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Proteomic Analysis of Microglia-Derived Exosomes: Metabolic Role of the Aminopeptidase CD13 in Neuropeptide Catabolism¹

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Vesicle transport is a fundamental mechanism of communication in the CNS. In this study we characterized a novel type of vesicle released by murine brain microglial cells: microglial exosomes. Analysis of their protein content identified several enzymes, chaperones, tetraspanins, and membrane receptors previously reported in B cells and dendritic cell-derived exosomes. Additionally, microglia-derived exosomes expressed the aminopeptidase CD13 and the lactate transporter MCT-1. Exosomal CD13 was metabolically active in cleaving leucine- and methionine-enkephalins peptides by releasing the N-terminal tyrosine. Cleaved neuropeptides were unable to bind to the neuronal opioid receptor as assessed by cAMP response. Microglial exosomal vesicles may represent an important, previously unrecognized, cellular communication system in an organ in which cell motility is highly restricted. *The Journal of Immunology*, 2005, 175: 2237–2243.

Microglia are brain-resident cells derived from bone marrow elements infiltrating the brain early during the fetal and neonatal development periods (1–4). The role of microglial cells has best been characterized under degenerative and inflammatory conditions, where they function as APCs. However, microglial function in normal brain physiology is largely unknown. Microglia may have a role in CNS maintenance and homeostasis, as suggested by their global gene expression profile, which revealed mRNA transcripts for several growth factors, proteoglycans, and neuropeptides as well as receptors for neurotransmitters, neuropeptides, and endorphins (5). The expression of surface receptors for neuropeptides and neurotransmitters on microglial cells suggests modulation of microglial functions by neuronal cells. In this respect, it has been previously shown that neuronal activity is a strong down-modulator of class II MHC protein expression (6, 7). In contrast, the expression of transcripts encoding proteoglycans and growth factors suggests a potential influence of microglial cells on neuronal growth and maintenance (5). Together these data underscore the existence of a bidirectional communication network between glial cells and neurons.

Cell-cell communication can rely on soluble mediators or direct membrane contact between the signaling and the targeting cells. Another mechanism has been recently described, which involves

the release and uptake of exosomes, small vesicles, 40–100 nm in diameter, secreted by different cell types and used as small packages of information to be transferred to target cells. Exosomal vesicles have been described in several bone marrow-derived cell types (8) and are well characterized in APCs (9–13). Because of the hemopoietic origin of microglial cells, the potential secretion of exosomal vesicles was hypothesized.

Electron microscopic analysis revealed that microglial cells produce exosomes. Their protein content was analyzed by mass spectroscopic peptide mapping, Western blotting, and enzymatic analysis. The analysis identified several proteins already reported in B cell- and dendritic cell (DC)³-derived exosomes as well as exosomal proteins not previously described, including the aminopeptidase CD13 and the lactate transporter MCT1. These results led us to test exosomal function in neuropeptide degradation, glucose catabolism, and lactate production. The ability of exosomes to carry out these processes independently highlights the existence of a novel vesicular delivery system in an organ in which cell motility is highly restricted.

Materials and Methods

Cell culture

N9, a well-characterized murine microglial cell line (14), and LS.102.9, a well-characterized B cell line, were cultured in hybridoma-serum-free medium (Invitrogen Life Technologies). NIE-115 a neuroblastoma cell line (American Type Culture Collection) was cultured in 10% DMEM. Primary microglial cells were prepared from mixed glial culture of SJL/J mice as previously described (15).

Exosome isolation and electron microscopic analysis

Exosomes were isolated from supernatant of freshly purified microglial cells or the N9 microglial cell line by serial centrifugation or by floatation on sucrose gradient (9) and analyzed by electron microscopy or Western blot for protein staining. For large-scale preparation of exosomes, cells were grown for 2 days in serum-free medium (Invitrogen Life Technologies) starting at a concentration of 3×10^5 cells/ml. One liter of cell supernatant was filtered over 0.22 μ m (11) and concentrated 10 times using

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³ Abbreviations used in this paper: DC, dendritic cell; Ii, invariant chain; MS, mass spectrometry; Lamp, lysosomal-associated membrane protein.

