Dendritic cells as potential adjuvant for immunotherapy in adrenocortical carcinoma

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Summary

Objective Adrenocortical carcinoma (ACC) is a rare malignancy associated with a dismal prognosis. Dendritic cells (DCs) are professional antigen-presenting cells leading to an antitumour immune response. The aim of this study was to elaborate two methods of antigen delivery to DCs and to evaluate an immunotherapy protocol in ACC patients.

Design/patients Autologous DCs were pulsed with autologous tumour lysate (TL). Fusion of DCs with tumour cells was based on a polyethylene glycol method. Two patients with metastasized hyper-secretory ACC were vaccinated twice.

Measurements In vitro data were quantified by measurement of PBMC (peripheral blood mononuclear cell) responses and cytokine secretion and by flow cytometry analyses. Clinical response was monitored by CT scan of tumour mass and measurement of angiogenic factors.

Results The maximum loading of TL was obtained at 24 h as 48-2% (± 26-8%) of DCs were TL-positive. The DC/tumour cell fusion efficacy was ∼45% as shown by double positive staining for ACTH receptor and DC-specific CD83. In vivo DC vaccination resulted in positive delayed-type hypersensitivity skin reactions reflecting specific memory T-lymphocyte reaction. In vitro analyses revealed specific T-cell proliferation in patient 1 (stimulation index: 5-7 compared to pretreatment) and induction of cytotoxic granzyme B secreting T cells in patient 2 (0·41% CD8 + cells vs. 0·06% pretreatment) as indicators of specific cytotoxic T cells. Although angiogenic serum markers could be stabilized, no impact on tumour growth could be observed.

Conclusion Our data demonstrate that autologous dendritic cells induce antigen-specific Th1 immunity in adrenocortical carcinoma. The clinical outcome, however, was not improved in the patients studied here.

Introduction

Adrenocortical carcinoma (ACC) is a rare malignancy associated with a dismal prognosis. In treated ACC, overall 5-year survival rates range from 20% to 60% in different series. Due to poor survival rates and the low incidence of ACC, the treatment of patients with ACC has never been adequately standardized and the treatment options are very limited. Complete tumour removal offers the best chance for long-term survival. However, despite tumour resection, most patients develop local recurrence or distant metastases. Mitotane, an analogue of the insecticide DDT, has been used in the treatment of ACC either alone or in combination with other cytotoxic agents. However, the average response rate of mitotane and conventional chemotherapy is at best about 30%. This low response rate coupled with considerable adverse effects of these drugs, emphasizes the need for novel treatment modalities such as immunotherapy.

The major goal for all vaccination strategies against cancer is to break the state of tolerance. This challenge is even greater in immunotherapy targeting ACC as the tumours often secrete large amounts of presumably immunosuppressive glucocorticoids. Dendritic cells (DCs) are professional antigen-presenting cells (APCs), derived from haematopoietic progenitor cells, acting as sentinels of the immune system and playing a pivotal role in the induction of the immune response. Based on these properties, DC vaccines have been developed as an approach for inducing antitumour immunity. Strategies to introduce tumour antigens into DCs have included loading of individual tumour peptides or proteins and transfer of tumour-specific DNA or RNA. Supporting this strategy, we have demonstrated that polypeptide hormones, shown for calcitonin in medullary thyroid carcinoma, may serve as specific targets to induce tumour-specific immunity. In general, however, the use of antigen-specific vaccines is potentially limited by the paucity of identified tumour antigens, their uncertain immunogenicity,
and the potential evasion of immunological targeting through their down-regulation by tumour cells. In this context, the use of whole tumour cell extracts have been established including pulsing of DCs with tumour lysate (TL), apoptotic bodies, or tumour RNA.

Another strategy for the generation of DC vaccines is based on the fusion of autologous tumour cells with DCs. In this approach, the entire repertoire of tumour antigens, including those yet to be identified, is expressed with the immune-stimulating machinery of DCs. The fusion cell vaccine allows for induction of helper T cells and cytotoxic T lymphocyte's (CTL) responses by class II presentation of exogenous protein and class I presentation of newly synthesized endogenous protein. Vaccination with fusion cells has eradicated established tumours in a number of animal models. In human MUC1 transgenic mice, vaccination with fusion cells reverses immunological unresponsiveness to MUC1 and results in the rejection of MUC1-positive tumours. Preclinical studies with patient-derived breast cancer cells and DCs have also demonstrated that fusion cells induce tumour-specific CTL responses and lysis of autologous tumour cells. Recently, Avigan and coworkers confirmed these results by demonstrating a Th1 immunity and clinical response in patients with metastatic breast and renal cancer.

