

Telomerase-pulsed dendritic cells: preclinical results and outcome of a clinical phase I/II trial in patients with metastatic renal cell carcinoma

Telomerase-beladene dendritische Zellen: Resultate einer klinischen Phase I/II-Studie bei Patienten mit metastasierendem Nierenzellkarzinom

Abstract

Objective: Therapeutic vaccination with dendritic cells (DC) showed promising results in first clinical trials in cases of metastatic renal cell carcinoma (RCC). Human telomerase reverse transcriptase (hTERT) could be a potential target because it is detectable in more than 85% of human tumors including RCC.

Design: 10 patients with progressive metastatic RCC were enrolled in a clinical phase I/II trial using DC pulsed with hTERT-peptide. Beside toxicity and feasibility aspects, a complex immune monitoring including *in vitro* data were evaluated. In addition to detection of tumor-specific effector cells we investigated their functionality like IFN- γ secretion and cytotoxic activity against tumor cells.

Results: The vaccine was well tolerated. Two patients showed a mixed response (MR) and one patient a stable disease (SD). Interestingly, responders showed cytotoxic activity already before start of therapy and there was a significant increase in cytotoxic activity of effector cells from all responders (SD and MR patients) after the first vaccination. In contrast non-responders showed no cytotoxic activity before and during treatment. Therefore, cytotoxic activity might be used as a predictive marker in the future. Tetramer staining detected higher amounts of tumor-specific cytotoxic cells in responding patients compared to non-responders. Also, responders possessed increasing amounts of IFN- γ producing immunological effector cells.

Conclusion: Telomerase-pulsed DC could enhance a tumor-specific immune response against RCC.

Keywords: dendritic cell vaccination, telomerase, immunotherapy, renal cancer, T-cell responses

Zusammenfassung

Ziel: Therapeutische Impfungen mit Antigen-beladenen dendritischen Zellen (DC) zeigten in ersten klinischen Versuchen bei Patienten mit metastasierendem Nierenzellkarzinom (RCC) vielversprechende Ergebnisse. Die menschliche Telomerase-reverse-Transkriptase (hTERT) könnte ein mögliches Zielantigen sein, da sie in 85% aller menschlichen Tumore einschließlich dem Nierenzellkarzinom nachweisbar ist.

Methodik: 10 Patienten mit fortschreitend metastasierendem RCC wurden in einer klinischen Phase I/II-Studie untersucht, nachdem sie mehrere intratumorale Impfungen aus hTERT-beladenen autologen DCs erhalten haben. Neben Überprüfung der Verträglichkeit wurden verschiedenste *in vitro* Analysen durchgeführt, die Aussagen über den Immunstatus der Patienten geben sollen. Hierbei wurde sowohl nach tumorspezifischen Effektorzellen geschaut, als auch deren Funktionalität in

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Form von IFN- γ Sekretion oder Zytotoxizität gegenüber Tumorzellen untersucht.

Ergebnisse: Die Impfungen wurden von allen Patienten gut vertragen. Zwei Patienten zeigten eine Mischantwort (MR) mit Rückbildung der punktierten Metastase und ein Patient zeigte eine Stabilisierung (SD) mit unverändertem Krankheitsverlauf. Erstaunlicherweise konnte bei den ansprechenden Patienten schon vor Studienbeginn eine Zytotoxizität von Effektorzellen nachgewiesen werden, die nach der 1. Impfung signifikant anstieg. Demgegenüber zeigten die nicht-ansprechenden Patienten vor, während und nach der Behandlung keine zytotoxische Aktivität gegenüber Tumorzellen. Folglich könnte die Zytotoxizität von Effektorzellen in Zukunft als Prognosemarker verwendet werden. Die ansprechenden Patienten besaßen im Vergleich zu den nicht-ansprechenden Patienten eine höhere Anzahl an Tumor-spezifischen zytotoxischen Zellen sowie IFN- γ produzierenden Effektorzellen.

Schlussfolgerung: Wir konnten zeigen, dass eine Impfung mit Telomerase-beladenen autologen DCs eine in geringem Ausmaß vorhandene Tumor-spezifische Immunantwort signifikant verstärken kann.

Schlüsselwörter: Dendritische Zellen, Telomerase, Immuntherapie, Nierenzellkarzinom, T-Zell-Antwort

Introduction

Metastatic RCC has a poor prognosis with a median survival of less than one year [1]. Systemic treatment with cytotoxic chemotherapy is usually ineffective. Since RCC is known for its immunological susceptibility [2], several immunotherapeutical approaches have been developed. These include immunomodulatory cytokines, allogeneic blood stem cell transplantation [3] and vaccination with DC [4], [5]. DC are the most potent stimulators of T lymphocytes and are therefore expected to be of great importance in the antitumoral immune response. Recently, DC were used in several studies to increase the cytotoxic activity of immunologic effector cells in vitro and in vivo [6], [7], [8].

