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RAPID COMMUNICATION

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In vitro induction of a bladder cancer-specific T-cell response by mRNA-transfected dendritic cells

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Abstract ***Purpose:*** To design a tumor-specific immunotherapeutic strategy for treating tumors for which no specific antigens are described (such as bladder urothelial carcinoma), we attempted to activate tumor-specific T-cells by dendritic cells transfected with tumor-derived mRNA. ***Methods:*** Dendritic cells were generated from a patient's peripheral blood and loaded with mRNA derived from the urothelial carcinoma tissue of the same patient. Autologous T-cells were incubated twice on these dendritic cells and tested for their ability to lyse tumor cells. ***Results:*** Dendritic cells transfected with tumor-derived mRNA were able to activate T-cells that recognized autologous tumor cells. Cytotoxicity was around 26% for an effector:target ratio of 50:1. Tumor-infiltrating lymphocytes did not kill the autologous tumor cells in vitro, but after a single stimulation with the transfected dendritic cells, they induced tumor cell lysis of 35.7% at an effector:target ratio of 50:1. ***Conclusions:*** These results indicate that dendritic cells transfected with tumor mRNA containing messages for one or more tumor antigens could serve for the ex vivo activation of effector T-cells or directly as vaccines for a wide range of human neoplasias.

Key words Bladder cancer · Immunotherapy · Dendritic cells · Urothelial carcinoma · Tumor vaccine

Abbreviations *CEA* Carcinoembryonic antigen · *E:T ratio* Effector/target cell ratio · *DC* Dendritic cells · *FACS* Fluorescence-activated cell sorting · *GM-CSF* Granulocyte-macrophage colony-stimulating factor · *PBL* Peripheral blood lymphocytes · *TIL* Tumor-infiltrating lymphocyte

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Introduction

Tumor vaccination with antigens recognized by tumor-specific T-cells is considered to be a promising strategy for cancer immunotherapy. However, this possible approach is hampered by several obstacles. Most of the known epitopes recognized by cytotoxic T-cells (CTL) are associated with melanoma (reviewed in van den Eynde and van der Bruggen 1996; Wang and Rosenberg 1999), and knowledge about T-helper epitopes is even more restricted. Nevertheless, many efforts have been made during the last few years to use dendritic cells (DC) as vaccines. Due to their powerful immune-stimulatory properties as antigen presenting cells (APC), DC are able to induce strong specific T-cell responses (reviewed in Gilboa et al. 1998). In anti-tumor trials, DC have been loaded with peptides, recombinant protein or tumor lysates or were transfected with antigen-coding DNA (Celluzzi et al. 1996; Nestle et al. 1998; Cochlovius et al. 1999; Philip et al. 2000; Wei et al. 2000). However, the results were somewhat disappointing, and in certain cases severe side effects were observed (Ludewig et al. 2000).

In addition, it is still unclear whether the dominant antigens in an anti-tumor immune response are shared tumor antigens or patient-specific individual antigens (Shrivastava 1996). Unfractionized tumor-derived material (mRNA, peptides, lysates) has therefore been used to circumvent the drawbacks of a strategy limited to only one or a few tumor antigens (Nair et al. 1997; Fields et al. 1998).

Recently, a method for generating tumor vaccines by transfecting DC with CEA mRNA was published (Nair at al. 1998). With this technique in mind, we were interested to see if DC transfected with unfractionated

mRNA derived from a freshly removed human tumor (bladder urothelial carcinoma) are able to induce an immune response against the autologous tumor by activating the patient's T-cells.

Material and methods

Tissues and primary cell cultures

Bladder urothelial carcinoma tissue and healthy bladder urothelial tissue of the same patient (male, 67 years old) were used for the establishment of primary cell cultures. All solutions used were treated with diethylpyrocarbonate. Tissues were washed in HBSS (Hank's balanced salt solution without Ca^{2+} and Mg^{2+}, Gibco Life Technologies, Karlsruhe, Germany), incubated for 1 h at 37 °C in solution A (5 mg/ml collagenase, 0.5 mg/ml hyaluronidase, 20 μg/ml DNase in RPMI cell culture medium, supplemented with 10% fetal calf serum), and then for 24 h at room temperature in enzyme solution B (1 mg/mg collagenase, 0.1 mg/ml hyaluronidase, 20 μm/ml DNase in RPMI/10% fetal calf serum). The solution was filtered and washed in HBSS. Afterwards, a standard Ficoll gradient centrifugation was performed to separate tumor cells from tumor-infiltrating lymphocytes. Both subpopulations were harvested, washed, and treated with erythrocyte-lysis buffer (0.37 mg/ml EDTA, 10 μg/ml $KHCO_3$, 82.5 μg/ml NH_4Cl in distilled water). Adherent tumor cells were cultured in RPMI/10% fetal calf serum/2 mM L-glutamine for 12 days prior to experiments, and tumor-infiltrating lymphocytes were maintained in Iscove's minimal essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 U/ml rhIL-2 for 4 days prior to experiments. A portion of the tumor cells and all cells from the normal bladder epithelial tissue were used for mRNA isolation immediately after preparation.

