J. Neurovirol. (2011) 17:448–454
DOI 10.1007/s13365-011-0048-z

Cytotoxicity of glioblastoma cells mediated ex vivo by varicella-zoster virus-specific T cells

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Received: 14 January 2011 / Revised: 30 April 2011 / Accepted: 12 July 2011 / Published online: 27 July 2011

Abstract Clinical or laboratory evidence of varicella-zoster virus (VZV) infection has been consistently associated with lower glioma risk in case–control studies, suggesting a protective effect of VZV against glioma. We formulated the following explanatory hypotheses: reactivated VZV preferentially infects and kills gliomas compared to normal parenchyma; and VZV-specific cytotoxic T lymphocytes (CTL) cross-react with gliomas. We established an ex vivo model of VZV infection, which showed that glioma cell lines and primary astrocytes were equally permissive to VZV infection and had similar 15% average decrease in viability upon infection. In co-cultures, the relative growth of glioma cells and astrocytes was not affected by the VZV infection. However, VZV stimulated, but not mock stimulated, peripheral blood mononuclear cells from VZV-seropositive individuals recognized and killed HLA class I-matched glioma cells (mean±SE decrease in viability of 26±12%, p=0.04), but not matched astrocytes. VZV infection of the glioma cells did not affect the T cell-mediated killing. Taken together, these data suggest that ex vivo VZV infection has similar direct effects on glioma cells and astrocytes. The protective effect of prior VZV infection against the incidence of glioma may be mediated by CTL that recognizes epitopes shared by VZV and glioma cells.

Keywords Glioma · Glioblastoma multiforme · Varicella-zoster virus · Cytotoxic T lymphocytes · Astrocytes

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Introduction

Gliomas account for over 70% of 18,000 incident cases of primary malignant central nervous system (CNS) tumors diagnosed annually in the USA (Wen and Kesari 2008). Gliomas include a multiplicity of tumor types, but the most common ones are glioblastomas multiforme. The only established risk factor for gliomas is exposure to ionizing radiation. Studies of genetic, demographic, environmental, and developmental factors associated with adult glioma etiology and prognosis over the past 20 years consistently show significant inverse associations of glioblastomas with chickenpox [odds ratio (OR)=0.53; 95% confidence intervals (CI)=0.34–0.84] documented by history (Wrensch et al. 2001, 1997a) or by anti-varicella-zoster virus (VZV) antibodies (OR=0.51; 95%CI=0.34–0.76) (Wrensch et al. 2005). Furthermore, VZV-seropositive glioma patients had lower anti-VZV antibody titers compared with seropositive non-glioma controls (Wrensch et al. 2005). Among a long

list of additional demographic, environmental, and genetic characteristics, including serologic evidence of latent infection with other herpesviruses, the only additional finding that correlated with lower incidence of glioblastomas was a high IgE serum titer (Wrensch et al. 1997b). Other risk or protective factors, including genetic traits, are likely to play a role in the incidence of glioma and are currently being investigated.

VZV is a ubiquitous herpesvirus, which, after primary infection (varicella, chickenpox), establishes latency in cranial and dorsal root sensory ganglia. Symptomatic VZV reactivation, which clinically presents as herpes zoster, a painful vesicular rash of dermatomal distribution, is commonly accompanied by VZV invasion of the CNS including parenchymal lesions (Gilden et al. 2000; Haanpaa et al. 1998). It has long been suspected that latent VZV may undergo asymptomatic reactivations. This hypothesis has been proven using polymerase chain reaction (PCR) techniques and intensive monitoring (Mehta et al. 2004; Quinlivan et al. 2007; Wilson et al. 1992). During asymptomatic reactivations, VZV may invade the CNS as with herpes zoster, which is strongly suggested by studies that found VZV deoxyribonucleic acid in the cerebrospinal fluid of patients with clinically unsuspected VZV infection (Tang et al. 1997).

