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Glioblastoma derived exosomes contribute to tumor immune evasion

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Abstracts

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, but its role has not been fully elicited. GBMs harvested *ex vivo* were analyzed by flow cytometry to determine CMV antigen expression. Distinct expressing subsets of cells, such as the myeloid lineage and CD133+ cells, were identified. CMV antigens US28, pp65, IE1, and gB were also present within four individual and fully characterized human GBM-derived glioma cancer stem cell (gCSC) populations as ascertained by flow cytometry. These gCSCs produce CMV IL-10 in a range from 5.62 to 111.11 pg/mL/10^6cells/day. When human CD14+ monocytes, precursor cells to macrophages/microglia, were exposed to gCSC-conditioned medium or recombinant CMV IL-10, there was a marked increase in the expression of immune-suppressive factors such as B7-H1, p-STAT3, VEGF, and TGF-beta. Concurrently, anti-tumor and pro-immune MHC II and CD86 were down-regulated and the expression pattern of immune markers was similar to the phenotype of glioma-associated macrophages/microglia. Exposure of CD14+ cells to CMV IL-10 induced the up-regulation of IE1, a marker of transcriptional activity of CMV. Chemotaxis assays revealed that the supernatant from CD14+ cells exposed to CMV IL-10 was able to induce the migration of gCSCs compared with the supernatant from CD14+ cells cultured in medium alone. This result indicates that CMV subverts GBM-associated macrophages/microglia to support the immune-suppressive microenvironment by shifting their phenotype to the immune-suppressive M2. The shift is subsequent to activation of the STAT3 pathway, resulting in the propagation of glioma angiogenesis via an increase in VEGF and by increasing glioma invasion. Therapeutic strategies involving immune-mediated cytotoxic responses now include strategies to reverse tumor-mediated immune suppression. This study suggests that including CMV as a target could enhance the effectiveness of immunotherapy.

IR-02. IMMUNE-MODULATORY PROPERTIES OF GLIOBLASTOMA MULTIFORME EXOSOMES

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The cellular immune response in patients with glioblastoma multiforme (GBM) displays abnormalities, and the aggressive nature of these tumors might be related to their capacity to evade the anti-tumoral response. Because most of the cells involved in the cellular response are not exposed to the tumor, it has long been thought that tumors secrete factors that modify the immune system. We hypothesized that exosomes are (at least some of) these secreted factors. Exosomes are microvesicles 50-100 nanometers in size that are derived from the endosomal pathway and, upon their release into the extracellular milieu, have been shown to be capable of modulating their microenvironment via the transfer of (micro)RNAs and proteins. GBM tumor-derived exosomes (TEXs) have been found in the serum of patients. It was recently shown in cell culture models that GBM TEXs can stimulate angiogenesis and cell proliferation, suggesting an important role for TEXs in tumor biology. To assess the effects of GBM exosomes on the cellular immune response, we isolated exosomes from the supernatant of GBM cell lines, GBM primary cell cultures, and serum of GBM patients. Peripheral blood mononuclear cells from healthy donors were exposed to the exosomes *in vitro* and were subsequently 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 with a purified monocyte population, indicating a direct effect. These results correspond to the changes in the phenotype of monocytes in peripheral blood of GBM patients and suggest 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 2008 and October 2010. CMV serologies were obtained by chemiluminiscent 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 IgG immune response was defined as HCMV IgG >100 UA/ml. All clinical and pathological 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 had intense response (31%). Two patients had an acute HCMV reactivation with positive values for IgM. In univariate analysis, HCMV IgG >100 UAI/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 UAI/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 2003 and April 2009. Immunohistochemical staining was performed for CD3, 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, 11-18.7 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. The intra-tumoural immune response occurred frequently 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-05. INTERLEUKIN-4 RECEPTOR ALPHA CHAIN (IL-4R-ALPHA) PROMOTES THE IMMUNOSUPPRESSIVE ACTIVITY OF GLIOMA-INFILTRATING MONOCYTES

