

Inflammatory stroke extracellular vesicles induce macrophage activation

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Abstract

Background and Purpose: Extracellular vesicles (EVs) are protein-lipid complexes released from cells, as well as actively exocytosed, as part of normal physiology, but also during pathological processes such as those occurring during a stroke. Our aim was to determine the inflammatory potential of stroke EVs.

Methods: EVs were quantified and analysed in the sera of patients after an acute stroke (<24 hours; OXVASC). Isolated EV fractions were subjected to untargeted proteomic analysis by LC-MS/MS, then applied to macrophages in culture to investigate inflammatory gene expression.

Results: EV number, but not size, is significantly increased in stroke patients when compared to age-matched controls. Proteomic analysis reveals an overall increase in acute phase proteins, including C-reactive protein. EV fractions applied to monocyte-differentiated macrophage cultures induced inflammatory gene expression.

Conclusions: Together these data show that EVs from stroke patients are pro-inflammatory in nature and are capable of inducing inflammation in immune cells.

Introduction

Inflammation plays a crucial role in the pathophysiology of stroke. Within the core of the infarct neurons rapidly depolarize and die, and these cells are phagocytosed by microglia and infiltrating circulating macrophages. Propagation of deleterious signals and processes from within the core of the infarct, to the circulation can result in the infiltration of systemic immune cells, significantly worsening infarct evolution.¹

A number of mechanisms are purported to communicate CNS injury signals to the periphery,² the most recent of which is the circulating extracellular vesicle (EV). EV is an umbrella term encompassing fragments blebbed from the membrane (microvesicles) as well as actively exocytosed vesicles (exosomes).

Whilst there is an increasing presence of EVs in the literature, the focus has thus far been on quantification and association, rather than investigation of structure and function. Their role as novel biomarkers in cancer,³ as well as in metabolic⁴ and cardiovascular disease⁵ has begun to be explored, but there remains a paucity of knowledge regarding their specific role in CNS disease, in particular acute CNS injury. On this background, we aimed to determine the potential for EVs to communicate injury and inflammatory signals after a stroke.

Materials and Methods

38 patient samples were selected from the Oxford Vascular Study (OXVASC) cohort – a population-based study of all acute vascular events in approximately 92,000 residents of Oxfordshire.⁶ EVs were isolated using ultracentrifugation techniques and analysed using NanoSight Tracking Analysis, electron microscopy and Western blotting for canonical EV markers (Tsg101, Alix, CD9).⁷ Proteomics was performed using untargeted liquid chromatography mass-spectrometry/mass-spectrometry (LC-MS/MS) and pathway analysis (IPA – Qiagen). To determine functionality, human THP-1 cells were treated with stroke and control EVs and mRNA expression levels of *tnf*, *il-1 β* , *cxcl-1* and *ccl2* were

studied by qPCR. For more detailed methods see Data Supplement. All research was conducted according to the principles of the Declaration of Helsinki.

Results

Acute stroke results in increased numbers of EVs which associate with CRP and NSE

Patients from the OXVASC study had a median age of 75.4 (IQR 68-80 years), had a minimum NIHSS score of 5, and were age and sex-matched with controls. NTA of EVs showed a standard distribution with an elevated number, but not size, in stroke patients (Fig. 1A). Characterization revealed a mixed population of vesicles expressing canonical vesicle markers (Fig. 1B). Number of EVs increased in stroke compared to age-matched controls (Fig. 1C). Correlation analysis showed a significant correlation between EV number and C-reactive protein (CRP; Fig. 1D).

EVs from stroke patients have an inflammatory proteomic profile

Of the 381 proteins found, 75 were consistently and significantly different in stroke EVs, when compared to age-matched controls, and hierarchical cluster analysis revealed an overall increase in inflammatory proteins (Fig. 2A; CRP is an example highlighted in Fig. 2B and confirmed with Western blot in Fig. 2C). Pathway analysis (Ingenuity; Qiagen, UK) performed on average-fold change showed an overall up-regulation of proteins associated with the acute-phase response including C-reactive protein (Fig. 2D).