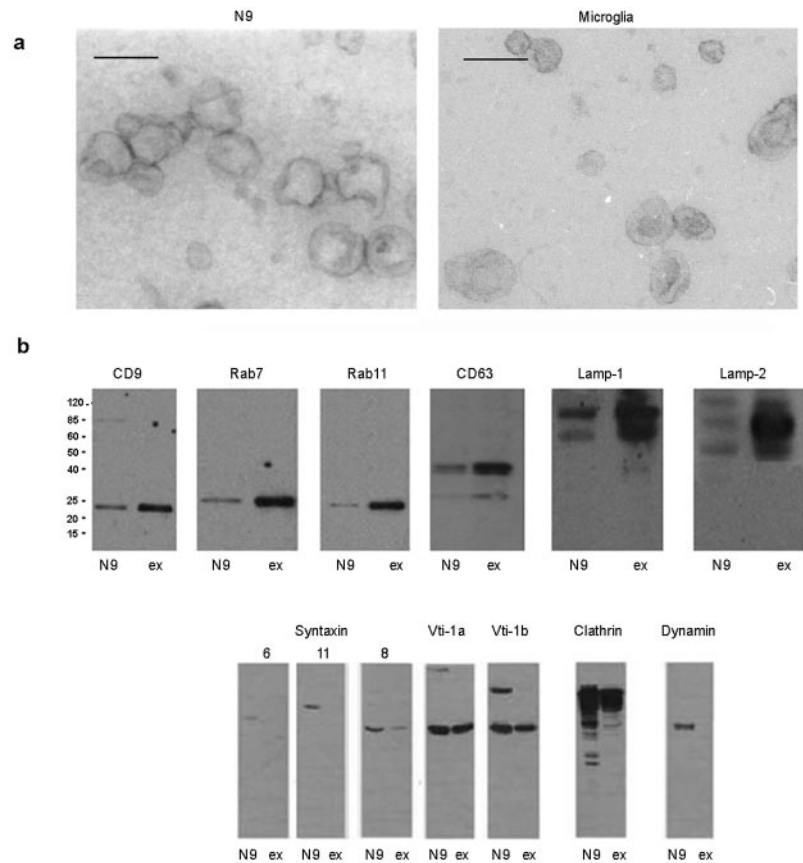


FIGURE 1. Microglial cells produce exosomes. *a*, Ultramicroscopy of exosomes derived from the fetal murine microglial cell line N9 and primary microglial cells. *b*, Western blot analysis of protein lysate from N9 postnuclear supernatant and N9 exosomal preparation as described in *Materials and Methods*.

ultrafiltration membranes NMWL 10000 (Millipore). Exosomes were pelleted by ultracentrifugation for 1 h at $100,000 \times g$ and washed once with PBS. For electron microscopic analysis, exosomes were fixed in 2% PFA and loaded on Formvar/carbon-coated electron microscopic grids. Contrast was performed in 2% uranyl acetate, pH 7, and 2% methylcellulose/0.4% uranyl acetate, pH 4. Class II MHC proteins were labeled using Y3P (American Type Culture Collection) mAb or CD13 mAb (BD Pharmingen), followed by gold-protein A. Alternatively, 100 μ g of exosomal proteins were loaded on 12% SDS-PAGE, and the gel was stained with Coomassie Blue for the MALDI TOF analysis.

Western blot analysis and flow cytometry

Eighty micrograms (80 μ g) of total protein lysate from the exosomal preparation or N9 postnuclear supernatant was run on a 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Western blot analysis was performed using the following mAbs: Syntaxin 6, 8 and 11, Vti-1A and Vti-1B, Rab-7, Rab 11, clathrin, and dynamin (BD Transduction Laboratories); anti-mouse invariant chain (I α) CD74 (clone In-1), lysosomal-associated membrane protein (Lamp)-1, Lamp-2, CD9, and CD63 (BD Pharmingen); anti-mouse class II MHC (clone KL-295; American Type Culture Collection); and anti-mouse cathepsin S serum (16). All secondary mAbs were HRP conjugated (BD Transduction Laboratories). Proteins were visualized by chemiluminescent detection.

CD13 surface staining on N9 and primary microglial cells was performed as previously described (15) using the anti-aminopeptidase N, FITC-labeled anti-CD13 mAb (BD Pharmingen). Class II MHC staining on N9 cells was performed using the Y3P mAb (15).

CD13 aminopeptidase activity measurements

Exosomes from N9 and LS102.9 cells were preincubated in the presence or the absence of the protease inhibitor phebestin (Sigma-Aldrich) at a final inhibitor concentration of 17 μ g/ml. They were then mixed with 200 nmol of leucine-*p*-nitroaniline (Sigma-Aldrich) in a 96-well plate (2- μ g/ml total exosomal protein in 150 μ l of PBS/well). The release of *p*-nitroaniline at 10, 60, 120, 180, and 240 min was used to follow aminopeptidase activity by measuring the absorbance at 405 nm.