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IL-4R-alpha is expressed on immunosuppressive cells of monocyte lineage and mediates their production of transforming growth factor (TGF)-beta in response to interleukin-13. We thus hypothesized that IL-4R-alpha expression on monocytes plays a significant role in glioma development. Analyses of human glioma-infiltrating leukocytes revealed that glioma-infiltrating monocytes, but not peripheral blood $CD14^+$ monocytes, express high levels of IL-4R-alpha, suggesting the unique up-regulation of IL-4R-alpha in the glioma microenvironment. We next sought to address the functional significance of IL-4R-alpha in a murine *de novo* glioma model. We induced gliomas in BALB/c-background mice by intracerebroventricular transfection of oncogenes using the *Sleeping Beauty* transposon system. *Il4ra*$^{-/-}$ mice exhibited significantly prolonged survival compared with wild-type (WT) mice. Consistently, gliomas induced in WT mice were infiltrated with higher numbers of $CD11b^+Gr1^+$ monocytes than were gliomas induced in *Il4ra*$^{-/-}$mice. We subsequently isolated glioma-infiltrating $CD11b^+Gr1^+$monocytes to address their functions. RT-PCR and ELISA revealed that the monocytes derived from WT mice expressed significantly higher levels of TGF-beta. Additionally, depletion of these cells using anti-Gr1 antibody in mice significantly prolonged survival after tumor challenge. Analysis of *in vitro* cultured bone marrow cells demonstrated that compared with cells derived from WT mice, *Il4ra*$^{-/-}$mouse-derived cells contained lower numbers of $CD11b^+Gr1^+$ monocytes with lower arginase and TGF-beta expression as well as a decreased ability to suppress T-cells *in vivo* and *in vitro*. Because type-1 skewed T-cells in *Il4ra*$^{-/-}$ animals could have contributed to the observed better survival compared with WT mice, we next depleted $CD4^+$ and $CD8^+$ T-cells by using antibodies. Although T-cell depletion shortened the overall survival of WT and *Il4ra*$^{-/-}$ mice, T-cell-depleted *Il4ra*$^{-/-}$ mice still exhibited enhanced survival over T-cell-depleted WT mice. These data suggest that IL-4R-alpha expression on glioma-infiltrating monocytes promotes the immunosuppressive microenvironment of gliomas through a variety of mechanisms, including TGF-beta production and T-cell inhibition, thereby facilitating glioma development.

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 metastasis 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 presence of metastatic tumor cells. These observations were accompanied by marked up-regulation of the inflammatory chemokines S100A8, S100A9, and serum amyloid A 3 (SAA3) in the pre-metastatic brains. Elevated levels of the cytokines tumor necrosis factor alpha and vascular endothelial growth factor, which could induce MDSCs, were detected 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. The cyclooxygenase-2 inhibitor celecoxib reduced MDSC infiltration as well as S100A8, S100A9, and SAA3 expression in the pre-metastatic brains of 4T1-bearing mice. On the other hand, neither MDSC accumulation nor up-regulation of S100A8, S100A9, or SAA3 was detected in the brains of mice bearing JC breast cancer cells, which are not metastatic. Our results suggest that tumor cells with high metastatic activity in their primary site induce immunosuppressive conditions in the distant target organs, such as brain, and thereby promote metastasis. Anti-CCL2 and celecoxib treatments might be used to prevent the formation of pre-metastatic immunological soil. Further understanding of the mechanisms underlying the immunological soil will allow us to develop effective strategies to prevent cerebral metastasis of breast cancer.