Several viruses preferentially infect and lyse glioma cells by comparison with normal brain cells (Wollmann et al. 2007, 2005). Of these, vesicular stomatitis virus seemed to have the highest oncolytic potential and cytomegalovirus had the lowest oncolytic potential. The low glioma–cytolytic potential of cytomegalovirus is consistent with the frequent isolation of this virus from glioma cells (Cobbs et al. 2002; Mitchell et al. 2008). Other herpesviruses, including herpes simplex, Epstein–Barr, and human herpesviruses 6 and 7, also have the ability to invade the central nervous system (Ansari et al. 2004; Fodor et al. 1998; Weinberg et al. 2005; Weinberg et al. 2002) but their glioma–cytolytic potential has not been studied. Another mechanism used by viruses to modulate CNS inflammation is through the activation of cytolytic T lymphocytes (CTL) (Ji et al. 2010). Virus-specific CTL may recognize brain tissue if they encounter cross-reactive epitopes or if they are equipped with dual T cell receptors, one for the virus and another that is tissue specific (Ji et al. 2010).

To gain insight into the protective effect of prior VZV infection against the incidence of glioma, we formulated two hypotheses: (1) VZV replicates more efficiently in glioma cells than in normal tissues and mediates the clearance of nascent tumors either through a direct cytopathic effect or by triggering a glioma-specific immune response; (2) VZV-specific adaptive immune response elements recognize gliomas in a cross-reactive fashion. The goal of this study was to test these hypotheses.

Material and methods

Cells and viruses

Glioblastoma cell lines U251MG and U118MG were obtained from ATCC. Additional lines including UPN0145, D3, D19, D33, and G12 were isolated from surgical specimens (Donson et al. 1999). Primary human astrocytes (N=2) were purchased from Clonexpress, Inc. Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy volunteers as previously described (Weinberg et al. 1998). VZV vOka strain was propagated in primary human lung fibroblasts following conventional procedures (Weinberg et al. 1996). A single viral stock, which was used throughout the entire study, was prepared by quick freeze and thaw cycles of the infected fibroblasts. The stock was stored in liquid nitrogen divided into multiple aliquots. One aliquot was used to determine the viral titer. Briefly, triplicate wells of a 24-well plate containing 75% to 80% confluent human lung fibroblasts were inoculated with tenfold serial dilutions from 10^{-2} to 10^{-6} of the stock virus in replicate wells. VZV was allowed to grow for 72 h in a 37°C, 5% CO_2, humidified atmosphere. The plate was fixed with a 70% methanol solution in acetic acid and stained with 0.8% crystal violet, which concentrates in infected cells. Viral plaques were counted under a CK2-inverted microscope (Olympus). The titer was calculated by identifying a set of replicate wells containing >20 and <100 plaques/well and multiplying the average number of plaques in those wells by the dilution factor and dividing the product by the inoculation volume. The titer of the VZV stock used in this study was 4.73×10^5 plaque-forming units (PFU)/ml.

Cell growth and infectivity assays

Glioma in Roswell Park Memorial Institute (RPMI) 1640 medium (Cellgro) with 10% fetal calf serum (Gemini) and/or primary human astrocytes in DMEM/F12 (Invitrogen) with 5% fetal calf serum, 10 ng/mL EGF, bFGF (Invitrogen), 25 μg/mL gentamicin, and 2.5 μg/mL amphotericin B were infected with VZV at the pre-optimized multiplicity of infection (MOI) of 1 plaque-forming unit/cell. After 24, 48, 72, and 96 h of incubation at 37°C and 5% CO_2, cells were trypsinized, counted, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-VZV gpI monoclonal antibodies (Chemicon) according to the manufacturer's instructions. The proportion of infected cells was measured using an FC500 instrument (Beckman Coulter) and Summit 4.3 (Dako).

Apoptosis determination

Glioblastomas and/or primary astrocytes were infected with VZV at a MOI of 1. At 48 h post-infection, VZV-infected

cells were gently trypsinized and stained for flow cytometric analysis using mouse anti-VZV gpI (Chemicon) at 1:5,000 and APC-conjugated goat anti-mouse antibodies (BD) at 1:500; and with propidium iodide (PI), a fluorescent dye that concentrates in necrotic cells, and AnnexinV-FITC, which binds to the membranes of apoptotic cells (TACS Apoptosis detection kit, R&D). Cells were analyzed as above.