Generation of dendritic cells from peripheral blood

Human DC were generated from peripheral blood following our standard protocol (Cochlovius et al. 1999). Freshly harvested PBL (2×10^6/ml) were resuspended in IMEM containing 10% autologous serum, seeded into 24-well plates and incubated for 90 min at 37 °C. Non-adherent cells were removed and adherent cells were cultured in IMEM supplemented with 10% autologous serum, antibiotics, 150 U/ml rhGM-CSF, 50 U/ml rhIL-4, and 50 U/ml rhIFNγ (Sigma, Munich, Germany) for 10 days.

Isolation of mRNA from tissue

The Oligotex Direct mRNA Kit (Qiagen, Hilden, Germany) was used for the isolation of mRNA from tissue. Briefly, tissue lysates were incubated with the Oligotex resin and resulting complexes were precipitated by centrifugation, washed, and resuspended in RNase-free water. DC were infected with mRNA by adding mRNA to the culture of DC (10 μg/10^6 DC) at day 7 (immature DC) or day 10 (mature DC), respectively, for 8 h in the presence of an RNase inhibitor.

Activation of T-cells and cytotoxicity assay

T-cells were enriched from the patient's PBL by non-adherence on nylon wool and subsequently activated by cocultivation with autologous mRNA-transfected DC (or non-transfected as a control). In 24-well plates, autologous DC and PBL were seeded in a relation of 1:20 for 4 days in IMEM supplemented with 10% autologous serum and antibiotics, removed and cultured for 2 days alone, then restimulated by a second cocultivation with DC for three more days. Subsequently, cells were harvested, washed, and used for further investigations. TILs were isolated from the tumor and activated by cocultivation on mRNA-transfected DC for 4 days. To test the cytotoxic potential of the activated PBL, a standard JAM test (Matzinger 1991) was performed. [^{3}H] thymidine-labeled target cells (10^4) were coincubated with effector cells for 4 h in 96-well plates. Cells were harvested and radioactivity measured using a scintillation β-counter. Each experiment was carried out in triplicate. Cytotoxicity (related to the observed apoptosis-induced DNA fragmentation) was calculated as % specific cytotoxicity = [(retained DNA in the absence of effector cells experimentally retained DNA)/(retained DNA in the absence of effector cells)] × 100.

Flow cytometry

Flow cytometric analysis was performed following standard protocols. Briefly, cells were washed, stained with the first antibody solution for 1 h at 4 °C, washed three times, then incubated with the secondary antibody solution for 1 h at 4 °C, washed three times, and then used for FACS analysis using a FACSCalibur (Becton Dickinson, Mountain View, USA).

Results and discussion

Bladder urothelial carcinoma and normal bladder urothelial tissue was obtained from a 67-year-old male patient. We established short time cultures for both of these. mRNA was isolated immediately after single cell suspensions were prepared. DC from the patient's PBL were generated following our standard protocol (Cochlovius et al. 1999). DC were generated and matured in vitro using GM-CSF, IL-4, and IFNγ. Flow cytometric analyses and microscopy showed that CD80, CD83, and CD86 were upregulated during maturation. The mature DC displayed high expression levels of MHC class I and class II molecules, CD40 and CD44s, were positive for CD11a, CD54, and CD58, and negative for CD14 (data not shown). mRNA isolated from tumor and normal tissues was transfected into DC with 10 μg at day 7 (immature DC), or at day 10 (mature DC), respectively.

Autologous PBL, enriched for T-cells, were stimulated twice for 4 plus 3 days on mRNA transfected DC. We observed an activation of T-cells only when using DC transfected at day 7 of their maturation, not with DC transfected at day 10. This observation is in line with the fact that immature monocyte-derived DC display a higher ability for antigen-uptake compared to mature DC (Peters et al. 1996). Dendritic cells loaded with mRNA display a higher expression of CD83 (32.1% positively stained cells in FACS analysis) after maturation compared to untransfected mature DC (18.36%). From previous experiments, where we used mRNA from a human melanoma cell line, it was known that DC transfected with total mRNA are able to activate T-cells. We observed that PBL stimulated by cocultivation with mRNA-transfected DC, upregulate activation markers such as CD25 and CD71 on levels comparable to low-dose IL-2 activated cells (W.E. Schmitt, unpublished data). These results correspond to those of the group of Eli Gilboa showing an impressive T-cell activation using

DC transfected with mRNA coding for the tumor antigen CEA (Nair et al. 1998, 1999).

