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Proteome characterization of small extracellular vesicles from spared nerve injury model of neuropathic pain

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Abstract

Exosomes are 30-150 nm extracellular vesicles mediating intercellular communication. Disease states can alter exosome composition affecting the message carried and thereby, its functional impact. The objective of this study was to identify proteins present in these vesicles in a mouse model of neuropathic pain induced by spared nerve injury (SNI). Small extracellular vesicles (sEVs) were purified from serum four weeks after SNI surgery and the protein composition was determined using tandem mass spectrometry and cytokine array. Proteomic analysis detected 274 gene products within sEVs. Of these, 24 were unique to SNI model, 100 to sham surgery control and five to naïve control samples. In addition to commonly expressed sEVs proteins, multiple members of serpin and complement family were detected in sEVs. Cytokine profiling using a membrane-based antibody array showed significant upregulation of complement component 5a (C5a) and Intercellular Adhesion Molecule 1 (ICAM-1) in sEVs from SNI model compared to sham control. We observed a differential distribution of C5a and ICAM-1 within sEVs and serum between sham and SNI, indicating changes from local or paracrine to long distance signaling under neuropathic pain. Our studies suggest critical roles for cargo sorting of vesicular proteins in mediating signaling mechanisms underlying neuropathic pain.

1. Introduction

Neuropathic pain is initiated by nerve injury or dysfunction and can result in allodynia (pain from a non-painful stimulus) and hyperalgesia (heightened sensitivity to pain). Unlike acute

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pain, where pain ceases after the damaged nerves or tissue heal, neuropathic pain can often be chronic. The differences between acute and chronic pain indicate that pain is not generated by a hardwired system, but rather results from the engagement of highly plastic molecules and circuits [1].

Exosomes are 30-150 nm extracellular vesicles (EVs) that can transport mRNAs, miRNAs, proteins, and lipid mediators to recipient cells via circulation [2]. Cells use these vesicles to communicate with both adjacent and distant cells. Their molecular composition reflects their origin in endosomes as intraluminal vesicles. In addition to a common set of membrane and cytosolic molecules, exosomes harbor unique subsets of proteins linked to cell type-associated functions [3]. Exosome secretion of proteins and RNAs has been proposed to be a fundamental mode of communication in the nervous system, supplementing the known mechanisms of anterograde and retrograde signaling across synapses [4, 5].

Exosome contents vary depending on the cellular homeostasis of the donor cells. However, not everything that is present in the parent cell is incorporated into the exosomes, suggesting that this well-regulated process is dynamically altered by signaling events. Thus, exosomes do not contain a random set of proteins, as would be the case for cell debris. Vesicular proteomes provide information on the nature of EVs and help decode the molecular mechanisms involved in vesicular cargo sorting and biogenesis as well as their diverse physiological and pathological functions [6]. Proteomic analyses of mammalian EVs have allowed several thousand vesicular proteins to be cataloged [7, 8], including cytosolic, nuclear, endosomal, and membrane proteins [9], but the role of many are unclear.

Disease states can alter exosome composition but the role of exosomes in circulation under chronic neuropathic pain is not well understood. As a first step in understanding the role of exosomes in neuropathic pain, we undertook a proteomics approach to determine the protein composition of exosomes purified from mouse serum four weeks after spared nerve injury (SNI). EVs constitute all particles with a lipid bilayer that are naturally released from the cell including exosomes and microvesicles [10]. Exosomes are 30-150 nm and released by cells upon fusion of multivesicular bodies with the plasma membrane. Microvesicles or ectosomes are 100-1,000 nm vesicles assembled at and released from the plasma membrane [11]. The International Society for Extracellular Vesicles has recommended the term small EVs (sEVs) for particles < 200 nm instead of specifying them as exosomes. This is because exosome purification will also result in the inclusion of microvesicles of comparable sizes and cannot be separated due to the lack of distinguishing markers [10]. Since exosomes used in our studies described here can include similar sized microvesicles, we will use the term sEVs. The proteome signature of sEVs from mouse SNI model can help in postulating potential roles for these vesicles released under chronic neuropathic pain.

2. Methods

2.1. Generation of spared nerve injury (SNI) model

All the studies were performed using eight-week-old male C57BL/6 mice (Jackson laboratory, Bar Harbor, MN). Experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Animal Care and

Use Committee. Animals were kept in a standard temperature and humidity-controlled room on a 12 h light/dark cycle. Mice had access to food and water ad libitum and habituated in the testing room two to three days before experiments. SNI surgery was performed as previously described [12]. Mice were anesthetized under 2-2.5% isoflurane; following incision through skin and muscle, the sural, common peroneal and tibial branches of the sciatic nerve of the left hind paw were exposed. A silk suture was used to ligate the common peroneal and tibial branches and 2-4mm of the distal ends were removed. The sural nerve was left intact. The surgical procedure in sham mice was identical to that performed on the SNI group, but without ligature and section. Mechanical sensitivity was measured using a series of von Frey filaments (North Coast Medical, Inc., San Jose, CA) and Dixon's up-down method [13] before and at different time points after surgery. Blood samples were collected at sacrifice four weeks after surgery.