Cytotoxic assays

PBMC from healthy adult volunteers, glioma and astrocytes had MHC class I typing performed by PCR amplification and sequencing at the University of Colorado Clinical Immunology, CAP- and CLIA-certified laboratory. PBMCs were stimulated with VZV, CMV, or mock-infected control antigens at 1:200 for 6 days in RPMI 1640 with 10% human AB serum (Gemini), 10 mM Hepes (Cellgro), 1% glutamine (Gibco), and 1% pen/strep solution (Gibco) at 37°C and 5% CO_2. Two days prior to the assay, glioma and astrocytes were infected with VZV as above. On the day of the assay, VZV-infected and uninfected glioma and astrocyte targets were labeled with carboxyfluorescein succinimidyl ester **(**CFSE), a fluorescent dye that is incorporated into the cellular membrane, and co-cultured overnight with mock- or VZV-stimulated PBMC at effector-to-target ratios of 3:1 or 30:1. Effectors and targets were matched at ≥1 MHC class I locus. After the incubation, cells were stained with AnnexinV-APC (BD) and PI (BD) and analyzed by flow cytometry as above.

Statistical analysis

The statistical analysis was performed using Prism 4 and Instat software (GraphPad). Data distribution was checked and parametric tests were used for normally distributed data. Significance was defined by $p \leq 0.05$.

Results

Kinetics of VZV infection and apoptosis of glioma cells

At an MOI=1 (100,000 glioma cells incubated with 100,000 VZV PFU), all 5 glioma lines tested (UPN0145, U251MG, D3, D19, and G12) were permissive to VZV infection (Fig. 1). To eliminate the potential bias associated with differential growth kinetics of each glioma line, the kinetics of VZV infection was assessed by the ratio of infected-to-uninfected cells in each culture. The peak of infection occurred between 24 and 48 h post-infection, with mean±SE infected: uninfected cell ratios of 1.4±0.1 at 48 h, followed by 0.9±0.1 at 72 h (p=0.02, paired T test). At 72 h, the absolute number of VZV-infected cells/well increased compared with 48 h. However, the total number of glioma cells/well increased even more, such that the proportion of infected-to-uninfected cells decreased. This did not completely rule out the possibility that VZV infection decreased the viability of infected cells compared with that of uninfected cells contributing to the decrease in the proportion of infected-to-uninfected cells.

