Paracrine Diffusion of PrP\textsuperscript{C} and Propagation of Prion Infectivity by Plasma Membrane-Derived Microvesicles

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**Abstract**

Cellular prion protein (PrP\textsuperscript{C}) is a physiological constituent of eukaryotic cells. The cellular pathways underlying prions spread from the sites of prions infection/peripheral replication to the central nervous system are still not elucidated. Membrane-derived microvesicles (MVs) are submicron (0.1–1 \( \mu \)m) particles, that are released by cells during plasma membrane budding processes. They are usually liberated from different cell types, mainly upon activation as well as apoptosis, in this case, one of their hallmarks is the exposure of phosphatidylserine in the outer leaflet of the membrane. MVs are also characterized by the presence of adhesion molecules, MHC I molecules, as well as of membrane antigens typical of their cell of origin. Evidence exists that MVs shedding provide vehicles to transfer molecules among cells, and that MVs are important modulators of cell-to-cell communication. In this study we therefore analyzed the potential role of membrane-derived MVs in the mechanism(s) of PrP\textsuperscript{C} diffusion and prion infectivity transmission. We first identified PrP\textsuperscript{C} in association with the lipid raft components Fyn, flotillin-2, GM1 and GM3 in MVs from plasma of healthy human donors. Similar findings were found in MVs from cell culture supernatants of murine neuronal cells. Furthermore we demonstrated that PrP\textsuperscript{Sc} is released from infected murine neuronal cells in association with plasma membrane-derived MVs and that PrP\textsuperscript{Sc}-bearing MVs are infectious both in vitro and in vivo. The data suggest that MVs may contribute both to the intercellular mechanism(s) of PrP\textsuperscript{C} diffusion and signaling as well as to the process of prion spread and neuroinvasion.

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**Introduction**

Prion diseases are a complex group of fatal neurodegenerative disorders that affect humans and a wide variety of animals and are characterized by strong neuronal cell loss, spongiform vacuolation and astrocytic proliferation [1]. According to the “protein-only” hypothesis PrP\textsuperscript{Sc}, the misfolded form of normal cellular prion protein (PrP\textsuperscript{C}), is the infectious agent that may convert PrP\textsuperscript{C} to PrP\textsuperscript{Sc} in a self-propagating reaction [2]. Prions accumulate not only in the central and peripheral nervous system but also in extracerebral compartments, such as secondary lymphoid organs and muscles. However, the only organ system in which severe histopathological damage can be demonstrated as a consequence of infection with prions is the nervous system. Prion diseases are typically initiated by infection of peripheral sites, as in the case of bovine spongiform encephalopathy (BSE), variant Creutzfeldt–Jakob disease (vCJD), Kuru, and most cases of iatrogenic Creutzfeldt–Jakob disease (iCJD). The mechanisms by which prions spread from the site of peripheral exposure, such as the gastrointestinal tract, to the lymphoreticular system where a first replication phase occurs and subsequently to and within the central nervous system are still not completely elucidated [3,4,5]. Although different cell types of the immune system, such as B lymphocytes [6,7,8] follicular dendritic cells [9,10,11], macrophages [12,13] and dendritic cells [14,15,16,17], and the peripheral nervous system [18,19,20] have been recognized as key players in the process of prion neuroinvasion, relatively little information is available about the mechanism(s) underlying intercellular prion transfer.

Microvesicles (MVs) are submicron (0.1–1 \( \mu \)m) [21,22], membrane-bounded vesicles which are released both from the cell surface of normal healthy or damaged cells. Shedding of membrane-derived MVs is a physiological phenomenon that usually accompanies cell activation and growth. MVs are characterized by the presence of adhesion molecules, MHC I molecules, as well as of membrane antigens typical of their cell of origin [21–23]. They are normal constituents of blood (5–50 \( \mu \)g/ml), and are secreted by leukocytes, endothelium, platelets and erythrocytes. The number of MV circulating in peripheral blood increases during cell injury, apoptosis, inflammation, thrombosis and platelet activation [23]. Although the molecular basis of protein sorting during MVs formation is not fully understood, they result from an exocytotic budding process involving lipids and proteins metabolism. The
segregation of specific proteins is followed by blebbing of the membrane surface, leading to the formation of MVs and their release in the extracellular environment. Some evidence shows that MVs components mainly arise from lipid rafts, which therefore might be involved in setting up sorting platforms to concentrate specific proteins within MVs, which could be destined to extracellular secretion [24].

The functional role of MVs is still largely unknown, however recent evidence suggests that MVs are important modulators of cell-to-cell communication and play an important pleiotropic role in many biological processes. Indeed, MVs may possibly act as paracrine vectors of transcellular exchange of messages between circulating and endothelial cells [24], participate in a variety of intracellular adhesion processes, and induce cellular responses [23].

