published online: 28 October 2013

Abstract Dendritic cell-based active immunotherapies of cancer patients are aimed to provoke the proliferation and differentiation of tumor-specific $CD4^+$ and $CD8^+$ T-lymphocytes towards protective effector cells. Isolation and in vitro differentiation of circulating blood monocytes has been established a reasonable platform for adoptively transferred DC-based immunotherapies. In the present study the safety and tolerability of vaccination by autologous tumor cell lysates (oncolysate)- or carcinoembriogenic antigen (CEA)-loaded DCs in patients with colorectal cancer was investigated in a phase I-II trial. The study included 12 patients with histologically confirmed colorectal cancer (Dukes B2-C stages). Six of the patients received oncolysate-pulsed, whereas the other six received recombinant CEA-loaded autologous DCs. The potential of the tumor antigen-loaded DCs to provoke the patient's immune system was studied both in vivo and in vitro. The clinical outcome of the therapy evaluated after 7 years revealed that none of the six patients treated with oncolysate-loaded DCs showed relapse of colorectal cancer, whereas three out of the six patients treated with CEA-loaded DCs died because of tumor relapse. Immunization with both the oncolysate- and the CEA-loaded autologous DCs induced measurable immune responses, which could be detected in vivo by cutaneous reactions and in vitro by lymphocyte proliferation assay. Our results show that vaccination by autologous DCs loaded with autologous oncolysates containing various tumor antigens represents a well tolerated therapeutic modality in patients with colorectal cancer without any detectable adverse effects. Demonstration of the efficacy of such therapy needs further studies with increased number of patients.

Keywords Dendritic cell · Vaccination · Colorectal cancer · Autologous

Abbreviations

5-FU	5-Fluorouracil
AICD	Activation-induced cell death
AIMV	Serum free therapeutic grade cell culture medium
ALAT	Alanine amino transferase
AMA	Antimitochondrial antibody
ANA	Antinuclear antibody
APC	Antigen presenting cell

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APTI	Activated partial thromboplastin time
ASAT	Aspartate amino transferase
CA19.9	Cancer antigen 19.9
CEA	Carcinoembriogenic antigen
CRC	Colorectal carcinoma
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ECOG	Eastern cooperative oncology group/patient performance status
GM-CSF	Granulocyte-monocyte colony-stimulating factor
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFNγ	Interferon gamma
IL 12	Interleukin 12
IL 2	Interleukin 2
IL 1β	Interleukin 1beta
IL 6	Interleukin 6
INR	Internation normalized ratio (coagulation)
LDH	Lactate dehydrogenase
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
NCI	National Cancer Institute
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cells
PGE2	Prostaglandin E2
PHA	Phytohaemagglutinin
PI	Proliferation index
QLQ-C30	Quality of life questionnaire-Core 30
RBC	Red blood cell
SGOT	Serum glutamic oxaloacetate transaminase
SGPT	Serum glutamic pyruvate transaminase
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
Tc	T catalytic lymphocyte
Th	T helper lymphocyte
TIL	Tumor infiltrating lymphocytes
TNFα	Tumor necrosis factor-alpha
WBC	White blood cell

Introduction

Over the past decade it has become apparent that effector cells of both the innate and adaptive immune systems play an important role in the recognition and elimination of neoplastic cells. It has also become evident that human tumors express a wide array of protein antigens that can be recognized by the immune system [1]. The mechanism(s) by which various tumors evade immune recognition and the development of large tumor masses challenged the design of new immunotherapeutic protocols. Antigen-specific targeting and the concomitant potentiation of effector cells of the immune system to induce the regression of and/or long term protection against tumor development and metastasis belongs to these strategies.

The goal of active and specific immunotherapies of patients with advanced, recurrent or metastatic cancers is to prime tumor-associated antigen (TAA)-specific T-lymphocyte proliferation and differentiation towards protective effector cells, which are able to kill neoplastic cells with minimal systemic toxicity [2, 3]. Tumor antigens recognized by human T-lymphocytes were first identified in 1989 [4] followed by the description of a wide range of TAAs. This knowledge established the molecular background for the design of tumor-specific immunotherapies [5, 6], which utilized killed tumor cells, tumor-associated recombinant proteins or synthetic peptides comprising known MHC-restricted epitopes. Furthermore, co-administration of cytokines, which are able to enhance the differentiation and/or function of dendritic cells (DCs) and generation of T-cells has also been used for increasing the efficacy of tumor antigen-specific vaccination.

