Dendritic cells (DCs) have attracted wide interest because of their unique capacity to elicit primary and secondary antitumor responses. We have generated autologous tumor lysate-pulsed DCs from three patients with medullary thyroid carcinoma (MTC) and tested them for their ability to stimulate cytotoxic T-cell responses against autologous MTC tumor cells in vitro. The aim of our investigations was to evaluate the potential efficacy of DC-based immunotherapy in patients with MTC. DCs were generated from peripheral blood monocytes using GM-CSF and IL-4 (immature DCs) or GM-CSF, IL-4, and TNFα (mature DCs). Our results indicate that mature tumor lysate-pulsed DCs are able to elicit a human leukocyte antigen class I-restricted cytotoxic T-cell response against autologous MTC tumor cells, whereas immature tumor lysate-pulsed DCs do not stimulate significant antitumor activity. We feel that our data may be relevant for future clinical trials of active immunotherapy using tumor lysate-pulsed DCs in patients with MTC who have residual or distant disease after surgical treatment. The fact that mature DCs displayed a substantially higher capacity to stimulate autologous antitumor T-cell responses than immature DCs underlines the importance of a maturation step in immunotherapy protocols based on DCs.

**Stimulation of Autologous Antitumor T-Cell Responses Against Medullary Thyroid Carcinoma Using Tumor Lysate-Pulsed Dendritic Cells**


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MEDULLARY THYROID CARCINOMA (MTC) is a calcitonin-secreting tumor of the parafollicular C cells that accounts for approximately 5–10% of all thyroid malignancies (1, 2). It may occur sporadically, in a familial form without associated endocrinopathies, or combined with other endocrinopathies as multiple endocrine neoplasia type 2A or 2B with an autosomal dominant inheritance (2, 3). MTC has a slow but progressive clinical course with an early involvement of lymph nodes in the neck and mediastinum. At the time of initial diagnosis, at least one-quarter of patients has distant metastases (4). The treatment of choice for both the hereditary and sporadic type of MTC is thyroidectomy and central/lateral neck dissection to control localized disease because radiation and chemotherapy are only palliative once distant metastases have developed (2). Although many patients live with recurrent or residual tumor for up to a decade, 30–50% of patients die within 10 yr of diagnosis (5). As a result, there has been a search for alternative strategies to treat patients with disseminated MTC. Thus far, promising preclinical and clinical results have been obtained using different experimental approaches such as suicide gene therapy (6), immunogene therapy (7), or treatment with radiolabeled monoclonal antibodies (8).

Recently, it has become evident that dendritic cells (DCs) play a key role in the induction of primary immune responses owing to their properties as the most potent antigen-presenting cells for naïve T-cell activation (9, 10). DCs originate from the bone marrow and reside in a resting or immature state in nonlymphoid tissues, in which they efficiently capture and process antigen. Upon stimulation with bacterial products, inflammatory cytokines or CD40-ligation, DCs undergo a maturation process that results in enhanced antigen-presenting capacity, enhanced expression of MHC and costimulatory molecules, and migration into secondary lymphoid organs in which they prime naïve T cells (11–13).

Because of their unique capacity to stimulate restig T cells, DCs are a promising option for immunization protocols, particularly for the induction of antitumor immunity in patients with malignant diseases (14–20). The rationale for this approach is based on the observation that DCs can be pulsed with tumor antigen and subsequently administered as a cellular vaccine to induce a specific antitumor response (21–28). Large numbers of DCs can relatively easily be generated in vitro from peripheral blood monocytes using granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (29). These cells have the characteristic features of immature DCs and can be further induced to mature by inflammatory stimuli such as TNFα, IL-1β, or CD40-ligand (30–35). To date, several clinical trials using tumor antigen-pulsed DCs have been carried out, and promising results have been obtained for different tumors without significant side effects from the vaccinations (36–45).

In the present in vitro study, we have evaluated whether tumor lysate-pulsed DCs derived from patients with MTC are able to elicit a cytotoxic T-cell response against autologous MTC tumor cells. Our aim was to obtain initial preclinical evidence for the potential efficacy of immunotherapy.
using tumor lysate-pulsed DCs in patients with MTC. Importantly, a major objective of our study was to establish an experimental MTC model that would allow the evaluation and subsequent optimization of the immunostimulatory capacity of DCs under autologous conditions (i.e. by using both DCs and MTC tumor cells from the same individual), hoping that such an approach would increase the clinical relevance of our investigations.