Stroke EVs activate macrophages

EVs applied to monocyte-differentiated macrophages resulted in an overall mRNA expression of TNF was different in cells treated with EVs (Fig. 3A). This was reflected in an increase in TNF mRNA expression in cells treated with EVs from stroke patients compared to both EV controls and untreated controls. IL-1 β mRNA expression was changed after EV treatment (Fig. 3B), resulting in an increase in IL-1 β expression in stroke EV-treated cells compared to both untreated controls and age-matched EV

controls. Neutrophil chemoattractant CXCL-1 showed the most profound increase in mRNA expression, a 400-fold increase in stroke-EV treated cells compared to untreated controls (Fig. 3C). Levels of CXCL-1 mRNA were increased in stroke EV-treated cells when compared to control EVs and when compared to untreated controls. Monocyte chemoattractant CCL-2 mRNA was also altered by treatment with EVs (Fig. 3D). Post-hoc testing revealed that CCL-2 mRNA increased after cells were challenged with stroke EVs compared to when they were challenged with control EVs and compared to untreated cells.

Discussion

In this brief report, we have demonstrated, for the first time, that EVs released into the circulation after a stroke contain pro-inflammatory proteins, and are capable of inducing an inflammatory response. Stroke patients show increased levels of circulating EVs, which are a mixed population of large and small vesicles expressing canonical vesicle markers and inflammatory proteins. The mechanisms of communication between the CNS and the systemic immune system after stroke remain poorly understood. Insights such as these into the fundamental mechanisms of this communication pathway are vital to further early interventional studies in stroke.

Studies on the EV population in stroke patients are still relatively sparse, and focus largely on their thrombogenic activity.^{8, 9} Kuriyama and colleagues¹⁰ have suggested that their potential as either a point of intervention or as a biomarker is limited; however, this is likely to be due to the field still being in its infancy. Here, we have used dynamic light scattering to demonstrate a significant increase in EV numbers post-stroke which is not correlated with stroke severity (Supplementary S1), and have also demonstrated the presence of standard vesicle markers. To our knowledge, this is the first stroke paper on EVs to present data in this manner since the publication of the ISEV guidelines on EVs.⁷

An Increased number of EVs does not necessarily indicate that the vesicles are physiochemically different between the control and the stroke patients, or indeed whether the vesicles are functional.

It was important, therefore, for us to establish a functional role for what has previously been considered 'cellular debris'. To date, studies have found elevated numbers of platelet-derived 'microparticles' in the acute phase of cerebral infarction and suggested that their pro-thrombotic nature may result in infarct development without shedding further light on the precise molecular mechanisms.^{4, 9} Our data here suggest that the profile of EVs from stroke patients is different from that of age-matched controls. Considering the average age of stroke patients, and the association of age with an altered inflammatory profile,¹¹ this data suggests that the changes in the EV population reflects a disease specific process. However, it should be noted these patients were not 'disease-matched' and co-morbidities in the elderly may confound the interpretation of the data and remain the focus of ongoing work. Despite this, the proteomics data here show that there is a significant acute phase profile in the EVs from stroke patients, and when these were applied to human immune cells they caused a significant increase in cytokine and chemokine expression when compared to EVs from control patients. It is likely that EVs in stroke patients reflect the pro-inflammatory nature of the stroke, and as such can act as a mechanism for signalling CNS injury to the periphery. Whilst the mechanisms of this may be fundamental to a number of disease processes, including other ischemic insults such as myocardial infarction, the origins and functions of the EV population are likely to be unique, and the purpose of this remains the focus of our ongoing research.

In conclusion, these data demonstrate a change in the fundamental components of circulating EVs after a stroke, and a significant functional role for them in activating immune cells. Whilst this research is preliminary, it provides a promising candidate route for CNS to immune system communication after brain injury and paves the way for significant advances in the fields of both neuroimmunology and EV research.

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Disclosures

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All other authors declare no competing conflict of interest.

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Figure Legends

Figure 1. Characterization of the extracellular vesicle fraction in the circulation of stroke patients.