Circulating and tissue-resident dendritic cells serve as sentinels of the immune system. They continuously take up extracellular material including TAAs and apoptotic or necrotic tumor cells. Under inflammatory conditions, DCs get activated and become highly potent antigen presenting cells (APCs), which display their accumulated intracellular peptide content for both CD4+ helper (Th) and CD8+ cytotoxic/cytolytic (Tc/CTL) T-lymphocytes. Transfer of ex vivo manipulated autologous DCs to tumor-bearing hosts was shown to have the potential to stimulate tumor-specific immune responses and DCs loaded by apoptotic or necrotic tumor cells turned out to be more efficient than those pulsed with soluble tumor antigens [7]. Co-administration of cytokines promoting Th1 polarization, such as IL-12 [8], IFNγ [9] or IL-2 [10] could further enhance the therapeutic potential of DC-based vaccines. The role of DCs in human tumor immunity was also corroborated by the decline of circulating DC numbers and changes in the phenotypic and functional attributes of DCs in tumor bearing patients [11].

Isolation and in vitro differentiation of circulating blood monocytes, precursors of migratory and inflammatory DCs, has been established a reasonable platform for adoptively transferred DC-based immunotherapies. This strategy has been applied for the treatment of tumors of low immunogenicity, which included B-cell lymphomas and hematological tumors [12], prostate cancer, renal cell carcinoma [13], melanoma [14] and colorectal carcinoma (CRC) [15]. Initial studies utilized DCs pulsed with idiotypic proteins and/or major histocompatibility complex (MHC) class I-restricted peptides derived from prostate specific antigen, a number of melanoma-specific antigenic peptides

or crude tumor cell lysates derived from biopsy material, respectively. The completed studies demonstrated the safety, tolerability and in some cases the clinical efficacy of these approaches [16–18], but also indicated the variability of clinical efficacy of such immunotherapeutic regimens. It was also shown that DC-based cancer immunotherapeutic strategies appeared to be more effective against primary than advanced malignancies with high tumor burden or with metastasis. Nevertheless, these personalized immunotherapeutic strategies, in combination with refined surgical techniques, adjuvant radio- and/or chemotherapies offered novel adjunct to conventional anti-tumor therapies.

Colorectal carcinoma is the second most common human malignancy, the development of which requires a multistep process that involves genetic changes leading to neoplasia, and cancer-related inflammation supported by the reactive stroma, infiltrating leukocytes and their soluble factors [19]. Besides tumor infiltrating lymphocytes (TIL), tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC) and DCs are also abundant participants of the tumor tissue, and thus the interplay of the pro- and anti-tumor activities of these cell types has a great impact on disease outcome. In primary operable CRC systemic inflammatory responses to the tumor have been associated to a negative prognostic factor [20], whereas local inflammation around the tumor was linked to cancer survival [21].

In the present study patients suffering from Dukes B2-C primary operable CRC were selected for studying the effects of DC-based immunotherapy. The predicted relapse rate for such patients at 5 years is about 50 %, while the5-year survival rate is 67–46 % for colon cancer and 65–45 % for rectal cancer, respectively [22]. The primary objective of this study was to investigate the safety and tolerability of autologous whole tumor cell lysate- or recombinant carcinoembriogenic antigen (CEA)-loaded autologous DC vaccination in 12 patients with CRC. The secondary objective was the monitoring of possible autoimmune reactions and tumor-specific immune responses in the vaccinated patients. Long term follow up to detect the appearance and number of tumor recidivates was a tertiary objective.

Material and Methods

Clinical Studies

Study Permission This single center study was conducted at University of Debrecen, Medical and Health Science Center, Hungaryin accordance with the Declaration of Helsinki and the Edinburgh (2000) revisions; ICH Harmonized Tripartite Guideline for Good Clinical Practice, and Hungarian Laws and Regulations. The study was approved by the National Institute of Pharmacy (permission number: 29254/40/2003) and Central Ethical Committee (permission number: 51042-1/2003-1017 EKL) before prospective patients had been consented and screened.

