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The limited capacity of malignant glioma-derived exosomes to suppress peripheral immune effectors

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ABSTRACT

Tumor-derived microvesicular exosomes permit intercellular communication both locally and systemically by delivering a snapshot of the tumor cell's constituents. We thus investigated whether exosomes mediate malignant glioma's facility for inducing peripheral immunosuppression. In Western blot and RT-PCR analyses, glioma-derived exosomes displayed exosome-specific markers, but failed to recapitulate the antigen-presentation machinery, surface co-modulatory signals, or immunosuppressive mediator status of their parent tumor cells. Treatment with glioma-derived exosomes promoted immunosuppressive HLA-DR^{low} monocytic phenotypes, but failed to induce monocytic PD-L1 expression or alter the activation of cytotoxic T-cells from patients' peripheral blood by FACS and RT-PCR analyses. Our results suggest that malignant glioma-derived exosomes are restricted in their capacity to directly prime peripheral immunosuppression.

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1. Introduction

Malignant gliomas, which universally portend a median survival less than 15 months, exhibit a tenacious propensity to subdue immune responses both locally and systemically (Stupp et al., 2005). In the local tumor environment, gliomas readily release anti-inflammatory cytokines, employ immunosuppressive surface ligands, and down-regulate their surface antigen-presentation machinery in order to successfully evade immune surveillance (Albesiano et al., 2010; Han et al., 2012; Waziri, 2010). Simultaneously, malignant gliomas secrete as of yet unidentified soluble factors that coax circulating monocytes and T lymphocytes into infiltrating the tumor as their anti-inflammatory phenotypes: M2 macrophages that up-regulate IL-10 and programmed death ligand 1 (PD-L1) and regulatory T lymphocytes, which together culminate in the apoptosis of anti-tumoral T lymphocytes (Bloch et al., 2013; Crane et al., 2012; Parsa et al., 2014; Zou et al., 1999).

There is growing evidence suggesting that gliomas can influence cells both nearby and beyond the blood–brain barrier via the carefully regulated excretion of exosomes: 40–100 nm microvesicles formed during the normal cellular membrane sorting cycle by the inward budding of late endosomes (Ostrowski et al., 2010). These multi-vesicular endosomes are typically destined for recycling by lysosomes, but occasionally fuse with the plasma membrane and thereby release their microvesicle contents into the extracellular milieu. Exosomes contain a representative profile of the proteins, mRNAs, and microRNAs of their parent T-cells; in glioblastoma (GBM) this exosomal content reflects the tumor's unique signature of EGFR amplification, EGFRvIII mutation, IDH1 mutation R132H, TGF-β, and/or podoplanin status (Al-Nedawi et al., 2008; Graner et al., 2009; Henriksen et al., 2014; Li et al., 2013; Manterola et al., 2014; Noerholm et al., 2012; Shao et al., 2012). GBM-derived exosomes also may beckon angiogenesis during hypoxia, by delivery of proteins (e.g. metalloproteinase 8, IL-8, and PDGF AA/AB) and mRNA transcripts (e.g. IGF-binding protein, BCL-2, and N-myc downstream regulator 1) to vascular endothelial cells and pericytes (Kucharzewska et al., 2013; Svensson et al., 2011). MiR-1 microRNA dysregulation in GBMs, in addition to unleashing the oncogenic JNK, MET, and EGFR signaling pathways, has also been shown to uncheck the high levels of Annexin A2 packaged in GBM-derived exosomes, thus driving more aggressive GBM growth, neovascularization, and invasion (Bronisz et al., 2014). Tumor-derived exosomes have also been shown to contribute to immunosuppression in a number of other tumor types; notably, exosomes distilled from the sera of

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patients with advanced melanoma or colon carcinoma not only hindered monocyte precursor differentiation into dendritic cells, but in fact programmed the precursors into myeloid suppressive cells characterized by down-regulation of surface MHC class II expression, persistent CD14 pattern recognition receptor positivity, and suppression of T lymphocytes by way of TGF-β secretion; whereas exosomes derived from normal control patients' sera encouraged myeloid maturation and T lymphocyte stimulation (Valenti et al., 2006; Zhang and Grizzle, 2011).

With the growing body of evidence implicating exosomes as an intercellular mediator of GBM pathophysiology, we hypothesized that they may comprise the unidentified soluble factors that exit the blood–brain barrier and reprogram distant immune effectors into their immunosuppressive phenotypes. In order to directly prime T lymphocytes responses, glioma-derived exosomes would require both surface antigen presentation machinery (including either MHC class I or class II molecules) and requisite co-modulatory signals. Alternatively, immune effectors can also be activated by the direct uptake of antigens bound to heat shock protein chaperones (HSP) released during cell necrosis (Bendz et al., 2007; Cho et al., 2009). Gliomas robustly express a number of constitutive and inducible HSPs, particularly HSP70 and HSP90 families, which chaperone the folding of several drivers of oncogenesis (Bloch et al., 2014; Graner et al., 2007; Yang et al., 2010). HSP70 has also been observed on the glioma cell surface, where it directly interacts with natural killer cells and γδ T lymphocytes (Wachstein et al., 2012; Zhang et al., 2005). HSP72 was identified on exosomes derived from a number of tumor types, which enabled the tumor-derived exosomes to trigger immunosuppressive Stat3 pathways in myeloid-derived cells (Chalmin et al., 2010). Herein we investigated whether glioma-derived exosomes have the capacity to promote immunosuppressive phenotypes of effectors from myeloid or lymphoid lineages.

