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Microparticles generated during chronic cerebral ischemia deliver proapoptotic signals to cultured endothelial cells

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ABSTRACT

Circulating microparticles (MPs) are involved in many physiological processes and numbers are increased in a variety of cardiovascular disorders. The present aims were to characterize levels of MPs in a rodent model of chronic cerebral hypoperfusion (CCH) and to determine their signaling properties. MPs were isolated from the plasma of rats exposed to CCH and quantified by flow cytometry. When MPs were added to cultured endothelial cells or normal rat kidney cells they induced cell death in a time and dose dependent manner. Analysis of pellets by electron microscopy indicates that cell death signals are carried by particles in the range of 400 nm in diameter or less. Cell death involved the activation of caspase 3 and was not a consequence of oxidative stress. Inhibition of the Fas/FasL signaling pathway also did not improve cell survival. MPs were found to contain caspase 3 and treating the MPs with a caspase 3 inhibitor significantly reduced cell death. A TNF-α receptor blocker and a TRAIL neutralizing antibody also significantly reduced cell death. Levels of circulating MPs are elevated in a rodent model of chronic cerebral ischemia. MPs with a diameter of 400 nm or less activate the TNF-α and TRAIL signaling pathways and may deliver caspase 3 to cultured cells.

1. Introduction

The term small vessel disease (SVD) refers to a variety of pathological conditions affecting the vasculature. Arteriolosclerosis, the most common form, is strongly associated with risk factors such as ageing, diabetes and hypertension and tends to be systemic in nature, affecting primarily the brain, kidneys and retina [1]. Cerebral small vessel disease (CSVD) is a heterogeneous condition characterized by damage to small penetrating vessels in the brain. Leukoaraiosis, cerebral microbleeds and lacunar infarcts are the major pathological features of CSVD and are a common cause of cognitive impairment [2,3]. CSVD is progressive in nature [4,5] but mechanisms mediating progression are not well understood. As arteriolosclerosis is a systemic condition, damage in one vascular bed is a risk factor for damage to other vascular beds in the body [6] and there may be endogenous mechanisms and signaling pathways that mediate the progression of small vessel disease within one vascular bed and the spread to other organs.

Microparticles (MPs) are membrane bound structures reported to be in the range of 100 nm to 1 micron in diameter. They are released from the plasma membrane of most cell types upon activation or apoptosis and are found in virtually all bodily fluids [7]. Originally described as "platelet dust" [8], MPs are now known to be involved in a range of physiological processes, including angiogenesis, inflammation, the progression of some forms of cancer and the reprogramming of mesenchymal stem cells, as well as coagulation. Levels of MPs of endothelial, leukocyte and platelet origin have been found to be elevated in a variety of cardiovascular disorders and neurodegenerative diseases [9].

MPs may affect target cells in a variety of ways [10]. They may provide a receptor ligand and they may directly bind to the target cell plasma membrane and release their cytoplasmic contents into the target cell while their delimiting membrane becomes incorporated into the plasma membrane. MPs have been shown to contain mRNAs and microRNAs and these may serve to reprogram target cells. MPs may also be internalized by target cells and deliver a variety of biologically active molecules.

In the present study we demonstrate that levels of MPs of endothelial origin and of unidentified origin are elevated at various time points following surgery in a rat model of (CCH). MPs were isolated

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by centrifugation from the plasma of rats subjected to cerebral ischemia and found to cause an increase in apoptosis in cultured rat brain microvascular endothelial cells (RBMVECs). MPs did not impart oxidative stress to these cells but were found to contain caspase 3 and to activate receptors for TNF-α and TRAIL.

2. Materials and methods

2.1. Animals, surgical procedures and centrifugation protocol

All experiments conformed to the guidelines set forth by the Canadian Council for the Use and Care of Animals in Research (CCAC). Male Long Evans rats, 250–275 g, were obtained from Charles River and anesthetized with 2.5% isoflurane in 30% O_2/ 70% N_2O. The common carotid arteries were isolated from vagus nerves and permanently ligated with silk sutures. After a variable period of time rats were anesthetized and the heart exposed. Up to 10 mL of blood was withdrawn by cardiac puncture and placed in a BD Vacutainer Blood Collection Tube containing sodium heparin. Collection tubes were centrifuged at 1200*g* for 15 min to produce platelet poor plasma (PPP). PPP was spun at 13,000*g* for 3 min to remove apoptotic bodies and other cellular debris. The supernatant was spun at 18,000*g* for 20 min to produce a pellet enriched in MPs. The supernatant from this step was spun at 100,000*g* for 30 min to produce a pellet containing exosomes.