Cleavage of opioid neuropeptides by CD13

Neuropeptides methionine-enkephalin (Bachem) and leucine-enkephalin (Bachem; at a final concentration of 100 μ M of each peptide) were incubated in the presence or the absence of exosomes from N9 microglial cells or LS102.9 B cells (3 μ g of total protein) overnight in a total volume of 0.5 ml of PBS. Then exosomes were separated from the reaction mixture by spinning in a Microcon 10 filter apparatus (Millipore). The amount of remaining peptide was determined by reverse-phase HPLC using a C₁₈ column and a gradient of 2–80% acetonitrile in 60 min.

cAMP immunoassay

Three micrograms of N9 or LS102.9 exosomal preparations were incubated with or without 1 μ M leucine-enkephalin for 3 h at room temperature in 100 μ l of PBS. Samples, including a 1 μ M leucine-enkephalin exosome-free, positive control, were centrifuged through a Centricon membrane (10,000 cut-off; Millipore). The exosome-free flow-through for each condition was incubated in triplicate with the NIE 115 neuroblastoma cell line (CD13 negative) for 15 min at 37°C. At the end of the incubation, the NIE-115 cells were lysed in 0.1 M HCl, and the acetylated amount of cAMP present was determined by immunoassay with an anti-rabbit polyclonal Ab (R&D Systems).

Results

Microglial cells secrete exosomes

Among the brain-resident cell types, microglial cells are unique in being derived from bone marrow. Microglia function as APCs in brain-mediated immune responses. Other APCs have been shown to produce exosomes, and the role of exosomes in ferrying MHC class II peptide complexes and other proteins to target cells has been described (12). We were interested in investigating whether microglial cells could also be a source of exosomes, and whether such exosomes could have a physiological role in CNS homeostasis.

A well-characterized murine microglial cell line, N9 (14), was grown in serum-free medium to avoid potential contamination from serum-derived vesicles. Primary microglial cells, isolated from mixed glial culture, were grown in 5% FBS previously ultracentrifuged to avoid potential contamination from serum-derived vesicles. Exosomes were isolated from culture supernatant by both serial centrifugation and flotation on a sucrose gradient. Electron microscopy revealed the presence of numerous secreted vesicles varying in size from 40 to 120 nm (Fig. 1a). These vesicles have the typical exosomal density of ~ 1.15 g/ml and were similar in shape and dimension to the previously described exosomes derived from dendritic cells and B cells (9, 12).

Exosomes from N9 cells display endosomal markers

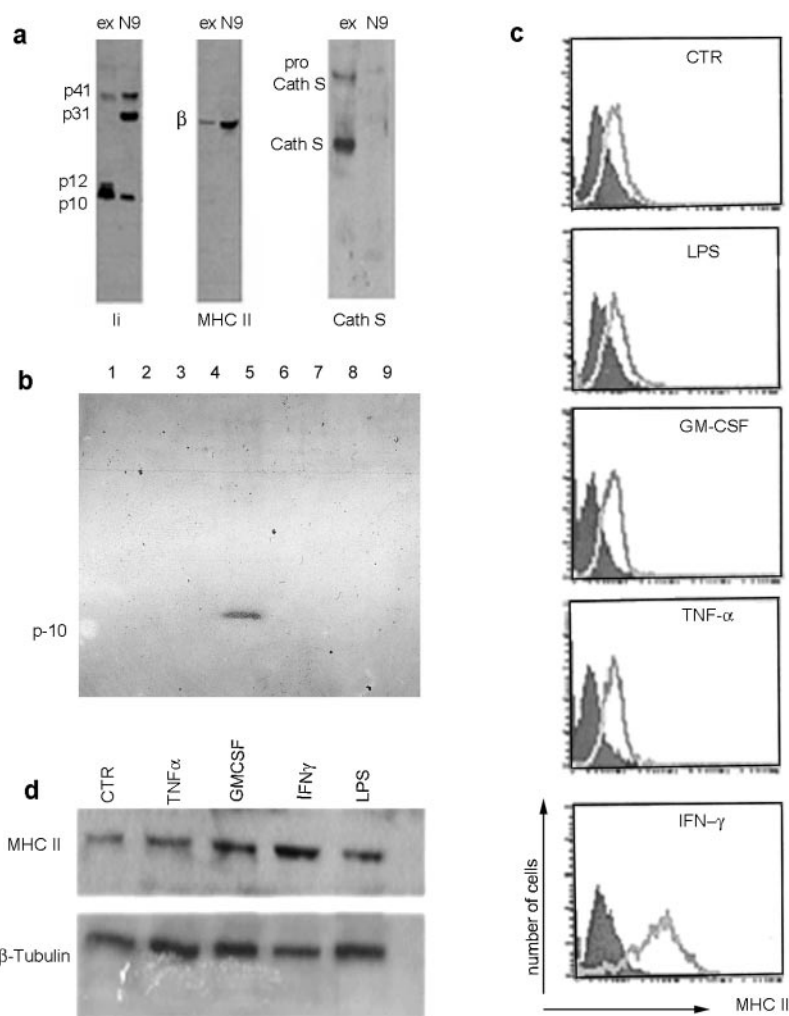
Exosomes were previously reported to be secreted from endosomal multivesicular bodies upon fusion with the plasma membrane (9). To evaluate whether microglia-derived exosomes were formed similarly, an exosomal protein lysate was probed by Western blot analysis for the presence of endosome-specific proteins. Several vSNARE proteins important for controlling endosomal trafficking were examined. Syntaxin 8, a SNARE molecule of the early/late endosomes (17), was found in the exosomal preparation as well as in the total N9 cell lysate (Fig. 1b). Syntaxins 6 (18) and 11 (19, 20), SNARE molecules of the trans-Golgi network and recycling endosomal compartments, were not detected in the exosomal preparation (Fig. 1b). Vti-1A and Vti-1B, which are Golgi/post-Golgi vesicle and late endosomal SNARE proteins (21), were present in