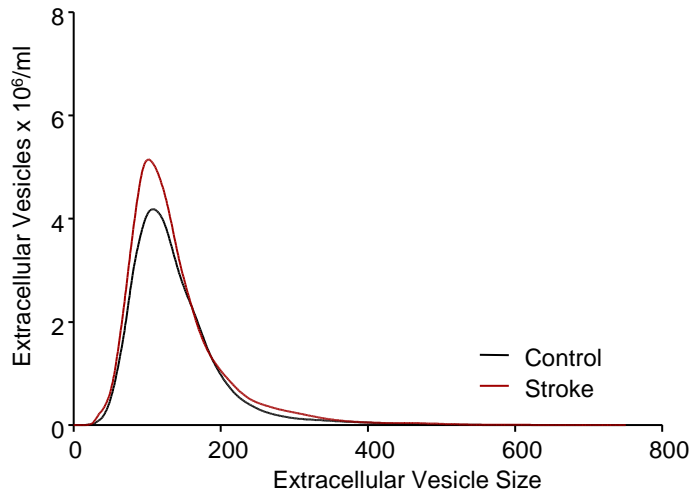
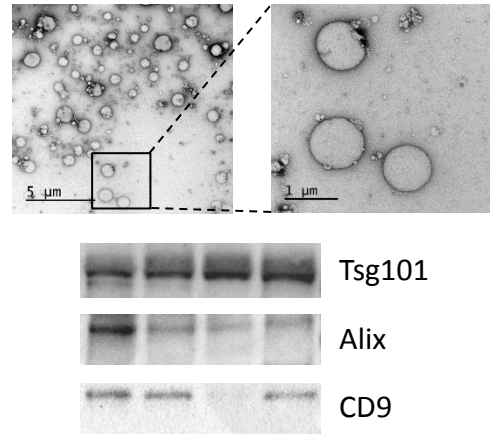
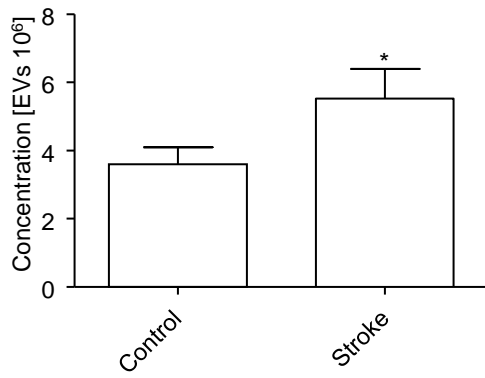
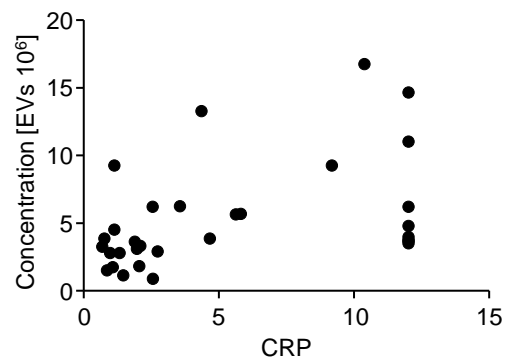
Nanosight Tracking Analysis (NTA) was used to determine (A) average distribution of vesicle size and number in the sera of stroke patients <24 hours post-stroke compared to age-matched controls. This population of extracellular vesicles was of mixed size when viewed by electron microscopy (B) and expressed common markers of a mixed EV population including Tsg101, Alix and CD9. NTA revealed an increase in number of vesicles per ml (C). EV number correlated with expression levels of and C-reactive protein (CRP; D). Data are mean \pm SEM (n = 38); *p<0.05.