2.2. sEVs isolation from serum

Whole blood was collected in serum collection tubes without coagulant (Grenier Bio-One, Monroe, NC) and incubated at room temperature for 30 min for clot formation. Approximately 500 μ l blood was obtained from each mice. Tubes were then centrifuged at 4°C at 2000 $\times g$ for 10 min. Serum was collected immediately by gently pipetting cleared supernatant into a fresh Eppendorf tube. Samples were stored at -80°C until further use. Isolation procedure for sEVs was performed as described previously [14, 15]. Briefly serum was diluted 1:1 with 1 $\times$ PBS (-) Mg²⁺ (-) Ca²⁺ (Corning; Corning, NY) and spun at 2000 $\times g$ for 30 min at 4°C. The supernatant was transferred to a polycarbonate centrifuge tube and spun at 12,000 $\times g$ for 45 min at 4°C. Samples were passed through a 0.22- μ m filter, collected in fresh polycarbonate ultracentrifuge tubes and spun at 110,000 $\times g$ for 2 h at 4°C. Following sEV pellet formation the supernatant was retained as sEV-deplete serum. The pellet containing sEVs was resuspended in 1 $\times$ PBS (-) Mg²⁺ (-) Ca²⁺ and spun for an additional 70 min at 110,000 $\times g$ at 4°C. The resulting pellet was resuspended gently in 50 to 200 μ l PBS and stored at -80°C.

2.3. Preparation of sEVs for Transmission electron microscopy (TEM)

This was performed as described previously with some modifications [16]. Briefly, 300-mesh carbon-coated polyvinyl formvar copper grids (Electron Microscopy Sciences, Hatfield, PA) were placed on droplets of purified sEVs resuspended in 2% paraformaldehyde (Electron Microscopy Sciences). The grids were washed with 0.1 M Sorensen's phosphate buffer (pH7.2; Electron Microscopy Sciences) and post-fixed in 1% glutaraldehyde (Electron Microscopy Sciences) and left to adsorb for 30 min. After excess buffer was removed, dry grids were washed with deionized water and stained with 1% aqueous uranyl acetate (Electron Microscopy Sciences) and embedded in 0.2% uranyl acetate-methylcellulose solution before TEM analysis. For immunolabeling, sEVs were resuspended in 2% paraformaldehyde and droplets were left to adsorb on 300-mesh carbon-coated formvar nickel grids for 20 min. After two washes in 0.1 M Sorensen's phosphate buffer (0.1 M PB) and four washes in 50 mM glycine in 0.1 M PB, grids were incubated with blocking buffer (5% BSA in 0.1 M PB) for 10 min. The grids were immunolabeled with goat anti-CD9 antibody (1:100; Sigma) in 1% BSA in 0.1 M PB for 30 min at room temperature. The unbound antibody was removed with six washes of 0.1% BSA in PBS

before grids were incubated with 10 nm gold-labeled anti-goat IgG antibody (1:25; EMS) for 20 min at room temperature. After the unbound antibody was removed with six washes in 0.1% BSA in 0.1M PB, grids were incubated in 1% glutaraldehyde for 5 min, washed with deionized water, stained with 1% uranyl acetate and embedded as above.

2.4. sEV number and size

The size, distribution, and concentration of purified sEVs were determined using a ZetaView nanoparticle tracking analysis system (Particle Metrix GmbH, Meerbusch, Germany) as described previously [17]. The corresponding ZetaView software (8.03.04.01) was used for data analysis. Nanosphere size standard 100 nm (Thermo Scientific) was used to calibrate the instrument prior to sample readings. Instrument pre-acquisition parameters were set to 23°C, a sensitivity of 65, a frame rate of 30 frames per second (fps), a shutter speed of 100, and laser pulse duration equal to that of shutter duration. Post-acquisition parameters were set to a minimum brightness of 25, a maximum size of 200 pixels, and a minimum size of 10 pixels. For each sample particle count were measured at five positions, with two cycles of reading per position. The cell was washed with PBS after every sample.

2.5. Protein Denaturation, Digestion and Desalting

Proteomics studies were performed twice, once using pooled samples from 6 mice each for the SNI, sham and naïve control group; a second study was performed using sEVs from individual mice from all three groups (n=4/group).

To determine the protein composition of sEVs, protein extracted was digested with sequencing-grade trypsin and analyzed by UPLC-MS/MS. Liquid chromatography (LC) was performed on an Easy-nLC 1200 UPLC system (Thermo Fisher Scientific, Waltham, MA) with a heated Easy-Spray column of 25 cm length. The LC was interfaced to a Q-Exactive HF-X quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) via nano-electrospray ionization. Mass spectrometry was performed by unbiased, data-dependent acquisition.