Telomerase is a ribonucleoprotein enzyme that plays a key role in maintaining chromosomal stability and cellular life span. Three components of human telomerase, human telomerase RNA component (hTERC), human telomerase protein 1 (hTEP1) and human telomerase reverse transcriptase (hTERT) have been identified. hTERT, the catalytic subunit of human telomerase, is expressed in 85% of human cancers but usually not in normal cells. Therefore, it seems to be an ideal candidate for an ubiquitous, specific tumor-associated antigen. Different antigenic epitopes from hTERT inducing tumor-specific cytotoxic T lymphocytes have been described [9], [10], [11].

Various clinical observations have suggested that the immune system may influence the prognosis of RCC. Unfortunately, in most patients the immune response to RCC is insufficient to control the disease. Although immunotherapeutical approaches to RCC have been promising, only a small number of patients could benefit from these treatments. This is discussed as tumor-induced

immunosuppression, which influences the immune status by raising the activation threshold or inducing anergy [12]. This could be crucial for the outcome of the individual patient and might be predictive for the success of immunotherapy. Immunotherapeutic strategies have so far only sometimes lead to clinical responses. Therefore, a predictive marker for response would be of major importance.

Here, we present in vitro data and results of a clinical phase I/II trial using DC pulsed with telomerase-peptide in patients with progressive metastatic renal cell carcinoma.

Patients and methods

Patient characteristics and evaluation

Patients with progressive metastatic RCC, Karnofsky score of 60%-100% and a haplotype of either HLA-A2, HLA-A3 or HLA-A24 were eligible for this study. Exclusion criteria included a time interval of less than 28 days to previous chemotherapy or cytokine treatment, severe heart disease, severe psychiatric disease, active hepatitis A, B, or C and HIV infection. Approval of local ethics committees was obtained (AZ 110/02).

Ten patients were enrolled in our clinical protocol (Table 1). Median age was 63.5 years (range 47 to 74) and all patients were male. Four patients had metastases in the lung, seven patients had bone metastases, four had metastases in lymph nodes, three had liver metastases and five patients had metastases in other viscera. The patients had an average score of 1.0 according to prognostic factors (prognostic scale of 0-2). Prognostic factors were calculated as determined by the Multicenter Group for Treatment of RCC based on serum level of CRP and LDH, number of neutrophil count, number of metastatic

Table 1: Characteristics of patients

Clinical outcome	Initials	Age	Haplotype	Date of diagnosis	Date of nephrectomy	Localisation of metastasis at date of diagnosis	Prior therapies	Localisation of metastasis at date of trial	Date of diagnosis of stage IV
PD	LS	62	A*02/A*24	08/91	08/91	no	immunotherapy (IFN-alpha/IL-2); immunotherapy (tumor lysate vaccination); immunotherapy (hybrid cell vaccination)	adrenal gland, liver, bone, lymph nodes, pancreas, peritoneal	01/99
PD	GS	51	A*24/A*31	01/95	01/95	no	Immunotherapy (hybrid cell vaccination); irradiation	bone	04/98
PD	HJ	47	A*03/A*03	01/02	01/02	lymph nodes	immunotherapy (IFN-alpha/IL-2)	lymph nodes	01/02
PD	HW	64	A*02/A*24	11/02	12/02	vertebral bodies	irradiation	lung, vertebral bodies	11/02
MR	HB	63	A*03/A*07	01/03	01/03	bones, liver	irradiation	lung, bone, liver	01/03
PD	AB	68	A*02/A*26	01/03	01/03	pleural metastases	----	lung, bone, pleura, lymph nodes	01/03
PD	WK	68	A*01/A*02	07/99	07/99	bones (multiple)	irradiation; chemotherapy; immunotherapy (IFN-alpha/IL-2)	bone, pleural	07/99
MR	AM	63	A*03/A*29	12/02	01/03	adrenal gland left, lung	----	lung, lymph nodes, liver, peritoneal	12/02
PD	WE	74	A*01/A*24	04/03	04/03	bones	----	bone	04/03
SD	GA	66	A*01/A*03	05/99	05/99	no	chemotherapy	paraaortal, mediastinal	06/02

(PD: progressive disease; MR: mixed response; SD: stable disease)

sites, presence of bone metastases and time from diagnosis of tumor to metastatic disease [13].