the exosomal protein lysate (Fig. 1b). Rab 7 and Rab 11 proteins of the late endosomal compartments as well as Lamp-1 and Lamp-2 and the tetraspannin CD9 and CD63 were also detected in the exosomes (22). Clathrin, an essential component of the clathrin-coat complex found in endocytosed and post-Golgi vesicles (23), was present in the exosomal preparation, whereas dynamin, a GTPase that plays an important role in vesicle formation through its ability to tubulate and constrict membranes at the plasma membrane and trans-Golgi network (24), was not evident in the crude exosomal protein extract (Fig. 1b). Thus, as previously reported for professional APCs, microglial-derived exosomes express specific markers of late endosomes and not other subcellular compartments.

Ii, class II MHC molecules, and cathepsin S are expressed in microglial-derived exosomes

In APCs, endosomal compartments are particularly enriched for MHC class II molecules, the MHC class II-associated chaperone Ii, and several proteases important for Ii degradation and Ag processing (25, 26). Western blot analysis was performed on microglial exosomes to investigate the presence of these proteins. A crude protein extract from N9 derived-exosomes revealed the presence of the p41 isoform of Ii as well as its cleavage product, p12 (Fig. 2a) (27). The full-length p31 isoform was not detected, although its proteolytic fragment, p10, was visible. Similarly, p10 was present in sucrose gradient fractions containing exosomes (Fig. 2b). This is in accordance with the observation that exosomes

FIGURE 2. Exosomes express MHC II, Ii, and cathepsin S. Exosomal MHC II is up-regulated by IFN- γ . N9 cells were cultured untreated or were grown for 48 h with different cytokines. *a*, Western blot analysis of protein lysate from N9 postnuclear supernatant and N9 exosomal preparation for MHC II (mAb KL-295), Ii (mAb In-1), and cathepsin S¹⁵. *b*, Analysis of Ii expression of exosomes isolated by flotation on sucrose gradient. The fraction in line 5 corresponds to a sucrose density of 1.15 g/ml. *c*, Cell surface expression of MHC II protein detected with the mAb Y3P. *d*, Western blot analysis of exosomal preparation of N9 cells treated with different proinflammatory cytokines as described in *c*.



originate from late endosomal compartments where partial cleavage of Ii is expected (16). Cathepsin S, the cysteine protease responsible for p10 cleavage to CLIP, was also detected by Western blot analysis (Fig. 2a). MHC class II molecules could also be detected by Western blot analysis in both total cell and exosomal protein extract (Fig. 2a). In the resting state, microglial cells have a low surface expression of MHC class II molecules (Fig. 2c). Consistent with their being nonprofessional APCs, microglia mostly up-regulate surface MHC class II in response to a 24-h stimulation with IFN- γ (Fig. 2c). A similar increase was observed in an exosomal preparation derived from IFN- γ -treated cells (Fig. 2d).

Analysis of microglial exosomal proteins

Electron microscopy and Western blot analysis established that microglial secreted vesicles could be identified as exosomes. To determine the protein composition of microglial-derived exosomes, total protein extracts from exosomal preparations were run on SDS-PAGE under boiling and reducing conditions. Gel lanes were sliced into 12 fragments (a to l) according to their m.w., and proteins were identified by MALDI-TOF mass spectrometry analysis after trypsin digestion (Fig. 3).