sEVs were prepared for digestion using the filter-assisted sample preparation (FASP) method [18]. Briefly, the samples were suspended in 5% SDC, 100 mM TEAB, pH 8.0, 3 mM DTT, sonicated briefly, and incubated in a Thermo-Mixer at 60 C, 1000 RPM for 20 min. Samples were centrifuged to clarify and the supernatant was transferred to a 30 kD MWCO device (Millipore) and centrifuged at 13 k g for 30 min. The remaining sample was buffer exchanged with 1% SDC, 100 mM TEAB, pH 8.0, then alkylated with 15 mM iodoacetamide. Samples were digested using trypsin at an enzyme to substrate ratio of 1:100, overnight, at 37 C in a Thermo-Mixer at 1000 RPM. Digested peptides were collected by centrifugation. A portion of the digested peptides, about 10 micrograms, were desalted using reversed phase (Empore SDB-RPS, 3M) stop-and-go extraction (STAGE) tips [19]. Peptides were eluted with 80% acetonitrile, 5% ammonium hydroxide and lyophilized in a SpeedVac (Thermo Savant) to remove volatile components, approximately 1 h.

2.6. Liquid Chromatography-Tandem Mass Spectrometry

Each digestion mixture was analyzed by UHPLC-MS/MS. LC was performed on a Easy-nLC 1000 UHPLC system (Thermo). Mobile phase A was 99.9% MilliQ water, 0.1% formic acid. Mobile phase B was 80% acetonitrile, 0.1% formic acid. The 90 min LC gradient ran from 0% B to 35% B over 70 min, then to 80% B for the remaining 20 min. Samples were loaded directly to the column. The column was 50 cm x 75 μ m I.D. and packed with 2 micron C18 media (Thermo Easy Spray PepMap). The LC was interfaced to a quadrupole-Orbitrap mass spectrometer (Q-Exactive, Thermo Fisher) via nano-electrospray ionization using a source with an integrated column heater (Thermo Easy Spray source). The column was heated to 50 C. An electrospray voltage of 2.2 kV was applied. The mass spectrometer was programmed to acquire, by data-dependent acquisition, tandem mass spectra from the top 20 ions in the full scan from 400 - 1200 m/z. Dynamic exclusion was set to 15s, singly-charged ions were excluded, isolation width was set to 1.6 Da, full MS resolution to 70,000 and MS/MS resolution to 17,500. Normalized collision energy was set to 25, automatic gain control to 2e5, max fill MS to 20 ms, max fill MS/MS to 60 ms and the underfill ratio to 0.1%.

2.7. Data Processing and Library Searching

Raw tandem mass spectral data obtained from the mass spectrometer were converted to mzML format using msconvert [20]. MGF files were generated from mzML using the Peak Picker HiRes tool, part of the OpenMS framework [21]. All searches were performed on Amazon Web Services-based cluster compute instances using the Proteome Cluster interface. All searches required 10 ppm precursor mass tolerance, 0.02 Da fragment mass tolerance, strict tryptic cleavage, up to 2 missed cleavages, fixed modification of cysteine alkylation, variable modification of methionine oxidation and protein-level expectation value scores of 0.0001 or lower. Mouse sequence library was obtained from the UniProtKB distribution [22] (release version 2018_09). Raw tandem mass spectral data obtained from the mass spectrometer were searched against the protein sequence libraries using X!Tandem [23] and OMSSA [24]. XML output files were parsed and non-redundant protein sets determined using Proteome Cluster based on previously published rules [25]. MS1-based isotopic features were detected and peptide peak areas calculated using the FeatureFinderCentroid tool, part of the OpenMS framework [21]. Identified proteins were required to have one or more unique peptides across the analyzed samples with an expectation value of 0.001 or less. The intensity, E-value, number of unique peptides, coverage, and spectral counts for each identified protein in each sample is reported in the supplementary file proteomicsdata.xlsx. Data for each sample was sum-normalized, where the intensity value of each protein was divided by the sum of the intensity values of all proteins in that sample.

Hierarchical clustering and principal component analysis are employed on the intensity values of the proteins to identify outlier samples. The sample Naive2 was determined to be an outlier based on being more than three scaled median absolute deviations from the median value in the first principal component; and removed from further analysis. In order to reduce redundancy in protein labels as well as to facilitate literature comparisons, protein products from the same gene are grouped together. Gene sets unique to each experimental

group were enriched for Gene Ontology biological processes using DAVID Bioinformatics Resources v6.8 [26].

2.8. Western blotting and cytokine array

sEVs were resuspended in radioimmunoprecipitation assay buffer (RIPA; Thermo Fisher Scientific) containing Halt protease inhibitor cocktail (Thermo Fisher Scientific) and the protein concentration was determined by Lowry assay (DC Protein Assay; Bio-Rad, Hercules, CA). Protein lysates were loaded and separated on a 10% SDS-PAGE (Novex/Life Technologies) for 40 minutes at 225 V. For western blotting, after transfer, the PVDF membrane was blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline and 0.1% Tween-20 (TBS-T) for 1 h, incubated with mouse anti-HSP70 (1:500; Santa Cruz Biotechnology, Dallas, TX) or rabbit anti-TSG101 (1:500; Genetex, Irvine, CA) overnight at 4°C. Primary antibodies were probed with goat anti-mouse IgG-HRP or donkey anti-rabbit IgG-HRP conjugated secondary antibodies (1:2500; Abcam). Proteins were detected using western HRP substrate (Immobilon Forte, Thermo Scientific) and blots were analyzed by enhanced chemiluminescence using the FluorChem M system (ProteinSimple, Santa Clara, CA). For cytokine array, the Mouse Cytokine Panel A Proteome Profiler arrays (R&D Systems, Minneapolis, MN) were used. A total of 100 µg of protein (25 µg/mice) were used for both serum and sEV samples and experiments were performed according to manufacturer's specifications. The arrays were scanned, and images acquired using FluorChem M System (ProteinSimple) and quantified using ImageJ software (NIH, Bethesda, MD).

