Biochemical and Biophysical Research Communications 453 (2014) 326–331

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inflammatory cytokines, interleukin-1 beta and tumor necrosis factor-alpha, upregulated in glioblastoma multiforme, raise the levels of CRYAB in exosomes secreted by U373 glioma cells

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ARTICLE INFO

Article history:
Received 15 September 2014
Available online 26 September 2014

Keywords:
IL-1β
TNF-α
GBM
Neuroinflammation
Cancer progression
CRYAB

ABSTRACT

In the brain, levels of inflammatory cytokines, interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), are elevated under traumatic brain injury, neuroinflammatory conditions and glioblastoma multiforme (GBM). In GBM, the levels of small heat shock protein, CRYAB (HspB5) are also reported to be elevated, where it has been shown to exert anti-apoptotic activity. Interestingly, CRYAB is secreted via exosomes by various cells. In order to understand the relation between inflammatory cytokines and CRYAB, U373 glioma cells, were stimulated with proinflammatory cytokines, IL-1β and TNF-α, and their effect on CRYAB levels in cells and secreted exosomes was studied. Our results show that U373 cells produce and secrete CRYAB via exosomes and that stimulation with IL-1β and TNF-α significantly increase the levels of CRYAB in not only the cells but also in the secreted exosomes. In addition, cytokine stimulation of U373 cells brings about changes in the secreted exosomal proteome, many of which are involved in cancer progression.

1. Introduction

Glioblastoma multiforme (GBM), a highly infiltrative and devastatingly aggressive astrocytoma is a common primary human brain cancer and is characterized by strong resistance to apoptosis by radiation and chemotherapeutic treatment regimens [1,2]. Studies have reported that levels of CRYAB, are elevated in many GBM specimens [3–6], and confers resistance to apoptosis by physically binding to and inhibiting the auto-proteolytic maturation and subsequent activity of caspase-3 [7,8].

Inflammation drives cancer progression [9–11]. Inflammatory cytokines like IL-1β and TNF-α (among others) play an important role in inflammation driven tumor growth and progression [12,13] and have been reported to be upregulated following radiation therapy in GBM patients [14–16]. While, IL-1β drives progression of neuroinflammation by upregulating expression of other pro-inflammatory cytokines [17], TNF-α induces expression of vascular endothelial growth factor (VEGF) in gliomas, leading to increased angiogenesis seen in GBM tumors [18].

Secreted exosomes are taken up by target cells [19], wherein the exosomal cargo may confer cytoprotective activity or may play a role in tumor invasion and progression [20,21]. Astrocytes secrete small discrete secretory vesicles called exosomes [22] and we show here that these secreted vesicles carry a distinct set of proteins, one, among which, is CRYAB.

Although inflammation is a hallmark characteristic in GBM tumors, and given their resistance to apoptosis, attributed to increased levels of CRYAB [7], there is no evidence, as of yet, for connection between increased levels of proinflammatory cytokines and CRYAB. The primary aim of our study was to determine the correlation, if any, between proinflammatory cytokines, IL-1β and TNF-α, and increased CRYAB levels observed in glioblastoma multiforme.

2. Methods and materials

2.1. Cell culture and reagents

Cell culture medium, phosphate buffered saline (PBS), penicillin/streptomycin were obtained from Invitrogen (CA), fetal bovine serum (FBS) from Atlas Biologicals Inc. (Fort Collins, CO), and the cytokines, IL-1β and TNF-α from Sigma. The human glioma cell line, U373, was a generous gift from Dr. Suraj Bhat, Jules Stein Eye Institute, UCLA.

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http://dx.doi.org/10.1016/j.bbrc.2014.09.068
0006-291X/

2.2. Cell culture and transfection

U373 cultures were maintained in complete EMEM media (Invitrogen CA) containing 10% FBS & 100 units/ml of penicillin/streptomycin (Invitrogen CA) at 37 °C in a 5% CO_2 incubator. Overnight cell cultures grown to 80% confluence were used for experiments. For cytokine studies, cells cultured overnight were washed 3× with PBS and fresh serum free EMEM media was added to the cultures along with either 10 ng/ml of IL-1β or 10 ng/ml of TNF-α, and then incubated for a further 18–24 h at 37 °C in a 5% CO_2 incubator. For control group, cells were cultured similarly, but without the cytokines.