As no specific tumour antigens have been identified in ACC so far, we aimed to evaluate two protocols for antigen delivery to DCs in order to elaborate a new therapeutic approach for ACC. On the basis of these results two patients with metastasized ACC were immunized with TL-pulsed DCs and were followed up for several months.

Materials and methods

Generation of dendritic cells

Dendritic cells were generated as recently described with minor modifications. Human monocytes were isolated by using leucapheresis followed by Ficoll density gradient centrifugation and an adherence step (for 2 h). Adherent cells were cultured for 6 days in X-VIVO 20 (Bio Witthacker, Walkersville, MD, USA) supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF; 10⁶ u/l; Leukine®, Immunix, Seattle, WA) and interleukin-4 (IL-4; R&D Systems, Wiesbaden, Germany) and an adherence step (for 2 h). Adherent cells were cultured for further analyses in cell suspension containing PEG was diluted within 10 min with RPMI 1640. After washing, cell hybrids were cultured for further analyses in serum-free medium at a 1 : 1 ratio. After low-speed centrifugation for 10 min, 1000 g; supernatant was passed through a 0-2 µm filter. Protein concentration was determined by Bradford method, and aliquots were stored at −80 °C until use.

Loading of dendritic cells with tumour lysate

As no established tumour antigen has yet been identified in ACC, we used whole autologous TL for antigen delivery (100 µg/1 × 10⁶ DC/ml medium) during maturation process of DCs (day 7). For exact quantification of TL uptake into DCs FITC-labelled TL was incubated with immature DCs (after 6 days of culturing) in the presence of the maturation cytokines for up to 48 h. The kinetic of uptake, performed at 37 °C, was monitored and compared to unspecific binding at 4 °C. Mature DCs were stained with phycoerythrin (PE) labelled anti-CD11c antibody.

Preparation of tumour lysate

Tumour samples from patients with ACC were prepared as described previously. In brief, after resection of tumour masses, tissue samples were cut into small pieces and dispersed in RPMI 1640. Cells were lysed by mechanical homogenization using a conical homogenizer (Merck, Frankfurt, Germany) and by repeated freezing in liquid nitrogen and thawing at 37 °C (five times, 10 min each). Larger particles were removed by centrifugation (10 min, 1000 g); supernatant was passed through a 0-2 µm filter. Protein concentration was determined by Bradford method, and aliquots were stored at −80 °C until use.

Preparation of dendritic cell/tumour cell hybrids

In parallel, a protocol for the fusion of DCs with tumour cells was established on the basis of the ACC cell line NCI-H295R and the DC-like cell line TF-1. Briefly, single cell suspension was held in serum-free medium at a 1 : 1 ratio. After low-speed centrifugation (200 g), cell pellets were stored at 4 °C for 30 min, and resuspended in 500 µl of 50% polyethylene glycol (PEG, Sigma, Deisenhofen, Germany) in phosphate buffered saline (PBS) or with PBS alone. The cell suspension containing PEG was diluted within 10 min with RPMI 1640. After washing, cell hybrids were cultured for further analyses in RPMI with 10% foetal calf serum together with 5 × 10⁵ iu/l GM-CSF.

Evaluation of fusion efficacy

Flow cytometry analyses. Cells or pellets of the cell hybrids (1 : 1) were compared to the controls of NCI-H295R and TF-1 alone. ACTH receptor expression as specific surface antigen of adrenocortical cells was determined by a rabbit anti-MC2R antibody (Abcam, Cambridge, UK) and visualized by fluorescein-labelled goat anti-rabbit antibody (Chemicon, Hofheim, Germany). FITC-labelled anti-CD83 (BD, Heidelberg, Germany) was used to detect DC surface antigens. FACS analysis was performed as described above.

Electron microscopy. For ultrastructural analysis (electron microscopy) pellets of all samples were fixed in 4% paraformaldehyde, 1% glutaraldehyde in 0·1 m phosphate buffer (pH 7·3) for 3 h, postfixed for 90 min in 2% OsO₄ in 0·1 m cacodylate (pH 7·3), dehydrated in ethanol, and embedded in epoxy resin. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and examined at 80 kiloVolts under a Phillips electron microscope 301 (Phillips, Rahway, NJ, USA).
Patients

Two ACC patients were treated with TL-pulsed DCs. Patient 1 (male, 60 years) was referred to our clinic in March 2003 because of a tumour (10 cm in diameter) of the left suprarenal region. Endocrinological testing revealed a cortisol-producing tumour. The diagnosis of ACC was confirmed following surgical resection of the tumour mass and tumour-infiltrated local lymph nodes (03/2003). Thereafter, mitotane therapy was initiated. After the diagnosis of metastatic spread (07/2003) with retroperitoneal and para-aortic lymph nodes swelling and diffuse hepatic lesions leucapheresis was performed and immunotherapy with TL-pulsed DCs was initiated.