2.2. Flow cytometry

Flow cytometry was performed at StemCore Laboratories in the Ottawa Hospital Research Institute. Endothelial derived and total MPs were isolated from plasma samples and enumerated by flow cytometry as previously described [11]. The supernatant following the 18,000*g* spin was aspirated and the microparticle-containing pellet was re-suspended in Annexin V binding buffer containing (in mM) 10 HEPES, pH 7.4, 140 NaCl, 2.5 $CaCl_2$. As MPs display externalized phosphatidylserine, MPs were identified using Alexa-647-labeled Annexin V (0.5 μg/mL, Biolegend) and endothelial MPs were identified using Annexin V and a FITC-labeled CD144 antibody (1:100). MPs were defined as fluorescence events of 0.1–1.0 μm in size and counted with a Beckman Coulter MoFlo.

2.3. Electron microscopy

Pellets immersed in glutaraldehyde (1.6% in 0.1 M cacodylate buffer) at 4 °C. Pellets were postfixed in 2% buffered osmium tetroxide, dehydrated in a graded ethanol series and embedded in Spurr resin using a Leica EM TP tissue processor. 70 nm thick sections were cut with a Leica UC6 ultramicrotome, picked up on copper grids, stained with uranyl acetate and lead citrate and examined with a Hitachi H7100 transmission electron microscope.

2.4. Cell culture

Primary rat brain microvascular endothelial cells (RBMVEC) were purchased from Cell Applications Inc. and cultured according to manufacturer's instructions. Cells in this study were used at passage 5. Normal rat kidney cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% fetal bovine serum, and 100 U/mL of penicillin/streptomycin, in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. Media were changed every 2–3 days and passaged at 90% confluence.

To treat cell cultures with MPs, platelet poor plasma from 3–6 rats was pooled and a specific volume spun at 13,000*g* and 18,000*g*. The resulting pellet was then resuspended in twice the volume of culture medium and applied to cultured cells. Thus, cultured cells were exposed to MPs at one half the concentration found in the plasma.

2.5. Western blot

Protein levels were determined by Western blotting using either activated caspase 3 or total caspase 3 antibodies at a concentration of 1:1000 as previously described [12].

2.6. Total RNA isolation

Cell cultures were lysed in Trizol reagent (Invitrogen) and total RNA was extracted following the manufacturer's instructions. The quality and concentration of the RNA were determined by measuring the absorbance at 260 and 280 nm.

2.7. Reverse transcription and PCR

Total RNA (2 μg) from the cell cultures was subjected to reverse transcription for 60 min at 42 °C using SuperScriptTM II (200 units, Invitrogen) and First Strand Buffer (Invitrogen), in the presence of 0.5 μg/μl oligo dT, 10 mM DL-dithiothreitol and 1 mM deoxynucleotide triphosphate solution (dNTP mix, Invitrogen) as previously described [13]. The sense and antisense primers used are described in Table 1

2.8. Cell death, cell survival assays

Cell death was assessed by lactate dehydrogenase (LDH) assay performed using the CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega) and expressed as a percentage of experimental LDH release/maximal LDH release. Cell survival was quantitatively assessed using Cell Cytotoxicity Assay Kit (Colorimetric) from Abcam.

2.9. Measurement of glutathione peroxidase (GPx) activity

GPx activity was measured by using a glutathione peroxidase assay kit (Cayman) according to manufacturer's instructions.

2.10. Treatments and reagents

Cells were pretreated with either VAS-2870 (Enzo Life Sciences; 10 μM), SPD304 (Cayman Chemical; 50 μM), Allopurinol (Cayman Chemical; 50 μM), L-NAME (Caymen Chemical; 10 μM), Apocynin (Caymen chemicals; 10 μM), AC-DEVD-CHO (Enzo Life Sciences; 20 μM), TRAIL neutralizing antibody (Abcam; 10 ng/mL) or FASL neutralizing antibody (Millipore; 500 ng/mL) for 1 h and then throughout the treatment period.