2.3. Exosome isolation

Conditioned media from U373 cultures was collected and exosomes isolated by sequential centrifugation, all steps done at 4 °C. In brief, collected media was sequentially centrifuged at 600*g* for 10 min, 3000*g* for 10 min, 10,000*g* for 30 mins, discarding any pellets formed. The supernatant media from the final step was then ultracentrifuged at 150,000*g* for 3–4 h. The resulting pellet was washed with PBS and ultracentrifuged again at 150,000*g* for 1 h and finally resuspended in 25 μl PBS for analysis.

2.4. Electron microscopy

3 μl of exosome suspension was added onto 200 mesh formvar coated grids and allowed to dry at room temp. The grids were washed with water, stained with 1% uranyl acetate solution for 5 min. After staining, the grids were washed once in 70% ethanol followed by 4× washes with molecular grade water. These grids were then loaded onto the sample holder of the electron microscope (FEI Tecnai F20 200 keV microscope) and exposed to 80kv electron beam for capturing images.

2.5. Enzyme-linked immunosorbent assay (ELISA)

We followed an indirect ELISA protocol. In brief, dilutions of recombinant CRYAB (0.5–20 ng) in 50 μl carbonate buffer were coated into the wells of a PVC 96-well microtiter plate at 4 °C overnight. Similarly, a volume of exosome suspension corresponding to 10 μg total protein was incubated with equal volume of DI-water at 37 °C for 1 h. The resulting mixture was mixed with carbonate buffer to make up the volume to 50 μl and coated onto the wells at 4 °C overnight. The coating solution was then removed, wells washed 3× with PBS. The remaining protein-binding sites in the coated wells were blocked by adding 100 μl blocking buffer (1% BSA prepared in tris buffered saline (TBS) containing 0.01% tween-20) and incubated at room temperature for 2 h. The plate was washed 2× with PBS and incubated with 50 μl primary mouse-anti-CRYAB antibody (1:1000 in 1% BSA, Sigma Aldrich) for 2 h. The wells were washed 4× with PBS and 50 μl of goat-anti mouse HRP conjugated secondary antibody (1:10,000 in 1% BSA) was added and incubated at room temperature for 2 h. The plate was then washed 4× with PBS and 50 μl of TMB was added for developing color. The solution was incubated at room temperature for 15 min till the color developed and the reaction was stopped by adding 100 μl of 2 M H_2SO_4. The OD was read at 450 nm in a plate reader.

2.6. SDS–PAGE and Western blotting

Equal amounts of exosomal pellets and cell lysates, determined by BCA protein estimation, were boiled with 4× laemmli's buffer containing 10% beta-mercaptoethanol and run on a 12% SDS–PAGE denaturing gel. The proteins were transferred to PVDF membranes using conventional transfer apparatus. Blots were blocked with 5% BSA dissolved in TBS containing 0.01% tween-20 (TBS-t) for 1 h at room temp. Blots were probed with primary antibodies [anti-CRYAB monoclonal antibody (1:1000) (Santa Cruz Biotech, Dallas, TX), anti-CD63, anti-CD9 (1:1000) (SBI Biosciences, Mountain View, CA) or anti-β-actin (1:5000) (Cell Signaling Technology, Beverly, MA)] diluted in blocking buffer for 1 h at room temp. Blots were washed 3× with TBS-t and then incubated with HRP conjugated anti-mouse or anti-rabbit antibody (1:10,000) (Santa Cruz Biotech, Dallas, TX) diluted in blocking buffer at room temperature for 1 h. Blots were washed thoroughly 3× with TBS-t and developed with a chemiluminescence reagent – Luminaforte developing reagent from Millipore.