2.9. Statistics

Statistical analyses were performed using Graph Pad Prism 7.0 (Graph Pad Software). All data are presented as averages $\pm$ SEM or $\pm$ SD (cytokine array) and were analyzed using one-way ANOVA followed by Tukey's multiple comparison test or repeated measures two-way ANOVA followed by Bonferroni's post hoc test for behavioral data. Differences between averages were considered statistically significant when $p < 0.05$.

3. Results

3.1. Confirmation of mechanical hypersensitivity after SNI surgery

Baseline measurements were performed before surgery and mechanical allodynia was determined using von Frey filaments. We observed a significant reduction in paw withdrawal threshold after surgery compared to sham-operated animals (Fig. 1A) indicating robust mechanical allodynia. There was an initial reduction in threshold in the ipsilateral paws of sham-operated mice that can be related to surgical inflammation, which quickly returned to baseline by day 2. There was no significant difference in contralateral paw withdrawal thresholds between SNI and sham-operated mice (Fig. 1B). These results indicate that the nerve injury induced mechanical allodynia confirming neuropathic pain, in these animals.

3.2. sEV characterization

We used TEM along with immunogold labeling to examine the specificity and morphology of sEVs purified from mouse serum. sEVs maintain a vesicular morphology with an

approximate diameter of 100 nm and show immunoreactivity for CD9, a tetraspanin protein found in sEVs and sEV membranes (Fig. 2A and 2B). Specificity of sEVs was further confirmed by western blot for the presence of HSP70 and TSG101 (tumor susceptibility gene). All sEV protein lysates showed specificity for HSP70 and TSG101 (Fig. 2C).

3.3. sEV number and size

Nanoparticle tracking analysis showed that the average size of mouse serum derived sEVs to be 135 nm and an average concentration of 3.08×10^8 sEVs/ml (Fig. 3). We did not observe a significant difference in the number or size of sEVs present in the serum from the SNI, sham and naïve control mice.

3.4. Proteomics studies using mass spectrometric analyses of sEVs

We detected a total of 274 genes (533 protein species) in all samples. There was a low correlation in the detected protein profiles between the pooled samples and the individual samples within the same experimental group (average Pearson correlation 0.10). The individual mice samples displayed a greater within-group consistency (average Pearson correlation 0.36) [27]. The low correlation between pooled and individual samples of the same experimental group was not due to the presence of 115 genes (217 protein species) that had only one distinct peptide identified; as removal of these genes did not increase the correlation between individual and pooled samples (average Pearson correlation 0.05) while the within-group correlation remained unchanged (average Pearson correlation 0.37). We attribute the low correlation between individual and pooled samples to a possible batch effect in mice and sample handling. In order to reconcile the variability among the samples, we consider a protein as expressed in an experimental group if it is observed in at least one sample (pooled or individual) from that experimental group.

The Venn diagram (Fig. 4) shows the distribution of the detected proteins across sets of experimental groups. Proteins from 82 genes were detected to be common to all conditions. The relative abundance of proteins common to all conditions was significantly higher than the abundance of proteins unique to one or two experimental groups (p-value = 0.01). Proteins expressed uniquely in samples of one experimental group included: 24 genes in the SNI model, 100 genes in sham, and five genes in naïve samples. Proteins unique to the sham samples included H2a histone family genes, which were significantly enriched in chromatin silencing, negative regulation of cell proliferation, and complement activation (enrichment p-value = 0.01). Of particular interest to this study were proteins exclusively detected in the SNI model. These proteins are listed in Table 1 and included dynein light chain 1, tubulin beta family of proteins, desmoplakin, junction plakoglobin, and plakophilin 1; which are enriched for cell-cell adhesion and intermediate filament assembly processes (enrichment p-value = 0.01). The relative abundance of proteins common to all conditions was significantly higher than the abundance of proteins unique to one or two experimental groups (p-value 1.3×10^{-4}). Similarly, relative abundance of proteins common to two experimental groups was significantly higher than the abundance of proteins unique to one experimental group (p-value 1.9×10^{-13}).