**Materials and Methods**

**Subjects and tumor cell lines**

After written informed consent, tumor tissue and peripheral blood samples were obtained from three patients with MTC. All were genetically proven sporadic cases: patient 1, 53 yr old, female; patient 2, 72 yr old, male; patient 3, 29 yr old, male (SinJ) (46). In addition, tumor tissue was obtained from a 51-yr-old female patient (MTC-SK) (47). However, because the patient died before initiation of our study, no peripheral blood samples could be obtained from this patient. Tumor tissue was collected and transported in PBS containing 100 IU penicillin/ml and 100 μg streptomycin/ml. Directly after transportation, tissue was treated for 20 min with PBS containing 1000 IU penicillin/ml and 1000 μg streptomycin/ml. Tissue was cut into small pieces, minced, and resuspended in erythrocyte lysis buffer for 15 min. The suspension was centrifuged and resuspended in nutrient mixture Ham’s F12 (Life Technologies, Inc., Grand Island, NY); supplemented with 10% FBS (PAA Laboratories, Exton, PA), 100 IU penicillin/ml, and 100 μg streptomycin/ml at an estimated cell number of 3-5 × 10⁶ cells/ml; and incubated at 37 C in a 5% CO₂ humidified atmosphere at 37 C. After a few weeks, antibiotics were omitted. Stromal fibroblasts were separated from tumor cells by repeated selective adhesion and selective detachment treatment (48). The resulting tumor cell line is growing continuously as suspensions of single cells and spheroid aggregates. Each cell line retains a high grade of differentiation, such as a positive immunoreactivity with antibodies to calcitonin.

**Preparation of DCs from CD14⁺ peripheral blood mononuclear cells (PBMCs)**

For preparation of DCs, PBMCs were isolated from peripheral blood of patients with MTC using Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation. CD14⁺ cells were purified using a magnetic bead-conjugated mouse antihuman CD14 monoconal antibody (CD14-MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD14⁺ cells, as determined by flow cytometry, was always more than 95%. The cells were seeded at 7.5 × 10⁶/ml in RPMI-1640 medium (Life Technologies, Inc.), supplemented with 1000 U/ml GM-CSF (Novartis Pharmaceuticals, Basel, Switzerland), 1000 U/ml IL-4 (Strathmann PBH, Hamburg, Germany) and 10% heat-inactivated FBS (Life Technologies, Inc.) in a 5% CO₂ humidified atmosphere at 37 C. On d 2, half a volume of growth medium containing freshly added cytokines was supplemented. On d 5, DCs were pulsed with 100 μg/ml autologous tumor cell lysate for 12 h at 37 C. Thereafter, pulsed DCs were cultured for another 48 h with either GM-CSF alone (immature tumor lysate-pulsed DCs) or were matured with GM-CSF + 100ng/ml TNFα, kindly made available by Dr. H. R. Alexander Jr. from the National Cancer Institute, Bethesda, MD (mature tumor lysate-pulsed DCs).

**Isolation of autologous T cells**

Autologous T cells were isolated by depletion of B cells, monocytes, natural killer cells, DCs, early erythroid cells, platelets, and basophils from PBMCs using a cocktail of hapten-conjugated CD11b, CD16, CD19, CD56, and CD56 antibodies in combination with a magnetic bead-conjugated anti-hapten antibody (Pan T cell isolation kit, Miltenyi Biotec). The purity of isolated T cells was always more than 95%. The T cells were cryopreserved at −70 C in RPMI-1640 (Life Technologies, Inc.) containing 10% dimethylsulphoxide and 20% FBS until further use.

**Phenotypic analysis of DCs by flow cytometry**

The expression of surface markers characteristic for DCs was determined by flow cytometry. Cell staining was performed using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mouse antibodies against CD11a, CD11c, CD14, CD40, CD80, CD83, CD86, HLA-ABC, and HLA-DR. Appropriate mouse IgG isotype controls were used to determine the levels of background staining. Approximately 10,000 cells/sample were analyzed using an Epic XL flow cytometer equipped with System II software (Beckman Coulter, Inc., Fullerton, CA).

**Preparation of autologous tumor cell lysate**

One to 2 × 10⁶ autologous tumor cells were washed in PBS (Life Technologies, Inc.) and subjected to five freeze (liquid nitrogen) and thaw (37 C water bath) cycles to obtain a crude lysate. After removal of larger particles by centrifugation (2000 g, 10 min, 4 C), the protein content was determined in the supernatant by the Comassie Plus Protein Assay (Pierce Chemical Co., Rockford, IL) and aliquots stored at −70 C until use.