Description of Patients Standard clinical diagnostic criteria were utilized to classify patients with CRC and 12 such patients with histologically confirmed tumors classified as stages Dukes B2-C, who satisfied all the inclusion and exclusion criteria, were enrolled in the study.

Inclusion Criteria

- Signed written informed consent prior to beginning specific protocol procedures, including expected cooperation of the patient for the treatment and follow up, must be obtained and documented according to local regulatory requirements.
- Age:17–70 years
- Histologically or cytologically confirmed colorectal carcinoma,
- Stage DUKES B2 or DUKES C,
- Expected survival minimum 12 months,
- ECOG: 0–2,
- Have clinically acceptable values on the laboratory screening tests and normal ECG.
- Hgb>90 g/l,
- Monocytes: 3.4–14 %

Exclusion Criteria

- Viral infections: HIV, hepatitis B, C
- Acute or chronic infectious disease
- Splenectomy
- Gravidity, lactation
- Allograft organ transplantation
- Heart attack within the last 5 years
- Uncontrolled or unstable diabetes mellitus
- Uncontrolled or unstable hypertension
- Unwillingness to sign the Written Informed Consent Form.
- Detectable organ metastasis of the colorectal cancer
- Medical history with second primary malignant disease except of curatively treated non-melanoma skin cancer, in situ carcinoma of the cervix
- Other known disease requiring hospitalization
- History of hypersensitivity to gentamycine
- History of significant neurologic or psychiatric disorders including psychotic disorders, dementia or seizures that would prohibit the understandidng and giving of informed consent.
- History of allergy or abnormal reaction to drugs in general.
- History of autoimmune disease

- Concomitant treatment with chemo-, immuno- (steroid, cyclosporine) or radiotherapy within 4 weeks at the expected time of the study vaccination
- Irradiation therapy before surgery
- Participation in a drug investigational study during the last 2 months.
- any contraindications of leukapheresis

Withdrawal Criteria

- patient non-compliance or a request to withdraw
- intolerable toxicity of vaccination in the treatment period
- intercurrent, non cancer related illness that prevents continuation of therapy or regular follow up
- DC cell number and/or function is insufficient for vaccination regimen

The selected 12 patients were divided into treatment groups A and B (both genders; 17–70 years of age). Six patients (group A) were treated with autologous oncolysate-loaded DCs, while the other six patients (group B) were treated with autologous DCs loaded with recombinant CEA. Personal data, demographic details, medical history, physical examinations, laboratory tests including immunomonitoring, ECG, chest X-ray, abdominal ultrasonography, computer tomography examinations, QLQ-C30 questionnaire were performed during the screening period. Before vaccination, all patients were treated with six cycles of 5-FU and leucovorin adjuvant chemotherapy. Prohibited drugs included steroids in any form and hematopoietic growth factors. The use of standard doses of other medications, e.g. anti-diabetic and/or cardio-respiratory drugs were not considered as exclusion criteria according to the patients' medical condition and irrespective of the vaccination study.

Monitoring of Adverse Events and Toxicity

At every visit patients were weighed, had temperature, pulse-rate, and blood pressure measured, had general bloodsamples taken, had urine analysed for glucose, protein and blood, had oxygen saturation measured by pulse oximetry, and finally went through a general clinical examination. Adverse events were graded according to the National Cancer Institutes common toxicity criteria and monitored throughout the entire study period. Oral cavity was inspected for erythematous and ulcerative lesions. Skin reactions including toxic and allergic cutaneous symptoms (i.e. maculopapular rashes, erythroderma, exfoliative dermatitis, fixed drug reactions, etc.) had also been carefully examined.

Routine Lab Tests Pre-study laboratory tests included routine hematology WBC and RBC counts, hemoglobin, hematocrit, platelets and differential blood counts, clinical chemistry tests (levels of Na^+, gamma-GT, K^+, total bilirubin, and Cl^-; total protein; SGOT (ASAT); glucose; SGPT (ALAT); urea; alkaline phosphatase; creatinine; LDH; hemostasis (INR; APTI) in blood and urine (Albumin, Ketones, Glucose, Blood, Urobilinogen). Serological tests included HIV, HBsAg, HCV, pregnancy and tumor marker tests (CEA, CA19.9). Clinical chemistry, hematology were repeated during the treatment and control period. Clinical evaluation, diagnostic procedures and laboratory tests relevant to the patient's condition were repeated after chemotherapy and within 14 days before DC-based vaccination.