2.7. Mass spectrometry

LC-MS/MS methods – Orbitrap Velos: protein gel bands from SDS–PAGE gels were excised and subjected to in-gel trypsin digestion as follows. Gel slices were destained in 50% methanol (Fisher), 100 mM ammonium bicarbonate (Sigma–Aldrich), followed by reduction in 10 mM Tris[2-carboxyethyl]phosphine (Pierce) and alkylation in 50 mM iodoacetamide (Sigma–Aldrich). Gel slices were then dehydrated in acetonitrile (Fisher), followed by addition of 100 ng porcine sequencing grade modified trypsin (Promega) in 100 mM ammonium bicarbonate (Sigma–Aldrich) and incubation at 37 °C for 12–16 h. Peptide products were then acidified in 0.1% formic acid (Pierce). Tryptic peptides were separated by reverse phase Jupiter Proteo resin (Phenomenex) on a 100 × 0.075 mm column using a nanoAcquity UPLC system (Waters). Peptides were eluted using a 40 min gradient from 97:3 to 35:65 buffer A:B ratio. [Buffer A = 0.1% formic acid, 0.5% acetonitrile; buffer B = 0.1% formic acid, 75% acetonitrile.] Eluted peptides were ionized by electrospray (1.8 kV) followed by MS/MS analysis using collision induced dissociation on an LTQ Orbitrap Velos mass spectrometer (Thermo). MS data were acquired using the FTMS analyzer in profile mode at a resolution of 60,000 over a range of 375 to 1500 *m*/*z*. MS/MS data were acquired for the top 15 peaks from each MS scan using the ion trap analyzer in centroid mode and normal mass range with a normalized collision energy of 35.0. Proteins were identified by database search using Mascot (Matrix Science). The Mascot results were viewed and analyzed by Scaffold 4 Proteomics Software 4.3.2. A protein threshold of 95%, minimum of 2 peptides and a 50% peptide threshold values were used as the cut-off values and resulting data exported into excel spreadsheets.

2.8. Quantitative analysis of exosomal proteins

In order to determine whether a protein was differentially expressed between different exosome samples, a label-free approach based on spectral counting was used [23,24]. Spectral count is the number of tandem mass spectra assigned to a given protein in all bands from a single gel lane. To determine the relative amount of a protein in a given gel lane, a normalized spectral abundance factor (NSAF) was calculated [23]. The NSAF was calculated as follows: $(\mathrm{NSAF})_\kappa = \frac{(\frac{SpC}{MW})\kappa}{\sum_{i=1}^{n}(\frac{SpC}{MW})i}$ where κ is a given protein, *SpC* is the spectral count, *MW* is the protein's molecular weight, and *n* is all proteins identified in the gel lane. A list of proteins in accordance to NSAF values of $\geqslant 0.5$, were identified and listed for each of the exosome samples. Of these, proteins with a fold increase greater than 2 were considered to be significant. Proteins differing in levels were then analyzed by the online Panther software [25] by their assigned GO annotations and classified based on their functional processes.

2.9. Statistical analyses

Unpaired, two-tailed Student's *t*-test was used to calculate the significance between CRYAB levels in cell lysates and exosomes. To determine statistical significance in quantification of CRYAB in cell lysates and in exosomes, significance was calculated using GraphPad Prism's one way ANOVA followed by Bonferroni's multiple comparison test. For each group, the average of three independent experiments was determined and results were expressed as mean ± SD. Value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Characterization of exosomes secreted by U373 cells

Electron microscopy analysis was used to confirm that the exosomal pellets isolated from U373 cultures were indeed discrete vesicles and not parts of damaged cellular membranes. TEM (80 keV) micropictographs of exosome pellets showed vesicles with a mean diameter of ~80 nm (Fig. 1A). Using Western blots to probe for exosomal markers CD9 & CD63, a family of proteins called the tetraspanins are found particularly enriched in the membranes of the intraluminal vesicles (ILVs) as opposed to the limiting membranes of the multivesicular bodies (MVBs) and hence considered to be excellent exosomal markers, showed that the exosomes were particularly enriched in CD9 (Fig. 1B) and CD63 (Fig. 1C) compared to the U373 cell lysates. Our above results indicate that the pellets isolated from conditioned media of U373 cultures by sequential centrifugation constitute a distinct set of secretory vesicles.