**In vitro proliferation assays**

T-cell proliferation assays were performed in round-bottom 96-well plates (Costar Corning, Inc., Acton, MA) by culturing 10⁵ freshly thawed naive autologous T cells with increasing numbers (from 1.25 × 10⁴ to 8 × 10⁴) of immature or mature tumor lysate-pulsed DCs (or unpulsed DCs as controls) in RPMI-1640 (Life Technologies, Inc.), supplemented with 10% FBS in a final volume of 200 μl. The proliferative response of the T cells was measured on d 6 by addition of 1 μCi [methyl-³²H]-thymidine to triplicate wells for 18 h. Thymidine uptake was counted on a liquid scintillation counter (Wallac, Inc., Turku, Finland). Values are expressed as mean counts per minute obtained for triplicate wells.

**In vitro cytotoxicity assays**

For stimulation of tumor-specific cytotoxic T cells, freshly thawed naive autologous T cells were cocultured with immature or mature tumor lysate-pulsed DCs at a T:DC ratio of 10:1 in RPMI-1640 + 10% FBS without addition of cytokines. On d 7, T cells were harvested and T-cell-mediated cytotoxicity against autologous MTC tumor cells was measured using a standard in vitro 4-h europium release assay. To do so, autologous target cells (5 × 10⁵) were labeled with europium for 15 min at room temperature. Subsequently, 5 × 10⁵ target cells and serial dilutions of effector cells at effector:target ratios ranging from 25:1 to 1:1 were incubated for 4 h in 200 μl RPMI-1640 medium (without phenol red) and 10% FBS in round bottom 96-well plates. Thirty microliters of the supernatant were harvested, and europium release was measured by time-resolved fluorometry (Wallac, Inc.). Specific cytotoxic activity was calculated by the following formula: percent specific release = (experimental release-spontaneous release)/(total release-spontaneous release) × 100. Spontaneous release of the target cells was generally less than 25% of total release as determined by detergent (1% Triton X-100).

**Blocking assay**

To determine whether tumor cell lysis was HLA class I-restricted, a blocking assay was performed in which tumor cells were pretreated with anti-HLA class I antibody (clone W6/32) or an appropriate isotype control antibody (DAKO Corp. A/S, Glostrup, Denmark) for 30 min at 4 C at a concentration of 50 μg/ml.

**Results**

**Cell surface phenotype of CD14⁺-derived DCs generated under different culture conditions**

Both culture conditions (GM-CSF + IL-4 as well as GM-CSF + IL-4 + TNFα)) resulted in the generation of cells with typical DC morphology. Flow cytometric analysis of DCs revealed significant differences in the expression of surface molecules crucially involved in the interaction of DCs with
immune effector T cells (Fig. 1): Compared with immature DCs (generated in the presence of GM-CSF + IL-4), mature DCs (generated in the presence of GM-CSF + IL-4 + TNFα) consistently showed a substantially enhanced expression of HLA-DR and costimulatory molecules such as CD40, CD80, and CD86. Furthermore, mature DCs expressed high levels of the DC maturation marker CD83, which was detectable only to a low extent on immature DCs.

To further characterize the DCs obtained under different culture conditions in our system, we examined the DCs for their ability to stimulate proliferation of autologous T cells in MLR assays. As representatively shown in Fig. 2, mature DCs displayed a higher T-cell stimulatory capacity than immature DCs. The most pronounced T-cell proliferative response was

**Fig. 1.** Phenotypic characteristics of CD14⁺-derived DCs. Cells were labeled with FITC- or PE-conjugated antibodies against surface markers, as indicated. Negative controls correspond to labeling with an isotype-matched control antibody. Logarithmic fluorescence intensity of FITC is plotted on the x-axis; logarithmic fluorescence intensity of PE is plotted on the y-axis. iDCs, immature DCs; mDCs, mature DCs.
obtained for triplicate wells. T-cell proliferation is expressed as mean counts per minute thawed T cells (1 to stimulate proliferation of autologous T cells in MLR assays. Freshly unpulsed DCs

Stimulation of cytotoxic T-cell activity by autologous DCs

To assess the capacity of mature unpulsed DCs of patients 2 and 3 to induce autologous cytotoxic T-cell responses was less prominent, being comparable with that of immature tumor lysate-pulsed DCs.