Clinical and Laboratory Follow Up Follow up studies of patients admitted as outpatients were performed on days 66, 84, 168, 252 post vaccination and twice a year thereafter.

Immunological Studies

Phenotypic analysis and determination of the frequency and absolute numbers of lymphocyte subpopulations expressing the CD3, CD4, CD8, CD56, CD19 cell type and the HLA-DR and CD69 activations markers was performed by standard flow cytometry protocols. Studies on the relative ratio of tumor antigen-activated lymphocytes, detection of autoantibodies ANA, AMA, thyroid peroxidase, thyroglobulin and rheumatoid factor were performed with blood samples obtained prior to, early after, during and post therapy.

Tumor Antigens

Individual tumor tissue samples were collected from patients belonging to group A during surgery. Tumor tissue was minced into 1 mm^2 fragments, transferred to cryo-vials and stored at −80 °C until use. To prepare the autologous oncolysate, the tumor tissue fragments were γ-irradiated (120 Gy), subjected to repeated freeze-thaw cycles, and the lysates were collected. Lipid and cell debris (<10 kDa) were removed using standard centrifugation and ultrafiltration techniques, and the protein/peptide enriched fraction was used for loading the autologous DC. The commercially available pyrogen-free recombinant CEA protein was purchased from Protein Sciences (Meriden, CT, USA).

Preparation of Dendritic Cells for Vaccine Delivery

The personalized vaccine was developed for each individual patient by the Cell Processing Laboratory (Omninvest Ltd., Hungary). Briefly, following standard surgical therapy but prior to chemotherapy, patients were subjected to leukapheresis to obtain sufficient numbers of circulating blood monocytes. The monocyte fraction (>95 %) was cultured in vitro at 2×10^6 cells/ml in serum free AIMV medium supplemented with 0.01 μg/ml recombinant interleukin-4

(IL-4) and 0.08 μg/ml of granulocyte-monocyte colony-stimulating factor (GM-CSF) all from Cellgenix, GmbH (Freiburg, Germany) for 5 days. Differentiating immature DCs were incubated either with autologous oncolysate containing 5 mg/ml protein (group A) or 1 mg/ml recombinant CEA (Protein Sciences Co., Meriden, CT, USA) for 4 h at 37 °C. The oncolysate- or CEA-pulsed immature DCs were activated by an inflammatory cocktail containing 5 μg/ml prostaglandin E2 (PGE2) (Sigma-Aldrich, Munich, Germany), 50 ng/ml interleukin-1β (IL-1β), 500 ng/ml interleukin-6 (IL-6), 50 ng/ml tumor necrosis factor-α (TNF-α) all from Cellgenix GmbH (Freiburg, Germany), to induce terminal differentiation of DCs. The quality control studies of the vaccine included cell counting, viability, analysis for microbial sterility and an aliquot subjected to phenotypic analysis by standard flow cytometry. The personalized autologous oncolysate- and CEA-pulsed DC vaccines were cryopreserved in liquid nitrogen until vaccination.

Immunization

The oncolysate- or CEA-pulsed autologous DC vaccines consisted of 5×10^6–2×10^7 DCs suspended in 1 ml buffered saline and were administered subcutaneously to 4 different sites (0.25 ml/site) in the upper arm. Aliquots of the vaccine batches containing the same doses were administered on days 1, 14, 28 and 56 for the treatment of the patients.

In Vitro Investigation of Vaccine-Induced Immune Responses

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Histopaque (Sigma, St Louis, MO, USA) gradient centrifugation. 1×10^6/ml cells were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum and 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin in 5 % CO_2 milieu at 89 % humidity for 72 h. Cultures were set up in triplicates and the aliquots were incubated with the culture medium alone, in combination with 1.0 μg/ml CEA (Sigma, St Louis, MO, USA) or 10, 1 or 0.2 μg/ml phytohaemagglutinin (Sigma, St Louis, MO, USA) as control. Cell proliferation was measured by ^{3}H-thymidine uptake and proliferation indexes (PI) were determined by dividing mean uptake of ^{3}H-thymidine of cultures incubated with CEA or PHA by the mean cpm values measured in the cultures incubated in medium. PI values>1.8 was considered as significant.