Many of the proteins identified by mass spectrometry (MS)/MS were cytoskeleton and cytoskeleton-associated proteins (actin, tubulin, cofilin, etc.; Fig. 3). Also, several cytoplasmic proteins of the GTPase family as well as tRNA binding protein could be detected in both preparations. Several chaperone molecules, which have been previously identified in DC-derived exosomes (11), were found in the microglial preparation, including members of the

heat shock protein family (HSC73 and HSP86 and three isoforms of 14-3-3 (β , ϵ , and ζ), which are multifunctional proteins that bind and modulate the function of a wide array of cellular proteins (28). Several enzymes involved in glycolysis and lipid metabolism were also identified (Fig. 3).

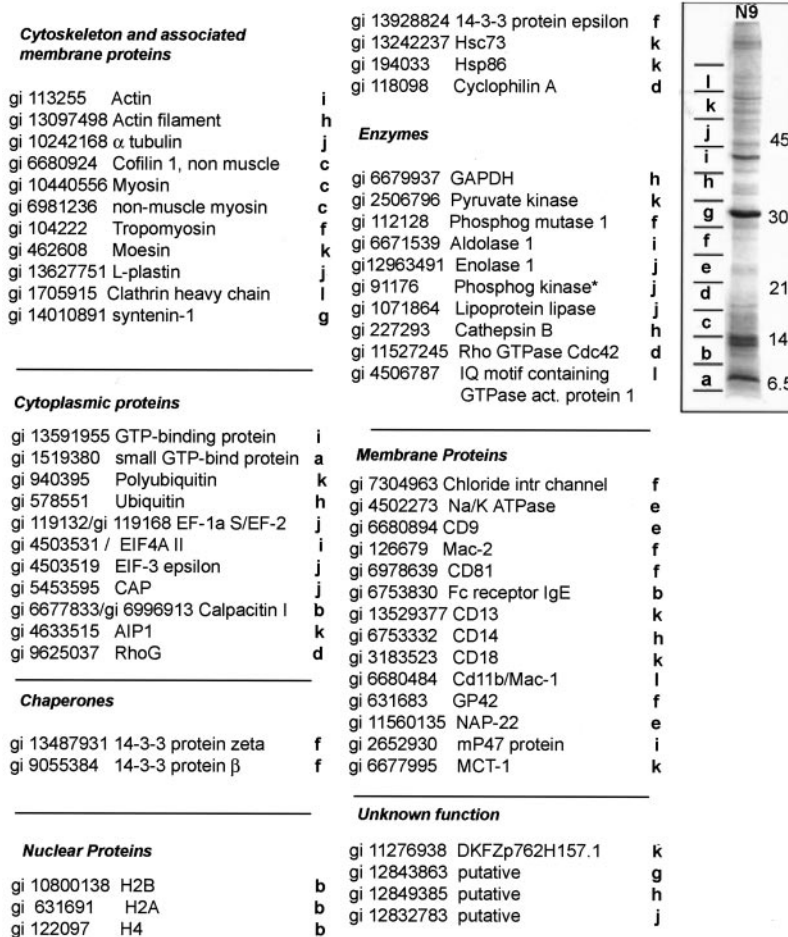
Integrins, such as Mac1 and Mac2 Ags, and tetraspanin proteins, such as CD9 and CD81, were expressed in microglia. Both classes of proteins are involved in Ag presentation by promoting adhesion and membrane organization in microdomains. The pattern recognition receptor CD14, important for innate immunity, was also identified by MS/MS, as was the FcR for IgE, and GP42, a membrane glycoprotein structurally related to the FcR family (29). Also notable is the presence of NAP-22, a calmodulin-associated protein previously described on the external surface of the synaptic vesicle membrane and involved in vesicle cycling (30).

Role of the exosomal CD13 aminopeptidase

A protein not previously identified in exosomes derived from B cells and DC was the aminopeptidase N or CD13 (Fig. 3). CD13 is a cell surface-bound peptidase, which cleaves N-terminal amino acids with the exceptions of proline and arginine. CD13 was detected by flow cytometric analysis on the surface of N9 cells, but not the LS 102.9 B cell line (Fig. 4a). Its expression in multivesicular endosomal compartments was confirmed by electron microscopy (Fig. 4b). By cryoimmunogold, the presence of CD13 at the limiting membrane of microglial exosomes (from both primary cultures and the N9 line) was also confirmed (Fig. 4c).