Exosomes, which measure about 50-90 nm in diameter, are membrane vesicles released into the extracellular environment upon exocytic fusion of multivesicular endosome with the cell surface. They have a particular composition [26] reflecting their origin in endosomes as intraluminal vesicles. It is assumed that exosomes are non-plasma-membrane-derived vesicles. Moreover, exosomes may “hijack” infectious particles such as the immuno deficiency virus (HIV) from the cytoplasm of the releasing cells [27,28] and possibly even whole intact organelles such as the mitochondria [29].

The release of PrPSc and infectious PrPSc by prion infected epithelial, neuronal and neuronal cells in association with exosomes has recently been highlighted [30–32], suggesting that PrPSc-bearing exosomes may provide a mechanism for intercellular transmission of infectious prions in addition to cell–cell contact. It has furthermore been shown that endogenous PrPC is associated with exosomes released by blood platelets [33], and a number of studies have demonstrated that prions are present in blood and blood components,uffy coats, plasma, and platelets in animal models [34,35]. It has also been demonstrated that blood as well as plasma of animals experimentally infected with TSEs can efficiently transmit prion infection by transfusion [35–37]. Of concern, is the finding that vCJD is most probably efficiently transmitted between human patients by blood transfusion [38–40]. This infection process, in the absence of sensitive vCJD blood tests for screening of blood components donations, could lead to a horizontal spread of vCJD within the human population.

The aims of this study were to evaluate the potential role of plasma membrane-derived MVs in the mechanism(s) of PrPSc diffusion and prion infectivity transmission in vitro and in vivo and to characterize the interactions of PrPSc with lipid raft components in MVs. Here, we demonstrate that PrPSc is associated with MVs from plasma of human healthy donors and with MVs shed by murine neuronal cells. We further show that in MVs PrPC associates with lipid raft components such as Fyn, flotillin-2, GM1 and GM3. Furthermore, we demonstrate for the first time that mouse neuronal Neuro-2a cells, endogenously expressing murine PrP, release PrPC and PrPSc in association with plasma membrane-derived MVs when infected with a mouse-adapted scrapie strain. Moreover, we show that PrPSc-bearing MVs can transmit prion infectivity both in vitro and in vivo, indicating that MVs contribute both to the intercellular mechanism(s) of PrPSc diffusion and to the process of infectious prions intercellular trafficking. We therefore postulate that MVs may act as carriers of prion infectivity during prion neuroinvasion and may also contribute to blood transfusion-mediated transmission of prion diseases.

**Materials and Methods**

**Cell lines**

Marine Neuro-2a cells (American Type Culture Collection ATCC CCL 131) were maintained in DMEM (Sigma, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Germany), 4 mM L-Glutamin (Sigma, Germany) and antibiotics (200 U/ml penicillin and 0.2 mg/ml streptomycin (Sigma, Germany) at 37°C in a humidified 5% CO2 atmosphere. Murine Neuro-2a PK1 cells and Rocky Mountain Laboratory strain (RML)-infected Neuro-2a PK1 cells [41] were maintained in Opti-MEM (Invitrogen, Germany) supplemented with 10% (FCS), 4 mM L-Glutamin and antibiotics (200 U/ml penicillin and 0.2 mg/ml streptomycin). The cell lines were maintained at 37°C in a humidified 5% CO2 atmosphere.

**Antibodies**

Monoclonal anti-PrP antibody 6H4 (Prionics Switzerland), Anti-mouse immunoglobulin-colloidal gold (Biocell, UK), Monoclonal anti-PrP antibody SAF 32 (SPI Bio, Italy), Polyclonal anti-PrP antibody C-20, polyclonal anti-PrP PE-conjugated antibody (C-20 PE), polyclonal anti-Fyn antibody FYN3, polyclonal anti-Flotillin-2 antibody, polyclonal anti-Tsg101 (M-19) were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). Monoclonal anti-GM2 and anti-GM3 [42] were purchased from Seikagaku (Corp. Chuo-ku, Tokyo, Japan). PE-conjugated anti-mouse IgG, HRP-conjugated cholina toxin, B subunit (HRP-CTxB), HRP-conjugated anti-mouse IgG (anti-mouse IgG-HRP), HRP anti-mouse IgM (anti-mouse IgM-HRP), HRP-conjugated anti-rabbit IgG (anti-rabbit IgG-HRP), HRP anti-goat IgG (anti-goat IgG-HRP), IgG from goat serum with irrelevant specificity (IgG irrelevant), monoclonal anti-GAP 43 antibody, were purchased from Sigma Chem. Co. (St Louis, MO, USA). B-PE-conjugated IgG from goat serum with irrelevant specificity (IgG B-PE irrelevant) was purchased from ICN Biomedicals (Milano, Italy). Monoclonal anti-Alix IgG1 3A9 was purchased from Cell Signaling (USA) Antibody (anti-a-synuclein). IgG1 3A9 was kindly provided by Dr Jean Gruenberg. PE-conjugated anti-human MHC-I was purchased from eBioscience (San Diego, CA, USA).

**Preparation of platelet-free plasma samples for MVs analysis**

For platelet-free plasma preparation, 5 ml blood samples were drawn by venipuncture into 0.129 M trisodium citrate. MVs were separated from whole blood within 1 h by two sequential centrifugations: 15 min at 1500 g, followed by a 1 min decantation at 13000 g to remove all the residual platelets or cell fragments of similar size [43].