3.2. Proteomic analysis of exosomal pellet

Equal quantities of total exosomal protein extracts of exosomes isolated from untreated and cytokine treated (IL-1β & TNF-α) U373 cells was determined by mass spectrometry and analyzed by the scaffold 4 software. MS analysis of U373 secreted exosomes identified a total of around 961 different proteins (NSAF > 0.5) among which 108 were detected exclusively in exosomes (unpublished data). A further 853 proteins were found to be common to both exosomes and U373 cells lysates, while 1113 proteins were detected only in the cell lysates (Fig. 2A). Exosomal markers, CD9 & CD63, were detected in the exosome preparations. Absence of Calnexin and GM130, markers for endoplasmic reticulum and golgi, respt., (data not shown), confirmed that exosome preparations were not contaminated with cellular debris. We also identified CRYAB as one of the proteins secreted in exosomes. A total of 5 exclusively unique peptides with 5 exclusive unique spectra (Fig. 2B) were identified, which covered 25% of the CRYAB sequence (43/175 amino acids), strongly confirming its identification.

3.3. αB-crystallin is secreted by U373 cells into culture media via exosomes

To validate the MS detection of CRYAB in exosomes, equivalent quantities of total exosomal proteins and total U373 cell lysate proteins were analyzed by Western blot. Antibody detected CRYAB in exosomes secreted by U373 cells (Fig. 2C). The exosomes were particularly enriched with CD63, an exosomal marker. Employing a modified indirect ELISA technique, we determined the concentration of CRYAB to be 7.3 ± 0.7 ng in exosomes derived from $1 * 10^6$ cells. Quantification of equivalent quantities of cell lysates and exosomes, determined by BCA protein assay, showed that levels of CRYAB were found to be significantly higher in the exosomes than in the cell lysates (Fig. 2D).

3.4. Effect of inflammatory cytokines on CRYAB levels in U373 cells

IL-1β and TNF-α, pro-inflammatory cytokines in primary GBM tumors, also found elevated in response to radiation exposure [16], are known to play an important role in progression of neuroinflammation, inflammation driven tumor growth and neuronal cell death. It has also been shown that CRYAB levels are increased in the CNS under various stress and inflammatory conditions. To determine the relation between pro-inflammatory cytokines (IL-1β & TNF-α) and CRYAB levels, we stimulated the U373 cells with these cytokines. Western blots results showed that cells treated (Fig. 3A) with either 10 ng/ml of IL-1β or 10 ng/ml of TNF-α overnight (18–20 h), significantly increased the levels of CRYAB (Fig. 3B). We then isolated exosomes from these cytokine stimulated cells and analyzed their CRYAB levels. Our analysis of immunoblots showed that stimulation of U373 cells with the inflammatory cytokines IL-1β and TNF-α significantly increased the quantity of αB-crystallin secreted in exosomes (Fig. 3B & C),