2.11. Statistical analysis

Data were displayed as a mean with error bars indicating standard error of the mean. All multiple comparisons were tested for significance using one-way ANOVA followed by Tukey's post hoc test in Prism GraphPad. A *P* value of less than 0.05 was considered statistically significant.

Table 1
Primer sequences, annealing temperatures, product sizes and sources.

Primer name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Anneal temp. (°C)	Product size (bp)	Source
GAPDH	CATGGCCTTCCGTGTTCCTACCC	CCT CGG CCG CCT GCT TAC	60	200	[13]
TNF-α R1	AATGGCACCGTGACAAT	CTGAAGGCTGGGATAGA	58	377	GenBank M63122.1
TNF-α R2	ATGGTGCCTCATCTGCC	GGACCTGCTCATCTTTG	58	373	GenBank AF498039.2
TRAILR4	AGCTGTGGTTGTGGTTGG	GGGTCAAGTACTGGACTG	50	498	[14]

3. Results

3.1. Microparticle numbers are increased in plasma of rats subjected to chronic cerebral ischemia

When PPP from sham operated rats or CCH rats is centrifuged at 13,000*g* the resulting pellet contains a variety of membrane bound vesicles, up to 5 microns in diameter, that appear to be apoptotic bodies and fragments of cells (Fig. 1B). When the resulting supernatant is centrifuged at 18,000*g* the pellet contains membrane bound vesicles of varying size and density. Most were 100–400 nm in diameter, with a few less electron dense vesicles up to 1 micron in diameter (Fig. 1C). When the supernatant from plasma spun at 13,000*g* is passed through a syringe filter with a 200 nm mesh the mixture of vesicles appears similar to that following centrifugation at 18,000*g* (Fig. 1D) and the physiological properties are similar (data not shown). When the supernatant following centrifugation at 18,000*g* was spun at 100,000*g* the pellet contained electron dense vesicles up to 25 nm in diameter and smaller particles that could be exosomes, poorly fixed vesicles or immune or lipoprotein complexes.

Following surgery to induce CCH there was a significant increase in numbers of both annexin V positive and VE-cadherin positive particles in the 18,000*g* pellet (Fig. 1A).