Fifty μl of platelet-free plasma were labeled with 2 μg anti-PrP (C-20 PE), at +4°C. The fluorescence intensity was analyzed with a IL Coulter cytometer (Coulter Electronics, Hialeah, FL) and compared with a sample of platelet-free plasma, labeled with goat IgG B-PE irrelevant. The size distribution of MVs was 0.1 to 1 μm; MVs were gated on the basis of forward angle light scatter and 90° light scatter parameters, using polystirene latex beads (Sigma, St Louis, MO, USA) 0.8 μm as standard. To distinguish eventual residual platelets in our sample, we compared the MVs preparation with a platelet-enriched plasma, stained by anti-CD41 MoAbs (data not show).

Separately, in parallel experiments MVs, collected by centrifugation at 20000×g, were resuspended in 50 μl of sample buffer and analyzed by Western Blot [44], using the anti-PrP 6H4, and then anti-mouse IgG-HRP, anti-Fyn (FYN3) and then anti-rabbit IgG-HRP, or anti-flotillin-2 and then anti-goat IgG-HRP. Immunoreactivity was assessed by chemiluminescence reaction using the ECL Western blocking detection system (Amersham Biosciences). As a control, cells were separated from human peripheral blood (PB) by centrifugation and resuspended in cold
lysis buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS]. Lysates were pelleted at 20000×g for 10 min at 4°C and supernatants were transferred to a clean microfuge tube. Protein concentration for cell lysates and MVs was determined by Bradford assay as recommended by the manufacturer’s guidelines (Roth, Germany).

Isolation of MVs from cell culture supernatants

Neuro-2a or Neuro-2a PK1 cells (2–4×10⁷ cells) were cultured for 2–3 days prior to MVs isolation by sequential centrifugation protocol [45,46]. Cellular debris was removed by two consecutive centrifugation steps at 4500×g for 5 min at RT. Supernatants were centrifuged at 20000×g for 1 h at 4°C. MVs were pooled, washed in either PBS or Opti-MEM (Invitrogen), repelleted and then either resuspended in Opti-MEM for in vitro immunoblot analyses. MVs were isolated both from uninfected (MVs uninfect.) and RML-infected (MVs infect.) Neuro-2a PK1 cells as previously described and resuspended in Opti-MEM. MVs were incubated for 48 hrs with noninfected Neuro-2a PK1 cells. RML and mock brain homogenates (1% w/v homogenates) were included as positive and negative controls, respectively. Prior infection was assayed by the presence of PrPsc by cell blot assay.

Immunochemistry

Neuro-2a PK1 cells were incubated with the anti-PrP 6H4 antibody for 1 h at 4°C. Cells were washed in PBS and then were fixed with 1% paraformaldehyde for 1 h at 4°C, washed and labeled with anti-mouse immunoglobulin-gold colloid (15 nm) for 30 min at RT and then in sodium tetroxide 1% in Veronal acetate buffer, pH 7.4, for 2 h at 4°C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone and embedded in Epon 612. Samples were examined under an electron microscope (Zeiss, Germany).

Western blot analysis

Eighty micrograms of total protein of each cell lysate and 20 µg of each MVs were electrophoresed through a 12% SDS polyacrylamide gel. When proteinase K digestion was performed prior to immunoblot analysis, 250 µg of cell lysates and 20 µg of MVs preparations were digested for 1 h at 37°C (0.1 µg or 0.3 µg or 1 µg PK (Roche Diagnostics NL, Germany) per 20 µg total protein). Proteinase K digestion was stopped with 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma, Germany). Proteins were transferred to PVDF membranes (Immobilon-P; Millipore, USA) by semi-blotting. Membranes were blocked at RT for 1 h with Tris-buffered saline/0.05% Tween 20 (TBST)/5% nonfat dry milk, incubated with the appropriate primary antibody diluted in Tris-buffered saline/0.05% Tween 20 (TBST)/1% nonfat dry milk (for PrP, anti-PrP SAF-32 or anti-PrP 6H4; anti-GAP-43; anti-flotillin-2; anti-Fyn FYN3; anti-Alix 3A9; anti-Tsg101 (M-19), overnight at 4°C. After washing with TBST, membranes were incubated for 1 h at room temperature to horseradish peroxidase-conjugated secondary antibody diluted in the same buffer as above. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia, Germany).

Immunoprecipitation experiments

Briefly, blood plasma-derived and Neuro-2a cells-derived MVs were lysed in lysis buffer (20 mM HEPES, pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, including protease inhibitors). After preclaring, the supernatant was immunoprecipitated with anti-PrP polyclonal antibody C-20 plus protein A-acrylic beads. The immunoprecipitates were split into two aliquots.