Generation of dendritic cells

DC were generated according to the protocol published by Schuler et al. [14]. In brief, peripheral blood mononuclear cells (PBMC) from leukopheresis products were isolated by Ficoll density gradient centrifugation. After two adhesion steps cells were cultivated in RPMI media with 750 U/ml GM-CSF and 500 U/ml IL-4. Maturation was induced using 1000 U/ml TNF- α , 750 U/ml IL-6, 100 U/ml IL-1 β and 1 μ g/ml PGE₂. Cells were harvested on day +8 and frozen at aliquots of 0.5-1.5x10⁷ cells/ml in 90% autologous serum and 10% DMSO. On the day of vaccination, 10⁶ DC were pulsed with 10 μ g of the respective telomerase peptide HLA-A*02: ILAKFLHWL, HLA-A*03: KLFGLRLK, HLA-A*24: VYAETKHFL from Jerini AG (Berlin, Germany) and 50 μ g/ml endotoxin free KLH as adjuvant for 2 hours at 37 °C. The maturation state of DC was controlled by flow cytometry.

Treatment of patients

All patients had been nephrectomized before start of vaccination. One half of DC was administered intradermally, the other half of the vaccine was injected directly into the tumor under CT-guidance. This procedure was repeated on days 8, 22, 36 and 50. One patient only received intradermal applications. In most cases, patients received 1-2x10⁷ cells per vaccine. In addition, a low dose of IL-2 (4x10⁶ IU/per day; Proleukin[®]S, Chiron-Behring, Marburg, Germany) was given on days 1-28. However, this dose was not tolerated in all patients.

Clinical outcome

Medical history was taken and the following baseline studies were performed before starting the treatment: physical examination, full blood count, blood chemistry basics and urine analysis. Blood was also subjected to immunological comprehensive testing as described below. Chest X-rays and CT scans of chest and abdomen were taken. Clinical assessment was repeated on days 8, 22, 36 and 50, a complete clinical and immunological screening comparable to the initial check-up was performed on day 50. Stable disease was defined as a decrease of measurable tumor manifestations of less than 50% or an increase of less than 25% [15] and a constant number of bone lesions. 'Measurable tumor manifestations' refers to diameters as determined in CT scans.

Immunological studies

Delayed-type hypersensitivity (DTH)

To test for a telomerase-specific immune response, 10 μ g from the corresponding telomerase peptide were administered intradermally before and after treatment. A positive skin-test reaction was defined as an induration of more than 2 mm in size after 48 hours.

Preparation of peripheral blood lymphocytes (PBL)

PBL were obtained and isolated from heparinized peripheral blood by centrifugation with BD Vacutainer CPT Cell Preparation Tubes[®]. Cells were cryo-preserved for further analysis.

Flow cytometric analysis

Fresh peripheral blood from patients and from healthy donors of similar age was stained using various monoclonal antibodies directed against human cell surface antigens, including those against human CD1a, CD3, CD4, CD8, CD11c, CD13, CD14, CD16, CD19, CD23, CD25, CD28, CD40, CD56, CD62, CD69, CD80, CD83, CD86, CD123, CD154, CD158a, CD158b, and HLA-DR (Immunotech Hamburg, Germany; BD Biosciences Pharmingen, Heidelberg, Germany; Beckman Coulter, Krefeld, Germany). In addition, patients' blood was screened for the presence of peptide-specific cells. Tetramers specific for the respective telomerase peptides were purchased from Prolimmune (Oxford, U.K.). Freshly drawn blood was stained with tetramers and antibody against CD8.

In addition, DC were generated as outlined above and stained for CD1a, CD14, CD40, CD80, CD83 and CD86 (Immunotech Hamburg, Germany; BD Biosciences Pharmingen, Heidelberg, Germany; Beckman Coulter, Krefeld, Germany).

Analysis was performed on a BD FACSCalibur™.

Cytotoxicity assay

A standard chromium release assay was used to determine the cytotoxic activity of PBL. In brief, T2 target cells were loaded with 50 µg/ml from the corresponding HLA-A*02 telomerase peptide and then labeled with 100 µCi ⁵¹Cr for two hours. HLA-matched allogeneic RCC cells as target cells were directly labeled with 100 µCi ⁵¹Cr for two hours. 5000 target cells per well were incubated with PBL at different effector to target cell ratios. After four hours, the supernatant was collected and counts per minute were determined. Each experiment was performed in triplicate and the mean value was calculated. Maximum release was obtained by incubating the target cells with 0.1% IGEPAL® (anionic detergent from Sigma). Target cells without effector cells served as a negative control (spontaneous release). Cytotoxicity calculations were performed using the following formula:

$$\text{Percent cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release of target cells}}{\text{maximal release} - \text{spontaneous release of target cells}}$$

Proliferation assay

The non-radioactive proliferation assay „EZ 4 U“ (Bio-medica, Vienna, Austria) was performed according to the manufacturer's instructions. PBMC were stimulated with 5 µg/ml of the respective telomerase peptide for three hours. PBL were harvested and the proliferation rate of 2.5x10⁴ cells was determined in triplicate after 24 hrs as the amount of turnover of yellow tetrazolium salt to red formazon. Absorbance at 490 nm was measured with an ELISA reader.

