Glioblastoma derived exosomes contribute to tumor immune evasion

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Glioblastoma derived exosomes contribute to tumor immune evasion
IMMUNOLOGY RESEARCH

IR-01. CYTOMEGALOVIRUS SUBVERTS THE MONOCYTE LINEAGE TO BECOME GLIOMA PROPAGATING
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We have identified a mechanism by which cytomegalovirus (CMV) interleukin-10 (IL-10) is utilized by glioblastoma multiforme (GBM) to maintain the immunosuppressive microenvironment. CMV has been ubiquitously detected within high-grade gliomas, and the aggressive nature of these tumors is not fully understood. GBMs harvested ex vivo were analyzed by flow cytometry to demonstrate CMV antigen expression. Distinct expression subsets of cells, such as the myeloid lineage and CD133+ cells, were identified. CMV antigens were detected in both GBM cell lines and GBM primary cell cultures, and the supernatant from these cultures induced the up-regulation of IL-10, a marker of transcriptional activity of CMV antigens. Chemotaxis assays revealed that the supernatant from CMV IL-10 induced the up-regulation of IE1, a marker of transcriptional activity of CMV. The cellular immune response in patients with glioblastoma multiforme (GBM) displays abnormalities, and the aggressive nature of these tumors is not fully understood. GBMs harvested ex vivo were analyzed by flow cytometry. We observed higher CD14 expression and lower HLA-DR expression on monocytes after 3 days of exposure of the peripheral blood mononuclear cells to GBM exosomes than on monocytes exposed to medium alone. The effect on monocytes was also observed in a purified monocyte population, indicating a direct effect. This result corresponds to the changes in the phenotype of monocytes in peripheral blood of GBM patients and suggests an important immunomodulatory role for brain tumor exosomes.

IR-03. INTENSE HUMAN CYTOMEGALOVIRUS (HCMV) IMMUNE RESPONSE IN Glioblastoma Patients: A PROGNOSTIC FACTOR FOR SURVIVAL
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INTRODUCTION: HCMV is a ubiquitous human herpesvirus found in nearly all humans worldwide, with persistent infection occurring in over 70% of adults. The virus has been implicated in the development of several human malignancies owing to the oncomodulatory effects of HCMV infection. There is growing scientific evidence about an association between HCMV and malignant gliomas. To study the prognostic value of the anti-HCMV immune response, we prospectively assessed the levels of serum HCMV immunoglobulins M and G (IgM and IgG) in newly diagnosed glioblastoma patients and correlated the results with the clinical course.

METHODS: We analyzed serum from 32 glioblastoma patients treated with standard chemoradiotherapy at our institution between November 2005 and October 2010. Serum serologies were obtained by chemoluminescent quantitative analyses. HCMV IgM >0.5 UA/ml was considered diagnostic for acute HCMV infection, and HCMV IgG >16 UA/ml was regarded as positive for latent infection. Intense HCMV immune response was defined as HCMV IgG >100 UA/ml. All clinical and pathologic data were recorded in a database system using the SPSS version 13.0 statistics package. RESULTS: After a median follow-up of 18.2 months, 24 patients (75%) have died. HCMV IgG was positive for latent infection in 23 patients (72%). 10 of whom (31%) had intense immune response (51%). Two patients had an acute HCMV reactivation with positive values for IgM. In univariate analysis, HCMV IgG >100 UA/ml demonstrated a strong significant association with a longer overall survival (p=0.0087). Positive HCMV IgG was found to be marginally associated with survival (p=0.07). In multivariate analysis, HCMV IgG >100 UA/ml retained statistically significant as a prognostic factor for longer survival (hazard ratio, 0.18; 95% CI 0.04-0.81; p=0.02). CONCLUSION: Intense HCMV IgG immune response is significantly associated with longer overall survival in our series. Larger studies are required to validate HCMV IgG as a prognostic factor for survival in glioblastoma patients.