Results

Clinical Results

Patients immunized with autologous oncolysate-loaded DCs (group A) were followed for 7 years. Except one patient, who exhibited ventricular adenocarcinoma as a secondary tumor and died after 57 months following the surgical elimination of the colorectal tumor (Table 1A), all patients remained tumor free for the 7–8 year follow up period. Three patients immunized with CEA-pulsed live DCs remained tumor free in the course of the follow up period, whereas the remaining 3 patients died as a consequence of the relapse of the primary tumor (Table 1B, Fig. 1).

Characterization of Serum Samples and Peripheral Blood Lymphocytes

As DCs act as highly potent antigen presenting cells and patients of group A received whole autologous tumor cell lysates, we performed serological studies for monitoring the possible induction of autoantibodies in the sera of the treated

Table 1 Clinical data of patients treated with oncolysate-loaded DC vaccines (group A) and CEA-loaded DC vaccines (group B)

Gorup A patients	N. I. (1)	V. J. (2)	S.J. (3)	G. J. (4)	Sz. J. (5)	V. L. (6)
Gender	male	male	male	male	female	female
Age (years)	52	53	63	55	64	60
Location of tumor	coecum	rectum sigma	sigma	sigma	sigma	sigma
Stage of disease	C1, G3, pT3	B2, G2, pT3	B2, G2, pT3	B2, G2, pT3	B2,G2, pT3.	B2,G2, pT3
Clinical outcome	excellent	excellent	exit	excellent	excellent	excellent
Survival time after surgery (month)	89	88	57	86	82	82
Group B Patients	N. L. (7)	T. I. (8)	K. L. (9)	B. J. (10)	Cs.S. (11)	B. K. (12)
Gender	female	female	male	female	male	female
Age (years)	57	60	58	67	51	17
Location of tumor	sigma	coecum	rectum	rectum	recto sigma	coecum
Stage of disease	B2, G3, pT3	B2, G2, pT3.	B2, G2, pT2	B,2 G2, pT3	B2, G3,pT2.	B2, G3, pT3
Clinical outcome	excellent	exit	exit	excellent	exit (suicide)	excellent
Survival time after surgery (month)	101	40	35	98	29	93

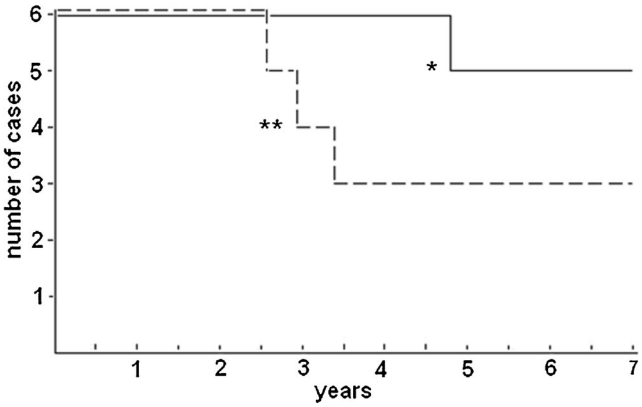

Fig. 1 Survival time of patients. ____ Group A, patients treated with oncolysate loaded DC vaccine); - - - - - Group B, patients treated with CEA-loaded DC vaccine. *2nd cancer; **suicide

patients. Serum samples were collected 14 days before, and on days 0, 56 and 168 post vaccination, and tested for antibodies against thyroid peroxidase, thyroglobulin as well as rheumatoid factor. None of the tested sera exhibited the signs of autoreactivity (data not shonwn). The penotypic analysis of lymphocytes by flow cytometry was aimed at determining changes in the ratio and/or absolute numbers of $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells and $CD3^+/HLA\text{-}DR^+$ and $CD3^+/CD69^+$ activated T cells. No significant differences in these cell populations could be detected in the blood samples drawn before and during the post vaccination period (data not shown). These results indicated that DC-based vaccination had no systemic effects on humoral and cellular autoimmunity.