The diagnosis of ACC in patient 2 (male, 34 years) was made after resection (08/2002) of an adrenal tumour mass (10 cm in diameter). Thereafter, oral therapy with mitotane was initiated. After diagnosing a rapidly growing local tumour mass and two new hepatic lesions the patient was referred to our clinic. Since the patient was already treated with mitotane an exact endocrine assessment was not feasible. However, a highly elevated basal 17-OH-progesterone level (22 nmol/l; normal range: 1·3–10 nmol/l) indicated an endocrine active adrenal cancer. After resection of all tumour masses (and asservation of the diagnosis of ACC) the patient again presented in our clinic in October 2003. At that time, magnetic resonance imaging of the liver revealed multiple liver metastases and metastases in the lumbar spine. Leucapheresis was performed and immunotherapy with TL-pulsed DCs was initiated.

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The study protocol was approved by the Ethics Committee of the University of Dusseldorf.

Treatment of patients

Both patients received two injections with a median of 3·4 × 10^7 mature DCs (range 2·3–4·1 × 10^7 DCs) per vaccine with an interval of 2 and 3 weeks, respectively. DCs were injected intradermally into the upper arm.

Evaluation of immunological response

Delayed-type hypersensitivity (DTH) was defined as positive when erythema and induration was > 5 mm in diameter 24 h after intradermal vaccine injection. In patient 2, we additionally tested DTH reactivity by intradermal injection of pure TL (10 µg in 100 µl isotonic NaCl/irradiated with 3000 rad).

T-cell proliferation assay was performed as recently described. Briefly, cryo-preserved PBMCs (peripheral blood mononuclear cells) were cultured in 96-well plates in triplicates (1 × 10^5 cells per well in 20 µl IL-2 containing T-cell medium) in the presence of TL or albumin (25 mg/ml) for 5 days. On day 4 cells were pulsed with 1 µCi [3H] thymidine per well 18 h before harvesting. Thymidine incorporation was assessed and cellular proliferation was expressed in absolute counts per minute.

For intracellular cytokine staining autologous, cryopreserved PBMCs (1 × 10^5 cells/ml) were cultured in T-cell medium in the presence of TL or human albumin (each 100 mg/l). After 16 h, Brefeldin A (10 mg/l) was added. Cell staining was conducted with PerCP-labelled anti-CD4 antibody, followed by cell permeabilization with Cytofix/Cytoperm and staining with PE-labelled anti-IL4 or FITC-labelled anti-IFNγ antibodies, respectively. In parallel, cells were stained with FITC-labelled anti-CD8 antibody, followed by intracellular staining with PE-labelled antigranzyme B antibody. Afterwards FACS analysis was performed as described above.

Evaluation of clinical response

Metastatic lesions were evaluated by computed tomography scans. Moreover, a number of angiogenic serum markers were measured as indirect parameters of tumour cell growth: antiangiogenic factor tissue inhibitor of metalloproteinase 2 (TIMP-2) as well as proangiogenic factors angiogenin, vascular epithelial growth factor (VEGF), and β-homodimer of the platelet derived growth factor (PDGF-BB) were determined as recommended by the manufacturer (R&D Systems).

Safety parameters (ANA, pANCA, cANCA, anti-TPO-Ab, anti-Tg-Ab, anti-GAD65-Ab) and hormones were measured in routine assays at regular intervals.

Statistical analysis

The results were analysed for statistical significance by paired t-test (using the PRISM computer software GraphPad Software Inc., San Diego, CA, USA).

Results

Generation of dendritic cells and tumour lysate uptake

As previously described, mature DCs expressed moderate to high levels of costimulatory CD80 (mean 60–2%; range 36–94%) and CD86 (89–1%; 63–96%), of DC-specific CD83 (65–7%; 37–88%), and antigen-presenting cell-specific HLA-DR (96%; 93–99%) (Fig. 1). The yield of DCs compared to the number of monocytes used for DC generation was 3-6% (range 2-5–6-2%).

![Fig. 1 Maturation of autologous dendritic cells (DC). Mature DCs were generated for both patients twice by using GM-CSF and IL-4 for 6 days and a maturation cocktail for one additional day. Mature DCs were stained with phycoerythrin-labelled antibodies and FACS analyses were performed to determine CD80, CD83, CD86, and HLA-DR positive cells.](image-url)
To investigate the efficacy of antigen incorporation by DCs, we used FITC-labelled TL. We could demonstrate that FITC-labelled TL was continuously absorbed by DCs with maximum intake of 48.2% (±26.8%) 24 h after initiation (mean fluorescence intensity: 65–100). After 48 h no further increase could be detected, which is in line with the maturation status of these cells (Fig. 2). For control, DCs were also incubated with TL at 4 °C without significant TL intake.

