Long-term Complete Remission following Radiosurgery and Immunotherapy in a Melanoma Patient with Brain Metastasis: Immunological Correlates

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Running title: cancer immunosurveillance restored

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Abstract

A melanoma patient with brain metastases was treated by gamma-knife radiosurgery and immunotherapy with autologous tumor-lysate-loaded dendritic cells (DC). Ten years after the combined treatment, the patient remains in complete remission. Remarkable immunological correlates to the clinical development were the transient induction of NY-ESO-1 antibody and the durable expansion of MAGE-A1\textsubscript{p161-168} EADPTGHSY-specific CD\textsuperscript{8}\textsuperscript{+} T cells. While the induction of NY-ESO-1 antibody most likely resulted from gamma-knife mediated ‘auto-vaccination’, the persistence of circulating MAGE-A1-specific T cells, which are still detectable \textit{ex-vivo} in the absence of any tumor manifestation, coincide with DC-based vaccination administered monthly until today.

Introduction

The discovery of ‘cancer-testis’ (CT) antigens, such as MAGE-A1 and NY-ESO-1, has led to the development of vaccine-based interventions against cancer that rely on the stimulation of effective tumor-specific immune responses (1-3). The recent clinical success of the dendritic cells (DC)-based cancer vaccine Sipuleucel-T (Provenge; Dendreon) for prostate cancer and the immunomodulatory anti-CTLA-4 antibody Ipilimumab (Yervoy; Bristol-Myers Squibb) for advanced melanoma has renewed interest in immunotherapy targeting cellular responses to cancer antigens (4, 5). Although promising results on prolongation of overall survival have been reported, the optimal strategy for cancer immunotherapy to control tumor growth has yet to be
determined. Mounting evidence suggests that immunotherapy might synergize with radiotherapy and is most successful in patients with a limited tumor burden (6, 7). In this regard, radiotherapy and the use of DC-based vaccines represent a promising treatment combination capable of inducing de novo and enhancing pre-existing tumor-specific T-cell and antibody responses (8). Melanoma is one of the most frequent metastatic cancers with increasing incidence worldwide and very limited treatment options in advanced stages. Patients with melanoma brain metastases carry a very poor prognosis with a median overall survival of about 4-5 months (9).

We report here on a brain metastatic melanoma patient, who experienced complete and sustained remission to the aforementioned treatment combination of radiosurgery and immunotherapy. In our approach, the vaccine consists of autologous tumor lysate-loaded dendritic cells (TL-DC) delivered into the skin by intradermal injections. Focusing on MAGE-A and NY-ESO-1, we provide a 12-year immunological and clinical follow-up with remarkable correlations between treatment-induced immune responses and long-term disease-free survival.

Material and Methods

Preparation of the TL-DC vaccine

DC were generated from CD4 and CD8 T cell-depleted peripheral blood mononuclear cells (PBMC) by adherence to plastic in 25 cm² tissue culture flasks and cultured in serum-free X-VIVO 15 medium (BioWhittaker) supplemented with GM-CSF and IL-4 (1000U/ml). After 5 days, 5 x 10⁵ DC were pulsed with 10µl tumor lysate (TL) for 6-8 hours. The TL was prepared from the patient’s lymph node metastasis by 3 freeze-thaw cycles of pulverized and homogenized tumor tissue (200mg/200µl water). For safety the TL was irradiated at 200 Gy. TL-DC were matured for 12-24 hours by adding a cytokine cocktail consisting of IL-6, IL-1β and
TNF-α (10ng/ml). Matured TL-DC were washed twice and used for intradermal vaccination. Remaining TL-DC were frozen for subsequent vaccinations.

**Monitoring of immune responses**

Longitudinal measurement of serum antibodies was performed by standard ELISA as described previously (10). *Ex vivo* or *in vitro* analysis of T cells was performed by interferon gamma (IFNγ) enzyme-linked immunospot (ELISPOT) assay and by fluorescence-activated cell sorting (FACS) using peptide-MHC class I tetramer complexes as described previously (11). Further details and information regarding these analyses are provided online in the Supplementary Appendix as SI Methods.