Detection of Carcinoembryonic Antigen

CEA is used as a tumor marker in colorectal carcinoma, CA19.9 may be elevated in many types of gastrointestinal cancers including colorectal cancer. Histologic analysis of tumor sections of the patients showed detectable levels of CEA expression (data not shown). Serum levels of the CEA and CA19.9 markers measured in serum samples collected prior to and 3 month after initiation of the vaccination program are given in Table 2. The final measurement was performed during the last 3 months of the survival period or at the time point of the last clinical investigation. Results of these comparative studies had no prognostic value for the outcome of the disease irrespective of the treatment groups A and B. (Table 2).

In Vitro Lymphocyte Proliferative Responses to CEA-Loaded Dendritic Cells

The proliferative response of autologous PBMC to autologous CEA-pulsed DCs was measured in the individual patients 28 days post immunization. CEA-induced cell proliferation was detected in co-cultures of lymphocytes with CEA-loaded

Table 2 Serum levels of CEA and CA19.9 tumor markers in individual patients

	CEA			CA 19.9		
	Before V[a]	3 month after V	Final[b]	Before V[a]	3 month after V	Final[b]
Group A						
N. I.	5,6	5,3	5,4	9,7	15,4	9,0
V. J.	1,8	1,0	1,0	8,8	8,3	7,6
S. J.	2,1	1,6	1,6	6,1	6,8	20,5
G. J.	1,1	0,8	1,0	4,5	4,3	4,4
Sz. J.	7,5	7,7	7,2	0,6	0,6	0,6
V. L.	1,1	1,4	1,5	8,5	6,0	9,7
Group B						
N. L	1,1	1,0	0,6	11,4	10,8	11,4
T. I.	1,0	1,2	1,2	5,0	3,2	1,3
K. L.	3,1	3,6	17,9	10,7	11,1	25,8
B. J.	2,9	2,1	1,6	12,9	13,9	18,1
Cs.S.	3,0	2,3	1,6	16,6	21,1	18,1
B. K.	1,2	1,9	1,3	35,2	31,9	31,2

[a] *V* vaccination

[b] Final investigations were carried out during the last 6 months of the survival period, or during the last 6 month before death. Reference values: CEA <3,4 μg/l; CA 19.9<34 KU/l

DCs of patients from both groups A and B (Table 3). The PI values ranged from 2.8 to 14.4 and were consistently higher in the co-cultures of oncolysate-loaded DCs (group A) as compared to group B.

Local Skin Reactions

Two hours after intra-cutaneous injections of the DC-based vaccines a slight erythema was detected at the site of injection of each patient, which disappeared within 8 h after the first immunization. However, a persistent erythematous reaction

Table 3 Proliferation indexes calculated from ^3H thymidin incorporation assays of co cultures of autologous lymphocytes and DCs pulsed with oncolysate (group A) or CEA (group B

Patients	Group A Treatment with oncolysate loaded DC vaccines	Patients	Group B Treatment with CEA loaded DC vaccines
N.I. (1)	5.5	N.L. (7)	ND
V.J. (2)	3.4	T.I. (8)	ND
S.J. (3)	14.4	K.L. (9)	1.8
G.J. (4)	2.8	B.J. (10)	2.8
Sz.J. (5)	7.7	Cs.S. (11)	0
V.L. (6)	7.2	B.K. (12)	3.9

Reference value: > 1.8.; *ND* not done

was detected at the site of immunization following the 2nd, 3rd, and 4th doses of immunization in all but one patient (B.K. (N°12) in Group B), which gradually developed into a site showing signs of cellular infiltrates. In contrast to the vaccine injection site, the control injection with physiological saline (K) did not result in any signs of erythema and the site remained normal (Fig. 2).