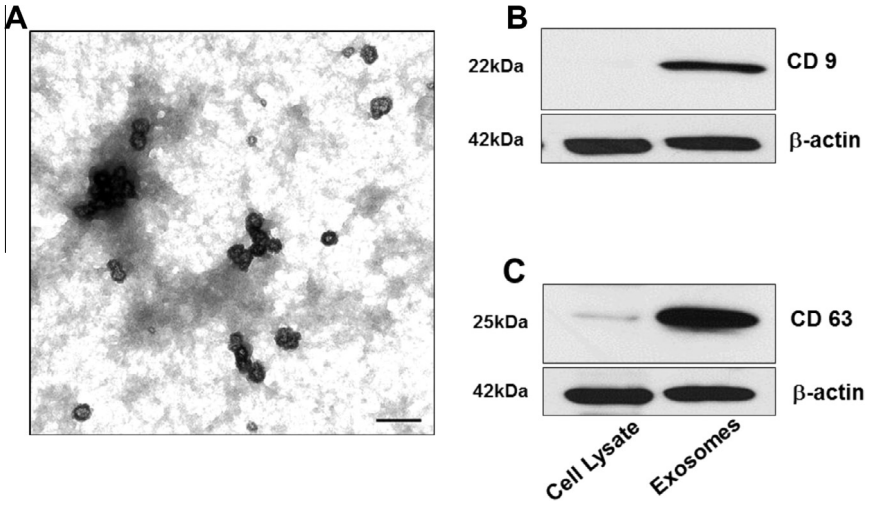
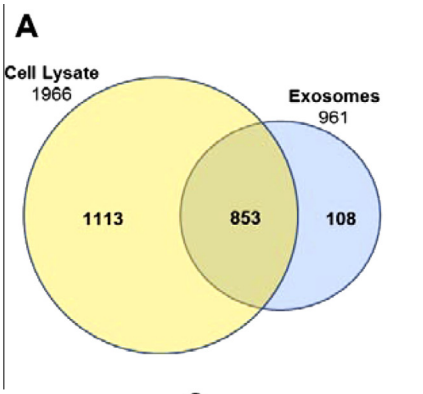


Fig. 1. Characterization of U373 derived exosomes. (A) TEM of exosomes isolated from media of U373 cells: EM showed a uniform set of distinct vesicles with an average diameter of ~80 nm, consistent with values (30–100 nm) described for exosomes. (*Scale bar: 200 nm*). Western blot analysis of equivalent amounts U373 lysates and exosomes showed enrichment of CD9 (B) and CD63 (C), tetraspanin molecules used as exosomal marker proteins, in U373 derived exosomes compared to total cell lysates.



B

P02511 (100%), 20,159.5Da: Alpha-crystallin B chain OS=Homo sapiens 5 exclusive unique peptides, 5 exclusive unique spectra, 5 total spectra, 43/175 amino acids (25% coverage)			
Sequence	**Observed m/z**	**Actual Mass**	**Residue #**
(R)APSWFDTGLSEMR(L)	748.8463	1,495.68	57-69
(K)HFSPEELK(V)	493.7505	985.4864	83-90
(K)HFSPEELKVK(V)	405.2238	1,212.65	83-92
(R)QDEHGFISR(E)	544.7596	1,087.50	108-116
(R)EEKPAVTAAPK(-)	570.8168	1,139.62	164-174

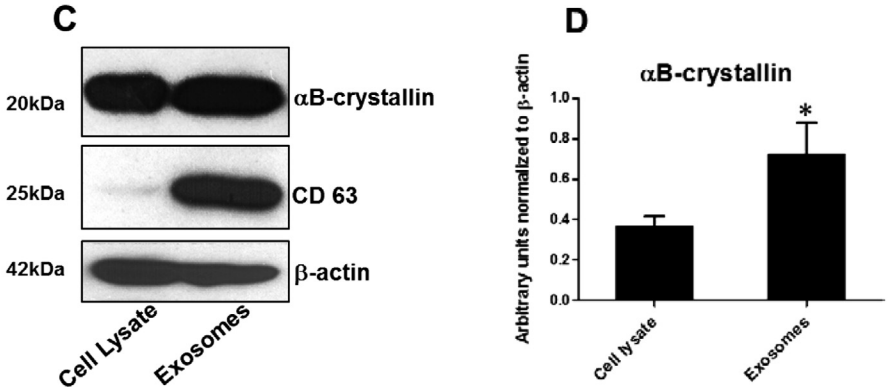


Fig. 2. CRYAB in exosomes & U373 cells. (A) Venn diagram showing proteins present in U373 cells and their secreted exosomes. The MS analysis identified 961 proteins in exosomes and 1966 proteins in U373 cells. Further MS analysis showed a set of 108 distinct proteins detected only in exosomes. (B) A total of 5 unique peptides with a total of 43 amino acids of CRYAB were identified by MS analysis. Among these, one fragment was from the N-terminal domain, 2 were from the core alpha-crystallin domain and two were from the C-terminal domain. (C) Western blot analysis of exosomes isolated from serum free, conditioned media of U373 cells detected presence of CRYAB. CD63, a highly enriched tetraspanin molecule in exosomes was used as an exosomal marker protein. (D) Quantification of the Western blots of equivalent amounts of U373 cell lysate and isolated exosomes showed presence of significantly higher levels of CRYAB in exosomes over cell lysates. β-actin was used as loading control. Error bars, SD ($p < 0.05$).

