Original paper

Toxic effects on astrocytes of extracellular vesicles from CSF of multiple sclerosis patients: a pilot *in vitro* study

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Multiple sclerosis (MS) is an autoimmune and degenerative disorder of the central nervous system (CNS) that causes a progressive loss of motor and cognitive performances. Moreover, since the earlier phases, axonal loss as well as neuronal degeneration and a failure of oligodendrocytes to promote myelin repair have been demonstrated. In previous studies, it has been shown that the treatment of rat neuronal primary cultures with serum from MS patients can be toxic for neurons. Here we report a pilot investigation showing that CSF from patients contains extracellular vesicles (EVs) able to induce cell death in rat cultured astrocytes. Although these data are still preliminary, they suggest at least two notable considerations: i) EVs can be instrumental to pathology, and their concentration in CSF might be proportional to MS severity; ii) astrocytes can be part of the degenerative process. As a consequence, we propose that cultured astrocytes can be used as a model to study the toxicity of EVs from patients affected by MS at different stages. In addition, we suggest that EVs and their cargoes might be used as biomarkers of MS severity.

Key words: multiple sclerosis, extracellular vesicles, astrocytes.

Introduction

Multiple sclerosis (MS) is an autoimmune and degenerative disorder of the central nervous system (CNS) causing a progressive loss of motor and cognitive performances in affected people. The disease represents a big challenge because, from the earlier phases, axonal loss as well as neuronal degeneration and a failure of oligodendrocytes to promote myelin repair has been demonstrated [1, 2, 3, 4, 5, 6]. The disease course is characterized by phenotypic heterogeneity, most patients experiencing a relapsing remitting disease course from the onset, while a smaller proportion of people present a progressive disease from the beginning of symptoms [7]. It is not yet fully explained whether inflammation alone is responsible for the whole cascade of events leading to neurodegeneration or whether neurodegeneration in MS is an independent/parallel mechanism, even if correlated with inflammation. In all MS patients, the contribution of glial cells has been clearly shown since the early studies [8, 9, 10, 11]. The complex nature of this disorder is challenging, not only in the diagnostic phase but also for monitoring disease activity, progression, and treatment efficacy.

Interestingly, it has also been found that the prevalence of MS tends to increase with increasing latitude, but the underlying causes of such distribution still remains elusive, although it has been recently hypothesized that the disruption of circadian rhythms might be involved [12].

At the cellular level, it is still unclear whether other cells, in addition to neurons and oligodendrocytes, are involved and through which mechanisms.

Previously it has been shown, however, that in inflammatory processes, the functional disturbance of astrocytes is one of the crucial mechanisms in the earliest phases. These phenomena, occurring at the perivascular glia limitans, are also associated with expansion of the inflammatory response (mainly innate immunity), and with active immune-mediated myelin and axonal damage [13].

Extracellular vesicles (EVs) are membrane-bound structures that can either directly bud from the plasma membrane (ectosomes, or microvesicles – MVs), or originate from exocytosis of multivesicular bodies (MVBs), components of the endosomal compartment (exosomes) [14]. EVs contain many different molecules (proteins, lipids, and nucleic acids), some of which are specifically sorted to them by a variety of mechanisms [15, 16, 17, 18]. Once released from a given cell, EVs may be recognized and bound by specific receptors present on the surrounding cells, or may fuse with the latter; alternatively, they can break, releasing their content into the extracellular matrix. Thus, EVs can be used to discard waste molecules but also have the potential to deeply affect the phenotype of the surrounding cells [19, 20, 21, 22, 23, 24, 25, 26].

In the central nervous system (CNS) of mammals, EVs are involved in a variety of physiological processes, such as establishment of synapses, neuronal plasticity, metabolic exchanges, and so on [27, 28, 29]. However, under pathological conditions, EVs can also be involved in spreading pathological proteins [30, 31, 32]. Moreover, EVs can be found in biological fluids such as serum, saliva, amniotic fluid and synovial fluid, breast milk and urine [33, 34, 35, 36], and, most important, they are able to cross the blood-brain barrier (BBB) [37, 38]. Therefore, it has been proposed that they and their contents can be used as biomarkers of specific brain pathologies [39, 40, 41, 42, 43].