Discussion

Most tumor cells do not act as APCs and thus fail to trigger primary T-lymphocyte responses. DCs have been identified as highly potent professional APCs that play an essential role in the induction and maintenance of self tolerance while having the potential to prime and boost antigen-specific T lymphocyte responses. The recently characterized DC subtypes and subsets however, may exhibit different functional activities in terms of provoking protective immune responses against tumors [23]. Based on the accumulated knowledge on DC biology and their role in tumor-specific immune responses clinical trials have been designed for using DCs as natural adjuvants of TAAs [24, 25]. These clinical trials have established the feasibility and safety of DC-based vaccination approaches [26–29], and in some but not all cancer patients have been associated with immune responses [30], which not always correlated with the immunological results. This discrepancy was often attributed to the low numbers of DCs migrating to the draining lymph nodes [31, 32]. To avoid activation-induced cell death (AICD) of T-lymphocytes and the elimination of activated DCs by Tc, the critical importance of the frequency and number of injections was also pointed out [33].

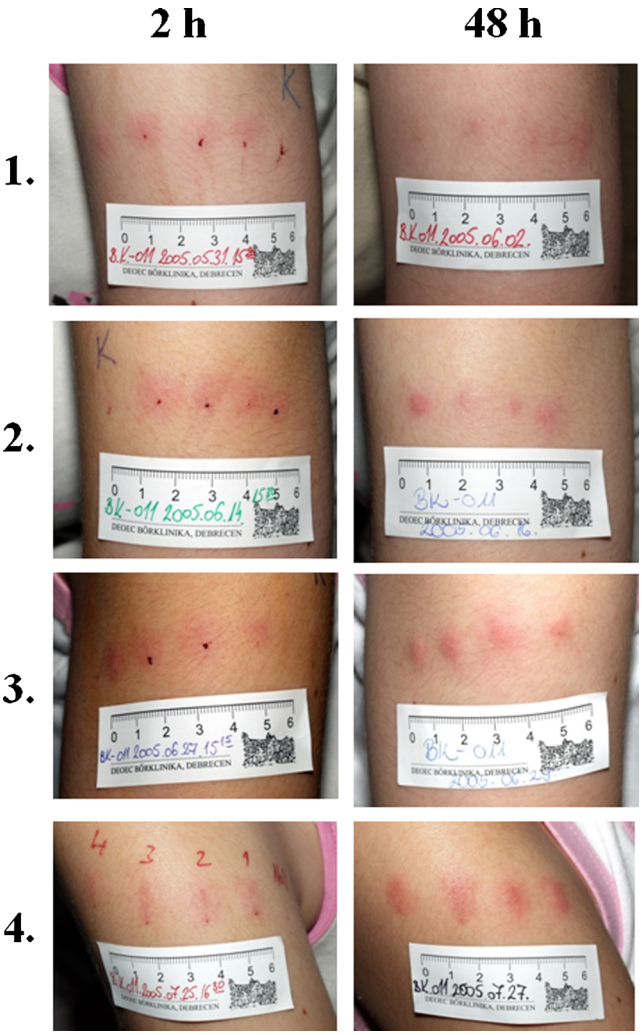

Fig. 2 Skin reactions following immunization. Pictures were taken 2 h and 48 h after vaccination. Intracutaneous injections containing tumor-antigen loaded mature DCs were injected at 4 different sites in 0.25 ml portions at day 1 (1), 14 (2), 28 (3) and 56 (4) of the study. K represents the saline control. No visible difference in skin reaction was detected by CEA and oncolysate loaded cell vaccination

Despite recent advances in the treatment of CRC, the overall survival rate of patients with advanced disease remains less than 50 %. Systemic adjuvant chemotherapy has improved the survival of these patients, but additional treatment modalities are still needed. The safety and feasibility of active tumor-specific immunotherapies has been tested in patients with CRC in various clinical settings [34–41], and the National Cancer Institute (NCI) in U.S.A. is currently enrolling patients with metastatic CRC for DC-based Phase I/II trials using peptides encompassing mutants of Ras and p53, or CEA RNA for DC loading (http://www.cancer.gov/clinicaltrials/search/results?protocolsearchid=6252091&vers=1).