In the case of the bladder urothelial carcinoma, we stimulated T-cells from the peripheral blood of the patient twice with total unfractionated mRNA-transfected DC. After activation, the T-cells were used as effector cells in a cytotoxicity assay with the autologous tumor cells as targets (Fig. 1). We observed a specific, induced target cell apoptosis of 6.4%, 23.4%, 25.9%, and 37.7% at E:T ratios of 3:1, 25:1, 50:1, and 100:1, respectively. Other human tumors were not lysed (Raji, BLM, HeLa) or only slightly lysed (Jurkat: 7% at E:T ratio of 50:1), whereas the xenoreaction against the murine melanoma B16 was quite high (71% at E:T ratio of 50:1). This strong reaction against murine cells might be due to the fact that the used T-cells were still very inhomogeneous, and the tumor-specific T-cells represented only a small portion of the whole population. T-cells activated by tumor-mRNA-transfected DC or by normal-mRNA-transfected DC did not lyse the autologous normal tissue. On the other hand, T-cells activated by normal-mRNA-transfected DC attacked the autologous tumor cells (12.7% at E:T of 50:1). This latter result is puzzling and additional experiments are necessary to investigate the maintenance or breakdown of tolerance using DC vaccines. However, taken together, our data suggest that unfractionated total tumor-mRNA-transfected DC are able to efficiently activate T-cells in a tumor-specific manner.

In addition to PBL, we used TILs isolated from the tumor to lyse autologous tumor cells. TILs did not lyse the autologous tumor in vitro. After activating them overnight with rhIL-2 (100 U/ml) we obtained a target cell apoptosis of 37.1% at an E:T ratio of 50:1. If the TILs were activated once by tumor-mRNA-transfected autologous DC, they also were able to attack the autologous tumor cells (35.7% at an E:T ratio of 50:1).

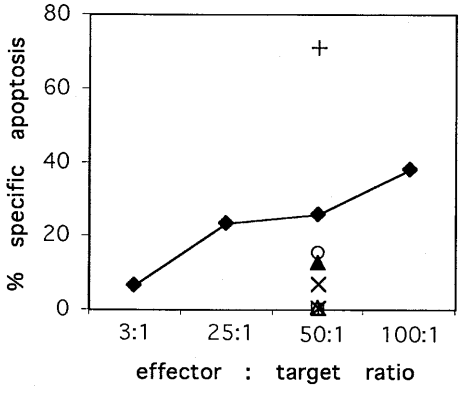

Fig. 1 Cytotoxicity of T-cells activated by mRNA-transfected DC. A standard JAM test was performed. T-cells were activated with DC transfected with mRNA derived from normal tissue (*filled triangle*) or with tumor mRNA (all others). Autologous tumor cells (*filled diamonds, filled triangle*), HeLa (*open square*), BLM (*open triangle*), Jurkat (*X*), Raji (*striped X*), K562 (*open circle*), and B16 (*cross*) served as target cells. Percentages of specific induced target cell apoptosis are presented

This effect was not seen when TILs were cocultured with untransfected DC. These results clearly indicate that tumor-mRNA-transfected DC are able to activate non-reactive TILs. Thus, tumor-mRNA-transfected DC could be a vaccine for (re-) activating the patient's own inadequate anti-tumor immune response. Mouse experiments have been planned to explore this possibility.

With the impressive data of Nair et al. in mind, showing that DC transfected with CEA mRNA were able to induce a strong T-cell response, we tried to elicit a specific T-cell response using unfractionated total mRNA derived from a tumor which is not known to be particularly immunogenic. The use of unfractionated total mRNA might be expected to induce a weaker T-cell response, like the use of one particular mRNA coding for a known tumor antigen as performed by Nair and colleagues (Nair et al. 1998, 1999). However, it has been assumed for many years that virtually every tumor, independent of its origin, is potentially immunogenic, and contains not only one single immunogenic tumor antigen but several (Sahin et al. 1995). The data described here suggest that the use of unfractionated total mRNA derived from a tumor is sufficient to induce a tumor-specific T-cell response when transfected into DC. These results, obtained using human primary tumor that is not particularly immunogenic as a model, are in line with very recently published observations, using the murine B16/F10.9 melanoma system, showing that DC transfected with total tumor-derived mRNA induce a CTL response in vitro and even in vivo (Boczkowski et al. 2000). Taken together, the use of tumor-mRNA-transfected DC seems to represent a particularly feasible and clinically suitable strategy for treating minimal residual disease of virtually all kinds of human neoplasms. Future studies will be carried out with additional patients and also other non/weak-immunogenic tumors.

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