**HLA class I specificity of T-cell-mediated lysis of autologous MTC tumor cells**

To test whether the cytotoxic T-cell response elicited by mature tumor lysate-pulsed DCs was HLA class I restricted, a blocking experiment using a monoclonal antibody specific for nonpolymorphic determinants of HLA class I molecules (clone W6/32) was performed. As shown in Fig. 4, pretreatment of tumor targets with W6/32 antibody led to approximately 70% inhibition of T-cell-mediated cytotoxic activity against autologous tumor cells, compared with pretreatment of tumor targets with an appropriate isotype control preparation (anti-mouse IgG2a).

**Discussion**

The question addressed by the present in vitro study was whether autologous tumor lysate-pulsed DCs derived from patients with MTC are able to elicit a cytotoxic T-cell response against autologous MTC tumor cells. We have found that autologous tumor lysate-pulsed DCs that have been matured with TNFα are able to stimulate a cytotoxic T-cell response against autologous MTC tumor cells, whereas immature tumor lysate-pulsed DCs do not stimulate significant autologous antitumor activity. Furthermore, the cytotoxic T-cell response induced by mature tumor lysate-pulsed DCs was strongly inhibited by a monoclonal antibody specific for nonpolymorphic determinants of HLA class I molecules. This provides evidence that HLA class I-restricted cytotoxic T cells were the main effectors of autologous antitumor activity in our experiments.

DCs are recognized as the most efficient antigen-presenting cells for the induction of primary immune responses and thus represent a highly promising therapeutic option for cancer immunotherapy (15, 20, 22, 49, 50). Several clinical studies using autologous tumor antigen-pulsed DCs in patients with advanced malignancies (e.g., malignant melanoma, prostate carcinoma, renal cell carcinoma, and hematological malignancies) have already been performed, and encouraging results have been obtained (36–44). Recently, also a patient with metastasized parathyroid carcinoma has been treated with autologous tumor lysate-pulsed DCs and both in vitro and in vivo immune responses have been observed (45). It has thus been suggested that immunotherapy with tumor antigen-pulsed DCs might be generally applicable to patients with other advanced, radio- and chemotherapy-resistant endocrine malignancies, including metastasized surgically incurable MTC. Of particular interest,
MTC may be especially suited for cell-mediated immunotherapy with tumor antigen-pulsed DCs because it could be demonstrated that a high proportion of patients with (familial) MTC exhibit cellular immune reactivity to autologous MTC tumor antigen (51).

To the best of our knowledge, the present report is the first to demonstrate in human MTC that autologous tumor lysate-pulsed DCs are able to induce an HLA class I-restricted cytotoxic T-cell response against autologous MTC tumor cells. It should be noted that we performed our experiments under autologous conditions (i.e., using an experimental *in vitro* system in which both DCs, T-cells, and MTC tumor cells were derived from the same individual). To optimally activate a broad repertoire of tumor-specific T cells in our experiments, we used whole-tumor freeze-thaw lysates as a source of antigen for pulsing the DCs. Importantly, the use of tumor lysates has the potential advantage of including antigens associated with the tumorigenic process of a given individual and thus offers the advantage of inducing a broader T-cell response to tumor-associated antigens than could be achieved by pulsing the DCs with a single or several defined synthetic tumor peptides (28, 52).

A key finding of our investigations was the fact that the capacity of DCs to induce a cytotoxic T-cell response against autologous tumor cells was strongly dependent on the maturation status of the DCs: We were able to show that mature tumor lysate-pulsed DCs are more potent stimulators of autologous antitumor activity than immature DCs. A conceiv-
of mature (even though unpulsed) DCs probably led to the cytotoxic T-cell response observed against the autologous but not the allogeneic tumor in this patient.

In conclusion, we have established an experimental tumor model for MTC that offers the possibility to test the immunostimulatory capacity of DCs under autologous conditions. By means of this tumor model, we have been able to show that mature tumor lysate-pulsed DCs obtained from patients with MTC can prime HLA class I-restricted antitumor T-cell responses against autologous tumor cells. Our data might therefore be relevant for future clinical trials of active immunotherapy using DCs in patients with MTC who have residual or distant disease after surgical treatment. The fact that mature tumor lysate-pulsed DCs displayed a substantially higher capacity to stimulate autologous antitumor T-cell responses than immature tumor lysate-pulsed DCs underlines the general importance of a maturation step in immunotherapy protocols based on DCs. Several in vitro studies are therefore currently underway in our laboratory to further optimize the maturation conditions for DCs that are to be used in a clinical trial.

Acknowledgments

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