IR-04. TUMOUR-INfiltrATING T-CELL SUBpopulations IN GliobLASTOMAS
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This study was designed to determine the incidence and prognostic value of various populations of tumour-infiltrating T cells in glioblastomas. We also evaluated the difference in T-cell populations after conventional treatment. Sixty-seven patients with glioblastomas underwent surgery between 2001 and 2009. Immunohistochemical and staining was performed for CD4, CD8, and FoxP3, and the average number and percentage of positive cells were calculated. From eight patients, the average number of subpopulations was compared between the specimens obtained during the first and second operations. Age, gender, Karnofsky performance status, RTOG-RPA class, extent of removal, treatment modality, MGMT methylation status, and immunopositivity for CD3, CD4, CD8, and FoxP3 were analyzed as prognostic factors. There was an average of 12.8 ± 1.8 CD3+ T cells, 1.5 ± 0.5 CD4+ T cells, 6.8 ± 1.3 CD8+ T cells, and 0.6 ± 0.2 FoxP3+ T cells. The percentage of positive T-cell subpopulations was 89.6%, 22.4%, 77.6%, and 34.3% for CD3, CD4, CD8, and FoxP3, respectively. Among the eight patients there was no difference in the subpopulations between the first and second operations. The median progression-free survival was 7.0 months (95% CI, 5.2-8.9 months) and the overall survival was 14.8 months (95% CI, 11.18-17.3 months). Univariate analysis showed a statistically significant difference in progression-free survival for CD8 (p=0.02) and in overall survival for
RTOG-RPA class ($p = 0.003$), extent of removal ($p = 0.01$), and MGMT promoter methylation status ($p = 0.005$). On the basis of the multivariate analysis results, RTOG-RPA class was significantly associated with longer overall survival of intra-tumoral infiltrated and extratumoral infiltrated tumors in glioblastomas and there was a consistent response, even after conventional treatment. There was a statistically significant difference in progression-free survival for CD8 in immunologically privileged central nervous systems.

**IR-06. IMMUNOLOGICAL SOIL AND PREVENTION OF BREAST CANCER BRAIN METASTASIS**

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As therapies for systemic cancer improve and patients survive longer, the risk of cerebral metastases increases. Therefore, the cerebral metastases of cancers is a major obstacle that must be overcome before cancers can be cured by any means. In our recent data, mice bearing 4T1 breast cancers in the primary site (mammary pad) showed accumulations of myeloid-derived suppressor cells (MDSCs) in the brain before their brains demonstrated any signs of glioma. Analysis of the MDSCs showed that they were present in the pre-metastatic brain of 4T1-bearing mice, and anti-CCL2 treatment reduced MDSC infiltration. The cyclooxygenase-2 inhibitor celecoxib reduced MDSC infiltration as well as their immunosuppressive effect in the sera of 4T1-bearing mice. Chemokine CCL2 was also up-regulated in the pre-metastatic brain of 4T1-bearing mice, and anti-CCL2 treatment reduced MDSC infiltration. We showed that the MDSCs are present in GBM tumors and that the mouse model overexpressing the pre-metastatic soil will allow us to determine the functional significance of IL-4R-alpha in the glioma microenvironment. We next sought to address the functional significance of IL-4R-alpha in a murine glioma model. We induced gliomas in BALB/c-background mice by intracerebroventricular transfection of oncogenes using the Sleeping Beauty transposon system and found that the cells suppress T-cell function. Neutrophilic MDSCs predominate in the tumors than in normal brain tissue ($p = 0.003$), extent of removal ($p = 0.01$), and MGMT promoter methylation status ($p = 0.005$). On the basis of the multivariate analysis results, RTOG-RPA class was significantly associated with longer overall survival of intra-tumoral infiltrated and extratumoral infiltrated tumors in glioblastomas and there was a consistent response, even after conventional treatment. There was a statistically significant difference in progression-free survival for CD8 in immunologically privileged central nervous systems.