The first one was subjected to ganglioside extraction according to the method of Svennerholm and Fredman [47]. The eluted glycosphingolipids were dried and separated by high-performance-thin-layer-chromatography (HPTLC) aluminium-backed silica gel 60 (20×20) plates (Merck, Darmstadt, Germany). Chromatography was performed in chloroform:methanol:0.25% aqueous KCl (5:4:1) (v/v/v). Plates were immunostained for 1 h at room temperature with HRP-CTxB, or, alternatively, with anti-GM2 IgM or anti-GM3 IgM and then with anti-mouse IgM-HRP. Immunoreactivity was assessed by chemiluminescence reaction using the ECL Western blotting detection system (Amersham, Buckinghamshire, UK). Alternatively, the immunoprecipitates were subjected to Western Blot analysis by anti-Fyn FYN3 and then with anti-rabbit IgG-HRP.

The immunoprecipitates were checked by Western blot, using the anti-PrP monoclonal 6H4 antibody.

In vitro infectivity assay

Noninfected Neuro-2a PK1 cells were seeded in six-well plates 24 hrs prior to infection. MVs were isolated from uninfected (MVs uninfect.) and RML-infected (MVs infect.) Neuro-2a PK1 cells as previously described and resuspended in Opti-MEM. MVs were incubated for 48 hrs with noninfected Neuro-2a PK1 cells. RML and mock brain homogenates (1% w/v homogenates) were included as positive and negative controls, respectively. Prior infection was assayed by the presence of PrPsc by cell blot assay.

Cell blot assay

Cells were cultured on glass coverslips (Roth, Germany) until confluence, washed with CMF-PBS, and placed cell side down on lysis buffer I [50 mM Tris-Cl, pH 8.0; 150 mM NaCl, 0.5% sodium deoxycholate; 0.5% Triton X-100]–soaked nitrocellulose membranes (Hybond-C-Extra, Amersham, Germany) that were previously laid on a lysis buffer-soaked filter paper. Membranes were then processed as described [41]. Briefly, membranes were dripped at RT, coverslips were carefully removed and then the membranes were incubated in a Proteinase K solution [5 µg/ml PK in lysis buffer] for 90 min at 37°C. After twice wash in dH2O, PK digestion was stopped by incubating the membranes in 2 mM PMSF for 10 min at RT. Membranes were then treated with 3 M guanidinium thiocyanate in 10 mM Tris-Cl (pH 8.0) for 10 min. After extensive washing in dH2O, membranes were processed for PrPsc detection by the use of the anti-PrP 6H4 as described above.

In vivo infectivity bioassay

MVs were isolated both from uninfected and RML-infected Neuro-2a PK1 cells, as previously described, and resuspended in PBS, 0.2 ml. Uninfected and infected Neuro-2a PK1 cells were collected and resuspended in 1 ml PBS. MVs and cells were subjected to five consecutive cycles of freeze and thawing. Protein concentration for cell lysates and MVs was determined by Bradford assay, as recommended by the manufacturer’s guidelines. Samples were then adjusted to 10 µg total protein per 30 µl with PBS/5%BSA. Different dilutions of mouse brain homogenate infected with the Rocky Mountain Laboratory (RML) scrapie strain (passage 5.0, 1×10⁷ LD50/ml 10% brain homogenate) [48] in PBS/5% BSA were used as positive controls, whereas mock infected brain homogenate was included as negative control.

Thirty microfilters for each sample were administered intracerebrally to groups of four tga20 mice [49]. Disease in animals was diagnosed when at least three of the following symptoms were observed: foot claspings of hindlegs when mice were lifted by the tail, plastic tail; decreased motor activity; mincing gait, disorientation; mild hind leg paresis, ataxia; kyphosis. Incubation time to terminal scrapie sickness was determined and infectivity titers were calculated by using the relationship $y = 11.45 - 0.088 x$, where $y$ is logLD50/ml.
homogenate and x is incubation time in days to terminal disease, was used to calculate infectivity titres. The presence of a protease-resistant isoform of PrP (PrP<sup>Sc</sup>) in the infected brains was investigated on proteinase K-treated (20 μg/ml; 30 min; 37°C) homogenates by Western blot analysis, as described above.

**Results**

**Presence and association of PrP<sup>C</sup> and lipid raft components in human plasma-derived microvesicles**

MVs are normal constituents of blood plasma and are secreted by leukocytes, erythrocytes, platelets and endothelium [50]. To investigate the presence of PrP<sup>C</sup> in cell membrane-derived MVs from plasma obtained from healthy human peripheral blood donors, MVs were isolated by sequential centrifugations from platelet-free plasma preparations. Presence of PrP<sup>C</sup> in the blood plasma-derived MVs was first detected by immunoblot analysis by the use of the monoclonal anti-PrP 6H4 antibody; PrP<sup>C</sup> was recognized such as shown in Figure 1A. As shown in Figure 1B, PrP<sup>C</sup> expression in MVs was confirmed by flow cytometry: staining with the anti-PrP polyclonal antibody C-20 PE revealed a specific PrP<sup>C</sup> reactivity. These findings indicate the presence of PrP<sup>C</sup> on MVs isolated from human plasma. In order to check the purity of MVs under the chosen test condition, two control stainings were performed: (i) anti-LBPA, which is an endosome-specific marker [51], thus representing an useful tool to identify exosomes, but not MVs; (ii) anti-MHC I as a positive control for MVs. As expected, MVs were virtually negative for anti-LBPA staining, demonstrating the absence of exosomes in our preparation. However, a specific anti-MHC I staining was detected, confirming both the isolation of MVs and the purity of the MV preparation (Fig. 1).