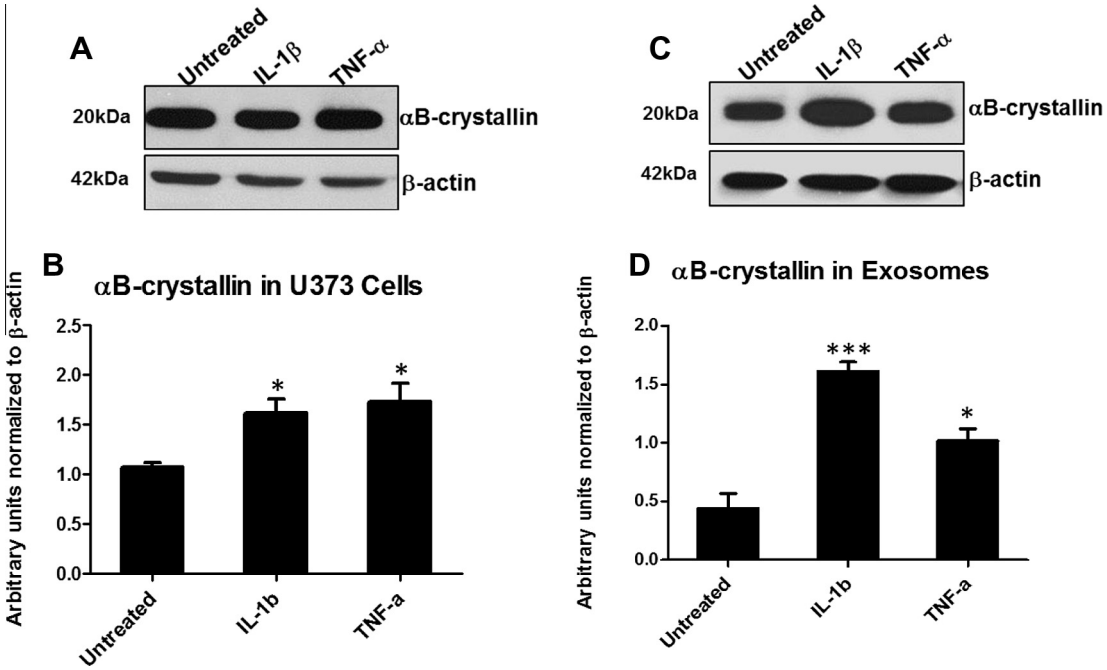


Fig. 3. Inflammatory cytokines, IL-1β and TNF-α, significantly increase levels of CRYAB in U373 cells and also its secretion of via exosomes. (A) Exposure of U373 cells to inflammatory cytokines, IL-1β and TNF-α, for 24 h showed a significant increase (B) in CRYAB levels compared to untreated cells. β-actin was used as loading control. Error bars, SD ($^*p < 0.05$). Similarly exosomes isolated from U373 cells exposed to inflammatory cytokines, IL-1β or TNF-α, were analyzed by Western blot (C) for CRYAB levels. (D) Quantification of the Western blots showed a significant increase in levels of secreted CRYAB in exosomes isolated from IL-1β and TNF-α treated cells. β-actin was used as loading control. Error bars, SD ($p < 0.0005$).

reflecting its effect on the protein levels observed in the cell lysates (Fig. 3A & B).