While the cellular origins of the sEVs harvested from the whole blood cannot be accurately identified or quantified, it is worthwhile to compare the protein composition of the sEVs with the protein profiles observed in different tissues. We compared our findings with PainProteome database [28], which provides a comprehensive catalogue of proteins enriched in peripheral nervous system (PNS) and spinal cord (SC). PainProteome also provides a list of proteins that were found to be significantly differentially expressed in an SNI model, in the SC, dorsal root ganglia (DRG), and the sciatic nerve (SN). We observed that at least 70% of the SNI-specific proteins we have detected in the sEVs are also expressed in the PNS or SC of control mice. Six of these proteins are significantly upregulated in the DRG of the SNI mouse model; two of these six proteins, SERPING1 and HEMOPEXIN, are also significantly upregulated in the sciatic nerve (SN) of the SNI mice. A few proteins detected in the sEVs of the SNI mice were reported to be downregulated in tissues (nine proteins in SN and six proteins in SC) of SNI mice.

3.5. Cytokine array

We also investigated the differences in the levels of cytokines, chemokines, and acute phase proteins in sEVs purified from the serum of SNI and sham mice. We hypothesized that a protein present exclusively in sEVs could mediate signaling in adjacent cells and at a distant location whereas, proteins in the serum will predominantly act locally by diffusion gradient and will have a local mode of action. To test this, in addition to purified sEVs, we also investigated whole serum and sEV-deplete serum to differentiate proteins present exclusively in sEVs. Our cytokine array data comparing sEVs from SNI and sham mice showed that both C5a and ICAM-1 were upregulated in the sEVs purified from the serum of SNI mice (Fig. 5). Interleukin-6 (IL-6) was absent in sEVs from both SNI and sham animals but present in the serum. This demonstrates the selectivity in sEV protein packaging within a cell. Comparison of sEVs and serum composition indicate 1) compared to sham mice, there is a higher distribution of C5a with an increase in C5a within sEVs vs free C5a in serum of SNI mice 2) ICAM-1 is absent in sEVs from sham mice but upregulated in sEVs from SNI mice (Fig. 5). This upregulation of ICAM-1 in sEVs of SNI mice was accompanied by a small reduction of ICAM-1 in serum compared to sham mice. sEV-deplete serum isolated from SNI and sham mouse contained both C5a and ICAM-1 (Fig. 6). Interestingly, while SNI derived sEVs contained an increased level of C5a and ICAM-1, sEV-deplete serum fractions contained significantly less of each protein further indicating increased packaging of these cytokines into sEVs following SNI (Fig. 6).

4. Discussion

Proteomics in pain research have primarily focused on DRG and SC tissues in various rodent models of pain and cerebrospinal fluid in humans [28–30]. Different studies show partly overlapping but mostly inconsistent protein modulation. Possible reasons include the different nerve injury models employed, duration of the injury, and differences between the rodent strains or tissues analyzed. Technical variations in protein extraction or methods used in detection can also significantly impact the outcome [31]. For the SNI model, proteomic analysis of the dorsal horn of the spinal cord seven days after surgery identified modulation of 41 proteins. These proteins are mostly involved in energy metabolism, cellular structure,

signal transduction and DNA binding [32]. We employed mass spectrometry to investigate sEV proteome from SNI, sham and naïve control mice. Limited availability of total proteins makes this approach suitable [33]. EVs harbor a common set of vesicular proteins and cell-type-specific components [6]. Mass spectrometry-based proteomic analyses of mammalian EVs led to the identification of the important vesicular marker proteins, such as tetraspanins, 14-3-3 proteins, actin, annexins, heat shock proteins among many others. In addition to the common proteins cataloged for exosomes such as actin and 14-3-3 [34], our studies identified multiple members of both the serpin and complement families in sEVs. Some proteins were exclusively detected in the SNI model (Table 1). Some proteins were unique to sham control surgery. Presence of unique proteins in sEVs from sham control group suggests that sham surgery itself can induce molecular changes and reflected in the proteome composition and cargo packaged into sEVs.

Serpins are serine protease inhibitors with established roles in inflammation, coagulation, and complement regulation [35]. This broadly distributed superfamily is comprised of 16 'clades' (termed A-P). The proteins are named SERPINXy, where X is the clade and y the number within that clade. We detected SERPINA 1a, 1b, 1c, 1d, 1e, 3c, 3f, 3i, 3k and 3m in sEVs. SERPINA3n can attenuate neuropathic pain by inhibiting T cell-derived leukocyte elastase [36]. The mRNA expression of *Serpina3n*, *Serpina3g* and *Serpina3k* were upregulated in L3–L5 DRGs one day post-SNI but not at 3 weeks after injury. Mice lacking *Serpina3n* developed more mechanical allodynia than WT mice, and exogenous delivery of SERPINA3n attenuated mechanical allodynia in WT mice [36]. SERPINA3k is an inhibitor of the Wnt pathway and its anti-inflammatory activity was demonstrated in diabetic retinopathy model [37]. A decrease in SERPINA members from SNI model suggests there is a downregulation of protective protein cargo in sEVs released under chronic neuropathic pain.

Complement is another crucial component of the host defense system and we detected C3 and C4. This pathway involves a highly regulated cascade of proteolytic events that generate numerous proinflammatory cytokines and membrane attack complex [38]. All components of this complex pathway are designated by the letter C followed by a number. The products of the cleavage reactions are designated by added lower-case letters, the larger fragment being designated b and the smaller a. Typically the b fragments can opsonize (mark cells for phagocytosis) or lyse cells by binding covalently to the surface of the pathogen, and the smaller a fragments (C3a, C4a and C5a) are anaphylatoxins, serving as soluble danger messenger molecules that attract and activate phagocytes. C3 and C5 were previously reported in mouse and human sEVs respectively [39].