**IR-07. THE RCAS/Tv-A MODEL OF MURINE GLIOMA REPRODUCES IMMUNOSUPPRESSION PRODUCED BY MYELOID-DERIVED SUPPRESSOR CELLS IN HUMANS WITH GBM**

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Myeloid-derived suppressor cells (MDSCs) are a population of bone marrow-derived cells with potent immunosuppressive properties. We previously showed (Neurooncology, 2011) that MDSCs are found at elevated levels in the circulation of patients with glioblastoma multiforme (GBM) and that they produce reversible T-cell dysfunction. We now show that MDSCs are present in GBM tumors and that the mouse model overexpressing the pre-metastatic soil will allow us to determine the functional significance of IL-4R-alpha in the glioma microenvironment. We next sought to address the functional significance of IL-4R-alpha in a murine glioma model. We induced gliomas in BALB/c-background mice by intracerebroventricular transfection of oncogenes using the Sleeping Beauty transposon system and found that the cells suppress T-cell function. Neutrophilic MDSCs predominate in the tumors than in normal brain tissue ($p = 0.003$), extent of removal ($p = 0.01$), and MGMT promoter methylation status ($p = 0.005$). On the basis of the multivariate analysis results, RTOG-RPA class was significantly associated with longer overall survival of intra-tumoral infiltrated and extratumoral infiltrated tumors in glioblastomas and there was a consistent response, even after conventional treatment. There was a statistically significant difference in progression-free survival for CD8 in immunologically privileged central nervous systems.

**IR-08. GLIOBLASTOMA-DERIVED EXOSOMES CONTRIBUTE TO TUMOR IMMUNE EVASION**

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Glioblastoma multiforme (GBM) is the most frequent and lethal primary brain tumor in adults. Despite intensive biomedical research, the median survival after diagnosis is 15 months. One factor contributing to this poor prognosis is the immune protection afforded by the tumor microenvironment. Tumors have a diverse repertoire of immune-evasive techniques. One method of evasion not well explored is the release of tumor-derived exosomes. Exosomes are tiny membrane-bound vesicles of endocytic origin that contain viable mRNA and functional proteins that can affect the physiology of recipient cells. Exosome release has been reported for numerous cancer types, including GBM. Exosomes from colon cancer have been shown to carry Fas ligand (FasL) and to induce apoptosis of activated T cells. The metastatic study was to elucidate whether the same immune-evasive technique is used in GBM. GBM exosomes were isolated from the serum-free culture medium of U87 MG and U138 MG cells by using differential ultracentrifugation and were then resuspended in phosphate-buffered saline. The protein concentration of the resulting exosome pellet was determined, and subsequent exosome treatments were based on protein concentration. A3T T cells were plated at a concentration of 10,000 cells per well in 96-well plates and were treated with quantified exosomes or with recombinant FasL, and T cell proliferation was determined. Our data demonstrated that tumor-derived exosomes significantly inhibited the proliferation of T cells and that the cellular inhibition resulting from the exosomes was comparable to that seen with the recombinant FasL. These results suggest that targeting FasL or GBM could greatly decrease the amount of immune suppression that occurs at the tumor site.
IR-09. CHARACTERIZATION OF THE IMMUNE RESPONSE TO ONCOLYTIC ADENOVIRUS THERAPY FOR MALIGNANT GLIOMA
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The oncolytic adenovirus Delta-24-RGD has demonstrated highly effective anti-tumor efficacy in various intracranial xenograft models for glioblastoma. However, the species specificity of human adenovirus has restricted preclinical studies to immunocompromised animals. As a result, the role of the innate immune response has not been clearly delineated and may be greatly under-estimated in this type of therapy. Therefore, we set up a syngeneic immune-competent intracranial model with murine glioma cells found to be semi-permissive to human adenovirus replication and investigated both the innate and adaptive immune response to Delta-24-RGD treatment. C57BL/6 mice were injected stereotactically with GL261 cells and treated five days later with AdΔ24RGD or phosphate-buffered saline. At different early and late time points post-treatment, brain, blood and spleen samples were collected. Splenocytes were co-cultured with Delta-24-RGD or GL261 cells and assayed for interferon (IFN)-gamma production within 24 hours after virus treatment. Splenocytes from virus-treated animals co-cultured with Delta24-RGD produced high levels of IFN-gamma. Interestingly, splenocytes from virus-treated mice also produced IFN-gamma when co-cultured with adherent GL261 cells. Splenocytes from phosphate-buffered saline-treated mice did not respond to virus or to GL261 cells. High-level neutralizing antibodies were detected in the sera of mice starting 96 hours post-treatment. In conclusion, the GL261 murine glioma model offers a system to gain insight into the role of the innate and adaptive immune response to oncolytic adenovirus treatment in the brain. We demonstrated that splenocytes from Delta-24-RGD-treated mice recognized both virus and tumor antigens. We also demonstrated that a humoral response to virus is attempting to neutralize the activity of the virus. Future experiments will be performed to gain more insight into this response and to develop strategies to enhance the anti-tumor immune response.