We previously found that the serum from patients with secondary progressive multiple sclerosis (SPMS) has a damaging effect on isolated neurons, thus suggesting that neuronal damage in MS could be a primary event and not only secondary to myelin damage, as generally assumed. Moreover, SPMS affected the permeability of an *in vitro* BBB model [44]. To shed more light on the possible involvement of astrocytes, we exposed rat primary cultures of astrocytes to EVs isolated from cerebral spinal fluid (CSF) of MS patients, in order to study the potential ability of these EVs to induce, in the recipient cells, morphological and functional changes, such as modifications of the proliferation rate, and cell death. Our study also aimed at exploring whether EVs present in CSF might be used as early biomarkers of MS pathology.

Material and methods

Patient selection

Recruited patients were screened at the Department of Medicine, Neurosciences and Advanced Diagnostics (Bi.N.D.), University Hospital of Palermo. The study included 16 consecutive individuals who, as part of the diagnostic process, underwent lumbar puncture. After screening they were separated into two different groups: MS patients and controls. Diagnoses of MS were confirmed according to McDonald criteria as revised in 2010 by trained neurologists [45]. Recruited controls were individuals affected by other neurological disorders who underwent the spinal tap because of a non-inflammatory condition such as idiopathic intracranial hypertension or normal pressure hydrocephalus. Lumbar puncture was performed as part of the diagnostic work-up in fasting conditions and according to the AAN guidelines [46].

Ethics approval and consent to participate

Permission to conduct the study was obtained from the Ethical Committee of the University of Palermo (CE Palermo 1), which approved the research project. All the recruited patients gave signed informed consent to participate in a molecular study on multiple sclerosis.

Concerning primary cultures of astrocytes, no special permission was necessary because we did not use animals for this study; instead, aliquots of astrocytes purified in previous years and frozen were thawed and used for these experiments.

Isolation of microvesicles from CSF

Extracellular vesicles were prepared from CSF through a method already described, based on differential centrifugation [47]. Briefly, the various samples of CSF were first centrifuged at 2,000 × g for 10 min and then at 4,000 × g for 15 min, in order to eliminate major debris.

The 4,000 × g supernatant was centrifuged at 10,000 × g for 30 min. At the end, pellets containing larger vesicles (presumably MVs) were resuspended in 50 μl of PBS (Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+}) and frozen. The 10,000 × g supernatant was filtered through a 0.2-μm filter and ultracentrifuged (Beckman; rotorTi60) at 105,000 × g for 90 min, at 4°C. The final supernatant was eliminated, while the pellet containing smaller vesicles (presumably exosomes, Es) was suspended in 50 μl of PBS and frozen.

Protein concentration from both larger and smaller vesicles was determined using a Qubit Protein assay kit (Q33211, Invitrogen, OR, USA).

Cell culture, immunofluorescence and acridine orange (AO)/ethidium bromide (EB) assay

As mentioned above, in the present study we did not purify astrocytes ex novo, but, instead, used aliquots of astrocytes purified in previous years and frozen, which can be stored in liquid nitrogen almost indefinitely. In detail, these astrocytes (primary astrocytes) had been isolated from the brain cortices of 2-day old Wistar newborn rats (Harlan, Udine, Italy), and frozen in a solution containing 93% heat-inactivated fetal calf serum (FCS) and 7% dimethyl-sulfoxide (both from Sigma-Aldrich; Merck KGaA), as previously described [48]. For the present study, astrocytes were thawed and cultured in NIH (DMEM/Ham's F-12 (2/1), supplemented with 10% heat-inactivated FCS (Sigma-Aldrich; Merck KGaA), and 100,000 units of penicillin, 100 mg of streptomycin, and 250 μg of amphotericin B (Sigma-Aldrich; Merck KGaA) per liter. The cells were then maintained in humidified 5% CO_2/95% air, at 37°C.