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 platelet-derived growth factor subunit B in Nestin-tva/ink4a- arf/KO reproduces our observations with patients. We collected tumor tissue and blood from consented patients ($n = 3$) with newly diagnosed GBM. Peripheral blood mononuclear cells were isolated, and MDSC subsets were detected by fluorescence-activated cell sorting analysis. From the murine glioma model ($n = 18$ mice) we harvested glioma tumors, normal brain tissue, and hematologic tissues. Cells were dissociated, stained, and subjected to similar analysis. MDSCs were present in both human and murine gliomas. In murine tumors there were more monocytic MDSCs (Gr1low, >5%) than neutrophilic MDSCs (Gr1high, >3%), and both were present at much higher levels in the tumors than in normal brain tissue ($p < 0.016$). MDSCs were also higher in the circulation of mice with gliomas than of control mice, but there the Gr1high subset predominated. GBM patients also had MDSCs present in tumor tissue ($7.0 \pm 3.1\%$). Subclassification of the MDSCs in the human samples indicated that lineage-negative MDSCs (CD15-CD14-CD33 + HLA-DR-) were more prevalent than MDSCs with a neutrophilic subtype (CD15 + CD14-CD33 + HLA-DR-). We also found that the proliferation and intracellular interferon-gamma level of splenocytes isolated from normal mice were decreased in the presence of Gr1+ MDSC cells from murine gliomas, indicating that this model reproduces the MDSC-induced immunosuppression seen in our GBM patients. We showed that MDSCs are present in both human and mouse glioma tumors and that the cells suppress T-cell function. Neutrophilic MDSCs predominate in the circulation of both species, whereas in the tumors the monocytic MDSCs dominate in mice and the linage-negative subset dominates in humans.

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 intense 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 aim of this 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 exosome fractions 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 in 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 immune system has not been clearly delineated and may be greatly underestimated in this type of therapy. Therefore, we set up a syngeneic immunecompetent 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 serum. 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 assessed for interferon (IFN)-gamma production. Brain sections were immunohistochemically stained for various immune cells. Serum samples were tested for the presence of neutralizing antibodies. A rapid influx of CD45+ leukocytes and F4/80+ macrophages was detected 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 GL261 cells. Splenocytes from phosphate-buffered serum-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 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-10. STEREOTACTIC RADIOSURGERY COMBINED WITH DOUBLE IMMUNOTHERAPY WITH ANTI-CTLA-4 AND ANTI-4-1BB YIELDS LONG-TERM SURVIVAL AND PROTECTIVE ANTITUMOR RESPONSE IN A MOUSE ORTHOTOPIC GLIOBLASTOMA MODEL

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Despite the best available therapies for glioblastoma multiforme, prognosis for patients remains poor. We tested an immunotherapeutic approach using two monoclonal antibodies in combination with stereotactic radiosurgery: anti-CTLA-4 blockade and anti-4-1BB agonist. CTLA-4 downregulates pathways of T-cell activation, while signaling through 4-1BB triggers T-cell expansion. Evidence that radiation alters the tumor microenvironment to enhance recruitment of antitumor T cells supports the strategy to unite radiotherapy and immunotherapy. To establish orthotopic tumors, GL261 glioma cells transfected with luciferase were intracranially implanted into C57/BL6 mice. On day 7 after implantation, mice were stratified into four treatment groups using bioluminescent imaging: (1) isotype controls, (2) stereotactic radiation, (3) anti-4-1BB and anti-CTLA-4 antibodies, and (4) stereotactic radiation with anti-4-1BB and anti-CTLA-4 antibodies. Stereotactic radiation was delivered on day 10 after implantation under computed tomographic guidance on a small-animal irradiator using a 3-mm beam set to 10 Gy. We administered anti-4-1BB on days 11, 14, and 17 and anti-CTLA-4 on days 11, 17, and 23. Overall survival was quantified. Protective antitumor memory response in the long-term survivors was assessed using subcutaneous tumor re-challenge compared to a group of naïve animals. The median survival time was 18 days for control animals, 19 days in the anti-4-1BB and anti-CTLA-4 arm, and 23 days in the stereotactic radiation arm. As of day 100 post-implantation, 50% of mice in the treatment arm combining stereotactic radiation with anti-4-1BB and anti-CTLA-4 antibodies are classified as long-term survivors; thus, the median survival for this treatment group has not been reached. After tumor re-challenge, all naïve animals had palpable tumors by day 17, whereas the long-term survivors had no sign of tumor growth by day 50. Our study shows that double immunotherapy using anti-4-1BB agonist and anti-CTLA-4 blockade combined with stereotactic radiosurgery results in long-term survival with development of a protective memory response.