Analysis of IFN-γ producing immunological effector cells using ELISpot assay

IFN-γ production of PBL was determined using a human IFN-γ ELISpot kit according to the manufacturer's instructions (Hölzel, Cologne, Germany). In brief, PBMC were stimulated with 5 µg/ml of the respective telomerase peptide over three hours. PBL were harvested and plated on nitrocellulose 96-well plates in duplicates. IFN-γ secretion of 2.5x10⁴ cells was determined after 48 hrs. Spot analysis was performed using the KS 400 system (Carl Zeiss, Jena, Germany).

Statistical analysis

Non parametrical analysis (Mann-Whitney-U test) on SPSS 11.5 was used to analyze statistical significance. A p-value <0.05 was considered significant.

Results

Expression of cell surface antigens on DC

DC were generated as reported in the materials and methods section. DC were stained for various cell surface antigens and measured by flow cytometry. Both DC of the first vaccine and of the following vaccines (2.-5.) are shown in Figure 1. DC showed high expressions of DC typical markers such as CD1a, CD40, CD80, CD83 and CD86. In contrast, expression of CD14 was low (Figure 1).

Cytotoxicity of PBL collected during treatment

The cytotoxic effect of PBL during the treatment was monitored using peptide-loaded T2 cells or HLA-matched allogeneic RCC cells as targets. PBL from responders always showed a significantly higher cell lysis compared to cells from non-responders. This was true even before vaccination. Non-responder never showed any remarkable lysis (Figure 2). Responders also showed a significant increase in cytotoxic activity after the first vaccination compared to the initial situation (p=0.043; Figure 3). This activity dropped below the initial level after the first vaccination and then increased.

Number of leukocytes

Interestingly, the number of leukocytes was increased in non-responders compared to healthy donors. This was significant at most times (Figure 4). The number of granulocytes was increased significantly in non-responders compared to healthy donors. The number of granulocytes was higher in non-responders compared to responders before, during and after the trial (Figure 5).

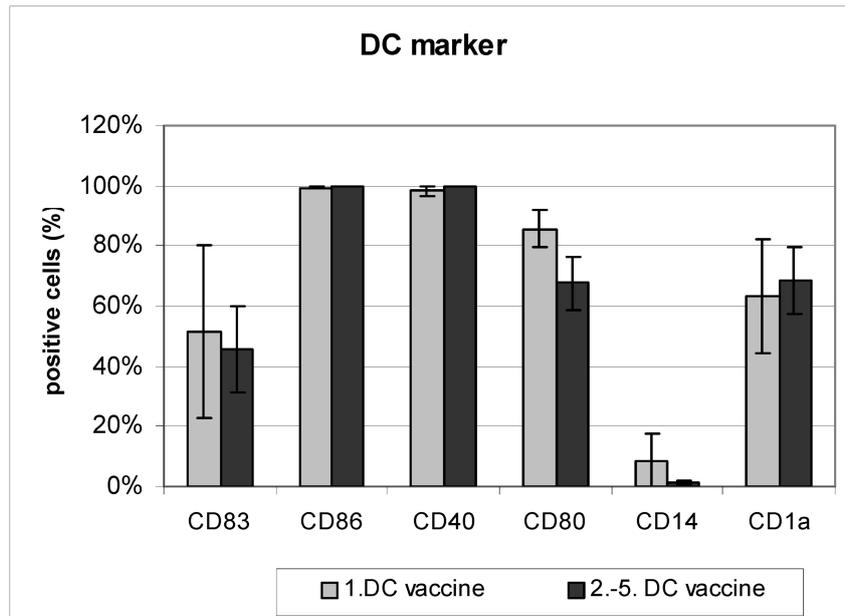


Figure 1: Expression of cell surface antigens on DC

DC were generated as reported in the materials and methods section. DC were stained for various cell surface antigens and measured by flow cytometry. Both DC of the first vaccine and of the following vaccines (2.-5.) are shown. Results are presented as mean of the different groups +/- standard error.