In order to analyze the effects on cells of the EVs isolated from CSF, primary astrocytes were counted in a Thoma counting chamber and plated in 24-well plates containing glass coverslips, at an initial concentration of 50,000/well. Before treating them, cells were cultured for 24 h. Moreover, after 24 h, a couple of coverslips were used for ascertaining that astrocytes were morphologically intact. With this aim, cells were fixed with 96% ethanol on ice for 10 min and permeabilized for 5 min with 0.1% Triton X-100 in PBS. Finally they were incubated with a polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Sigma-Aldrich; Merck KGaA; cat. no. G9269), used at 1:200 dilution. The secondary antibody was rhodamine-isothiocyanate-conjugated anti-rabbit-IgG (Sigma-Aldrich; Merck KGaA; cat. no. T6778; used at a 1:100 dilution). Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (H1200; Vector Laboratories, Inc.). Cells were finally observed using an Olympus BX-50 fluorescent microscope (Olympus Italia SRL, Segrate, Italy).

After confirming their good starting shape, astrocytes were treated with 15 μg of exosomes or 5 μg of MVs from patients or from the control group. After additional 24 h, cell death levels were evaluated by staining astrocytes with an acridine orange/ethidium bromide (AO/EB) mixture (100 μg/ml in PBS) [44]. Cells were finally observed with an Olympus BX-50 fluorescent microscope as above. Counting of viable cells was performed after dividing each picture into quarters. Cells in each quarter were counted by two different operators. Finally, the values were used to calculate a mean value.

Statistical analysis

The results were expressed as mean ± standard error (SE) of the mean. Differences were considered significant at $p \leq 0.05$.

Results

In order to be sure that primary astrocytes had the expected morphology after thawing and culturing for 24 h, some samples were immune-stained with an anti-GFAP antibody. As shown in Fig. 1, astrocytes showed the expected shape for astrocytes cultured in two dimensions [49].

The same morphology can be also observed in Fig. 2A (not treated, control astrocytes).

Notably, astrocytes exposed to MVs or exosomes (Es) purified from control CSF (Fig. 2B, D, respectively), or to MVs purified from MS patients (Fig. 2C) do not show significant vitality differences with respect to the untreated astrocytes. On the other hand, astrocytes treated with Es from MS patients (Fig. 2E) show a dramatic increase of cell death.

As described under "Materials and methods" viable cells were counted by two different operators, after dividing each picture into quarters. The counting results for each condition were used to calculate mean values. As reported in Table I, the vitality of untreated astrocytes was around 99%, similar to that of astrocytes exposed to MV or Es purified from control CSF. Moreover, although MVs from MS patients tended to induce a higher degree of cell death with respect to those prepared from the control CSF (89.3 ±18.0 vs. 99.3 ±1.8 of dead cells), the difference was not statistically significant (p value = 0.142). On the other hand, a clear toxic effect was highlighted when

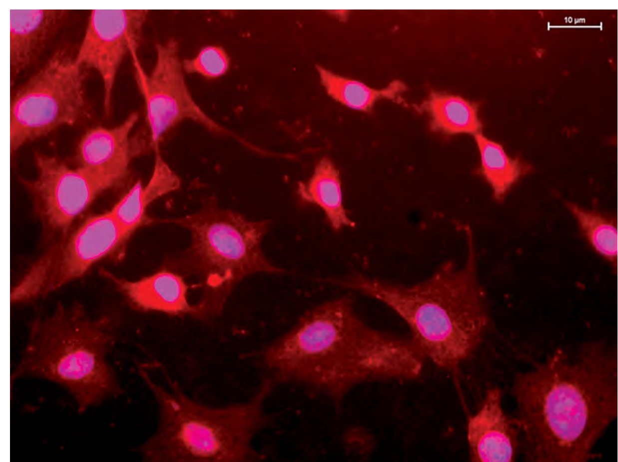

Fig. 1. Astrocytes cultured for 24 h on glass coverslips. Cells were then immune-stained with anti-glial fibrillary acidic protein antibodies, as described in the Material and methods section, and observed using an Olympus BX-50 fluorescent microscope (Olympus Italia SRL, Segrate, Italy). Scale bars, 10 μm