Having previously demonstrated that PrP<sup>C</sup> is strongly associated with gangliosides GM1 and GM3 within lipid rafts on the surface of lymphocytic cells [52,53], we investigated the presence of GM1 and GM3 in MVs from plasma of human healthy donors. Acidic glycosphingolipids extracted from MVs were immunostained by anti-GM3 antibody (MoAb GMR6) [42] or by HRP-CtxB, which stains GM1 [54]. As shown in Figure 2, the presence of GM3 (Fig. 2A) and GM1 (Fig. 2B) in isolated MVs was clearly detected. Since p59Fyn kinase as well as flotillin-2 have also been indicated as raft components, which may be associated with PrP<sup>C</sup> [53,55,56,57], we then analyzed the presence of both proteins in blood plasma-derived MVs. Western blot analyses showed a 59 kDa band specifically recognized by the anti-Fyn antibody (Fig. 2C) and a 42 kDa band specifically recognized by the anti-flotillin-2 antibody (Fig. 2D). The purity and enrichment of MVs was shown by electron microscopy (Fig. 2E).

Direct interaction of PrP<sup>C</sup> with gangliosides as well as with p39Fyn kinase in plasma-derived MVs was assayed by coimmunoprecipitation followed either by TLC immunostaining for the detection of gangliosides or conventional immunoblotting for p39Fyn kinase detection. As shown in Figure 3, PrP<sup>C</sup> associates in MVs with the gangliosides GM3 (Fig. 3A) and GM1 (Fig. 3B) and with the tyrosine kinase Fyn (Fig. 3C). These findings suggest that raft components, strictly associated with PrP<sup>C</sup> on the cell plasma membrane, such as gangliosides, p39Fyn and flotillin-2, are recruited during membrane blebbing which leads to MVs formation.

**Neuronal cells release membrane-derived MVs bearing PrP<sup>C</sup> and raft components**

In order to determine whether neuronal cells can also release plasma membrane-derived MVs and to investigate whether these MVs also contain PrP<sup>C</sup> associated with lipid rafts components, we isolated MVs from cell culture supernatants of a murine neuronal cell line (Neuro-2a) and from its subclonal line (Neuro-2a PK1) [41]. The isolation of MVs from cell culture supernatants was based on a sequential centrifugation steps protocol in which increasing centrifugal forces were used. Plasma membrane-derived MVs were collected at 20000 g, a centrifugal force not sufficient to enrich for exosomes. The presence of PrP<sup>C</sup> was shown in MVs released both by Neuro-2a (Fig. 4A) and Neuro-2a PK1 cells. Electron microscopy analyses demonstrated the presence of vesicles, often aggregated, with a size (from 100 nm to 1 μm) compatible with MVs similarly purified from other cells (Fig. 4B) [23]. In particular, immunogold labeling of Neuro-2a PK1 cells revealed that PrP<sup>C</sup> is unevenly distributed on the membrane of MVs (Fig. 4B).

To exclude the presence of contaminating exosomes within the MVs-enriched fraction, the levels of Tsg101 and Alix, both cytoplasmic proteins previously identified as specific markers for exosomes [46,58], were assessed by immunoblot analysis (Fig. S1). The signals obtained in the MVs-enriched fraction were lower than those observed in the exosome-enriched fraction.

Since we have shown that blood plasma-derived MVs contain lipid raft components, such as Flotillin-2, gangliosides and, most importantly, PrP<sup>C</sup>, we analyzed the presence of these components in MVs released by Neuro-2a cells by Thin Layer Chromatography (TLC) immunostaining and immunoblot analyses (Fig. 5). Since the main ganglioside constituent of Neuro-2a was GM2 [59], acidic glycosphingolipids extracted from MVs were immunostained with a highly specific anti-GM2 antibody. As shown in Figure 5A, the presence of GM2 in isolated MVs was detected. In addition, neuronal cells-derived MVs also contained Flotillin-2 and the axonal membrane protein GAP-43, a neuronal protein that binds to rafts via its acylated N-terminal. Since gangliosides have been shown to be components of the signaling complex within lipid rafts [60], we investigated the association of PrP<sup>C</sup> with ganglioside GM2 in MVs from Neuro-2a. MVs were isolated by sequential centrifugations as above and PrP<sup>C</sup> was immunoprecipitated with the anti-PrP monoclonal antibody C-20. Acidic glycosphingolipids extracted from the PrP<sup>C</sup>-immunoprecipitates were analyzed by Thin Layer Chromatography (TLC) immunostaining, using the anti-GM2 MoAb. The results indicated that PrP<sup>C</sup> associates with GM2 (Fig. 5B) within MVs rafts. The immunoprecipitate was revealed as PrP<sup>C</sup>, as detected by Western blot, using the anti-PrP monoclonal 6H4 antibody. In control samples the immunoprecipitation using IgG with irrelevant specificity, under the same condition, did not result in detectable levels of gangliosides.

