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EphA2 as a Glioma-Associated Antigen: A Novel Target for Glioma Vaccines[1]

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Abstract

EphA2 is a receptor tyrosine kinase and is frequently overexpressed in a wide array of advanced cancers. We demonstrate in the current study that the EphA2 protein is restrictedly expressed in primary glioblastoma multiforme and anaplastic astrocytoma tissues in comparison to normal brain tissues. To evaluate the possibility of targeting EphA2 in glioma vaccine strategies, we stimulated human leukocyte antigen (HLA) A2$^+$ peripheral blood mononuclear cells (PBMCs) obtained from healthy donors and glioma patients with autologous dendritic cells (DCs) loaded with synthetic EphA2$_{883-891}$ peptide (TLADFDPRV), which has previously been reported to induce interferon-γ in HLA-A2$^+$ PBMCs. Stimulated PBMCs demonstrated antigen-specific cytotoxic T lymphocyte (CTL) responses as detected by specific lysis of T2 cells loaded with the EphA2$_{883}$ peptide as well as HLA-A2$^+$ glioma cells, SNB19 and U251, that express EphA2. Furthermore, *in vivo* immunization of HLA-A2 transgenic HHD mice with the EphA2$_{883-891}$ peptide resulted in the development of an epitope-specific CTL response in splenocytes, despite the fact that EphA2$_{883-891}$ is an autoantigen in these mice. Taken together, these data suggest that EphA2$_{883-891}$ may be an attractive antigen epitope for molecularly targeted glioma vaccines. *Neoplasia* (2005) **7,** 717–722

Keywords: EphA2, glioma, cancer vaccine, cytotoxic T lymphocytes, human leukocyte antigen (HLA) A2.

Introduction

Immunotherapy, particularly active vaccination, may be developed as an effective and safe treatment modality for malignant gliomas, which continue to have a poor prognosis, despite advances in surgical techniques and adjuvant chemotherapy and radiotherapy [1]. Indeed, several groups, including ours, have demonstrated the safety and preliminary efficacy of whole glioma cell–based vaccine approaches [2,3]. However, the use of autologous glioma lysates or glioma cells as the antigen source may limit the feasibility and safety of the approach due to the cumbersome preparation procedures and theoretical concerns for inducing autoimmune encephalitis [4]. Although the number of well-defined glioma-specific cytotoxic T lymphocyte (CTL) epitopes has been limited, our recent report describing a human leukocyte antigen (HLA) A2–restricted CD8$^+$ T-cell epitope derived from the GAA interleukin-13 receptor α2-chain (IL-13Rα2) demonstrates the feasibility of identifying such loci, particularly when targeting a protein that is differentially expressed in glioma cells *versus* normal tissues [5]. Given the marked antigenic heterogeneity of gliomas, however, immunotherapy with a single tumor-specific T-cell epitope might merely promote transient stabilization of disease, prior to the progression of antigen loss variants [6]. We have therefore been dedicated to broaden the list of available CTL epitopes for integration into multiepitope-based vaccine strategies for glioma therapy. EphA2 [7–9] is a member of the Eph family of receptor tyrosine kinases, comprised of two major classes (EphA and EphB), which are distinguished by their specificities for ligands (ephrin-A and ephrin-B, respectively) [7–9]. It has been reported that EphA2 is frequently overexpressed and often functionally dysregulated in advanced cancers, such as metastatic lesions (reviewed in Ref. [10]). Because of the aggressive and invasive nature of malignant gliomas, we hypothesized that EphA2 might be expressed in this tumor entity and might serve as an attractive target for glioma vaccines. Indeed, T-cell immunoepitopes in EphA2 have been identified and characterized as potential targets and surrogate markers for cancer immunotherapy [11,12]. In the current study, we have chosen to evaluate one of the HLA-A2–restricted epitopes, EphA2$_{883-891}$, because it has been noted to elicit relatively

Abbreviations: CTL, cytotoxic T lymphocyte; DC, dendritic cell; E/T ratio, effector-to-target ratio; HLA, human leukocyte antigen; IL, interleukin; PBMC, peripheral blood mononuclear cell

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high level interferon (IFN) γ responses in cancer patients, in comparison to the other epitopes [12]. Here, we show that EphA2 protein is expressed in human malignant glioma and that stimulation with $EphA2_{883-891}$ can induce tumor-reactive CTLs in patient-derived HLA-$A2^+$ peripheral blood mononuclear cells (PBMCs) *in vitro* and by *in vivo* vaccination of mice transgenic for modified HLA-A2.1-β2 microglobulin single chain (HHD mice).

Materials and Methods

Cells and Cell Culture

The U251, SNB19, and A172 glioma cell lines [5] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all reagents from Life Technologies, Inc., Grand Island, NY).