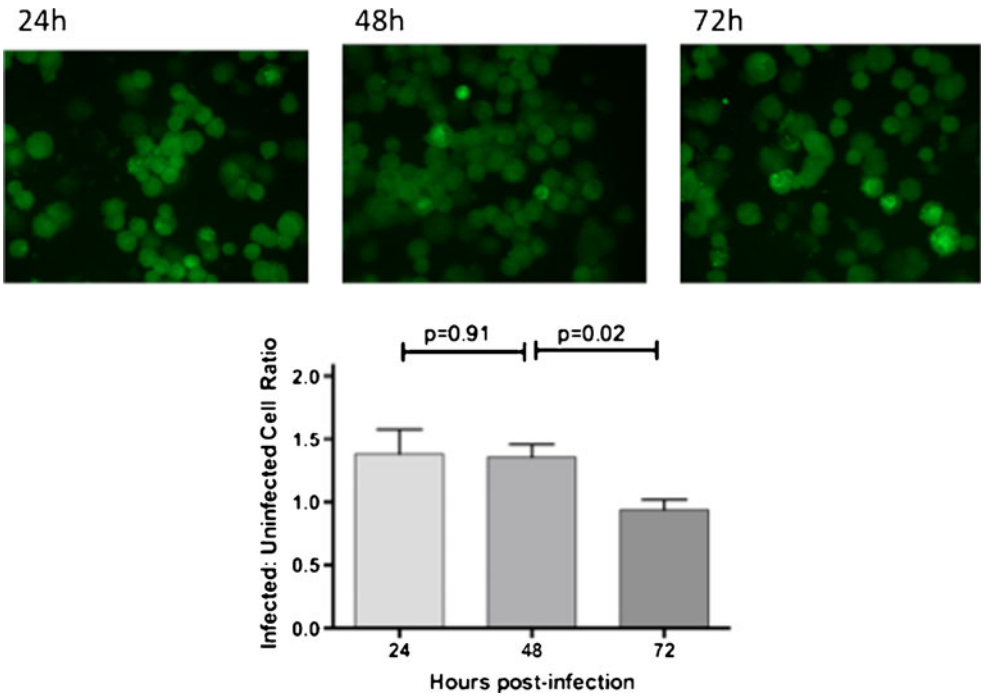

Fig. 1 Kinetics of ex vivo VZV infection in glioma cell lines. The *upper panel* shows a typical representation of the course of VZV infection of gliomas over time. The illustration shows UPN glioma. VZV-infected cells are in *green*. In the *lower panel*, data were derived from five glioma lines infected with VZV at a MOI of 1. *Bars* represent mean and standard errors. There was a significant decrease in the proportion of infected cells at 72 h of infection, indicating that the neoplastic cells multiplied faster than the viral infection

To determine the effect of VZV infection on the viability of glioma cells, we compared the annexinV-PI-% of VZV-infected and uninfected glioma cells in the same culture at 48 h post-infection and used primary human astrocytes as a reference (Fig. 2). VZV-infected glioma cells, identified by the expression of VZV gpI, had significantly lower viability than their uninfected counterparts (N=5, p=0.02, paired T test). However, the viability of VZV-infected glioma cells was not significantly different compared with infected astrocytes (N=5, p=0.69, one sample T test) suggesting that VZV infection may not handicap the growth of glioma by comparison with that of astrocytes.

We further performed co-culture experiments of glioma and CFSE-labeled astrocytes to determine if VZV infection differentially affected the growth of neoplastic compared with healthy astrocytes (Fig. 3). Between 0 and 48 h of co-culture, the proportion of normal astrocytes decreased from 90% to mean±SE of 74±2% and 71±6% in 8 VZV-infected and in 8 uninfected co-cultures, respectively (p=0.4, paired T test). VZV-specific staining of cells in co-cultures showed that similar proportions of glioma and astrocytes were infected by VZV (mean±SE of 49±5% and 50±2% infected cells, respectively; p=0.8, unpaired T test).

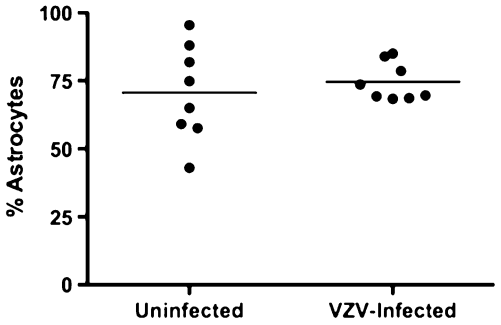

Fig. 3 Differential growth of glioma cells and primary astrocytes in VZV-infected and uninfected co-cultures. Data were derived from seven glioma lines and two primary astrocyte cultures at 48 h of co-culture. Each culture initially contained 90% astrocytes and 10% glioma cells in quadruplicate wells of 24-well plates. Half of the wells of each astrocyte/glioma combination were infected with VZV at an MOI of 1. At 48 h post-infection, the proportion of astrocytes decreased to a mean of 75% both in infected and uninfected cultures (p=0.4)