3.5. Pro-inflammatory cytokine induced changes in exosomal proteome

Having determined that CRYAB levels in cells and in their secreted exosomes were increased upon cytokine stimulation, we wanted to study overall changes in the proteome of exosomes secreted by cytokine stimulated cells. Elaborating on previously described MS analysis, we identified 942 proteins in exosomes derived from IL-1β treated cells and 1044 proteins in exosomes isolated from TNF-α treated U373 cells, apart from the earlier reported 961 proteins identified in exosomes from untreated cells (Fig. 4A, unpublished data). Among these, 707 proteins were common to all the three groups, 79 proteins were detected exclusively in exosomes secreted by IL-1β stimulated cells and 152 were found exclusively in exosomes from TNF-α treated cells. Since we had examined the levels of CRYAB following cytokine stimulation, we concentrated our efforts, on identifying and listing only on those proteins, with a fold change of 2 or greater (based on NSAF analysis), secreted in exosomes derived from cytokine stimulated cells (Supplementary data). The identified proteins were annotated with Gene Ontology (GO) functions based on the NCBI database and classified based on their biological processes by the web based Panther classification database [25] (Fig. 4B).

In exosomes secreted by cytokine stimulated cells these proteins (⩾2-fold or greater levels), were found to be involved, among others, in biological adhesion, regulation, developmental processes, response to stimulus & localization (Supplementary data), suggesting a role in tumor progression and invasiveness.

4. Discussion

Although some studies on GBM secretome [26–29] (which includes shedded microvesicles, apoptotic bodies and other secretory vesicles) have been previously reported, our study focuses solely on the effects of proinflammatory cytokines on the protein cargo of discrete secretory vesicles – exosomes. Exosomes differ from other secretory vesicles in that they are endosomal in origin and result due to release of ILVs into extracellular milieu by fusion of MVBs with the plasma membrane [30,31]. Exosomes, constituting a novel mode of intercellular communication, deliver not only proteins but also mRNA and miRNA across biological membranes [32,33]. The intercellular trafficking of such cargo, which differs depending on the microenvironment of the cells secreting them, can either be beneficial in exerting cytoprotective actions [21,34] or may promote disease development [35,36], tumor invasiveness and progression [20,37–40].

We report here that astrocytes, secrete CRYAB via exosomes. Our studies provide a direct link between inflammatory cytokines-IL-1β and TNF-α, seen in GBM specimens and CRYAB levels