PBMCs were obtained from glioma patients and healthy donors under an Institutional Review Board–approved protocol. The HLA-A2 expression on the PBMCs was determined by flow cytometry as a double-positive staining with monoclonal antibodies (mAbs) MA2.1 (against HLA-A2, B17) and BB7.2 (against HLA-A2, Aw69) (both from ATCC, Manassas, VA; www.atcc.org). The murine EL-4HHD [D^bxβ2 microglobulin (β2M) null, and transgenic for modified HLA-A2.1-β2 microglobulin single chain (HHD gene)] and control EL-4S3-Rob cells (D^bxβ2M null) were generous gifts from Dr. François A. Lemonnier (Pasteur Institute, Paris, France) [13]. The HLA-A*0201–transfected transporter associated with antigen processing (TAP)–deficient T2 cell lines [5], EL-4HHD and EL-4S3-Rob cells, were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all reagents from Life Technologies, Inc.).

Peptides

The synthetic $EphA2_{883-891}$ (TLADFDPRV) and control MART-1_{27-35} (AAGIGILTV) were synthesized by FMOC chemistry in the University of Pittsburgh Cancer Institute (UPCI) Peptide Synthesis Facility, and were >95% pure as indicated by analytic high-performance liquid chromatography and mass spectrometric analysis performed by the University of Pittsburgh Cancer Institute's Protein Sequencing Facility. Peptides were dissolved in phosphate-buffered saline (PBS)/10% DMSO at a concentration of 2 mg/ml and stored at -20°C until use.

Western Blot

Tumor cells ($5-10 \times 10^6$) were analyzed for EphA2 expression through Western blot analysis as described previously [12], with slight modifications. Cell lines were collected by scraping in PBS and were pelleted before lysis in a buffer containing the protease inhibitors, phenylmethylsulfonyl fluoride (100 μg/ml), aprotinin (1 μg/ml), and leupeptin (1 μg/ml) (all these reagents from Sigma, St. Louis, MO). Lysate protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Protein samples were denatured in gel-loading buffer at 99°C for 5 min. Equal amounts of proteins (40 μg) were applied to each well and electrophoresed on 7% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad) and blocked with 5% low-fat skim milk for 1 hour at room temperature. Blots were incubated with the antihuman EphA2 polyclonal antibody (c-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or actin (ACTN05; NeoMarkers, Fremont, CA), followed by species-specific secondary antibodies (mouse and rabbit, respectively; Santa Cruz Biotechnology, Inc.). Blots were imaged on a Kodak X-Omat Blue XB-1 film (NEN Life Science Products, Boston, MA) using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

Immunohistochemistry for EphA2 in Glioma Tissue

Glioma specimens were obtained surgically under an Institutional Review Board–approved protocol and paraffin-embedded. Normal brain sections were obtained from the Human Brain Tissue Bank, Evelyn F. and William L. McKnight Brain Institute of the University of Florida. Eight-micrometer sections were deparaffinized, rehydrated, and subjected to antigen retrieval by electric pressure cooker (DC2000; Biocare Medical, Walnut Creek, CA) in DakoCytomation Target Retrieval Solution (DakoCytomation, Glostrup, Denmark). Endogenous peroxidase was blocked with 3% H_2O_2 in PBS. Anti-EphA2 mAb (Ab 208, mIgG1; MedImmune, Inc., Gaithersburg, MD) or isotype-matched control mAb was incubated on sections for 2 hours at room temperature. After PBS washing, sections were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 20 minutes at room temperature, then incubated with avidin–biotin complex horseradish peroxidase (Vectastatin ABC kits; Vector Laboratories) for 10 minutes, developed with a Nova Red substrate kit (Vector Laboratories), then nuclei were counterstained with hematoxylin. The expression intensity of EphA2 immunostaining (negative, lightly diffuse, moderately diffuse, focally moderately diffuse, or strongly diffuse) was evaluated independently with a microscope under $\times$20 to $\times$40 magnification.

In Vitro Induction of CTL with the $EphA2_{883-891}$ Peptide

Induction of CTL responses in HLA-$A2^+$ PBMCs against HLA-A2–binding peptides was evaluated with a previously described method by us [5], with slight modifications. Briefly, autologous dendritic cells (DCs) (2×10^5 per well), which were preincubated with each peptide (10 μg/ml) for 2 hours, were cocultured with 2×10^6 $CD8^+$ T cells in a final volume of 2 ml (each well of 24-well plates) of AIM-V media supplemented with 10% human serum (both reagents from Life Technologies, Inc.) and 20 U/ml human rhIL-2 (R&D Systems, Minneapolis, MN). On day 7, lymphocytes were restimulated with autologous DCs pulsed with the peptide, and the stimulated T cells were analyzed for their specific CTL activity on day 14 by standard ^{51}Cr release assays. For the cold target (CT) inhibition assays, ^{51}Cr-labeled SNB19 cells (1×10^3 cells/well) and cold T2 cells (1×10^4 cells/well), pulsed with or without $EphA2_{883-891}$, were incubated with the effector cells.