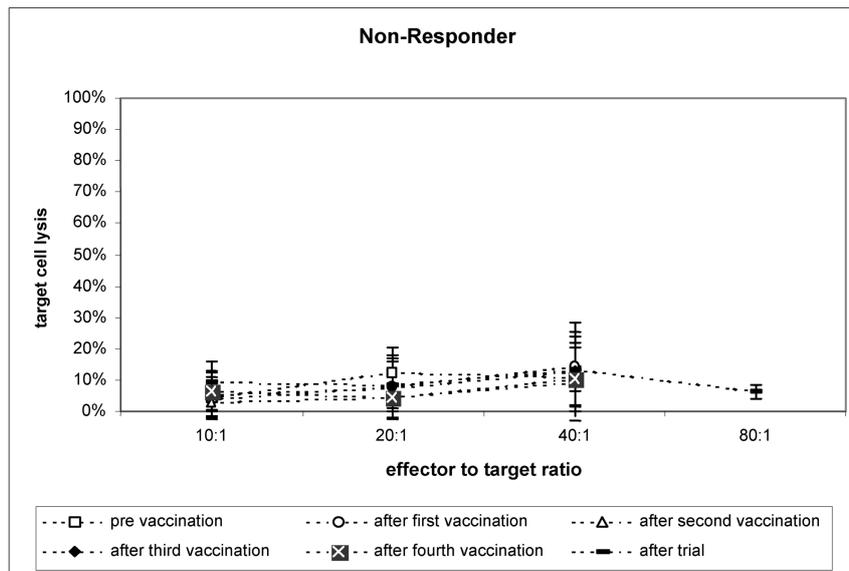


Figure 2: Cytotoxicity assay of PBL from Non-Responder

PBL were obtained from patients before, during and after treatment and investigated in a standard chromium release assay. A p-value <0.05 was labeled with an asterisk and means a significant increase in cytotoxic activity after the first vaccination compared to the initial situation. Results are presented as mean of the different groups.

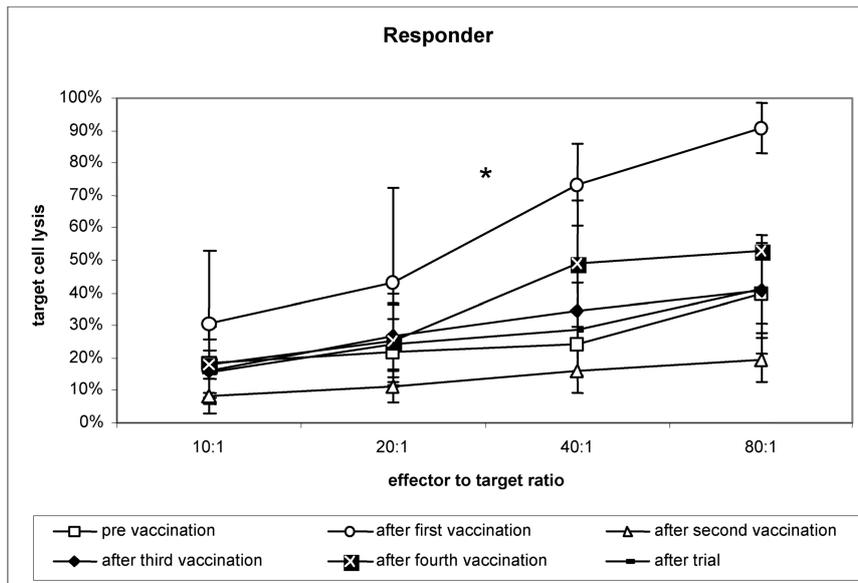


Figure 3: Cytotoxicity assay of PBL from Responder

PBL were obtained from patients before, during and after treatment and investigated in a standard chromium release assay. A p-value <0.05 was labeled with an asterisk and means a significant increase in cytotoxic activity after the first vaccination compared to the initial situation. Results are presented as mean of the different groups.

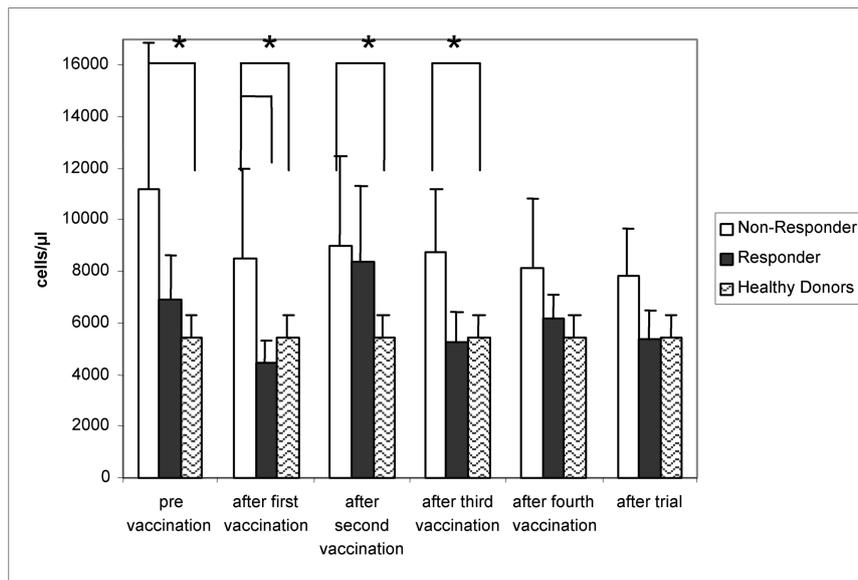


Figure 4: Number of leukocytes

Blood was drawn from patients before, during and after treatment and cell numbers were determined by FACS analysis. A p-value <0.05 was labeled with an asterisk. Results are presented as mean of the different groups.

