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In vitro induction of immune responses to shared tumor-associated antigens in rhabdomyosarcoma☆

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Abstract

Purpose: Currently, novel therapies to improve survival of patients with rhabdomyosarcoma (RMS) are being investigated. One of the new approaches involves immunotherapy using tumor-specific T-lymphocytes. An effective prolonged immune-mediated response against tumor cells is dependent upon the response of helper T-lymphocytes (HTLs) to tumor-associated antigens in the presence of histocompatibility lymphocyte antigen surface proteins.

Methods: Rhabdomyosarcoma tumor lysate-pulsed human dendritic cells were used to stimulate HTL precursors (naive CD4+ T-cells) in vitro. After 3 rounds of antigen stimulation with antigen-presenting cells, the T-cells were tested for reactivity (T-cell proliferation assays) against a large panel of tumor lysate-pulsed autologous antigen-presenting cells.

Results: Using peripheral blood mononuclear cells from normal naive donors, we have been able to generate HTL clones that recognize and proliferate to multiple tumor cell lines. The HTLs were induced using lysate from a single alveolar RMS tumor cell line (RMS13). The clones generated recognized all of the alveolar RMS cell lines (RMS13, Rh18, Rh28, Rh30, and Rh41), prostate cancer cell lines (LNCAP and LAPC4), melanoma cell lines (Mel 624 and G361), and breast cancer cell line (SKBR3). Helper T-lymphocytes recognition was also confirmed by interferon-γ production. The clones did not recognize colon, lymphoma, ovarian carcinoma, ERMS or Epstein-Barr virus (EBV) transformed B-cells. This recognition was histocompatibility lymphocyte antigen class II restricted and was not an allogeneic response.

Conclusion: The results of this work demonstrate that HTLs, exposed to RMS lysate, are able to recognize and respond to a broad range of tumor types suggesting that a common antigen exist among these different tumors. These findings suggest novel treatment strategies for patients with RMS using tumor lysate to induce antitumor immune responses.

Rhabdomyosarcoma (RMS) is the most common sarcoma and is the fourth most common solid tumor during childhood and adolescence [1]. It represents approximately 8% of all childhood tumors. The current therapy for patients with RMS consists of multiagent chemotherapy and radiation therapy and/or resection of the primary tumor for local control

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resulting in an overall survival rate of 70% at 5 years [2]. Patients with alveolar RMS (ARMS) have a significantly worse prognosis than those with embryonal because 50% of patients with ARMS already harbor metastatic disease at presentation, and their survival is only 20% at 5 years.

Immunotherapy of cancers is based on tumor rejection primarily mediated by the T-lymphocytes of the cellular immune response resulting in tumor necrosis. Both CD4 and CD8 responses are required to insure an adequate and prolonged tumor response in vivo [3]. Vaccination with tumor-associated antigen (TAA) loaded antigen-presenting cells (APCs) leads to the generation of T-lymphocytes with antitumor activity [4,5]. Dendritic cells (DCs) are considered the best APCs because they are able to internalize external peptides and express peptide fragments from these proteins on both major histocompatibility complex (MHC) class I and II molecules as well as express costimulatory molecules such as B7 and CD40 [6-8]. Thus, DCs have all the signals necessary to stimulate naive CD8 and CD4 T-cells to provoke tumor-specific immune responses to TAA. Data emerging from clinical trials indicate that DC vaccination can induce both immunological and, more importantly, clinical responses albeit in a very small number of patients [5,9-13].

It is accepted that the development of effective immune-based therapies for cancer will require induction of tumor-specific helper T-lymphocytes (HTLs) responses [14]. The HTLs recognizing TAA, in the context of MHC class II molecules, may have direct antitumor activity through cytolysis or by producing cytokines with antitumor activity. Recognition of TAA can take place via APC at the tumor site that captures, process, and presents TAA released by dead tumor cells. The second reason to assume that tumor-reactive HTLs will be critical is that these regulatory cells should amplify CTL responses by producing lymphokines required for cytotoxic t lymphosite (CTL) growth (IL-2), MHC expression by tumor cells (IFN-γ), and will facilitate establishment of long-term memory that is critical for prevention of tumor recurrences.