**Results**

**Case report**

In June 2001, a 44-year-old woman was diagnosed with a 1.2 mm Breslow primary nodular melanoma located in the right upper arm. She underwent wide local excision. The disease was classified as pT2NXMX, Clark level IV according to the AJCC 2001 staging system. Within one year the disease recurred twice in the right axilla and three times in the clavicular lymph node region. Lymph node metastases were surgically resected repeatedly. The disease further progressed to the brain. Four lesions with volumes of 1, 3.7, 0.1 and 0.1 cm³ were radiologically treated with 45 Gy each by gamma-knife surgery in August 2002. Brain metastases completely regressed and did not recur as confirmed by routine magnetic resonance tomography (MRT) images every year until today. Sixteen months later in December 2003, the patient relapsed again with several mesenteric lymph node metastases that were removed by partial resection of the small intestine. Since then, the patient has remained free of disease for nearly ten years now.
Apart from the primary surgical treatment the patient successively received adjuvant high-dose interferon-α2a (HDI) therapy for eight weeks in 2002, followed by four injections of melanoma-antigen-3 (MAGE-A3)-associated peptide within the LUD01-006 clinical study in June 2002. Due to rapid disease progression the patient was removed from the study. Subsequently, immunotherapy with autologous tumor lysate-loaded dendritic cells (TL-DC) was initiated in August 2002 on a compassionate-use basis. Routinely, $5 \times 10^5$ autologous TL-DC have been injected intradermally in turn on the left upper arm and left or right upper thigh once a month until today. Up to now the patient has received a total of 133 vaccines. The patient is still in complete remission today. The course of disease under treatment is presented in Figure 1A.

**Induction of NY-ESO-1 antibody after gamma-knife radiosurgery.**

By the time of tumor progression to the brain there were no antibody responses against the tumor antigens MAGE-A1, MAGE-A3 or NY-ESO-1, which were expressed as assessed by reverse transcriptase-PCR in previously resected lymph nodes (SI Table 1). After gamma-knife surgery of brain metastases the patient developed NY-ESO-1 antibodies that persisted for a year and a half until the removal of the mesenteric lymph node metastases in December 2003 (Figure 1B). During the time of antibody persistence, we identified NY-ESO-1 antibody specificities against two different antigenic B-cell epitopes. Induction of the NY-ESO-1 antibody against epitope p31-50 located in the N-terminal part of the protein coincided with tumor destruction of the brain metastases, and the presence of the NY-ESO-1 antibody against epitope p161-180 located in the C-terminal region of the protein coincided with the development and subsequent resection of mesenteric lymph node metastases. Thus NY-ESO-1 antibody responses of distinct specificities were triggered by different tumor manifestations and potentially through different ways of tumor antigen release (Figure 2).
Expansion of MAGE-A1\(_{p161-169}\) Specific CD8\(^+\) T Cells.