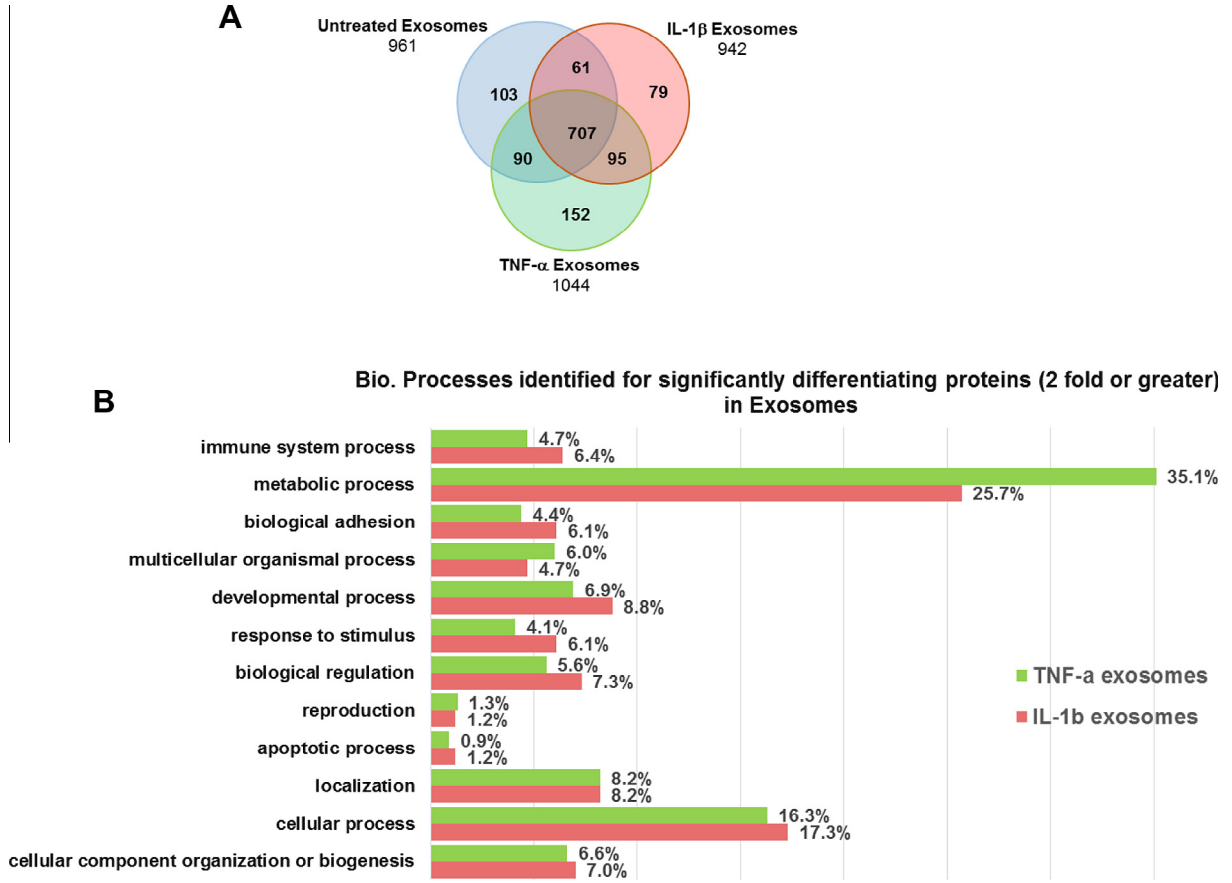


Fig. 4. Classification of exosomal proteins secreted by cytokine treated U373 cells. (A) Venn diagram of MS analysis comparing total number of proteins in exosomes derived from U373 cells untreated vs cytokine (IL-1β or TNF-α) treated U373 cells. The MS analysis identified 961 proteins in exosomes from untreated cells, 942 proteins in exosomes derived from IL-1β treated cells while 1044 proteins were identified in exosomes isolated from TNF-α treated U373 cells. 707 proteins were common to all the three groups. (B) Bar graph depicting biological processes for significantly differentiating exosomal proteins (⩾2-fold, cytokine treated vs untreated) secreted by cytokine treated U373 cells.

and that these cytokines increase levels of CRYAB in astrocytes. Increase in CRYAB levels in GBM coupled with its secretion via exosomes points to an important mode of intercellular communication which, in GBM, may confer resistance to apoptosis in surrounding cells following radiation and chemo-therapies.

Proinflammatory cytokines also bring about profound changes in the proteome of the exosome. Our preliminary analysis of MS data shows that following cytokine stimulation of U373 cells, levels of several proteins secreted via exosome are increased two fold or more. It may thus be suggested that increases in cytokine levels in GBM patient brains, either due to disease or radiation, leads to quantitative changes in the exosomal proteome. A detailed Gene Ontology analysis of these proteins with elevated levels reveals their roles in pathways which promote progression of inflammation, tumor invasiveness, angiogenesis and tumor progression, which in part explains GBM aggressiveness and resistance to apoptosis with anti-cancer therapies.

Acknowledgments

This study was supported by NIH/NEI Grant R01EY011352-17. The authors would like to acknowledge Jeffrey A. Kamykowski and UAMS Digital Microscopy Core for the electron microscopy images, and the UAMS Proteomics Core supported by the Arkansas IDeA Network for Biomedical Research Excellence (P20GM103429), the UA Center for Protein Structure and Function (P30GM103450), and the UAMS Center for Microbial Pathogenesis and Host Inflammatory Responses (P20GM103625) for proteomics studies. The authors would also like to thank Dr. Samuel G. Mackintosh, Ms. Linley Moreland and Dr. Alan J. Tackett for their help.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.068.

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