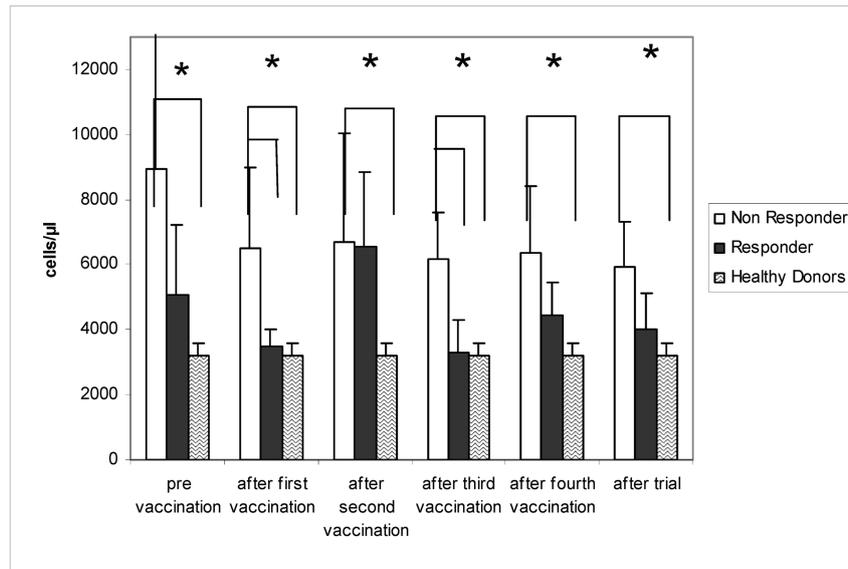


Figure 5: Number of granulocytes

Blood was drawn from patients before, during and after treatment and cell numbers were determined by FACS analysis. A p-value <0.05 was labeled with an asterisk. Results are presented as mean of the different groups.

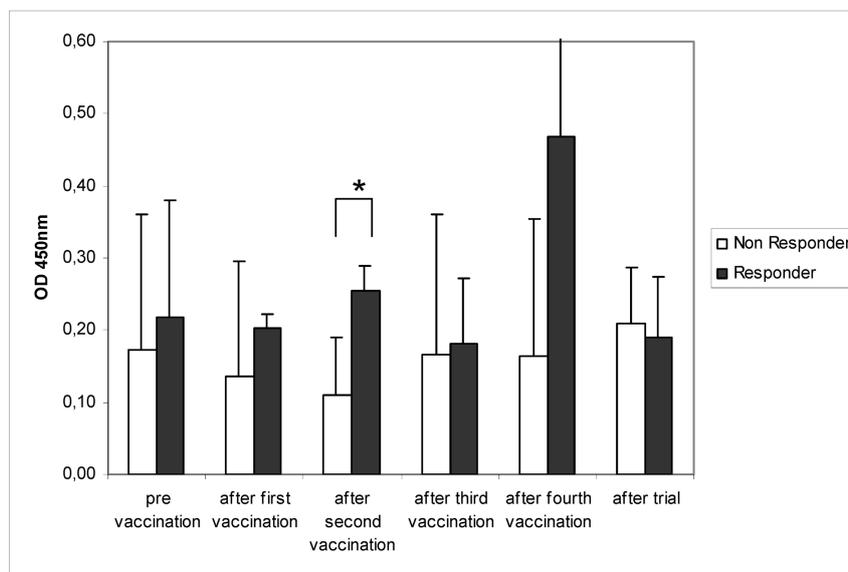


Figure 6: Proliferation assay of PBL

PBMC were obtained from patients before, during and after treatment. Mononuclear cells were stimulated with telomerase peptide and lymphocytes were investigated in an MTT proliferation assay. The OD (optical density) was determined at 450 nm. A p-value <0.05 was labeled with an asterisk. Results are presented as mean of the different groups.

Proliferation of PBL

Proliferation tended to be higher in responders compared to non-responders. This was significant after the second vaccination (Figure 6, $p=0.033$).

ELISpot results

Numbers of IFN- γ producing cells increased during vaccination with peaks after the third and fourth vaccination. This phenomenon was significantly higher in responders (SD and MR patients) compared to non-responders

($p=0.03$; Figure 7). Numbers of cells remained elevated after completion of the trial.

Tetramer analysis

In responders, numbers of telomerase-specific cytotoxic cells (CD3+CD8+) increased during vaccination compared to numbers before treatment with a peak after the third vaccination (Figure 8). These data tend to correlate with the ELISpot results ($p=0.06$).