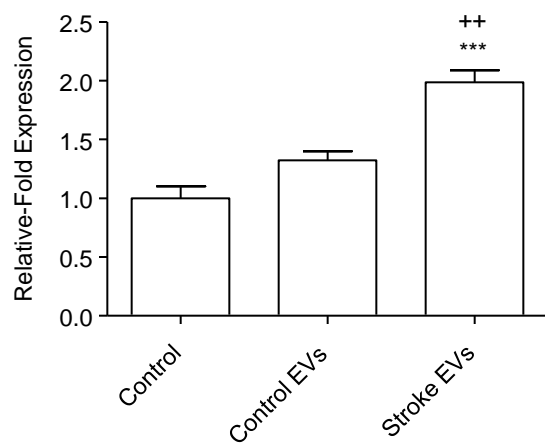
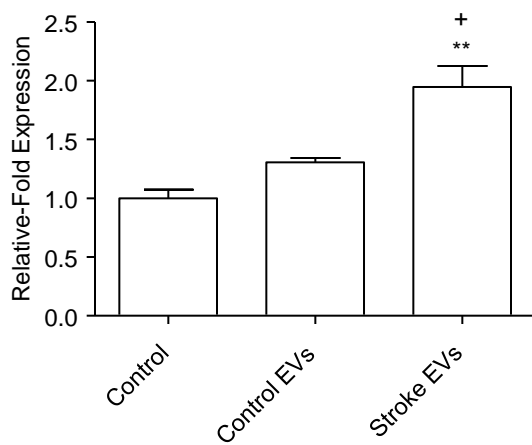
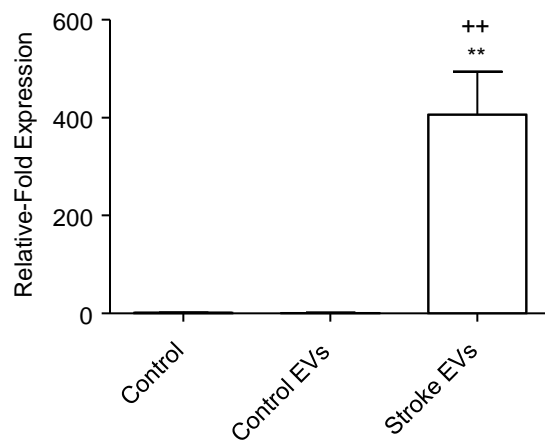
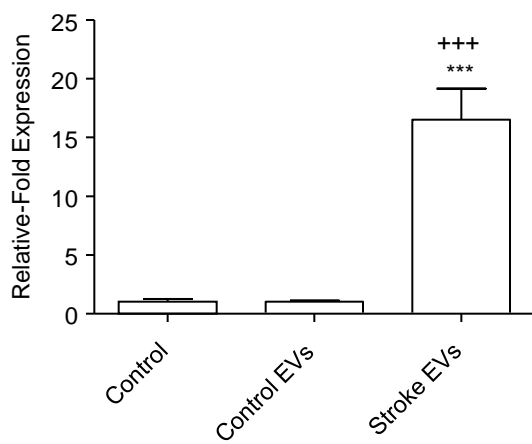
Figure 2. Proteomic profile of extracellular vesicles in stroke and control patients. (A) Heat map of

proteins found in extracellular vesicle populations from stroke patients and age-matched controls as determined by LC-MS/MS (represented by range of colour intensities – see colour key). Proteins are arranged by hierarchical clustering (depicted by dendrogram). Blue indicates an increase compared to control patients, red indicates a decrease. Log ratio stroke/control vs average fold change (B) shows a number of significantly upregulated proteins associated with the acute phase response, including C-reactive protein (highlighted in red). (C) Confirmation of CRP in stroke EVs by Western blot. Ingenuity pathway analysis of control vs stroke extracellular vesicle proteins indicates the acute phase response is one of the most highly altered pathways post-stroke (D). Red indicates proteins which are up-regulated in stroke, green indicates proteins which are down-regulated.

Figure 3. Cytokine and chemokine expression in monocyte-differentiated macrophage cells after

treatment with extracellular vesicles from control or stroke patients. mRNA expression of (A) TNF; (B) IL-1 β ; (C) CXCL-1 and (D) CCL-2 in THP-1 cells treated with EVs from stroke patients, or age-matched controls for 6 hours. Data are mean \pm SEM; n=3; **p<0.001 and ***p<0.0001 vs. controls; +p<0.05, ++p<0.001 and +++p<0.0001 vs. age-matched control EVs.