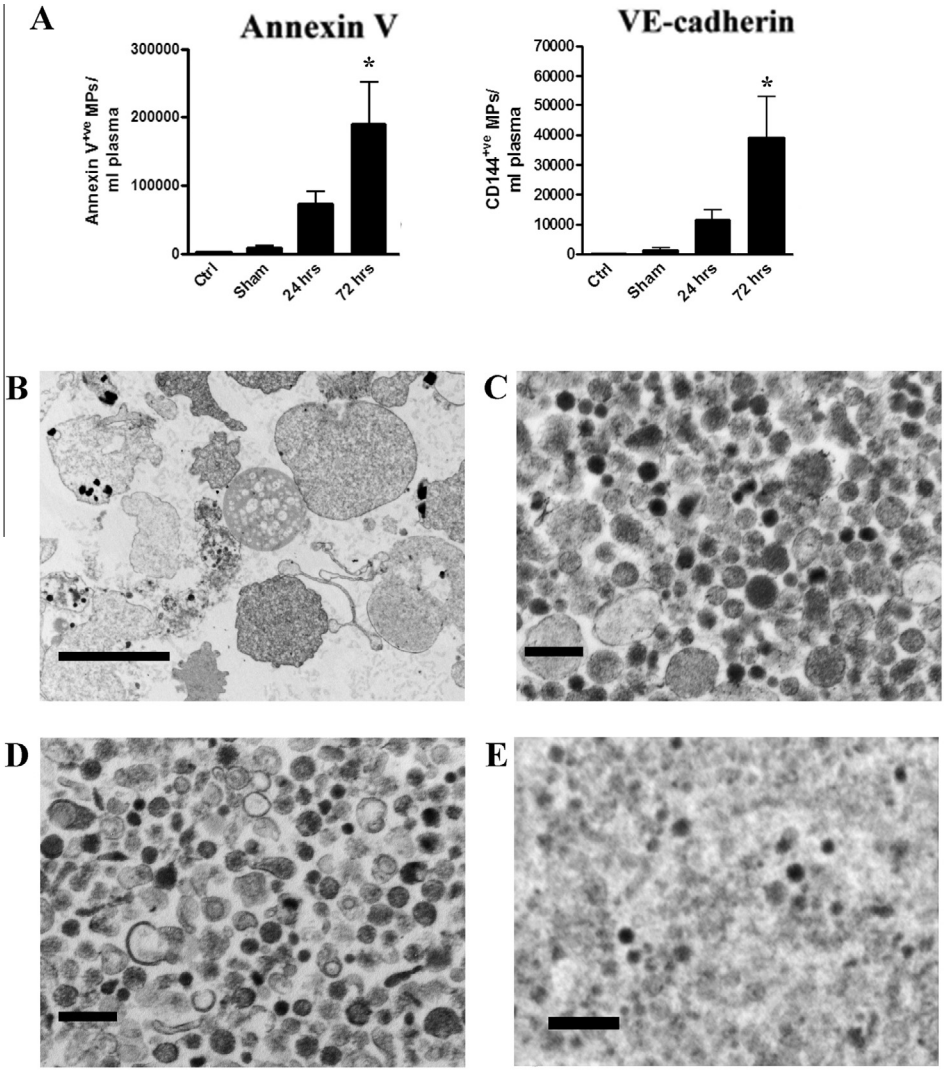

Fig. 1. Levels of MPs are significantly elevated in rats subjected to CCH. (A) The numbers of annexin V +ve and VE-cadherin +ve MPs are increased in the plasma. Pellets were examined by electron microscopy following centrifugation at 13,000*g* (B), 18,000*g* (C), after passing through a 200 nm mesh syringe filter (D) and following centrifugation at 100,000*g*. Scale bars: 5 μm (B), 500 nm (C and D) and 100 nm (E).

3.2. Microparticles induce caspase 3-dependent apoptosis in cultured cells

Pellets from centrifugation of plasma at 13,000*g*, 18,000*g* and 100,000*g* were resuspended and applied to cultured NRK cells and cell survival assessed 24 h later. Only the 18,000*g* pellet induced a significant amount of cell death (Fig. 2A) and this is the fraction containing MPs. MPs induce apoptosis in cultured RBMVECs in a time-dependent manner (Fig. 2B) and survival of NRK cells is improved by treatment with a caspase 3 inhibitor (Fig. 2C). Expression of caspase 3 protein and levels of activated caspase 3 were elevated in NRK cells following exposure to MPs (Fig. 2D). MPs themselves contain caspase 3 and equal numbers of MPs from sham operated rats and rats exposed to CCH, as determined by flow cytometry, contained similar levels of caspase 3 protein (Fig. 2D below). When MPs were treated with a caspase 3 inhibitor prior to being added to cultured cells there was a significant reduction in cell death 24 h later (Fig. 2E). These observations indicate that most of the cell death signals are in particles smaller than 400 nm and larger than exosomes.

In order to avoid the variability associated with flow cytometry equal volumes of plasma were centrifuged and the pellets resuspended in culture medium and applied to cultured RBMVECs (Fig. 3A). While MPs from sham operated rats initiate apoptotic cell death, MPs from an equal volume of plasma from rats subjected to CCH are much more lethal and the lethality of the plasma decreases with time following surgery (Fig. 3B).

3.2.1. MPs do not cause significant oxidative stress in cultured RBMVECs or NRK cells

The contribution of oxidative stress was assessed by treatment of cultured cells with oxidase inhibitors prior to the application of MPs. The NADPH oxidase inhibitors apocynin and VAS2870 both significantly increased cell death when applied alone and did not reduce the apoptosis initiated by exposure to MPs (Fig. 3C). The xanthine oxidase inhibitor allopurinol and the nitric oxide synthase inhibitor L-NAME did not reduce cell viability and did not protect against exposure to MPs (Fig. 3D and E) Finally, the application of MPs to cultured endothelial cells or NRK cells did not cause a significant increase in GPx activity (Fig. 3F).