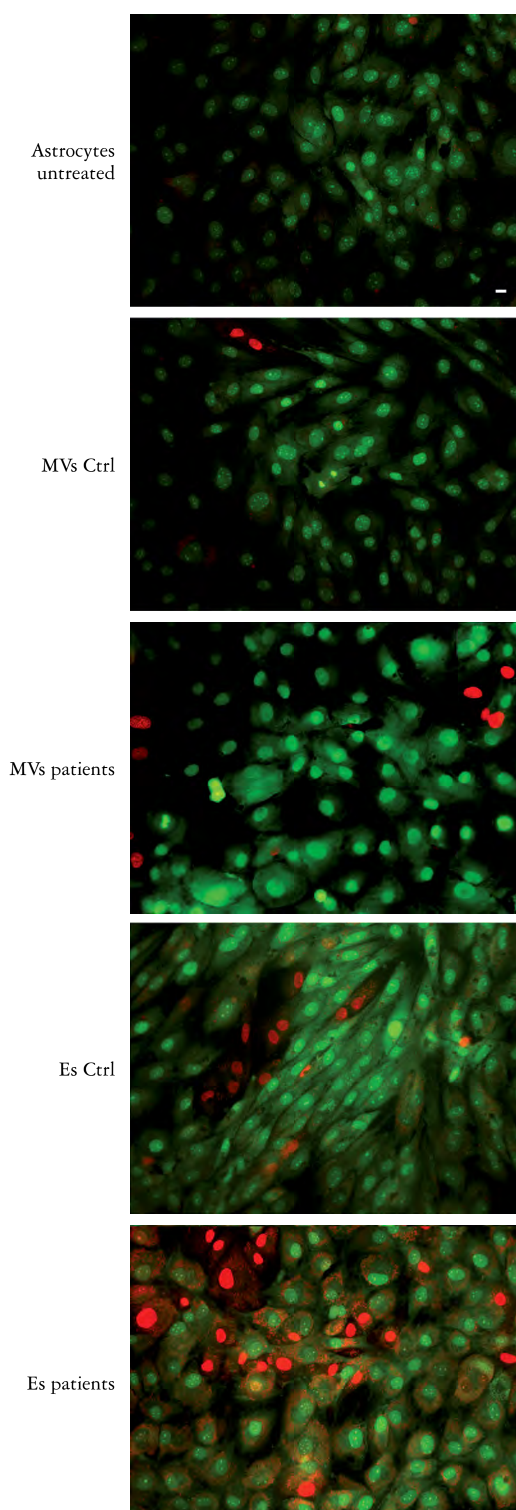

Fig. 2. Cell viability evaluation: 15 μg of putative exosomes or 5 μg of putative MV from patients or from the control group were added to astrocytes already cultured for 24 h. After an additional 24 h, cell death was evaluated by staining astrocytes with an AO/EB mixture (100 μg/ml in PBS). Cells were observed with an Olympus BX-50 fluorescent microscope (Olympus Italia SRL, Segrate, Italy). A) Untreated astrocytes; B) astrocytes treated with MVs from controls; C) astrocytes treated with MVs from MS patients; D) astrocytes treated with Es from controls; E) astrocytes treated with Es from MS patients

the astrocytes were treated with the exosome fraction from MS patients (Es): a significantly higher degree of cell death was indeed observed in comparison with the effects induced by Es from controls (71.2 ±28.2 vs. 94.3 ±14.6; p value = 0.0001). Notably, since some of the CFS samples had been frozen, while other samples were used fresh, we could also note that MVs and Es prepared from fresh MS CSF had a stronger effect on astrocytes' vitality than those from frozen CSF (51.9 ±28.9 vs. 70 ±21.21).

Discussion

Normally, all the cells of the central nervous system can produce EVs, probably involved in a special form of cell-to-cell communication, aimed at coordinating the responses of the entire brain to both external and internal signals and stresses [27, 28, 29]. Under pathological conditions, however, it is known that the same mechanism is used by altered proteins and aggregates to spread among the cells, thus horizontally transferring the pathology itself [30, 31, 32].