IR-11. MODULATION OF NEUTROPHIL ACTIVATION BY TUMOR AND TUMOR-ASSOCIATED NECROSIS IN GLIOBLASTOMA

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We recently identified an immunosuppressive mechanism in GBM patients through which neutrophilic degranulation and release of arginase I resulted in reversible T-cell dysfunction. We noted isolated instances of increased neutrophilic infiltration of GBM, but the mechanism through which significant numbers of activated neutrophils persist within the peripheral circulation of these patients remains unclear. We hypothesized that tumor-associated factors are responsible for the activation and subsequent alteration of surface-binding proteins of neutrophils within the tumor microvasculature. 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 neutrophilic 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 (1.7- 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 patterns of CD11b and CD66 on resting neutrophils were equivalent to those of normal donor neutrophils, and functional analysis using fMLP-induced activation confirmed parallel degranulation responses. As expected, degranulated neutrophils from GBM patients demonstrated elevated expression of CD66, consistent with activation. However, degranulated neutrophils expressed paradoxically low levels of CD11b and high levels of L-selectin (0.6- and 1.3-fold, respectively, compared with normal cells). Because CD11b is required for strong intravascular adhesion and L-selectin is normally shed prior to neutrophil transmigration, these data suggest that peritumoral activation may result in degranulation without effective intratumoral infiltration. These results provide a preliminary explanation for the prevalence of degranulated neutrophils within the peripheral circulation of GBM patients. In addition, we propose that factors present within the tumor microvasculature induce important changes in the functional binding characteristics of these cells.

IR-12. ELK-1 REGULATES INTERFERON-ALPHA-8 EXPRESSION VIA A POLYMORPHIC REGION IN INTERFERON-ALPHA-8 PROMOTER ASSOCIATED WITH THE PROGNOSIS OF GLIOMA PATIENTS

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Several studies have demonstrated a significant immunological impact of single nucleotide polymorphisms (SNPs) in innate immune response-related genes, such as Toll-like receptors 3 and 4 (*TLR3* and *TLR4*). We recently reported that among patients with WHO grade 2 and 3 gliomas, those with the AA genotype of the rs12553612 SNP in the interferon-alpha-8 (IFNA8) promoter region had better overall survival than did those with the AC genotype. On the basis of these observations we hypothesized that compared with the C allele, the A allele in the IFNA8 promoter allows for enhanced transcription factor binding and expression levels of IFNA8. Analyses of THP-1 cells transfected with a luciferase gene downstream of a short sequence containing the SNP in the IFNA8 promoter demonstrated that the A allele results in enhanced promoter activity. *In silico* analysis suggested c-Krox and ELK-1 as likely transcription factors that bind to the IFNA8 polymorphic region. Co-transfection of plasmids encoding the c-Krox or ELK-1 and the luciferase constructs revealed that ELK-1 negatively regulated promoter activity with the A allele but not the C allele, whereas c-Krox did not affect activity regardless of the allele. Transfection of ELK-1 small interfering RNA into THP-1 cells consistently resulted in increased promoter activity. To further identify factors that contribute to enhanced A allele promoter activity, we assessed the effects of drugs that inhibit intracellular signaling pathways: PD98059 (which inhibits MEK/ERK), SB203580 (p38), and PPase-2B (small molecules). Use of any of one of the inhibitors resulted in decreased activity of the A allele promoter, indicating that multiple proteins or a protein common to multiple pathways is involved. Taken together, our data demonstrate that the A allele in the IFNA8 promoter, which is associated with better survival for glioma patients, allows for enhanced promoter activity that in turn is negatively regulated by ELK-1 and further regulated by multiple signaling pathways.

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.