To assess the potential activity of CD13 in N9-derived exosomes, an exosomal preparation was incubated with leucine-pNA,

FIGURE 3. Proteomic analysis of microglial cell exosomes. One hundred micrograms of total protein lysate from N9 exosomes was separated by a 12% SDS-PAGE. Gel lanes were sliced into 12 fragments (a to l) according to their m.w. Excised bands were eluted, trypsin digested, and sequenced by microcapillary reverse-phase HPLC nanoelectrospray tandem MS on a Finnigan LCQDECA XP quadrupole ion trap mass spectrometer (LC-MS/MS). Sequence analysis was performed at the Harvard University microchemistry facility. Only proteins identified by three or more sequenced peptides are reported in the figure, with the exception of phosphoglycerate kinase. gi, GenBank accession number.



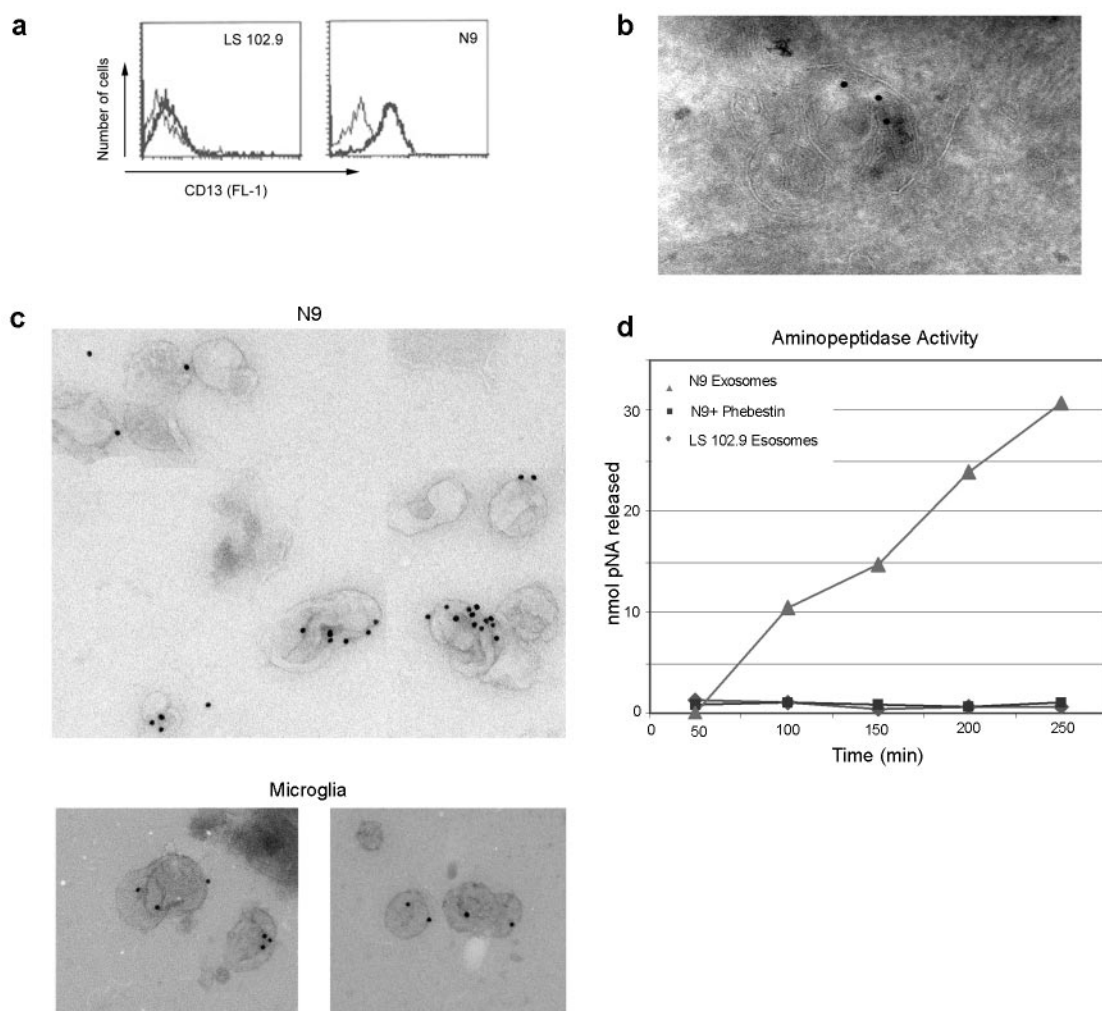


FIGURE 4. CD13 expression of microglial cells and aminopeptidase activity of microglial exosomes. *a*, CD13 is present at the cell surface of N9 microglial cells, but not in the control B cell line LS102.9. *b*, CD13 is expressed in endosomal compartments in the N9 microglial cell line. *c*, CD13 is highly expressed at the limiting membrane of microglia-derived exosomes (N9 cell line and primary microglial cell) as shown by electron microscopy. *d*, Microglia-derived exosomes (N9), and not B cell-derived exosomes (LS102.9), are able to cleave the chromogenic substrate leucine-*p*-nitroalaline. This activity is completely inhibited by the CD13-specific inhibitor, phebestin.