These findings clearly indicate that neuronal cells actively release membrane-derived MVs in addition to exosomes [61]. The secreted MVs contain lipid raft components, suggesting that also in the CNS MVs-based intercellular transmission of molecular information could occur.

**MV secretion by neuronal cells harbour both PrP<sup>Sc</sup> and prion infectivity**

In order to assess whether prion infected neuronal cells may release membrane-derived MVs that harbor the pathological isoform of the prion protein (PrP<sup>Sc</sup>) and prion infectivity, we isolated MVs from cell culture supernatants of Rocky Mountain Laboratory prion strain (RML)-infected and non-infected Neuro-2a PK1 cells. We then assessed the presence of PrP<sup>Sc</sup> by immunoblot analysis and that of prion infectivity by in vitro and in vivo infectivity transmission experiments. Specific detection of PrP<sup>Sc</sup> was achieved by the use of proteinase K (PK) digestion. The
abnormally folded PrPSc is partially resistant against PK treatment, whereas the cellular isoform PrPc is completely degraded by this protease [62]. As shown in Figure 6, MVs derived from infected cells harbored PK-resistant PrP, a clear indication for the presence of PrPSc. Interestingly, the levels of PrPSc detected in the purified MVs were higher than those observed in the parental cell line (Fig. 6), suggesting that PrPSc is enriched on the membrane of MVs. PrPSc was not detected, as expected, in MVs secreted by non-infected Neuro-2a PK1 cells (Fig. 6), confirming that PrPSc is released specifically from prion infected neuronal cells.

MVs-dependent transmission of prion infectivity was then first assayed by an in vitro infection approach. Noninfected Neuro-2a PK1 cells were incubated in the presence of MVs isolated from cell culture supernatants of infected and noninfected Neuro-2a PK1 cells, respectively. De novo amplification of PrPSc was then followed for longer than 3 months post infection by cell blot assay (Fig. 7). Conversion of the endogenous PrPc into the prion disease-associated pathological isoform PrPSc was only detected in Neuro-2a PK1 cells treated with MVs derived from prion-infected but not from non-infected donor cells (Fig. 7). Prion replication was detected up to 30 passages after infection, indicating that MVs-

![Figure 1. PrPc detection in MVs from human plasma.](https://example.com/figure1.png)

A: Western blot analysis with anti-PrP monoclonal 6H4 antibody of MVs compared with human peripheral blood (PB). B: Cytofluorimetric analysis with an anti-PrP (C-20 PE). MVs: MVs gated on the basis of forward and side scatter parameters using polystyrene beads 0.8 μm as standard. Anti-PrP: MVs stained by anti-PrP PE (C-20 PE) vs IgG PE with irrelevant specificity. Anti-LBPA: MVs stained by anti-LBPA, followed by PE-conjugated anti-mouse IgG. Anti-MHC-I: MVs stained by anti-human MHC I vs IgG PE with irrelevant specificity.

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dependent prion transmission generate a persistent infection in the recipient neuroblastoma cells and excluding that the detected PrPSc could just be due to the persistence of the initial inoculum.

In order to further confirm the association of prion infectivity with membrane-derived MVs and to determine prion titres, in vivo biossay was performed in which purified MVs from infected and non-infected Neuro-2a PK1 cells or cell lysates were intracerebrally inoculated into tga20 indicator mice [49]. Incubation times until development of terminal scrapie were determined and infectious titres were calculated by comparing incubation times against a calibration curve (Table 1). Indicator mice developed clinical prion disease after inoculation with Rocky Mountain Laboratory mouse-adapted prion strain (RML) at the three dilutions tested, but not when they were inoculated with mock control, as expected. Interestingly, membrane-derived MVs from infected but not from non-infected Neuro-2a cells were capable of transmitting prion disease to tga20 indicator mice, demonstrating that MVs are infectious not only in vitro but also in vivo. Furthermore, MVs-associated prion titres were higher than those detected as cell-associated (Table 1), a finding confirming the observation that MVs contain more PrPSc than the cells from which they are derived (Fig. 6). On the other hand, MVs seem to be a little less infectious than crude brain homogenate: indeed mice challenged with 6 × 10−7 dilution of RML (i.e. corresponding to 10 μg of total brain protein) developed clinical prion disease earlier than those challenged with MVs.

Discussion

MVs are normal constituents of blood plasma and are secreted by leukocytes, endothelium, platelets and erythrocytes [50]. Interestingly, only one-third of the PrPSc in human blood is cell
MVs from human plasma of healthy donors and that, among MVs, PrP\(^C\) is associated with other lipid raft components. Indeed, in MVs, not only PrP\(^C\), but also p59Fyn, as well as GM1 and GM3 gangliosides were revealed and co-immunoprecipitated with PrP\(^C\), demonstrating that all the signaling complex may be shed within MVs. The presence of lipid rafts-associated signaling complex(es) in MVs suggests a potential role for these microparticles in cell-to-cell communication through MVs shedding and fusion.