These observations have been fostering the idea that EVs can be part of the pathological processes. In a previous paper, we reported that the serum from patients with SPMS could damage isolated primary neurons in culture, thus suggesting that neuronal damage in MS could be a primary event and not only secondary to myelin damage [44]. We thus asked whether the toxic effects might be due to molecules carried by extracellular vesicles, and decided to start our analysis by purifying EVs from CSF. Moreover, in consideration of the fact that astrocytes form with neurons a metabolic and functional unit of the most importance for the CNS activities, and are probably involved in all neuronal functions and dysfunctions [50, 51, 52, 53, 54], we chose these cells for our analyses.

This pilot study suggests that EVs released into the CSF of patients affected by MS do contain molecules with effects toxic to astrocytes. We found that EVs (and, in particular, the smallest vesicles, probably exosomes) coming from the CSF of MS patients are significantly more toxic than those purified from CSF of patients with other, less critical disorders. Interestingly, toxicity was demonstrated after adding EVs to astrocytes, thus confirming that these cells, and not only oligodendrocytes and neurons, are sensitive to the damaging factors produced in MS patients.

As a pilot study, our analysis has a few limitations: first, two separate fractions of EVs were isolated through standard centrifugation methods, but we did not confirm their composition by biochemical analyses or by nanoparticle tracking analysis (NTA). In addition, although the existence of larger and smaller vesicles, as well as the different cellular origins of different EV classes, is widely accepted, there

Table I. Percentages (means ±SEM) of viable astrocytes after treatment with putative MVs (microvesicles) or Es (exosomes) from control CSF and from MS patients' CSF. As a starting control untreated astrocytes were also used

Untreated Astrocytes	Astrocytes treated with MVs			Astrocytes treated with Es		
	Ctrl	Patients	p Value	Ctrl	Patients	p Value
99.3 ±1.8	98.5 ±3.73	89.3 ±18.0	0.142	94.3 ±14.6	71.2 ±28.2	0.0001*

**Significant differences at the 0.001 levels*

is still an open controversy on the methods that can accurately allow one to distinguish among them, and on their nomenclature [55, 56]. For these reasons we tentatively identified our fractions as larger vesicles (putative MVs) and smaller vesicles (putative exosomes).

A second limitation comes from the small sample of patients enrolled.

On the other hand, it is worth noting that the CSF samples used in this study derived from individuals under the diagnostic process and, hence, not yet under any kind of disease-modifying therapy. Moreover, the patients underwent the lumbar puncture even before starting steroid therapy if they were potentially in a relapse phase to avoid the possibility that steroid treatments could impact the results of the diagnostic examination. Thus the present study not only confirmed that EVs have a pathogenic role in MS disease and act directly on cell survival, but suggested that this can happen even before a clear diagnosis of MS.

The observed effects on cell survival are particularly evident for the smallest vesicles (probably exosomes), and suggested that a correlation might exist between their concentration in the CSF and the severity of the pathology. Notably, EVs prepared from fresh MS CSF had a stronger effect on astrocytes' vitality than those from frozen samples.

On the basis of these results, we thus hypothesize that astrocyte damage is a further aspect of the sustained degenerative process found in MS, and that cultures of astrocytes could be used as *in vitro* models of EV toxicity and hence of MS degree. In addition, astrocyte cultures could be of help as further diagnostic criteria to distinguish MS from other autoimmune, inflammatory diseases of the central nervous system, such as neuromyelitis optica (NMO). This latter disorder is a demyelinating disease that affects the optic nerve and the spinal cord, and is distinct from MS, although the clinical manifestations of MS and NMO partly overlap [13, 57, 58]. In 2004, it was found that the serum from patients with NMO contains autoantibodies directed against the astrocytic protein aquaporin 4 (AQP4) [59]. More recently, it was suggested that these antibodies can interfere with the systemic and intra-cerebral synthesis of transforming growth factor β1 (TGF-β1), thus abolishing an important anti-inflammatory response [60]. Thus astrocytic cultures might also be used to study the specific molecular pathways triggered by serum/CSF as well as of EVs from patients affected by similar but different disorders.

Of course, first of all, further studies are necessary to confirm the present results, and to identify the toxic molecules present in EVs.

As a final comment, the ability of EVs to cross the blood-brain-barrier suggests that they and their cargoes might be used as biomarkers of MS (and of its severity) even before a clear diagnosis has been established.

The authors declare no conflict of interest.

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