**Fusion of dendritic cells and adrenal carcinoma cells**

To improve antigen delivery for future vaccination trials, we investigated the possibility of adrenal tumour cells to be fused with DCs. As expected the ACC cell line was negative for CD83 and positive for MC2R and the DC-like cell line vice versa. After fusion, 47% (±29%) of the hybridized cells were positive for MC2R and 45% (±26%) for CD83 (Fig. 3a,b) indicating an efficacy rate of about 45%. These results are in line with previously published data by others.

Electronmicroscopic imaging of NCI-H295/TF-1 hybrids clearly showed viable cells indicated by intact basal membranes around the hybrids (Fig. 3e). Most of the cell hybrids revealed all cellular compartments (black and white arrows) characteristic for both cell lines used for fusionizing (NCI-H295 is shown in Fig. 3c, TF-1 is shown in Fig. 3d). Treatment of ACC patients with autologous dendritic cells.

**Toxicity profile of vaccination**

Dendritic cell vaccinations were well tolerated by both patients. No adverse effects or any clinical signs of autoimmune reactions were seen. None of the laboratory autoimmune parameters were elevated during follow-up.

**Immunological responses**

Following the second immunization, both patients developed delayed-type hypersensitivity with erythema and induration suggesting the presence of TL-specific memory T lymphocytes. Additionally, the administration of 10 µg pure TL (without DCs) in patient 1 led to an equal skin reaction suggesting a specific immune response.

T-cell proliferation analyses using autologous PBMCs revealed a significant TL-specific T-cell proliferation in patient 1 following the second vaccination (5.7-fold increase compared to pretreatment) but not in patient 2 (Fig. 4a).

To monitor the induced immune response in more detail we also performed intracytoplasmatic cytokine and granzyme B staining. The analysis of intracytoplasmatic IL-4 and IFNγ did not demonstrate any significant differences during follow-up in both patients (Fig. 4b).

Interestingly, in patient 1 a strong intracytoplasmatic IFNγ staining was already detectable before initiating immunotherapy (0.57% TL-specific IFNγ-producing CD4+ T cells vs. 0.08% albumin-specific CD4+ T cells, P < 0.001). This high frequency of TL-specific Th1 T cells might indicate a pre-existing antitumour-specific immune response in this patient.

By using the more sensitive granzyme B measurement to detect a cytotoxic T-cell response a pre-existing CTL reactivity was seen in patient 1 (0.19% ± 0.04%) without any further increase during follow-up. In contrast, patient 2 developed a strong TL-specific CTL response after the second vaccination (0.41% granzyme B positive T cells after vaccination vs. 0.06% before treatment, Fig. 4c).

**Clinical monitoring of patients**

Due to the concomitant treatment with mitotane, steroid hormones could not serve as tumour markers. Therefore, we sought for alternative...
parameters. As angiogenic markers have already been reported to have considerable potential in the prognosis of malignant diseases, we investigated different angiogenic factors. Level in normal subjects are known to differ individually and have been shown in the literature as control groups for TIMP-2 and for angiogenin and for VDGF.

Both patients had normal or slightly increased levels of TIMP-2 (Fig. 5a) and angiogenin (Fig. 5b). Patient 1 had significantly elevated levels of VEGF (Fig. 5c), and of PDGF-BB (Fig. 5d) before therapy. During follow-up a decrease of VEGF was documented for 12 weeks after vaccination (Fig. 5b). Patient 2 had an almost normal and stable level of VEGF, whereby the level of PDGF-BB decreased 3 weeks after the initial therapy (Fig. 5d). Despite these results, both patients had increased metastatic spread and patients died 5 months (patient 1) and 7 months (patient 2) after first vaccination.

Discussion

The major aim of this study was to elaborate different methods of antigen delivery to autologous dendritic cells (DCs) in order to establish an immunotherapy approach for adrenocortical carcinoma (ACC). Based on these results two ACC patients were vaccinated to evaluate the feasibility and safety of an autologous DC vaccination in vivo and to test the option of autologous tumour lysate to serve as a source of antigen in this context.