1. Methods

1.1. Cell lines

The tumor cell lines used in this study were alveolar RMS (RMS13, Rh18, Rh28, Rh30, Rh41); prostate adenocarcinoma (LAPC4, LNCAP); melanoma (G361, Mel624), breast carcinoma (HBL100, SKBR3, T470); colon adenocarcinoma (CACO2); ovarian carcinoma (SW626); embryonal RMS (RD), undifferentiated sarcoma (A204); EBV transformed human peripheral blood mononuclear cells (PBMC) (MWF, MGAR, M35); lymphoma (Jurkat). Cell lines were kept in PRMI 1640 supplemented with 10% fetal bovine serum (vol/vol), L-glutamine, nonessential amino acids, sodium pyruvate, and gentamicin (complete RPMI medium). All of the culture materials were purchased from Life Technologies, Inc (Rockville, MD).

1.2. Generation of DCs and tumor lysate

The DCs were generated using PBMC from normal volunteers that had been purified using Ficoll gradient centrifugation as previously described [15]. The Institutional Review Board on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all volunteers. Monocytes were isolated and incubated with ex vivo medium containing 280 U/mL of granulocyte macrophage colony stimulating factor (GM-CSF) and 50 ng/mL of IL-4. On days 3 and 5, additional media + IL-4 and GM-CSF was added. The DCs were then ready for lysate loading on day 6.

Soluble lysate Ags were prepared by 3 freeze-thaw cycles ($-140^{\circ}C/37^{\circ}C$) of 1×10^8 tumor cells resuspended in 1 mL of serum-free RPMI 1640, with removal of cell debris by centrifugation at 12,000 rpm for 5 minutes (Biofuge Pico Bench centrifuge; Heraeus Inst.) [5]. Supernatant proteins were then added to DC cultures at a 1:1 tumor cell lysate equivalent/DC ratio in 1 mL complete medium (RPMI 1640 supplemented with 5% human male AB serum, 0.1 mmol/L MEM nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 50 μg/mL gentamicin) and left at $37^{\circ}C$ for 16 to 18 hours. Excess Ags were removed by washing the DCs as with complete medium $\times 2$.

1.3. In vitro induction of antigen-specific HTL lines with tumor lysate

The procedure selected for the generation of tumor antigen-reactive HTL lines by use of peptide-stimulated PBMC has been described in detail elsewhere, and the methods used for lysate stimulated PBMC is similar (16-18). Briefly, ARMS lysate (RMS13 or Rh30) pulsed DCs were irradiated (4200 rad) and cocultured with autologous purified CD4+ T-cells (using antibody-coated magnetic microbeads from Miltenyi Biotech, Auburn, CA) in 96-well round-bottom culture plates. Each well contained 0.25×10^5 DC and 5×10^5 CD4+ cells in 0.2 mL of complete medium.

At 7 and 14 days, the CD4+ T-cells were restimulated with lysate-pulsed irradiated autologous PBMC (1:1 ratio of tumor cell lysate equivalent to PBMC incubated 16-18 at $37^{\circ}C$), and 2 days later, human recombinant interleukin-2 was added at a final concentration of 25 IU/mL. At day 21, the T-cells were tested for their proliferative responses to lysate pulsed autologous PBMC as described below. Those cultures exhibiting a proliferative response to lysate (at least 2.5-fold over background) were expanded in 24- or 48-well plates by weekly restimulation with lysate-pulsed irradiated autologous PBMC. Where T-cell clones were used (as mentioned in the Results section), these were produced by limiting dilution as described elsewhere [16-18].

1.4. Measurement of antigen-specific responses

CD4+ T-cells (3×10^4/well) were mixed with lysate pulsed irradiated autologous PBMC (1×10^5/well)