A**B****C****D**

A**B****C****D**

SUPPLEMENTAL MATERIAL

Materials and Methods

Patients and Sample Collection: 38 patient samples were selected from the Oxford Vascular Study (OXVASC) cohort – a population-based study of all acute vascular events in approximately 92,000 residents of Oxfordshire. Briefly, patients were selected who had suffered an ischaemic stroke with an NIHSS score of >5 (i.e. a major ischaemic stroke) and had blood taken within 24 hours of symptom onset. Further selection criteria included inclusion in an OXVASC study investigating serum levels of various biomarkers, including C-reactive protein. All samples were spun and frozen within 2 hours of sampling and were stored at -80°C. Blood samples from healthy controls were also collected in OXVASC (usually from friends or spouses of patients) and samples were processed and stored in the same way. For the current study, controls were matched to ischaemic stroke cases by sex and by age to within 6 months.

Extracellular Vesicle (EV) Isolation for Nanoparticle Tracking Analysis (NTA): 100µl of serum was combined with 900µl of EV-free phosphate buffered saline (PBS) and centrifuged at 34,000rpm for 1 hour to isolate the extracellular vesicle fraction. This was re-suspended in 100µl PBS and further diluted 1:100 in PBS for nanoparticle tracking analysis (NTA).

NanoSight Tracking Analysis (NTA): A NanoSight LM10 (NanoSight Ltd., Amesbury, United Kingdom), was used to count and determine the mean size of the isolated particles. The system uses a finely focused laser beam that is introduced to the sample through a glass prism. The beam refracts at a low angle as it enters the sample, resulting in a thin beam of laser light that illuminates particles through the sample. Particles resident within the beam are visualized using a conventional optical microscope, fitted with a video camera, aligned normally to the beam axis, which collects light scattered from all particles in the field of view. The sample chamber is 500µm deep, but the beam depth is around 20µm at the point of analysis, matching with the depth of focus of the imaging optics. A video of typically 60 seconds duration is taken, with a frame rate of 30 frames per second, and particle movement is analyzed by NTA software (NanoSight Ltd.). The NTA software is optimized to first identify and then track each particle on a frame-by-frame basis, and its brownian movement tracked and measured frame to frame. The velocity of particle movement is used to calculate particle size by applying the two-dimensional Stokes-Einstein equation:

$$\langle x, y \rangle^2 = \frac{K_B T_{ts}}{3\pi\eta d_h}$$

Where $\langle x, y \rangle^2$ is the mean squared displacement, K_B is Boltzmann's constant, T is the temperature of the solvent in Kelvin, t_s is the sampling time (i.e., 1/30 fpsec = 33 msec), η is the viscosity, and d_h is the hydrodynamic diameter.

Western Blotting: Total protein was extracted from vesicle fractions using RIPA buffer and quantified using a micro BCA kit (Thermo Scientific, UK). 25µg total protein was loaded for electrophoresis and transferred onto a PVDF membrane. Blotting was performed overnight using antibodies against vesicle markers Tsg101 (4A10 Ab83; AbCam, Cambridge, UK; 1:250 in 5% BSA), Alix (3A9 ab117600; AbCam; 1:500 in 5% milk) and CD9 (ExoAb-1; Stratech, Suffolk, UK; 1:1000) in both stroke and control samples. Detection was performed using horse-radish peroxidase conjugated secondary antibodies (1:10,000) and blots were visualized using chemiluminescence and a ChemiDoc (Bio-Rad, UK).