Serpin and complement components were detected in our two independent studies, first using pooled samples and the second using EVs from individual mice from the three experimental groups. However, there were other proteins that were detected only in either pooled or samples from individual mice. It is well established that in a discovery-based mass spectrometry approach to detect all the proteins in a mixture, different subsets of the whole proteome is identified from independent experiments [27, 40]. We attribute this to the observed differences in peptides identified in EVs. We cannot rule out some of the differences to variability associated with mice too. A targeted approach to detect specific

peptide ions derived from proteins of interest using approaches such as selected reaction monitoring or multiple reaction monitoring are typically pursued to answer hypothesis driven questions. However, not all peptides are equally analyzed by mass spectrometry because some are better separated, ionized and detected than others owing to their physicochemical properties [41].

Mass spectrometry-based approach can identify peptides, but it is often difficult to differentiate cleaved fragments from precursor proteins in data analysis. We pursued a membrane-based antibody array for the parallel determination of the relative levels of mouse cytokines to monitor a select few proteins of interest with high sensitivity, reproducibility and quantitative accuracy. Systematic studies have not been conducted to determine the complete spectrum of EV associated cytokines. Additionally, the extent to which vesicular localization of cytokines affects conventional cytokine measurements has not been addressed [42]. We observed a significant upregulation of C5a in sEVs from SNI model.

The C5a peptide, which is generated following the proteolytic cleavage of C5 is integrally linked to central nervous system (CNS) diseases. C5a has pleiotropic inflammatory effects and can activate NF κ B, activator protein-1, and CREB protein signal transduction pathways [43]. C5a functions as a chemoattractant [44], signals through C5aR1 [45] and promotes inflammatory processes that can be detrimental to the CNS with increase in integrin expression and recruitment of leukocytes leading to inflammation [46]. C5aR1 activation promotes neuronal death, demyelination, and blood brain barrier (BBB) dysfunction [47], sEVs, such as exosomes, are able to cross BBB and transfer pathogenic and nonpathogenic biomolecules [2]. Activation of the complement cascade and the production of C5a play a significant role in neuropathic pain and its inhibition produces analgesic effects in several animal models of chronic pain [48]. Expression of C5 mRNA and that of the C5aR in the dorsal horn showed a progressive increase after SNI [49]. Whether C5a acts via the induction of downstream mediators such as proinflammatory cytokines and glial cell activation, or whether C5a can directly enhance pain is unknown [48].

Cell recognition molecules expressed on the surface of sEVs facilitate their selective targeting and uptake into recipient cells. Our cytokine array showed a significant upregulation of ICAM-1. ICAM is one of the major molecules required for the leukocyte recruitment, adhesion [50] and homing of sEVs, like exosomes [51]. Blockade of ICAM-1 by monoclonal antibodies, antisense oligonucleotides, or deletion of the ICAM-1 gene was shown to impair the migration of immunocytes to inflamed tissue and to decrease inflammation in animals and humans [50]. ICAM-1 regulates the homing of opioid-producing cells and the subsequent generation of analgesia within sites of painful inflammation [50]. Loperamide-encapsulated anti-ICAM-1 immunoliposomes exerted analgesic and anti-inflammatory effects exclusively in peripheral painful inflamed tissue [52]. The authors proposed that engineering targeted nanoparticles can be beneficial in treating pain disorders [52]. Exosomes released by monocytes induce ICAM-1 and cytokines via activation of NF κ B [53]. Major histocompatibility class II and ICAM-1 are required for mature exosomes to prime naïve T cells and changes in protein composition and priming abilities of exosomes reflect the maturation signals received by dendritic cells [51]. These findings suggest pathogenic as well as therapeutic use of sEVs.

Although the detailed molecular mechanisms by which proteins are loaded into EVs during their biogenesis are not fully understood, increasing evidence indicates that the clustering, oligomerization, or protein–protein interactions among vesicular proteins play critical roles in cargo sorting [6]. Though present in the serum, IL-6 was absent in sEVs from both SNI and sham samples. An increase in SNI sEV C5a is accompanied by a decrease in whole serum and sEV-deplete serum C5a levels compared to sham control. ICAM-1 was upregulated in sEVs from SNI mice but absent in sEVs from sham mice. This ICAM-1 upregulation in sEVs from SNI was accompanied by a small reduction of ICAM-1 in whole serum and sEV-deplete serum. It is possible that under neuropathic pain, there is an upregulation in the packaging of C5a and ICAM-1 proteins into sEVs rather than an increase in protein production. Collectively, our observations indicate proteins in the cell are being selectively packaged into sEVs for release into the circulation. Protein cargo carried by the sEVs might possess an enhanced stability than their freely floating counterparts until they reach target tissues. This could be aided either by their protection against degradation by proteases or from reduced interactions to high concentrations of their cognate ligand/receptors, when they are enclosed within the vesicles. Hence, we hypothesize that a protein present exclusively in sEVs could mediate signaling in adjacent cells and at a distant location whereas proteins in the serum will predominantly act locally by diffusion gradient and will have a local mode of action.