which can be cleaved to form the bright yellow product *p*-nitroaniline in the presence of an aminopeptidase. Exosomes from N9 cells were determined to be proteolytically active, whereas exosomes from LS102.9 B cells were unable to cleave the chromogenic substrate (Fig. 4*d*). This activity was completely inhibited by phebestin (Fig. 4*d*), an inhibitor specific for aminopeptidase-N (CD13) (31).

CD13 has been implicated as an important enzyme responsible for degradation of the neuropeptides methionine- and leucine-enkephalin (31). To investigate whether exosomal CD13 was active in the degradation of these neuropeptides, cleavage was monitored by reverse phase HPLC (Fig. 5*a*). Methionine- and leucine-enkephalin were completely degraded after overnight incubation with exosomes from N9 cells, but remained intact after incubation with exosomes from LS102.9 cells. A shorter incubation (3 h) was sufficient to degrade >80% of 50 μ M leucine-enkephalin (data not shown).

Enkephalins bind with high affinity to opioid receptors expressed on the surface of neuronal cells (32). Activation of the opioid receptor leads to inhibition of adenylyl cyclase activity, with a subsequent decrease in neuronal cAMP levels (33) (Fig. 5*b*). To investigate whether enkephalin cleavage by exosomal CD13 could affect neuronal cAMP levels, exosomal preparations

from N9 or LS102.9 cells were incubated with or without 1 μ M leucine-enkephalin peptides for 3 h at room temperature. Samples were then centrifuged through a Centricon 10 (to remove the exosomes), and the flow-through was incubated for 30 min at 37°C with the neuronal cell line NIE-115 (which does express high levels of opioid receptors (34) and is CD13 negative (data not shown)). Neuronal cells incubated with leucine-enkephalin showed a 50% decrease in the basal level of cAMP (Fig. 5*b*). As predicted, exosomes alone did not alter cAMP levels (Fig. 5*b*). Leucine-enkephalin previously incubated with exosomes from LS102.9 was not cleaved and thus retained its ability to down-regulate cAMP. In contrast, leucine-enkephalin previously incubated with exosomes from N9 cells was cleaved and thus was unable to signal through the opioid receptors as assessed by a statistically significant decrease in neuronal cAMP (Fig. 5*b*).

Thus, the CD13 expressed by microglial exosomes is active in neuropeptide degradation and could have a distinct effect on local neuropeptide activity in the brain.

Discussion

Exosomal vesicles have been described in several different cell types, most of which are derived from bone marrow. In this study