IR-09. MODULATION OF NEUTROPHIL ACTIVATION BY TUMOR AND TUMOR-ASSOCIATED NECROSIS IN GliOBlastoma
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We recently identified an immuno-suppressive mechanism in GBM patients that is characterized by cell necrosis and release of arginase I (ARG1) in reversible T-cell dysfunction. We noted isolated instances of increased neutrophil infiltration of GBM, but the mechanism behind this significant number of activated neutrophils has been unclear. To investigate the role of tumor-associated factors in this response, we hypothesized that tumor-associated factors are responsible for the activation and subsequent alteration of surface-binding proteins of neutrophils within the tumor micro-vasculature. To initially explore this hypothesis, normal donor neutrophils were incubated with tumor-conditioned medium or necrotic material. Induction of degranulation was measured via flow-cytometric quantification of surface markers associated with neutrophil activation. Following incubation with conditioned medium or necrotic material, increased surface expression was observed for both CD11b (1.6- and 1.5-fold, respectively) and CD66 (11.7- and 1.4-fold, respectively), confirming tumor-specific induction. In further exploration of baseline characteristics of circulating neutrophils in GBM patients, resting and degranulated populations were purified from peripheral blood using a dual-density Histopaque gradient. Baseline expression of CD11b is increased in patients with high-grade GBM, whereas the long-term survivors had no sign of tumor growth by day 50. Additionally, we report that double immunotherapy using anti-4-1BB agonist and anti-CTLA-4 antibodies are classified as long-term survivors; thus, the role of the innate immune response to Delta-24-RGD treatment in the brain. We demonstrated that splenocytes from Delta-24-RGD-treated mice recognized both virus and tumor antigens. We also demonstrated that a humoral response to virus is attempting to neutralize the activity of the virus. Future experiments will be performed to gain more insight into this response and to develop strategies to enhance the anti-tumor immune response.
IR-13. COMPREHENSIVE CHARACTERIZATION OF HUMAN CYTOMEGALOVIRUS INFECTION IN THE LONG-TERM INFECTED T98G CELL MODEL
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HCMV is the leading viral cause of birth defects, affecting primarily the central nervous system. It is also strongly associated with glioma in adults and plays a role via oncomodulation. Previously we created an HCMV-infected T98G cell line long-term infection model (J Virol 2007). We investigated HCMV infection by culturing infected cells longer term without passaging and viral genome persistence in infected cells with continuous passaging. HCMV Ag+ cells formed clusters during long-term culture without passaging. Maintenance of the viral genome in T98G cells was determined by PCR, nested PCR, and FISH, and viral genome copy number was analyzed by qPCR. PCR and nested PCR results showed that viral genome lasted to passage 7 (24 days post-infection) and to at least passage 13 (42 days post-infection), respectively; there was a high copy number of the viral genome at earlier passages, and the copy number was maintained around 400 from passages 10 through 13. To confirm that the viral genome was retained in Ag- (GFP-IE2+ ) cells, GFP-fused virus (WT-J+eGFP) was used for infection, purified Ag- cells were collected and cultured with continuous passaging, and viral genomes were detected by FISH. All cells had about 30 spots of HCMV genome in the nuclei. To investigate the HCMV infection feature in T98G cells, cells were transfected with p53 (host factor) and the IE1 protein-positive rate increased one-fold; for viral factors, pp71 mutant virus (T223A) and clinical isolates (TR and Tolledo) were used to infect cells. Cells with the mutant virus infection had a higher IE1 protein-expressing rate; clinical isolates went into latency directly or faster. Thus, HCMV infection in T98G cells without the disturbance of passaging forms Ag+ clusters, indicating that infection in the brain without interference may become worse. High copy number, HCMV genome persistence, and cellular and viral factors affected HCMV gene expression but could not change the infection, indicate a promising latent infection model that would be useful in glioma oncomodulation studies.