Our data indicate that autologous DCs can be applied without side-effects and without signs of autoimmune diseases. After administration of the second vaccination, both patients developed a delayed-type hypersensitivity skin reaction as an indicator of an antigen-specific memory T-cell response. Detailed in vitro analyses revealed a TL-specific Th1-driven PBMC response and the presence of cytotoxic immunity. Hereby, we could prove that it is feasible to stimulate an ACC-specific immune response in patients with a glucocorticoid secreting tumour (patient 1). In contrast, both patients experienced growing metastatic disease and died at the end of follow-up period. Irrespective of the clinical outcome, however, this study demonstrates, for the first time, that autologous DCs may serve as adjuvants for immunotherapy in ACC, which potentially represents the basis for future vaccination trials in ACC.

The main obstacle for an effective immunotherapy is that no established tumour antigen has yet been identified for ACC. Recently, an approach in a murine model gave evidence that steroidogenic acute regulatory protein (StAR) potentially serves as an immunotherapeutic target. But StAR is present only in a subset of patients with ACC. We therefore used whole TL as antigen source in our study. We were able to demonstrate that DCs continuously absorb FITC-labelled TL in vitro with high efficacy rate about 24 h after initiation. A TL-based approach had already been applied in vivo in different malignancies such as highly malignant melanoma or in metastatic renal cell carcinoma. In the context of endocrine malignancies, we applied TL-pulsed DCs in a patient with parathyroid carcinoma leading to a specific in vitro immune response.

Dozens of other studies have reported on specific CTL reactivities and some clinical responses following administration of DCs loaded with tumour-specific antigens. Most of these studies were performed in immunogenic cancers such as malignant melanoma and malignant lymphoma, which indicates that TL in general might serve as antigen donor in immunotherapy concepts. Our study clearly indicates that this approach might be suitable for ACC as well.
Fig. 4 Proliferative response, IFN-γ-specific intracytoplasmatic cytokine staining and Granzyme B staining in response to tumour lysate. (a) Proliferative response: cryo-preserved PBMC (1 × 10^5 cells) were cultured in triplicates in the presence of TL (25 mg/l) for 5 days. Cells were pulsed with 1 μCi[^3H] thymidine and incorporation was assessed using a microscintillation counter. Cellular proliferation is expressed in counts per minute (c.p.m.). Results are demonstrated before therapy (hatched bars) and after second vaccination (black bars). (b) IFN-γ-specific intracytoplasmatic cytokine staining of CD4 lymphocytes were determined after 20 h of culturing with 100 mg/l tumour lysate for both patients before therapy (hatched bars) and after second vaccination (black bars). (c) Induction of cytotoxic T lymphocytes: granzyme B staining of CD8 lymphocytes as an indicator of cytotoxic immunity was performed after 20 h of culture of PBMCs with TL (100 mg/l) using an intracytoplasmatic staining protocol. Results are demonstrated before therapy (hatched bars) and after second vaccination (black bars).

Fig. 5 Pro- and antiangiogenic serum markers. Pro- and antiangiogenic serum markers [TIMP-2 (5a); angiogenin (5b); VEGF (5c); PDGF-BB (5d)] were evaluated before (white bars; day 0), after first vaccination (hatched bars; for patient 1: day 19; for patient 2: day 17) and after immunotherapy (black bars; day 79/day 66). Whereas for TIMP-2 and angiogenin only minor changes were seen during the follow-up, the VEGF level was decreased in patient 1 after the first vaccination and a trend to improvement was seen in patient 2 for PDGF-BB. The normal values according to the literature are shown by dotted lines.
Also, we here established a method to fusion DCs with tumour cells based on a polyethylene glycol method with high efficacy rate. This is of potential interest for future vaccination strategies, as the whole antigenic repertoire of tumour cells together with the strong immunostimulatory capacity of DCs will be unified in one cell type. For ethical reasons we could not use this approach in our patients with adrenocortical carcinoma in vivo. However, our in vitro results are in line with other studies in murine cancer models as well as in a preclinical study with patient-derived breast cancer cells leading to tumour cell lysis. Recently, Avigan et al. reported on tumour regression or disease stabilization, respectively, in 8 of 23 patients with breast cancer and renal cell carcinoma. Our in vitro results based on two tumour cell lines indicate that hybrid cell generation may represent an alternative approach in ACC as well.

In summary, we here report for the first time on dendritic cell immunotherapy in two patients with ACC in advanced disease stages. Irrespective of the unfavourable clinical outcome, our in vitro results strongly support the ability of DCs to induce tumour-specific immune responses in endocrine malignancies. Our in vitro fusion studies may serve as a promising technique for future DC vaccination trials in ACC patients, where no established tumour antigens have been identified thus far.

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References

human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. *Proceedings of the National Academy of Sciences of the USA*, 95, 6279–6283.