Functional annotations of vesicular proteins indicate that specific biological processes are highly enriched or depleted in mammalian EVs when compared with other intracellular compartments suggesting EVs are extracellular organelles that are distinct from other intracellular organelles and compartments [6, 54]. Proteomic studies can facilitate biomarker discovery based on the protein signature of the originating cells [54]. A recent study using sEVs purified from cultured primary satellite glial cells stimulated with lipopolysaccharide to mimic inflammation identified myosin-9 as a novel biomarker [55]. Proteome profile can differ between serum and sEVs depending on whether they are packaged or released locally. Secretion of sEV would allow information transfer from one location to the entire body; such a mechanism would have a profound effect if dysregulated. Future studies will be crucial in determining how they impact homing and signaling and thereby identify novel mechanisms involved in mediating neuropathic pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Approximately 100 million U.S. adults are burdened by chronic pain. Neuropathic pain resulting from injury or dysfunction of the nervous system is challenging to treat. Unlike acute pain that resolves over time, chronic pain persists resulting in changes in the peripheral and central nervous system. The transport of biomolecular cargo comprised of proteins and RNAs by small extracellular vesicles (sEVs) including exosomes has been proposed to be a fundamental mode of intercellular communication. To obtain insights on the role of exosome-mediated information transfer in the context of neuropathic pain, we investigated alterations in protein composition of sEVs in a mouse model of neuropathic pain induced by spared nerve injury (SNI). Our studies using mass spectrometry and cytokine array show that sEVs from SNI model harbor unique proteins. We observed an upregulation of C5a and ICAM-1 in exosomes from SNI model compared to control. There was a differential distribution of C5a and ICAM-1 within exosomes and serum, between control and SNI suggesting a switch from local to long distance signaling. Our studies suggest critical roles for cargo sorting of vesicular proteins in mediating signaling under neuropathic pain.

Highlights

- Proteomic analysis of 30-150 nm small extracellular vesicles or exosomes in mouse spared nerve injury (SNI) model of neuropathic pain.
- Of the 274 gene products detected, 24 were unique to exosomes from SNI model.
- Cytokine profiling showed upregulation of C5a and ICAM-1 in exosomes from SNI model compared to sham control.
- Distribution of C5a and ICAM-1 within exosomes and serum differed between sham and SNI.
- Alterations in cargo sorting of vesicular proteins observed under neuropathic pain.

Figure 1.

Confirmation of mechanical hypersensitivity after SNI. Mechanical allodynia was measured using von Frey filaments in eight week-old male C57BL/6 mice. Paw withdrawal thresholds were measured before and after SNI surgery in both the ipsilateral (A) and contralateral (B) paws. Treatments were compared across time using a repeated measures two-way ANOVA with Bonferroni's post hoc test. Data are presented as $\pm$ SEM. * $p < 0.05$ compared to sham control at the same time point.

Figure 2.

Characterization of small extracellular vesicle (sEVs). A) Representative transmission electron microscopy (TEM) image of sEVs isolated from SNI or sham control mice. sEVs purified from the serum of SNI model mouse were fixed, mounted and stained on copper mesh grids (scale=100 nm). TEM images showed sEVs are intact and in the expected size range. Specificity of sEVs was confirmed by B) immunogold labeling for CD9 (scale=100 nm) and C) Western blot of sEVs lysates showing the two commonly detected exosome marker proteins HSP70 and TSG101.

Figure 3.

Nanoparticle tracking showing size distribution and concentration of sEVs isolated from serum of naïve, SNI model and sham surgery control mice (n=6) using a ZetaView nanoparticle tracking analysis system. A) Size distribution and B) concentration of sEVs isolated from SNI, sham control or naïve mice. Data are presented as $\pm$ SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparisons test.

Figure 4. Venn Diagram summarizing the findings from mass spectrometric analyses of proteins in sEVs purified from the serum of SNI, sham and naïve control mice four weeks after surgery.

Figure 5.

Differential transport of sEV-associated inflammatory proteins in SNI model compared to sham control mice. A) Representative arrays incubated with sEVs or serum from SNI or sham control mice. B) Quantification of protein levels (normalized to background) of sEVs or serum from SNI or sham control mice. Assessment of 40 proteins using the Proteome Profiler mouse cytokine array showed upregulation of C5a and ICAM-1 in sEVs from SNI mice compared to sham control. An increase in vesicular C5a was accompanied by a decrease in serum C5a compared to sham control. ICAM-1 is absent in sEVs from sham mice and upregulation of ICAM-1 in sEVs in SNI was accompanied by a small reduction of ICAM-1 in serum. IL-6 was detected only in serum. Serum or sEVs from four mice were pooled for each experiment (n=2). A total of 100 µg of protein (25 µg/mice) were used for both sEVs and serum samples. Data represent mean ± SD from two independent experiments. One-way ANOVA with Tukey's test was used for analysis * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Figure 6.