Protein Digest: 90µl of EVs from the NTA were spun at 120,000g for 2 hours to deplete serum proteins from the pellet. The pellet was briefly air dried and frozen on dry ice. Frozen EV pellets were re-suspended in 100µl lysis buffer (ThermoFisher, Loughborough, UK) with 20mM DTT and incubated for 30 minutes at room temperature. Samples were then diluted to 180µl with ultra-pure water and

alkylated with 20µl 200mM iodoacetamide for 30 minutes at room temperature. Proteins were precipitated using methanol/chloroform extraction as described elsewhere.¹ Precipitated proteins were re-suspended in 50µl 6M urea and protein digestion was carried out with 0.6µg trypsin (Promega, Southampton, UK) after dilution to 1M urea with ultra-pure water. Samples were desalted on C18 solid-phase extraction cartridges (SOLA C18, Thermo), dried and re-suspended in 2% acetonitrile 0.1% formic acid for analysis by LC-MS/MS.

Liquid Chromatography – Mass Spectrometry/Mass Spectrometry (LC-MS/MS): LC-MS/MS analysis was performed in technical duplicates using a Dionex Ultimate 3000 UPLC coupled on-line to a Q Exactive HF mass spectrometer (Thermo Scientific). Samples were separated on an EASY-Spray PepMap C18 column (500mm x 75µm, 2µm particle size, Thermo Scientific) over a 60 minute gradient of 2-35% acetonitrile in 5% DMSO 0.1% formic acid at 250nl/min. MS1 scans were acquired at a resolution of 60,000 at 200 m/z and the top 15 most abundant precursor ions were selected for HCD fragmentation.

Protein Quantification: Protein quantitation was performed using Progenesis Q1 for Proteomics (Non-linear Dynamics, version 2.0). MS/MS data was searched using Mascot (Matrix Science, version 2.5.1) against the human Swissprot database (retrieval date 15.10.14) allowing 1 missed cleavage. Mass tolerances were 10ppm for precursor and 0.05 Da for fragment masses. Carbamidomethylation of cysteine was set as a fixed modification. Oxidation of methionine, deamidation of asparagine and glutamine were set as variable modifications. Identified peptides scoring below 20 following application of a 1% false discovery rate were discarded and the Mascot search results were imported back into Progenesis for label-free protein quantitation.

Cell Culture: Human monocyte cell line (THP-1) were maintained under normal cell culture conditions (37°C; 95% CO₂; 5% air) in RPMI supplemented with 10% FCS, L-glutamine, and antibiotic/antimycotic and 2-mercaptoethanol. For stimulation paradigms cells were plated in 6-well plates at 1 x 10⁶ cells per well and differentiated into macrophages with phorbol 12-myristate 12-acetate for 24 hours. EVs from clinical stroke samples and age-matched controls were added to cells and allowed to incubate for 4 hours. Analysis was performed on whole RNA for expression levels of inflammatory cytokines and chemokines.

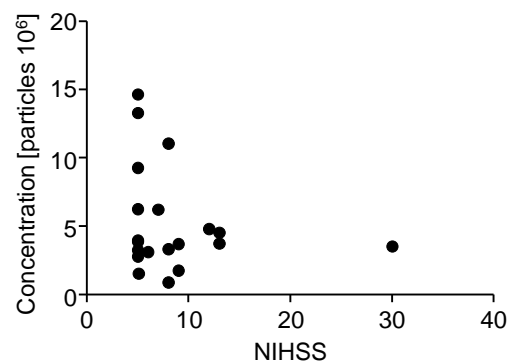
RNA Extraction and qPCR: RNA was extracted from whole cells according to manufacturers' instructions (QIAGEN, UK) and converted to cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, UK). qPCR was performed using the SYBR green based technology (PrimerDesign Ltd., UK) and analysed using the Pfaffl method.² Expression levels were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase and normalised to expression levels in control cells.

Statistics: The normalised intensity values of the protein concentrations were converted into z scores for subsequent analysis. The heat map and hierarchical cluster analysis was generated using the heatmap.2 (gplots, version 3.0.1) function in Rstudio (version 0.99.893). Clustering was performed in both the x and y direction to investigate the clustering of the samples and the individual proteins. All other data were analysed using Prism 6.0 and presented as mean ±SEM with data being considered significant at p<0.05.

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Supplemental Figure I



Supplemental Figure I: Extracellular vesicle number plotted against NIHSS score. Data points represent individual patients. n=17.