VZV-stimulated T cell cytotoxicity of glioma cells

To study the effect of VZV infection on T cell-mediated cytotoxity of glioma cells, we measured apoptosis of glioma and astrocytes after overnight incubation with MHC class I-matched, VZV-stimulated, or mock-stimulated PBMC (Fig. 4). Glioma cell lines with ≥1 allele of the MHC type A01, A02, A24, or A33 were incubated with PBMC from three donors with ≥1 allele of the corresponding MHC type. Five unique glioma/PBMC pairing options were identified. Glioma cells incubated with VZV-stimulated PBMC at effector: target ratios of 30:1 had marginally significant higher levels of late apoptosis (N=5; mean±SE difference= 23±12%; p=0.06) and significantly lower viability (N=5; mean±SE difference=26±12%; p=0.04) than glioma cells incubated with mock-stimulated PBMC at the same effector-to-target ratios. The specificity of this effect was demonstrated by the fact that the apoptosis of glioma cells incubated with VZV-stimulated PBMC at effector-to-target ratios of 3:1 was significantly lower than at effector-to-target ratios of 30:1 (N= 5; mean±SE difference=27±13%; p=0.05). Furthermore, at effector-to-target ratios of 30:1, glioma cells incubated with mock-stimulated PBMC maintained a significantly higher viability than glioma cells incubated with VZV-stimulated PBMC at the same effector-to-target ratio. This observation ruled out a nonspecific allotypic reaction between PBMC and gliomas. Astrocyte cytotoxicity was <10% and did not

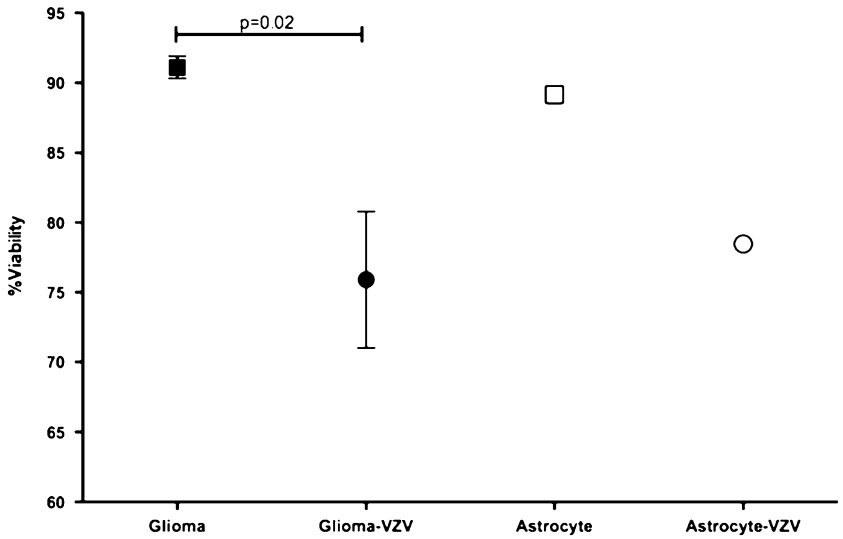

Fig. 2 Effect of ex vivo VZV infection on viability of glioma and primary astrocytes. Data were derived from five gliomas and two primary astrocyte cell cultures at 48 h post-infection. *Dots* indicate mean and standard error of the percent viable cells in the glioma cultures and mean only in the astrocyte cultures. VZV-infected glioma cells had significantly lower viability compared with uninfected cells. Similar trends were observed for the astrocytes