It has previously been shown that neuronal cells release exosomes containing numerous proteins and lipids similar to those present in the membranes of the cells from which they originate, including typical neuronal proteins such as the GluR2/3 subunits of glutamate receptors, the cell adhesion molecule L1 and, interestingly, PrP\(^C\) [61]. Here, we report for the first time that neuronal cells also release plasma-derived MVs. Indeed, MVs were isolated from the cell culture supernatants of two murine neuronal cell lines (Neuro-2a and Neuro-2a PK1 cells). Electron microscopy analyses of MVs preparations demonstrated the presence of vesicles with a diameter ranging from 100 nm to 1 \(\mu\)m, a size compatible with that described for MVs purified from other cells [23].

In this study, the association of PrP\(^C\) with MVs shed by murine neuronal cells was demonstrated. The presence of PrP\(^C\) in MVs released from both the Neuro-2a and the Neuro-2a PK1 cell lines was shown by immunoblot analysis. Furthermore, IEM analysis of Neuro-2a PK1 cells, a murine neuroblastoma cell line endogenously expressing PrP\(^C\), revealed the unevenly distribution of PrP\(^C\) on the membrane of MVs, as well as on the cell plasma membrane. Heterogeneity of PrP\(^C\) molecules is attributed mainly to various degrees of N-glycosylation on asparagine residues [64,65]. Interestingly, MV-associated PrP\(^C\) displayed an electrophoretic mobility pattern on SDS-polyacrylamide gels resembling if not overlapping the one detected for cell-derived PrP\(^C\), suggesting that in MVs fully processed and mature PrP\(^C\) molecules are incorporated during membrane shedding. However, in a recently published work, a novel processing pathway that involves the N-terminal modification of PrP\(^C\) and the selection of distinct PrP\(^C\) glycoforms for incorporation into exosomes has been described [31]. Thus, a more accurate and detailed analysis of MV-associated PrP\(^C\) should be performed in order to verify whether a similar processing pathway also occurs during incorporation of PrP\(^C\) into plasma-derived MVs. The presence of lipid raft components, such as the p59Fyn kinase, flotillin-2 and the ganglioside GM2 was furthermore detected in MVs released by the murine Neuro-2a cell line, suggesting that raft components strictly associated with PrP\(^C\) on the cell plasma membrane are recruited during the formation of MVs in neuronal cells. The neuronal origin of the analyzed MVs was confirmed by the

associated, and the remaining two-third is present in plasma [63]. MVs are shed from the plasma membrane of eukaryotic cells, mainly from those undergoing activation or apoptosis [22]. In this context, it has previously been shown that thrombin stimulation triggered a transient shedding of microvesicles from the platelet surface. Of note, PrP\(^C\) is released initially in small quantities on microvesicles and subsequently in higher levels on exosomes [33].

In the present study we demonstrated that PrP\(^C\) is present in MVs from human plasma of healthy donors and that, among MVs, PrP\(^C\) is associated with other lipid raft components. The functional role of MVs is still largely unknown, however, some evidence show that MVs components mainly arise from lipid rafts, which therefore might be involved in setting up sorting platforms to concentrate specific proteins within MVs, that could be destined to extracellular secretion. It suggests that they may participate in a variety of intercellular adhesion processes and induce cellular responses [24]. Moreover, it has been demonstrated that blood as well as plasma of animals experimentally infected with TSEs can transmit TSE infection by transfusion [35,37]. Our results support and extend these findings, showing the presence of PrP\(^C\) in MVs from human plasma, as revealed by Western blot and cytofluorimetric analysis results, and demonstrating that the whole signaling complex is represented in these microparticles. Indeed, in MVs, not only PrP\(^C\), but also p59Fyn, as well as GM1 and GM3 gangliosides were revealed and co-immunoprecipitated with PrP\(^C\), demonstrating that all the signaling complex may be shed within MVs. The presence of lipid rafts-associated signaling complex(es) in MVs suggests a potential role for these microparticles in cell-to-cell communication through MVs shedding and fusion.

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detection of the axonal membrane protein GAP-43, a neuronal protein which binds to rafts via its acylated N-termini.

The growing interest in the presence of PrPSc in MVs is due to the potential role of these microparticles in the propagation of the disease. The release of PrPSc and infectious PrPSc by prion infected epithelial (Rov cells), neuroglial (Mov cells) and neuronal cells (GT1-7 cells) in association with exosomes has recently been highlighted [30,31], suggesting that PrPSc-bearing exosomes may provide a mechanism for intercellular transmission of infectious prions in addition to cell-to-cell contact. Indeed it was found that both PrPSc and PrPSc are present in cell culture supernatants in a secreted, exosome-associated form and that exosomes bearing PrPSc were infectious both in vitro and in vivo [30,31].