3.2.2. MPs activate TNF-α and TRAIL receptors in cultured cells

The activation of cell death receptors by MPs was assessed by treating cultured NRK cells with inhibitors of the TNF-α, TRAIL and Fas signaling pathways. Activation of the Fas signaling pathway is not required for MP induced cell death as no change in survival was observed when NRK cells were treated with a neutralizing antibody to Fas ligand prior to the addition of MPs (Fig. 4A). When cultured cells were exposed to the TNF-α inhibitor SPD-30 or a neutralizing antibody to TRIAL an increase in cell survival was seen. When both SPD304 and the TRAIL peptide were applied together a significant increase was seen in cell survival above using either of the inhibitors alone, however, cell survival was still not 100% of the control value (Fig. 4B). Treatment of MPs with the TACE inhibitor TAPI-0 resulted in a significant increase in cell survival (Fig. 4C). PCR analysis of RNA extracted from cultured RBMVECs indicates the presence of transcripts coding for TNF-α receptor 1 and receptor 2, with TNFR1 being much more abundant, and TRAIL receptor 4 (Fig. 4d).

4. Discussion

Following the induction of chronic cerebral hypoperfusion in the rat there is a significant increase in the total number of MPs circulating in the plasma and in the number of MPs of endothelial origin at 72 h post surgery compared to sham operated rats. There is a

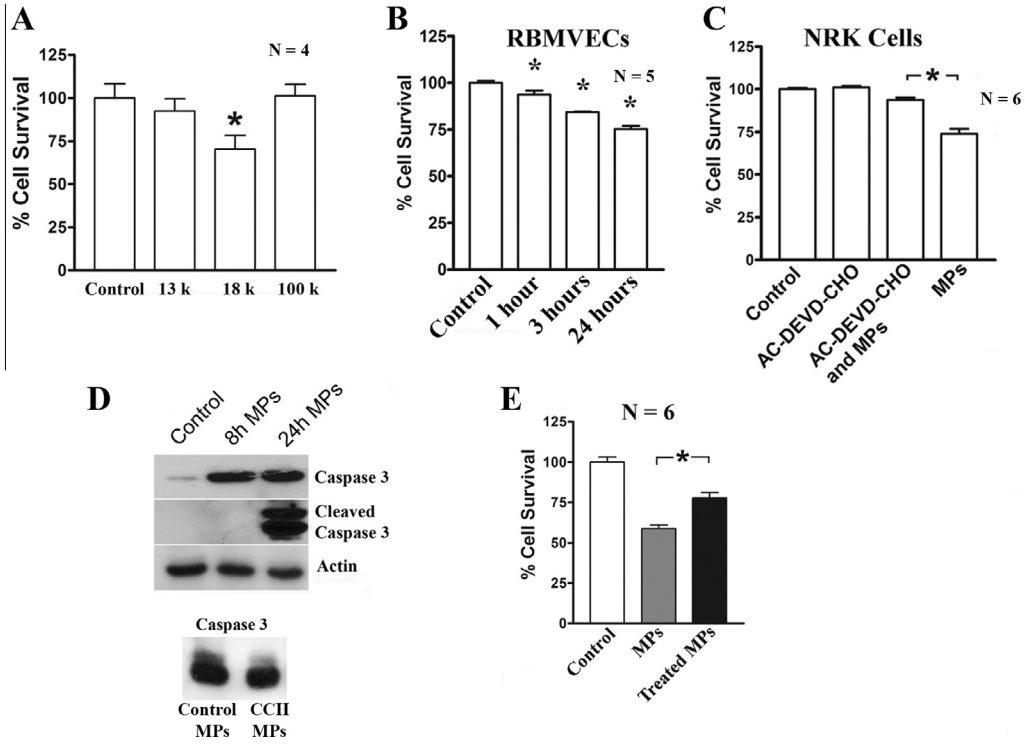

Fig. 2. Microparticles induce apoptosis in cultured RBMVECs and NRK cells. (A) Pellets from each centrifugation step were resuspended and applied to cultured NRK cells. (B) Exposure of cultured endothelial cells to MPs causes cell death in a time-dependent manner. (C) Caspase 3 inhibition protects NRK cells from MP-induced cell death. (D) Caspase 3 protein expression and activated caspase 3 are increased in NRK cells following treatment with MPs for 8 and 24 h and caspase 3 is present in the MP fraction (below). (E) MPs treated with AC-DEVD-CHO prior to treating NRK cells induce significantly less cell death.