2. Methods

2.1. Cell culture

Established glioma cell lines U87 and U251 were obtained through the UCSF Brain Tumor Research Center and cultured in Dulbecco's modified Eagle medium H21 (UCSF Cell Culture Facility). Primary GBM lines from two patients, denoted as GBM1 and GBM2, were derived from patients' fresh tumor tissue collected during initial resection and cultured for no more than 15 passages in RPMI-1640 media supplemented with 10 mmol/L non-essential amino acids. All glioma cultures were additionally supplemented with and 1% penicillin–streptomycin and 5% fetal bovine serum (FBS) depleted of exosomes by ultracentrifugation at 110,000 *g* at 4 °C for 4 h and careful collection of the resulting supernatant. Normal human astrocytes (NHA) were acquired from Sciencell Laboratories and cultured in Lonza proprietary media with exosome-depleted FBS.

2.2. Exosome isolation

Conditioned media were harvested after 48 h from cultures at 80–90% confluence and centrifuged at 600 *g* at 4 °C for 10 min to precipitate cell debris. The supernatant was then centrifuged at 16,500 *g* at 4 °C for 20 min and passed through a 0.22 μL filter (Milipore). The resulting supernatant was ultracentrifuged at 110,000 *g* at 4 °C for 1 h to pellet the exosomes, which were then resuspended in a phosphate-buffered saline (PBS) wash and ultracentrifuged again at 110,000 *g* at 4 °C for 1 h. In order to maximize detection of the effects of exosomes in our experiments, concentrations many magnitudes higher than found in circulation were used. Additionally, protein was precipitated from supernatant fractions of exosome-depleted conditioned media by using 20% trichloroacetic acid on ice, thrice washed with 1:1 acetone and ethanol, and pelleted by 16,500 *g* at 4 °C for 20 min. Exosome and protein pellets were resuspended in either PBS for cell treatment or lysis buffer for Western blot analysis.

2.3. Western blot analysis

Cells and exosomes were lysed by radioimmunoprecipitation assay buffer (RIPA; UCSF Cell Culture Facility) supplemented with protease inhibitors (Roche Diagnostics) on a 4 °C shaker for 45 min. Protein concentrations were determined by BCA Protein Assay Kit (Pierce) and loaded in equal amounts for polyacrylamide gel electrophoresis, with either SDS or non-reducing conditions as specified by the antibody manufacturer, then transferred to PVDF membranes for blocking and subsequent probing with primary antibodies against: CD81 (Santa Cruz Biotech), CD9 (Abcam), HSP70 (Santa Cruz Biotech), HSP90 (Abcam), GAPDH (Cell Signal), CTLA4 (Abcam), CD80 (Abcam), CD86 (Abcam), PD-L1 (Abcam), HLA-ABC (Biolegend), and HLA-DR (Biolegend). Secondary antibodies conjugated to horseradish peroxidase visualized the proteins by way chemilluminescence (ECL Western blotting substrate; Pierce).

2.4. Cell sorting

As previously described, peripheral blood leukocytes (PBL) were isolated from primary GBM patients' and healthy naïve donors' whole blood samples by Ficoll-Paque Plus (GE Healthcare) centrifugation.(Bloch et al., 2013; Crane et al., 2012) Monocytes were extracted by CD14 + selection using magnetic nanoparticles (EasySep, Stem Cell Technologies) and T lymphocytes by negative magnetic nanoparticle selection, then suspended in RPMI-1640 media supplemented with 1% penicillin–streptomycin, 1 mmol/L sodium pyruvate, 10 mmol/L nonessential amino acids, and 2.5% exosome-depleted FBS.

2.5. Treatment of cells

Monocytes were plated at 2×10^5 cells per well in a 24 well plate and incubated at 37 °C for 24 h with escalating doses of exosomes from established or primary glioma lines, as compared to positive controls treated with glioma conditioned media and untreated controls, in triplicate. T lymphocytes were plated at 3.1×10^5 per well in a 96 well plate and incubated at 37 °C for 24 h with Golgi plug protein transport inhibitor (BD Bioscience) and either exosomes from established or primary glioma lines, compared to treatment with NHA-derived exosomes or untreated controls.

2.6. Flow cytometry analysis

Monocytes were stained extracellularly with CD45 FITC (clone HI30, eBioscience), CD11b PeCy (clone ICRF44, eBioscience), HLA-DR APC (clone LN3, eBioscience), and PD-L1 PE (clone MIH1, eBioscience) or isotype control (eBioscience) and T lymphocytes extracellularly with CD3 PerCP (eBioscience), CD4 PE (eBioscience), and CD8 FITC (BD Pharmingen), and intracellularly with IFNγ APC (eBioscience) in saponin-containing buffer (PermWash, BD Pharmingen), all in PBS with 2% bovine serum albumin on ice for 30 min, washed, fixed with 2% paraformaldehyde, and read using a BD FACScaliber flow cytometer with CellQuest Software (Beckton Dickinson), as previously described (Bloch et al., 2013; Crane et al., 2012). PBLs were treated in triplicate. The gate strategy for identifying monocytes included forward vs side scatter gates for size, then CD45 vs CD11b gates for monocytic phenotype, with CD11b vs PDL-1 to assess PDL-1 expression levels, and CD11b vs HLA-DR to evaluate monocyte phenotype changes. CD8 + T-cells were identified by forward vs side scatter for size, and forward scatter vs CD3 to confirm T-lymphocytic phenotype, with CD8 vs CD4 for identifying CD8 + T-cells and CD8 vs IFNγ for assessing changes in CD8 + T-cell activation levels.