CEA is over expressed in all CRC and thus has become the first immunological target of TAA-specific DC-based cancer vaccines. The results with CEA protein, HLA-restricted CEA peptides, epitope-encoding vectors or transfection of CEA mRNA showed that the peptide- and epitope-based vaccines induced CEA-specific immune responses. No difference in the effects of peptide loading and transfection of CEA mRNA was detected [42], and despite in vitro CEA-specific T-lymphocyte proliferation, clinical responses remained limited [43, 44]. Recent studies also revealed that the high density of TAM along the invasive margin of the tumor was associated with improved prognosis[45], while other studies demonstrated the role of TAM and MDSC in blocking T-lymphocyte activation by suppressive mediators [46], or induced by $CD4^+$ [47] and $CD8^+$ Treg cells with immune suppressive potential [48]. It was also shown that the survival of CRC patients with high DC infiltration was significantly longer than those with low DC densities[49].

In the present study the clinical outcome of vaccination by oncolysate- and CEA-loaded DCs showed that i) the oncolysate vaccine conferred long term protection against CRC recurrence; ii) no adverse effects of the two DC-based vaccines could be shown by monitoring Ab responses to a panel of autoantigens and the phenotypes of peripheral blood lymphocytes; iii) levels of the CEA and CA-19.9 tumor markers did not change during the follow up period, and had no prognostic value for the

outcome of the disease; iv) results of the in vitro T-lymphocyte proliferation assay correlated with the long term survival of patients vaccinated by oncolysate-loaded DC.

Patient N° 3, vaccinated by the oncolysate-containing vaccine died 57 months after the surgical elimination of the CRC due to a secondary ventricular adenocarcinoma (Table 1A), but the other 5 patients remained tumor free for 6-years. In contrast to these clinical results, 3 out of the 6 patients treated with CEA-loaded DCs died because of tumor relapse indicating the higher clinical efficacy of the oncolysate-based vaccine as compared to that of the CEA-based strategy.

The adverse effects of DC-based vaccines reported to date were restricted to transient inflammation indicated by fever or local reactions. A potential risk of all DC-based tumor immunotherapies is the induction of pathological autoimmune reactions against'self' proteins present in the vaccine, exemplified by vitiligo observed in some melanoma patients after vaccination with whole cell lysates or melanocyte-related differentiation antigens [50]. This reaction however, was shown to correlate with clinical responses but not with autoimmune dysfunction. In line with these findings, results of the present study did not indicate antibody reactivities against a panel of self antigens measured 14 days before and up to 168 days of the post-vaccination period. Accordingly, no changes in the distribution and phenotype of peripheral blood lymphocytes could be observed.

Lymphocyte proliferation tests of the oncolysate-vaccinated patients measured in vitro 28 days after immunization demonstrated positive reactions in all patients however, the high variability of the PI values in both groups did not allow statistical analysis. Nevertheless, the PI values measured in Group A were consistently higher than those obtained in Group B (Table 3). As no signs of adverse effects could be observed during the long term follow up period, and patients of the oncolysate-vaccine group remained tumor free for more than 7 years, we concluded that the use of autologous tumor cell lysate-containing DC vaccine in CRC is safe and is able to provoke long term protection against tumor recurrence.

All of the removed tumors of the investigated patients were proved to be CEA positive. Kinetics of CEA and CA-19.9 serum levels demonstrated that CEA levels remained below the reference value throughout the whole follow up period without significant changes. No prognostic value of these data for the outcome of the disease could be shown (Table 2). In line with previous results the oncolysate-loaded DC-based vaccine used in this study induced local T-lymphocyte activation and infiltration visible as in vivo cutaneous reaction at the injection site tested 48 h after vaccination. Similar skin reaction was observed with CEA-loaded DCs. The in vitro T-lymphocyte proliferation test, however, resulted in a slightly elevated PI by oncolysate-loaded DCs.

Our results confirm the safety and tolerability of the use of live in vitro loaded autologous DC-based therapy. All patients developed a delayed type hypersensitivity skin reaction in the early phase of immunization, immediately after the second vaccination, but the production of autoantibodies and/or signs of autoimmune reactions were not detected. These results indicate that autologous DCs loaded with autologous "oncolysates" containing a wide spectrum of tumor-associated antigens represents a well tolerated therapeutic adjuvant modality in patients with CRC. Our results showed that after 7 years all patients treated with autologous oncolysate remained tumor free, whereas 3 patients treated with the CEA-loaded DC vaccine succumbed recidivates and died. Further trials with increased patient numbers are warranted to optimize the most effective combination, dose and delivery mode of CRC-associated antigen.

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