This work further extends these findings showing for the first time that stably infected neuronal cells endogenously expressing PrPSc shed plasma-derived MVs, which are carriers both of the pathological isoform PrPSc and of prion infectivity. Noteworthy, MVs isolated from cell culture supernatants of Rocky Mountain Laboratory prion strain (RML)-infected Neuro-2a PK1 harbored PK-resistant PrP, an indication for the presence of PrPSc. Interestingly, the levels of PrPSc detected in the purified MVs were higher than those observed in the parental cell line, suggesting that PrPSc is enriched on the membrane of MVs. PrPSc and PrPSc are GPI-anchored proteins known to partition into lipid rafts [66,67] and the lipid raft-like nature of MVs membranes argues for an efficient insertion of PrPSc in lipid raft-rich domains of the plasma membrane involved in MVs formation. Furthermore, the evidence that lipid rafts play a role in the formation of PrPSc in scrapie-infected culture cells [68,69] supports the hypothesis of a preferential distribution of PrPSc within lipid rafts.

On the other hand, PrPSc but not PrPSc was detected in MVs secreted by non-infected Neuro-2a PK1 cells, confirming that PrPSc is released specifically from prion infected neuronal cells. Thus, these findings clearly indicate that prion infected neuronal cells such as the Neuro-2a PK1 shed plasma membrane-derived MVs containing PrPSc.

The potential role of plasma membrane-derived MVs in prion infectivity transmission was tested both in vitro and in vivo infection models. This study demonstrates that PrPSc-bearing MVs can transmit prion infectivity both in vitro and in vivo, indicating that MVs contribute both to the intercellular mechanism(s) of PrPSc diffusion and to the process of infectious prions intercellular trafficking. Indeed, MVs isolated from cell culture supernatants of infected but not from uninfected Neuro-2a PK1 cells were capable of initiating de novo amplification of prions in the recipient cells. Interestingly, prion replication was detected up to 30 passages after infection, indicating that MV-dependent prion transmission generated a persistent infection in the recipient neuroblastoma cells. Hypothetically, PrPSc conversion may be initiated as a consequence of the binding of PrPSc-bearing MVs to acceptor cells. In this context, it is noteworthy that the topology of exosomal and, most probably, of MVs membranes is identical to that of the plasma membrane [70]. Alternatively, but not mutually exclusive, MVs captured by the target cells could fuse with the cell surface or be internalized by an unclear mode of entry to induce conformational change of PrPSc at the cell surface and/or in endocytic compartments, respectively. Moreover, in vivo biossay in transgenic mice that overexpress murine PrPSc and are highly susceptible to murine prions [50] clearly demonstrated that prion
membrane-derived MVs infectivity is associated with PrPSc-harboring MVs shed by prion carriers of prion infectivity during prion neuroinvasion and play a pivotal role in the propagation of prion infectivity from neuron to neuron, in this way contributing to the pathogenesis of the disease. However, it does not exclude the possibility that also exosomes may play an important role in secreted infectivity from the cells.

In conclusion, our data provide here evidence for the presence of PrPSc in MVs in vivo, in human plasma, suggesting that these MVs might be involved in the mechanism(s) of PrPSc paracrine as well as endocytic diffusion and may also contribute to blood transfusion-mediated transmission of prion diseases.

**Supporting Information**

**Table 1. PrPSc-bearing MVs transmit disease to Tga20 indicator mice.**

| Inoculum | Indicator mice succumbing to scrapie | Scrapie death Days (mean +/- STDEV) | Estimated infectivity titres (log LD50/ml) |
|----------|--------------------------------------|------------------------------------|----------------------------------------|
| Mock     | 0/4                                  | >150                               | <1.5                                   |
| RML      | 4/4                                  | 78 ± 2                             | 4.6                                    |
| RML 10^-2| 4/4                                  | 68 ± 2                             | 5.5                                    |
| RML 6x10^-2 | 4/4                                | 64 ± 2                             | 5.8                                    |
| MVNonLD | 0/4                                  | >150                               | <1.5                                   |
| MVLD    | 4/4                                  | 68 ± 2                             | 5.5                                    |
| Neuro-2a PK1 cells | 0/4                      | >150                               | <1.5                                   |
| Neuro-2 a PK1 inf. cells | 4/4                           | 76 ± 6                             | 4.8                                    |

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These new findings suggest that plasma membrane-derived MVs shed by prion infected cells could actively participate in the intercellular trafficking of PrPSc and contribute to the transmission of infectious prions. One may therefore postulate that MVs act as carriers of prion infectivity during prion neuroinvasion and play a pivotal role in the propagation of prion infectivity from neuron to neuron, in this way contributing to the pathogenesis of the disease. However, it does not exclude the possibility that also exosomes may play an important role in secreted infectivity from the cells.

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**Author Contributions**

Conceived and designed the experiments: VM TG RM FM. Performed the experiments: VM MGB VT TG AL FM. Analyzed the data: VM GMB VT TA KB JL FM. Wrote the paper: RM FM MS.

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