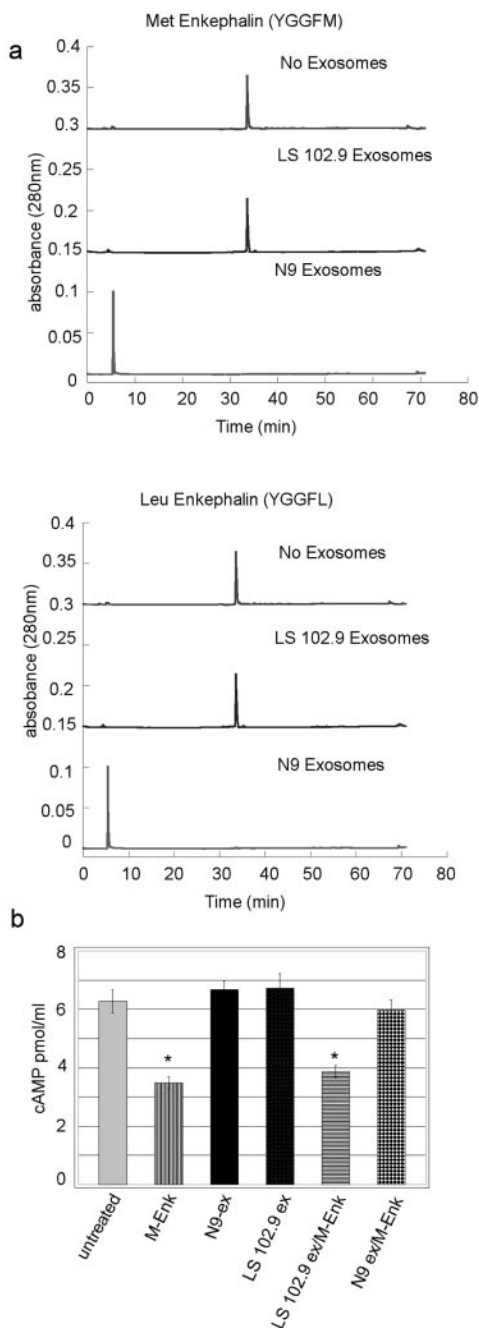


FIGURE 5. Exosoma-cleaved enkephalins decrease neuronal signaling, as detected by cAMP levels. *a*, Exosomes from microglial cells (N9) and B cells (LS102.9) were incubated with the neuropeptides methionine-enkephalin and leucine-enkephalin. Cleavage of the neuropeptides was monitored by HPLC. *b*, Methionine-enkephalin (1 μ M) was incubated for 2 h with exosomes from microglia (N9) or B cells (LS102.9). Enkephalin cleavage/inactivation was determined by measuring the levels of the second messenger cAMP using the neuroblastoma cell line NIE 115 that express opioid receptors and is CD13 negative.

we report that CNS microglia also secrete exosomes. The analysis of proteins expressed in microglial exosomes identified quantitative and qualitative differences in protein content between microglial exosomes and previously described exosomes from other cell types (12). The expression of CD13 and MCT-1 appeared to be an important feature of microglial exosomes, which also distinguish these vesicles from exosomes secreted in other hemopoietic cells. The role of microglial cells under physiological brain conditions is

still poorly defined, even though evidence has been accumulating that microglial cells are an integral part of a communication network within the CNS. One possible function of microglial cells is metabolic support to neurons. Glucose and its metabolites are the major substrates used in normal adult brain, and glycolysis and lactate transport are critical and tightly regulated functions in brain metabolism. Neurons, which predominantly express isoform 1 of lactate dehydrogenase, can oxidize lactate for energy production. Also, like other tissues which mainly consume lactate, neurons express the MCT2 lactate transporter (32). Conversely, glial cells preferentially release lactate and express the MCT1 transporter. Recently, a model has been proposed in which a large flux of lactate occurs from astrocytes to neurons, particularly during activation at the synaptic terminal (32). In this study we present evidence that microglia-derived exosomes contain an intact metabolic pathway with all the enzymes necessary for anaerobic glycolysis or lactate production. Exosomal release of lactate could function as a supplementary energy substrate by neurons during synaptic activity. Recently, a coupling between neural activity and oxidative metabolism has been established by showing that activation of single unit neuronal synapses produces a transient decrease in tissue oxygenation (35). In this respect, exosomes could function as a means to deliver energy substrates on demand to match increased energy needs during increased neuronal activity. The ability of exosomes to diffuse through the extracellular milieu would enhance their ability to fulfill this function.

As additional evidence of their postulated role in neuronal homeostasis, microglial exosomes also display enzymes important for protein degradation. CD13 is an aminopeptidase that functions as a membrane ectoenzyme in removing N-terminal amino acid residues from polypeptides (36). In this report, exosomal CD13 is shown to hydrolyze leucine- and methionine-enkephalins, thus regulating the ligand concentration for opioid receptors and ultimately the neuronal levels of the second messenger cAMP. Different enzymes, including aminopeptidases (N and A) and endopeptidases (enkephalinase, dipeptidyl aminopeptidase, and angiotensin II), are involved in the brain catabolism of enkephalins. However, the major mode of enkephalin inactivation *in vitro* and *in vivo* was shown to be the release of the N-terminal tyrosine by an aminopeptidase (37). More importantly, exosomal aminopeptidase activity is 20 times higher compared with plasma membrane surface activity. The microanatomy of neuropeptide release differs from that of neurotransmitter release. Neuropeptides can be released from the neuronal soma and neuronal dendrites and at the axonal level, whereas neurotransmitters are almost uniquely released at the axonal terminal. Neurons express CD13 on the pre-synaptic membrane, which could control the catabolism of neuropeptides released at the synaptic level. In contrast, glial and exosomal CD13 could control the catabolism of neuropeptides released at sites distant from the synaptic membranes.

In conclusion, proteomic and functional analyses of microglial exosomes indicate that they may have a role in attending functions such as neuronal metabolic support and neuropeptide catabolism. Such functions are also provided by parental microglial cells; however, there are physiological advantages in releasing exosomes, because they are mobile in an organ in which cell mobility is greatly restricted.

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Disclosures

The authors have no financial conflict of interest.

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