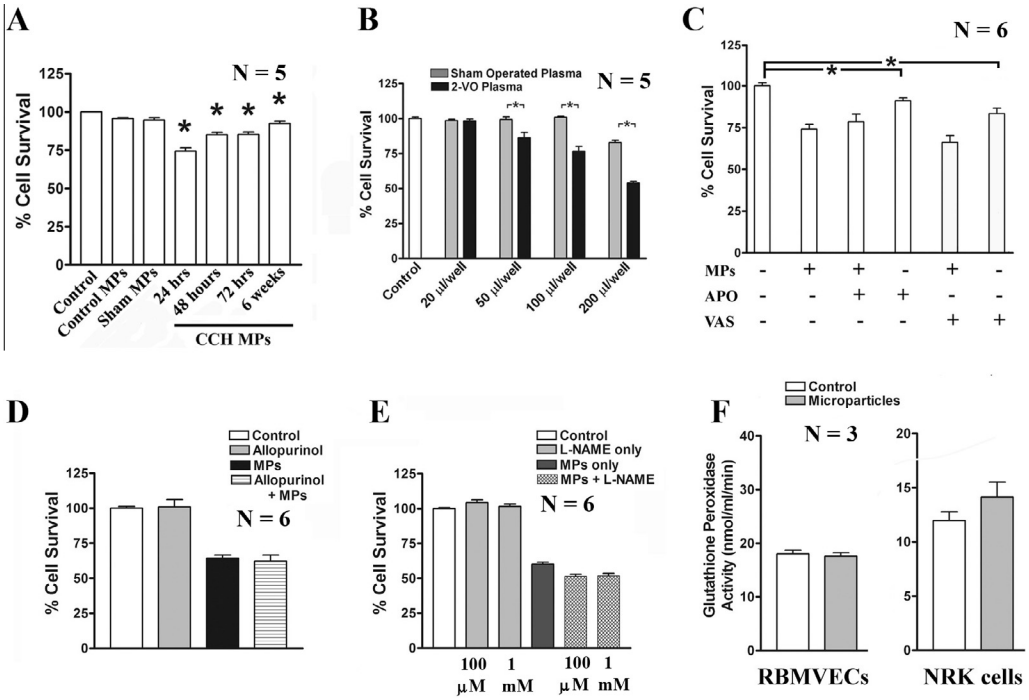

Fig. 3. (A) MPs CCH rats and sham rats induce apoptosis. (B) Plasma lethality is greatest 24 h following surgery and decreases thereafter. (C) Inhibition of NADPH oxidase does not protect against exposure to MPs. (D) Neither xanthine oxidase inhibition (D) nor nitric oxide synthase inhibition (E) is protective. (F) MPs do not increase glutathione oxidase activity in RBMVECs or NRK cells.

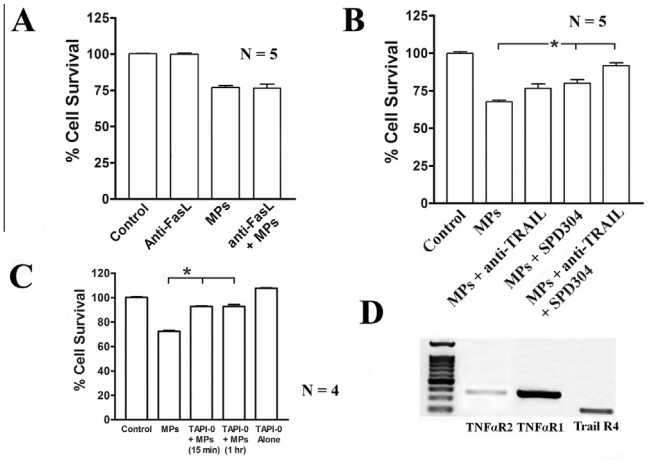

Fig. 4. Activation of cell death receptors by MPs. (A) A neutralizing antibody against Fas ligand did not affect cell survival following exposure to MPs. (B) Exposure of cultured RBMVECs to SPD-304 or a neutralizing antibody against TRAIL improved cell survival. (C) Treating MPs with a TACE inhibitor TAPI-0 improved cell survival. (D) mRNA encoding for TNFαR1, TNFαR2 and TRAIL R4 was detected in cultures of RBMVECs by PCR.