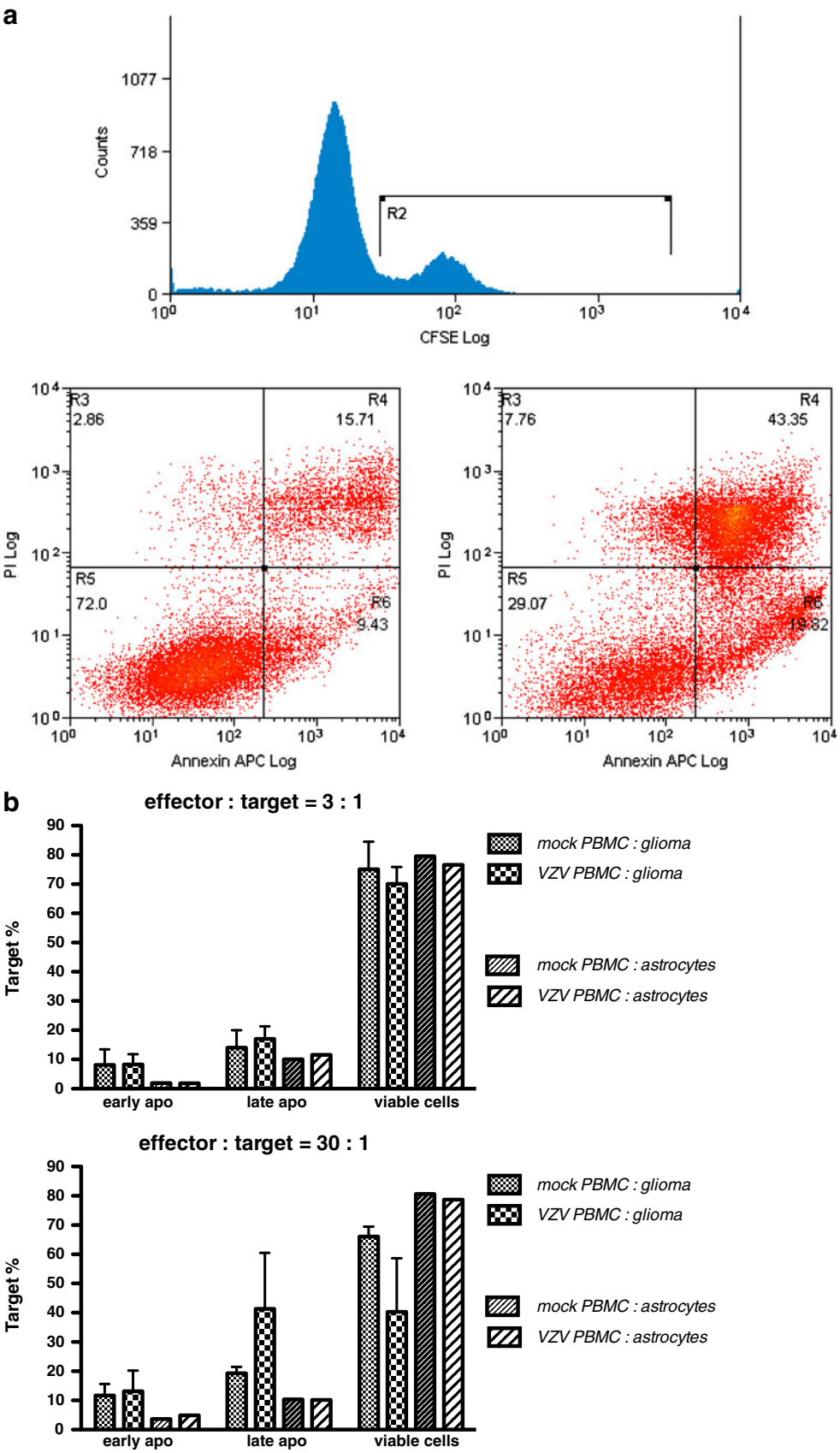

Fig. 4 VZV-stimulated cytotoxicity of glioma cells. **a** The figure illustrates the flow cytometric analysis of matched glioma and PBMC at effector-to-target ratio=30:1. The *upper panel* represents the gating strategy used to identify the CFSE + glioma cells (*R2*). CFSE cells consist of PBMC. The *lower panel* shows apoptosis of glioma cells incubated with PBMC. In the *left lower panel*, the PBMCs were mock stimulated, whereas in the *right lower panel*, PBMCs were stimulated with VZV prior to incubation. The numbers in each quadrant represent percent of the anchor gate, which contains the CFSE + glioma targets. Viable cells are captured in *R5*, early apoptosis in *R6*, late apoptosis in *R4*, and necrosis in *R3*. **b** Data were derived from five experiments in which VZV- or mock-stimulated PBMC were cultured overnight with HLA class I-matched glioma cells or primary astrocytes at effector-to-target ratios of 3 to 1 or 30 to 1. Killing was measured by target apoptosis (*apo*). *Bars* indicate mean±SE of glioma experiments and the results of two astrocyte primary cultures. At an effector-to-target ratio of 30:1, there was significantly lower viability of glioma cells cultured with VZV-stimulated compared with mock-stimulated PBMC (p=0.04, paired T test). VZV- or mock-stimulated PBMC did not appreciably kill primary astrocytes

differ between co-cultures containing VZV-stimulated or mock-stimulated PBMC (N=2). VZV infection of glioma or astrocytes did not alter the cytotoxicity of VZV-stimulated or mock-stimulated PBMC (data not depicted). Furthermore, cytomegalovirus-stimulated PBMC from three seropositive healthy donors, whose cells were also used in the experiments described above, did not show appreciably higher lysis of MHC class I-matched glioma cells compared with unstimulated PBMC (mean±SE viability difference=9±5%).