Based on the antigenic profile of the tumor and the patient’s HLA haplotype: A1, A11, B13, B35, Cw4, Cw6, we analyzed the CD8\(^+\) T-cell responses against NY-ESO-1, MAGE-A1, MAGE-A3, and MELAN-A. CD8\(^+\) T cells from different time points were pre-sensitized \textit{in vitro} with the following antigenic peptides: NY-ESO-1\(_{p91-110}\) (YLAMPFATPMEAELARRSLA), MAGE-A1\(_{p161-169}\) (EADPTGHSY), MAGE-A3\(_{p168-176}\) (EVDPIGHLY), and Melan-A\(_{p26-35}\) (EAAGIGILTV) (12). Cellular immune responses were detectable after \textit{in vitro} pre-sensitization in May 2002 early in the course of the disease (Figure 3A); however, the frequency of these antigen-specific CD8\(^+\) T cells was low and not detectable \textit{ex vivo} from the peripheral blood (Figure 3B). We analyzed lymphocytes of the first blood sample available after radiotherapy and immunotherapy with TL-DC in June 2004, and found that cellular responses were higher to all antigens tested, while levels of MAGE-A1\(_{p161-169}\)-specific CD8\(^+\) T cells had increased significantly and had become clearly detectable \textit{ex vivo} (Figure 3A/B). A frequency of 0.4% of circulating MAGE-A1\(_{p161-169}\)-specific CD8\(^+\) T cells was determined by both ELISPOT assay for IFN\(\gamma\) secretion and FACS analysis for peptide/MHC tetramer binding. The robust MAGE-A1-specific CD8\(^+\) T-cell response was maintained throughout immunotherapy and is still detectable \textit{ex vivo} in the patient’s peripheral blood without any evidence of detectable disease (Figure 1C). The ability of MAGE-A1\(_{p161-169}\)-specific CD8\(^+\) T cells to recognize naturally processed MAGE-A1 was shown at the clonal level by specific MZ2-MEL tumor cell lysis in \(^{51}\)chromium release assays as described in (1) (Figure 3C). To test whether the TL-DC vaccine could stimulate the patient’s peripheral blood lymphocytes, we assessed IFN\(\gamma\) secretion of circulating T cells in \textit{ex}...
vivo ELISPOT. As shown in Figure 3D, the vaccine was efficiently recognized by CD4 and CD8 T cells.

**Discussion**

The combination of radiotherapy with active immunotherapy may restore immunosurveillance in patients with advanced cancers resulting in long-term tumor control. The effective stimulation of the immune system in the patient presented here is mainly characterized by the induction of high levels of MAGE-A1\textsubscript{p161-169}-specific antitumor CD8\textsuperscript{+} T cells. This finding matches with early T-cell data of our historical melanoma patient MZ2 in whom MAGE-A1\textsubscript{p161-169}-specific T cells were first identified (13). In both cases, complete tumor eradication and long-term survival correlated with the presence of MAGE-A1-specific T cells, thus highlighting their potential to effectively control tumor growth. Analyzing early immune responses in the patient described herein we found that T cells responding to tumor-associated antigens MAGE-A1, MAGE-A3, NY-ESO-1, and Melan-A were present in the peripheral blood already in June 2002. However, the T-cell response at the time was ineffective and failed to prevent tumor progression. Upon local radiotherapy and immunotherapy with TL-DC, the patient’s immune response shifted towards tumor-recognition indicated by the development of NY-ESO-1 antibodies. It turned out that NY-ESO-1 antibody response was directed against two distinct epitopes, each of which was targeted at different time points and different tumor sites. Moreover, the antibody switch observed indicated an important evolution of the patient’s antitumor immune response as NY-ESO-1\textsubscript{p161-180}-specific antibody appeared independent of radiation, and spontaneously. Of note, subsequent to radiotherapy and vaccination with TL-DC the patient showed a prolonged time to disease progression of 16 months, which was potentially mediated by the initiation of effective immune responses.