high degree of variance in the flow cytometry which may be due to variability in the response of individual rats to CCH or in the isolation and counting procedures. Examination of pellets by EM indicates that the particles carrying cell death signals are in the range of 300–400 nm in diameter and this is at the very limits of resolution for the flow cytometer. The increase in numbers of VE-cadherin positive particles in not great enough to account for the increase in total numbers, indicating that cells other than endothelial cells are releasing MPs in response to ischemia.

MPs were found to initiate cell death when applied to cultured RBMVECs and NRK cells. MPs isolated under various conditions have been shown to induce dysfunction in cultured tissue and apoptosis in cultured cells and a variety of different mechanisms have been identified [15–18]. MPs isolated under some conditions contain NADPH oxidase and impart oxidative stress to cultured cells [11,19]. Huang et al. [20] also report that MPs isolated from hypertensive patients caused an increase in H_2O_2 production, cellular senescence and apoptosis in endothelial progenitor cells. MPs isolated in the setting of cerebral ischemia in this study do not induce oxidative stress in cultured cells.

Three independent proapoptotic signals were identified in MPs in the present study; caspase 3 and activation of TRAIL and TNF-α receptors. MPs derived from human umbilical vein endothelial cells [21] and platelets [22] have been previously reported to contain caspase 3 and Schneider et al. [23] have suggested that circulating MPs induce apoptosis by the transfer of caspases into target cells. Abid Hussein et al. [21] suggest that MPs could be phagocytosed causing the release of caspase 3 within the cell and initiating apoptosis. However, caspase 3 is involved in many other functions outside of cell death and could be initiating apoptosis in some of the cells in a culture and involved in other cellular processes in other cells [24]. In this study treatment of MPs with a caspase 3 inhibitor significantly improved cell survival indicating that the MPs are imparting a proapoptotic caspase 3 signal.

MPs from various sources have been reported to contain ligands that activate some members of the death receptor family [25–30].

Treating RBMVECs or NRK cells with a neutralizing antibody to Fas ligand did not improve cell survival while antibodies to TRAIL and aTNF-α receptor blocker did. PCR analysis indicates that mRNA for TNFR1, TNFR2 and TRAIL receptor 4 are present in the cells. TRAIL may activate the extrinsic apoptotic pathway but in some circumstances may activate prosurvival or proliferative pathways [31]. In this case the proapoptotic pathway is activated in at least some cells. TNF-α is synthesized as a type two transmembrane protein and the ectodomain cleaved off by the sheddase TACE (ADAM17) to release soluble TNF-α ,which may then activate TNF receptors. The observation that treating MPs with the TACE inhibitor TAPI-0 prior applying them to cultured cells provides a significant improvement in cell survival indicates that TACE is present and active in MP membranes. Little is known about how

TACE activity is regulated but it is reasonable to assume that the active TACE in MPs was present in the plasma membrane of the cells of origin, as conditions that promote MP release such as activation and apoptosis are associated with inflammation. Given the small size of MPs it is not likely that they could continue to shed soluble TNF-α for an extended period of time. A recent study of MPs generated under conditions of cardiac stress found an immediate increase in circulating MPs of platelet, erythrocyte and endothelial origin that subsided within 1 h [32].

The data presented here indicate that chronic cerebral hypoperfusion causes an increase in numbers of circulating MPs and these MPs are able to transmit biologically relevant signals to target cells. Thus, MPs are dynamic participants in the response of tissues to various stressful conditions and play a crucial role in mediating cytokine signaling. The observation that TNF-α can promote the release of MPs from endothelial cells provides a mechanism to amplify responses to stress [33]. Also, the observation that MPs may activate receptors for the cytokines TNF-α and TRAIL on NRK cells provides a mechanism for the transmission of signals from an ischemic vascular bed to another, which might partly explain the multi-organ involvement with vascular disease in affected individuals [6,34].

Conflict of interest

None declared.

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