sEV-associated proteins from sEV-deplete serum fraction. A) Representative proteome profiler mouse cytokine arrays incubated with sEV depleted serum from SNI or sham control mice. B) Quantification of protein levels from sEV-depleted serum from SNI or sham control mice showed downregulation of both C5a and ICAM-1 levels in SNI sEV-deplete serum fractions compared to sham sEV-deplete serum fractions. Serum was pooled from 4 mice prior to centrifugation and collection of sEV-deplete serum (n=2). A total of 100 µg of protein (25 µg/mice) were used. Data represent normalized mean pixel density $\pm$ SD from two independent experiments. Student's t-test was used for analysis * $p < 0.05$, ** $p < 0.01$.

Table 1

List of proteins that were exclusively detected in the sEVs from SNI model.

Gene	Description	SNI Log2-FC			Tissue Expression	Protein IDs
		DRG	SN	SC		
Serping1	Plasma protease C1 inhibitor	0.31	0.17		DRG,SN	P97290
Ces1c	Carboxylesterase 1C	0.29			ACC,AMY,CER,PFC,SC,DRG,SN	P23953
Hrg	Histidine-rich glycoprotein	0.23			SC,DRG,SN	Q9ESB3,A0A338P6H8,A0A0R4J039
Actn4	Alpha-actinin-4,Actinin alpha 4	0.18			ACC,AMY,CER,PFC,SC,DRG,SN	P57780,D3Z761,E9Q2W9,D3Z0L8,A0A1L1SV25,Q3ULT2
Hpx	Hemopexin	0.17	0.47		ACC,AMY,CER,PFC,SC,DRG,SN	Q91X72
Gc	Vitamin D-binding protein	0.16			ACC,CER,SC,DRG,SN	P21614
S100a11	Protein S100-A11		0.59		CER,SC,DRG,SN	P50543
Tubb2a	Tubulin beta-2A chain,Tubb2a protein,Beta-tubulin gene M-beta-2, 3' end		-0.26	-0.31	ACC,AMY,CER,PFC,SC,DRG,SN	Q7TMM9,Q99J49,Q62363
Jup	Junction plakoglobin		-0.34		ACC,AMY,CER,PFC,SC,DRG,SN	Q02257
Tubb2b	Tubulin beta-2B chain,Tubulin beta chain		-0.41	-0.7	ACC,AMY,CER,PFC,SC,DRG,SN	Q9CWF2,B2RSN3
Dynl1	Dynein light chain 1, cytoplasmic		-0.46		ACC,AMY,CER,PFC,SC,DRG,SN	P63168
Tubb5	Tubulin beta-5 chain,Tubb5 protein		-0.47		ACC,AMY,CER,PFC,SC,DRG,SN	Q8WUC1,G3UZR1,Q80ZV2
Tubb3	Tubulin beta-3 chain,Uncharacterized protein,Tubulin beta chain		-0.48		ACC,AMY,CER,PFC,SC,DRG,SN	Q9ERD7,Q9CRT0,D5MR34
Tubb4b	Tubulin beta-4B chain,Tubulin beta chain,Uncharacterized protein		-0.49		ACC,AMY,CER,PFC,SC,DRG,SN	P68372,Q9CVR0,Q9DCR1
Tubb6	Tubulin beta-6 chain,Tubulin beta chain,Uncharacterized protein		-0.51	-0.48	ACC,AMY,CER,PFC,SC,DRG,SN	Q922F4,Q3UMM1,Q3U9U3,Q9CUN8
Nefh	Neurofilament heavy polypeptide,MKIAA0845 protein		-0.84		ACC,AMY,CER,PFC,SC,DRG,SN	P19246,Q80TQ3
Dsp	Desmoplakin				PFC,SC,DRG,SN	E9Q557,E9PZW0
Kmt2e	Inactive histone-lysine N-methyltransferase 2E,NKp44L				N/A	Q9CVK6,M4KB43,A0A0G2JH19,A0A0G2JDX7
Krt20	Keratin, type I cytoskeletal 20				N/A	Q9D312
Pkp1	Plakophilin-1				N/A	P97350,A0A087WS37
Q61348	Beta-majglobin gene 5' flanking region					Q61348
Q61782	Type I epidermal keratin mRNA, 3' end					Q61782
Spr1a	Cornifin-A				N/A	Q62266

SNI Log2-FC						
Gene	Description	DRG	SN	SC	Tissue Expression	Protein IDs
Sytl4	Synaptotagmin-like protein 4				N/A	Q9R0Q1,G3UYC6

Proteins detected only in sEVs from SNI model were compared with the proteins in PainProteome database. PainProteome database [28] provides a compendium of proteins enriched in peripheral nervous system (PNS) and spinal cord (SC). PainProteome also provides a list of proteins that were found to be significantly differentially expressed in an SNI model, in the SC, dorsal root ganglia (DRG), and the sciatic nerve (SN). ACC Anterior cingulate cortex, AMY Amygdala, CER Cerebellum, PFC Prefrontal cortex.