A major goal of active cancer immunotherapy is to induce T cell-mediated antitumor immune responses with the potential to control tumor growth and to protect against tumor recurrence. To achieve this goal DC-based vaccines represent a promising treatment approach as DC play a key role in the initiation and regulation of cellular immune responses (14). In our case, immunotherapy with TL-DC was initiated one week after gamma-knife surgery of brain metastases. Analysis of the first post vaccination blood sample revealed a strong \textit{ex vivo} detectable MAGE-A1-specific antitumor CD8$^+$ T-cell response that was maintained at a relatively constant high level over time and is still present in the patient, who is alive without any evidence of disease. Therefore it is likely that the durable expansion of MAGE-A1_{p161-169}-specific T cells has been maintained and is still being boosted by continued vaccination with TL-DC. However, evidence that the vaccine still enables the patient’s immune system to protect against melanoma recurrence would only be provided by cessation of vaccination. Therefore it remains uncertain whether the induced T-cell immune response still controls the outgrowth of residual tumor cells, thus keeping the patient’s disease in a dormant state (15), or whether the strong anti-MAGE-A1 T-cell immune response has led to the complete elimination of residual tumor cells. The clinical efficacy of our treatment approach may be caused by several factors. The use of autologous whole tumor cell lysate as the source of antigens to load DC generates a broad immune response covering all relevant antigens of the individual’s disease (16). In this regard, the antigenic material obtained from the abdominal metastases in December 2003 was highly effective in stimulating T-cell responses \textit{in vivo} (Figure 3D). For the timing of vaccination it is assumed that patients with large tumor burdens are less likely to clinically respond to therapeutic cancer vaccines because of immunosuppressive mechanisms released by the tumor microenvironment. Furthermore, a minimum time is required to generate a sufficient immune response. Vaccination with TL-DC in our patient was initiated after gamma-knife surgery in a
status of minimal residual disease and was even continued beyond tumor recurrence and the subsequent surgery in December 2003. Finally, our data suggest that immunotherapy may synergize well with radiotherapy, especially hypofractionated radiosurgery. Our patient developed tumor antigen-specific antibody and T-cell responses that were not detectable before treatment suggesting that radiation has led to the release of tumor-associated antigens and DC activation, which in turn may have supported the initiation of a tumor-specific CD8+ T-cell response (17, 7). In mice, it has been shown recently that eradication of murine colon adenocarcinoma under immunotherapy was successful only when preceded by radiotherapy (18). The cancer immunoediting model by Dunn, Schreiber and Old describes the interaction between cancer development and a patient’s immune response resulting in tumor elimination, equilibrium, or escape (19). It has been established that the immunoediting process can be affected significantly by therapeutic manipulations (20). In our patient, it seems probable that after the failure of immunosurveillance, the rapidly growing tumor was locally controlled by surgery and radiotherapy. However, long-term disease-free survival may have resulted from the induction of an antitumor T-cell response triggered by auto-vaccination effects of radiotherapy durably sustained by immunotherapy with TL-DC.