Discussion

We showed that VZV stimulation expanded or selected PBMC with the ability to recognize and kill glioma cells, but not normal astrocytes. In contrast, mock-stimulated PBMC did not induce apoptosis of glioma cells or astrocytes. This suggests that VZV infection may prime T cells to recognize one or more cross-reactive epitopes expressed by glioma cells, but not by astrocytes. The specificity of the lytic activity is strongly supported by the fact that astrocytes were not lysed by the stimulated PBMC.

It is important to note that the effector cells in our assays were bulk PBMC. These typically contain 20% to 40% CD8+ conventional CTL. Furthermore, VZV-specific CD8+ T cells account for <5% of the total CD8+ cells in young adults (Patterson-Bartlett et al. 2007). Assuming that 5% of the VZV-stimulated PBMC used in the glioma cytotoxic assays in this study were VZV-specific CTL, the effector-to-target ratio was 1 VZV-CTL to 2 glioma cells. This suggests that only a few VZV-specific T cells are necessary to efficiently lyse glioma cells.

Since malignant transformation is known to increase viral infectivity, we explored the possibility that VZV preferentially infected glioma cells during asymptomatic reactivations. Asymptomatic VZV reactivations are not uncommon in immunocompetent hosts (Mehta et al. 2004) and they can be associated with CNS invasion (Tang et al. 1997). However, in our experiments, VZV infected glioma cells and primary astrocytes equally well. Although VZV infection decreased the viability of glioma cells as measured by apoptosis, it decreased the viability of astrocytes to the same extent. VZV infection of glioma and astrocyte co-cultures did not alter the overgrowth of glioma cells in comparison with astrocytes. Furthermore, the ex vivo VZV infection of glioma cells did not increase their susceptibility to CTL-mediated killing. Taken together, these data suggest that even if VZV invades the CNS during asymptomatic reactivations, it does not appear to preferentially infect tumor cells or to inhibit directly or indirectly their growth.

Our findings are limited by the nature of ex vivo experiments. Further studies are warranted to assess the prevalence of VZV-specific CTL in the CNS of VZV-seropositive individuals. Wakim et al. (2010) recently showed that memory T cells persist in the brain in the absence of continuous antigen presentation, very much as in other non-immune tissues. It is not known if antigenic re-exposure, such as it may occur during VZV reactivations, boosts the brain resident memory T cells. This is suggested by the inverse association between anti-VZV antibody titers and the incidence of glioma in the general population; frequent VZV reactivations or re-exposures would be expected to boost both humoral and cell-mediated immune responses. Equally important would be to determine if the VZV-specific memory T cells in the CNS could be expanded through vaccination or other interventions. Both low- and high-dose VZV vaccines are commercially available for varicella and herpes zoster prevention, respectively. These vaccines efficiently stimulate VZV-specific cell-mediated immunity (Levin et al. 2008; Watson et al. 1995). The live-attenuated Oka VZV vaccine strain was occasionally found to cause herpes zoster (Loparev et al. 2007) suggesting that the vaccine virus is capable of establishing latency. Whether these properties of the vaccine virus can be used in association with other therapeutic interventions to control the growth of gliomas is an important area for future research.

Acknowledgments The authors thank Karen Helm for technical support and Drs. Ken Tyler, Margaret Wrensch, Joe Wiemels, John Wincke, and Myron Levin for critical review of the manuscript. This work was supported by P30 CA046934 grant to the University of Colorado Cancer Center and by a subcontract to R01CA52689 (PI Margaret Wrensch). The contents are the sole responsibility of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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