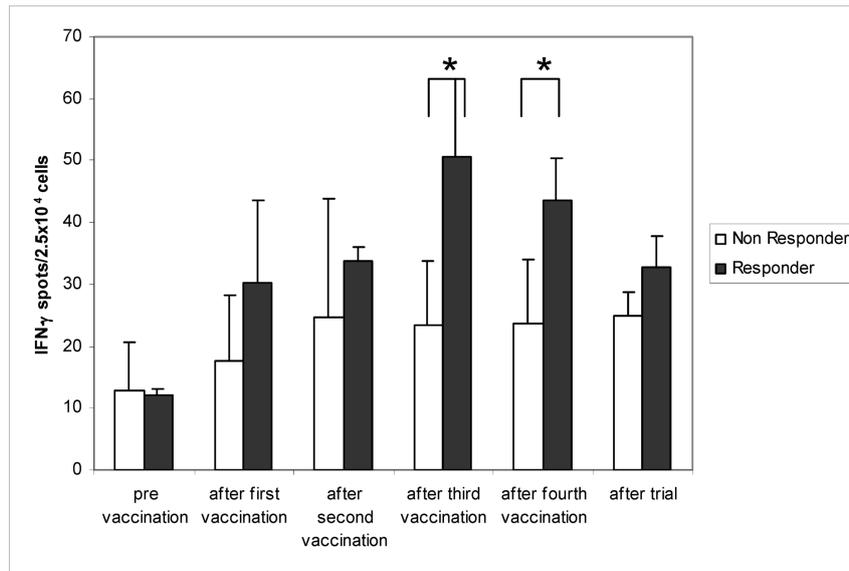


Figure 7: IFN-gamma ELISpot of PBL

PBMC were obtained from patients before, during and after treatment. Mononuclear cells were stimulated with telomerase peptide and lymphocytes were investigated for IFN-gamma secretion. A p-value <0.05 was labeled with an asterisk. Results are presented as mean of the different groups.

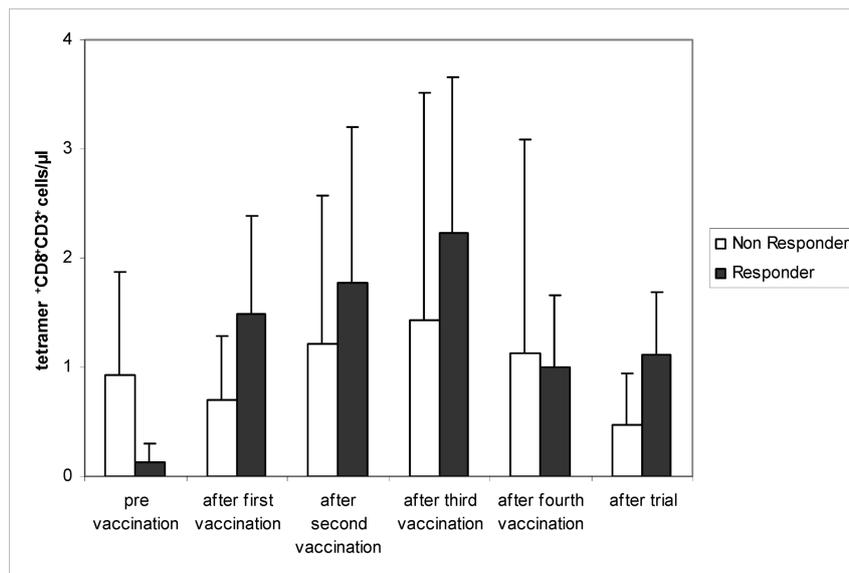


Figure 8: Tetramer analysis

Patients' blood was screened for telomerase-specific cytotoxic T cells before, during and after treatment. Cells were stained using tetramers. Cells from healthy donors were used as negative control. Data are shown as mean of the different groups.

Patient outcome

With respect to our protocol, clinical outcome was based on a comparison of CT scans before and after treatment, i.e. three months after entering the protocol. All ten patients were in progressive disease when entering our protocol. 2/10 patients showed a mixed response (MR) with tumor regression at the site of DC injection and progress of the untreated tumor. One patient showed a stable disease (SD) after vaccination and 7/10 patients remained in progressive disease (PD). The two patients belonging to prognostic group 0 (good prognosis) responded with SD or with MR, respectively. Of prognostic group

1, five of six patients remained in progression, one patient showed a MR. Of prognostic group 2 (poor prognosis), both patients remained in progression (Table 2). MR lasted for 92 and 145 days, respectively. SD lasted for 180 days. In general, the vaccine was well tolerated in all patients and no toxicity was observed. However, three patients died due to progression of tumor growth on days 59, 64, and 100 after start of treatment, respectively.