Based on the convincing evidence of effective tumor control following surgery, hypofractionated radiotherapy and immunotherapy with TL-DC, we suggest considering conventional cancer treatments to be combined with active immunotherapy to improve patient survival.
References


Figure legends

Figure 1: Positive Correlations between Disease Course and Treatment-induced Immune Responses

A: The course of disease is shown on top of the timeline. Before radio- and immuno-therapy with tumor lysate-loaded dendritic cells (TL-DC) the tumor rapidly progressed to regional lymph nodes (LN) and to the brain. After radiotherapy of brain metastases the patient showed a prolonged time to progression of 16 months. Since December 2003 the patient has remained free of disease. Immunotherapeutic treatments are shown below the timeline. HDI: high-dose interferon-α2a therapy; M3: MAGE-A3 peptide vaccination. Continuous vaccination with tumor-lysate-loaded dendritic cells correlated with long-term tumor control. B: Antibody responses against the cancer-testis antigens NY-ESO-1, MAGE-A1 and MAGE-A3. The patient developed NY-ESO-1 antibodies in 11/2002 after gamma-knife radiation of brain metastasis. After surgical removal of mesenteric lymph node metastasis in 12/2003, NY-ESO-1 antibody decreased and has not been detectable at all since 7/2004. Antibody responses to MAGE-A1 and MAGE-A3 were not observed at any time. C: Ex vivo tetramer analysis showing strong expansion of MAGE-A1p161-169-specific CD8+ T cells after radiotherapy and immunotherapy with TL-DC in 2004 that persisted throughout the course of immunotherapy and is still detectable ex vivo in the patient’s peripheral blood without any evidence of detectable disease.
Figure 2: Induction of NY-ESO-1 antibody after gamma-knife radiation. Changes in NY-ESO-1 antibody response were investigated by measuring serum IgG against recombinant full-length NY-ESO-1 protein and against 17 different synthetic 20mer overlapping NY-ESO-1 peptides as antigens in standard ELISA. Before radiotherapy the patient has not developed NY-ESO-1 antibodies. After gamma-knife radiation of brain metastasis the patient sero-converted for NY-ESO-1 antibody. B-cell epitope mapping of NY-ESO-1 antibody response revealed that the presence of NY-ESO-1 p31-50-specific antibody coincided with tumor cell destruction of brain metastasis. The presence of NY-ESO-1 p161-180-specific antibody coincided with the development and subsequent surgical removal of mesenteric lymph node metastasis in 12/2003. MRT images with coronal view on two of the brain metastasis are shown before gamma-knife radiation (7/2002) and two months (10/2002) and 14 months (10/2003) after radiotherapy. Upper images show the regression of the right occipital lesion, lower images show the transformation and subsequent regression of the right cerebellar lesion.
Figure 3: Specificity, frequency and effector function of CD8⁺ T-cell responses before and after radiotherapy/immunotherapy (RT/IT). A: IFN-γ secretion of CD8⁺ T-cells following in vitro pre-sensitization with peptides. Peptide-pulsed and unpulsed autologous monocyte-derived DC were used as antigen-presenting cells in ELISPOT. B: Dominant MAGE-A1-specific CD8⁺ T-cell response after RT/IT detectable ex-vivo by IFN-γ ELISPOT analysis. C: Lytic activity of MAGE-A1-specific CD8⁺ T-cell clone NW1751-7/9 against MAGE-A1_p161-169 peptide-pulsed autologous EBV-B-cells and against the MAGE-A1/HLA-A1-expressing tumor cell line MZ2-MEL in chromium release assay at 10:1 effector/target ratio. MAGE-A3_p168-176 peptide-pulsed autologous EBV-B-cells, the MAGE-A1-negative tumor cell line NW-LC-1 (HLA-A1⁺) and K562 were used as negative controls and were not recognized. D: Recognition of the vaccine by circulating CD4 and CD8 T cells is shown in ex-vivo IFN-γ ELISPOT assay. Unpulsed DC were not recognized.
Figure 1

A

Brain Metastasis

Primary Melanoma Surgery

LN Metastasis Surgery

Abdominal Metastasis Surgery

Complete Remission


B

Immunootherapy with Tumor-lysate-loaded Dendritic Cells

Antibody responses

OD 405 nm

0 0.2 0.4 0.6 0.8

CD8

Tetramer

HLA-A1/MAGE-A1

p161-169

CD8

6/2002
0.00%

6/2004
0.41%

9/2007
0.21%

9/2010
0.20%

11/2012
0.19%

CD8

CD8

CD8

CD8

CD8

CD8

CD8

CD8

CD8
Ab induction after radiotherapy
Ab switch after disease progression

Figure 2: Induction of NY-ESO-1 antibody (Ab) after gamma-knife radiation. Changes in NY-ESO-1 antibody response were investigated by measuring serum IgG against recombinant full-length NY-ESO-1 protein and against 17 different synthetic 20mer overlapping NY-ESO-1 peptides as antigens in ELISA. Before radiotherapy the patient has not developed NY-ESO-1 antibodies. After gamma knife radiation of brain metastasis the patient seroconverted for NY-ESO-1 antibody. B-cell epitope mapping of NY-ESO-1 antibody response revealed that the presence of NY-ESO-1 p31-50-specific antibody coincided with tumor cell destruction of brain metastasis. The presence of NY-ESO-1 p161-180-specific antibody coincided with the development and subsequent surgical removal of mesenteric lymph node metastasis in 12/2003. MRT images with coronal view on two of the brain metastasis are shown before gamma knife radiation (7/2002) and two months (10/2002) and 14 months (10/2003) after radiotherapy. Upper images show the regression of the right occipital lesion, lower images show the transformation and subsequent regression of the right cerebellar lesion.
Figure 3
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