Table 2: DTH reaction against telomerase peptide

Patient	State of disease after treatment	DTH reactivity against telomerase peptide	
		before	after
LS	PD	negative	positive
GS	PD	positive	negative
HJ	PD	negative	n.d.
HW	PD	negative	negative
HB	MR	negative	positive
AB	PD	negative	negative
WK	PD	negative	n.d.
AM	MR	negative	negative
WE	PD	negative	negative
GA	SD	negative	negative

(n.d.: not done; PD: progressive disease; MR: mixed response; SD: stable disease; DTH: delayed-type hypersensitivity)

DTH reactivity

DTH testing using telomerase peptide was performed before and after treatment. 2/8 patients showed reactivity after vaccination (Table 2).

Discussion

There is an urgent need for new therapeutic approaches for treatment of metastatic renal cell carcinoma. Immunotherapy could be a potential way to treat this immunogenic tumor entity, where telomerase could be a suitable tumor-associated antigen (TAA) [16]. So far, no other TAAs were found to have a major impact in immunotherapeutic approaches in RCC.

Here, we present data from a clinical phase I/II study using telomerase peptide-pulsed DC. This vaccination protocol was shown to be safe and feasible, although, unfortunately, three patients died during therapy due to rapid progression of disease. Although the clinical outcome was not the main focus of this study, we staged the patients after a follow-up of three months. 2/10 patients showed a MR, 1 patient had a SD for a median of 6 months.

The main focus of this trial was the complex immune monitoring. We compared the patients with MR and SD (responder) with patients with PD (non-responder) by statistical analysis. Interestingly, we saw significant differences in the immunological data between responders and non-responders. The most relevant result was the difference in cytotoxic activity of PBL between responders and non-responders. Responders showed cytotoxic activity prior to therapy, which could be enhanced further. In contrast, effector cells of non-responders were not able to lyse allogeneic RCC and peptide-loaded T2 cells at any time. Similar results were obtained in other clinical trials, where we used tumor lysate-pulsed DC or DC fused with tumor cells for vaccination [17].

In general, we observed a statistically significant increase in granulocytes and leukocytes in non-responders. However, an elevated number of leukocytes was not accompanied by an increase in activity. PBL from non-responders generally showed lower proliferation rates and lower

numbers of IFN- γ secreting cells after antigen restimulation. Also, we observed an increase in antigen-specific cytotoxic T cells and in IFN- γ secreting cells during therapy, which was more prominent in responders.

In our cytotoxicity experiments, we observed a decrease in activity after the second vaccination. This might indicate a downregulation in immunological function, which could be due to the fact that the interval between 1st and 2nd was short (1 week).

Our protocol included both intradermal and intratumoral injection of peptide-loaded DC. Interestingly, patients who showed a MR had tumor regression at the site of DC injection. This is in accordance with data from other groups who observed a good response after local administration of interleukin-2 [18]. The patient who had a stabilization of disease received no intratumoral injection due to technical reasons. Therefore, we conclude that intratumoral injection has a locally limited effect whereas intradermal injection leads to systemic activation.

Most interestingly, all non-responders showed no cytotoxic activity at any time during the study. In contrast, responders showed cytotoxic activity before start of therapy. This could be enhanced during the study. Therefore, cytotoxic activity may serve as a predictive marker for immunologic function.

In conclusion, the protocol described has produced encouraging results concerning toxicity and feasibility of DC-based vaccines in RCC. In addition, this study shows a potential role for cytotoxic activity of patients' effector cells as predictive marker. This aspect will be evaluated in further studies.

Acknowledgements

The authors would like to thank the Institute of Experimental Hematology of the University of Bonn for excellent cooperation, in particular Dr. K. Dengler, and PD Dr. G. Marklein from the Institute of Medical Microbiology & Immunology, the colleagues from the Department of Radiology in particular Dr. Meyer, Prof. Dr. Th. Bieber for helpful discussions, and our colleagues from the Department of Medicine of the University of Bonn. We would particularly like to thank A. Kling, Department of Surgery, Heidelberg, and Prof. Dr. P. Knolle, Institute for Molecular Medicine and Experimental Immunology, University of Bonn for proof-reading the manuscript. The authors also thank the H.W. & J. Hector foundation, Mannheim, Germany, for a generous grant.

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Please cite as

Märten A, Sievers E, Albers P, Müller S, Franchy C, von Ruecker A, Strunk H, Schild HH, Schmiedel A, Sommer T, Sauerbruch T, Schmidt-Wolf IG. Telomerase-pulsed dendritic cells: preclinical results and outcome of a clinical phase I/II trial in patients with metastatic renal cell carcinoma. *GMS Ger Med Sci*. 2006;4:Doc02.

This article is freely available from

<http://www.egms.de/en/gms/2006-4/000031.shtml>

Received: 2005-09-28

Published: 2006-02-13

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