Proteomic Analysis of Dendritic Cell-Derived Exosomes: A Secreted Subcellular Compartment Distinct from Apoptotic Vesicles

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Dendritic cells constitutively secrete a population of small (50–90 nm diameter) Ag-presenting vesicles called exosomes. When sensitized with tumor antigenic peptides, dendritic cells produce exosomes, which stimulate anti-tumor immune responses and the rejection of established tumors in mice. Using a systematic proteomic approach, we establish the first extensive protein map of a particular exosome population: 21 new exosomal proteins were thus identified. Most proteins present in exosomes are related to endocytic compartments. New exosomal residents include cytosolic proteins most likely involved in exosome biogenesis and function, mainly cytoskeleton-related (cofilin, profilin I, and elongation factor 1) and intracellular membrane transport and signaling factors (such as several annexins, rab 7, and 11, rap 1B, and syntenin). Importantly, we also identified a novel category of exosomal proteins related to apoptosis: thioredoxin peroxidase II, Alix, 14-3-3, and galectin-3. These findings led us to analyze possible structural relationships between exosomes and microvesicles released by apoptotic cells. We show that although they both represent secreted populations of membrane vesicles relevant to immune responses, exosomes and apoptotic vesicles are biochemically and morphologically distinct. Therefore, in addition to cytokines, dendritic cells produce a specific population of membrane vesicles, exosomes, with unique molecular composition and strong immunostimulating properties. The Journal of Immunology, 2001, 166: 7309–7318.

In addition to soluble proteins and mediators, cells also release membrane vesicles in the extracellular environment. Although their biological functions are still unclear, two types of secreted membranes involved in immune responses were recently analyzed in some detail: apoptotic blebs and exosomes (1, 2). Exosomes represent a population of membrane vesicles homogeneous in size (ranging from 60 to 90 nm) and shape (3, 4). They form by inward budding from the limiting membrane into the lumen of endosomes, which are then called multivesicular endosomes (5). Exosomes are most likely secreted upon fusion of multivesicular endosomes with the plasma membrane. Different cell types produce exosomes, including RBC, platelets, B and T lymphocytes, and dendritic cells (DCs) (2). Production of apoptotic blebs, on the other hand, is initiated early after induction of apoptotic cell death (6, 7). Apoptotic blebs and microvesicles represent heterogeneous populations of membrane vesicles, budding directly from the plasma membrane and carrying a number of nuclear, cytosolic, and endoplasmic reticulum (ER)-derived proteins (6, 8). Interestingly, because of their biogenesis, the membrane topologies of apoptotic microvesicles and exosomes are similar; the cytosolic side of the lipid bilayer is inside the vesicle, and the luminal part of the membrane is exposed.

Recent functional studies suggested that secreted membranes may indeed play specific roles in immune responses. Apoptotic blebs are efficiently phagocytosed by DCs and macrophages through specific receptors, including α, β/α, β3, and CD36 (9). Phagocytosis by macrophages results in degradation and clearance of apoptotic material, whereas phagocytosis by DCs results in efficient processing and presentation of Ags expressed in the apoptotic cell to CD4+ and CD8+ T lymphocytes (10).

Exosome’s biological functions, on the other hand, are starting to be unraveled. In reticulocytes, secretion of exosomes eliminates proteins that are not necessary for the function of differentiated RBC (11). B lymphocyte-derived exosomes bear abundant MHC class II molecules and stimulate CD4+ T lymphocytes in vitro (3). B lymphocyte-derived exosomes also concentrate high amounts of tetraspanins (i.e., CD63, CD81, CD37, and CD82), a protein family that also accumulates in late endocytic compartments and whose biological functions are unclear (12). Interestingly, recent results show that B lymphocyte-derived exosomes bind selectively to follicular DCs in vivo, suggesting a possible function for exosomes in humoral immune responses (13).

Exosomes produced by DCs bear not only MHC class II molecules, but also MHC class I and CD86, an important T cell co-stimulatory molecule (4). Tumor peptide-loaded DC-derived exosomes stimulate strong cytotoxic T lymphocyte-mediated anti
toxin-free FCS (Valbiotech, ABCYS, Paris, France) twice a week in 145-mm non-tissue culture-treated petri dishes (5 mg/ml medium from J558 (a GM-CSF-secreting plasmacytoma, provided by Dr. Véron, unpublished observations)). For large scale preparations of exosomes, the Coomassie-stained protein bands were excised from the gel, trypsin digested, and analyzed essentially as previously described (14, 17). Mass spectra of the peptide mixtures were acquired on a BiHex (Bruker-Franzen Analytik, Bremen, Germany) MALDI-TOF mass spectrometer equipped with a nitrogen laser for anion delayed extraction. The instrument was operated in the reflector mode. A mass list of peptides was obtained for each protein digest, and the appropriate software was used to identify the proteins (usually MS-FIT: http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm).

When a protein could not be confidently identified from its peptide mass map, the trypsin digest was extracted with acetonitrile and a 5% formic acid solution. The digest solution and the extracts were then pooled, dried in a vacuum centrifuge, and desalted with ZipTip C18 (Millipore, Bedford, MA) before the nanospray tandem mass spectrometry (MS/MS) analysis (18). A Q-TOF instrument (Micromass, Manchester, U.K.) was used with a Z-Spray ion source operating in the nanospray mode. About 3–5 μl of the desalted sample was introduced into a needle (medium sample needle, PROTANA, Odense, Denmark) to run MS and MS/MS experiments. The capillary voltage was set at an average voltage of 1000 V, and the sample cone was set at 50 V. Glutathionepetide was used to calibrate the instrument in the MS/MS mode. Amino acid sequences, sequence tags, or peptide ion fragments that could be determined were used to screen the protein databases with dedicated software: Peptrig (http://prow11 Rockefeller.edu/prow/pептрjpg.shtml), peptide search (http://www.mann-embL-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html), or BLAST for homology searches (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

Antibodies

The Abs used were: for FACS analysis: FITC-conjugated anti mouse CD11b (M1/70 clone), CD86 (GL1), H-2Kb (AF6-88.5.klon), I-A<sup>β</sup> (AF6-120.1 clone), CD11c (HL.3), and the corresponding FITC-conjugated isotype-matched controls rat IgG2b, mouse IgG2a, and hamster IgG (all from PharMingen, San Diego, CA), and rat and anti-mouse FcyR type II/III (2.4G2 clone), followed by FITC-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA); for FACS analyses, Western blotting: rat anti-mouse CD9 (KMCS clone) and Lamp2 (ABL-93 clone), both from PharMingen, followed by FITC-conjugated donkey anti-rat IgG (for FACS, Jackson ImmunoResearch) or HRP-conjugated donkey anti-rat IgG (for Western blotting, Pierce, Rockford, IL); and for Western blotting: rabbit antiserum anti-mouse MHC class II α-chain C terminus (14), anti-FcyR type II/III (provided by Dr. C. Gautes, Institut National de la Sante et de la Recherche Médical Centre, Unité 255, Paris, France), and anti-API/Alix (provided by R. Sadoul, Centre Hospitalier Universitaire, Grenoble, France), followed by HRP-conjugated donkey anti-rabbit IgG (Pierce).

**FACS analysis of cells and exosomes**

For FACS analysis, 30 μg of exosomes (or 30 μg of FCS proteins for negative control) were incubated with 10 μl of 4-μm diameter aldehyde/sulfate latex beads (Interfacial Dynamics, Portland, OR) for 15 min at room temperature in a 30–100 μl final volume, followed by 2 h with gentle shaking in 1 ml PBS. The reaction was stopped by incubation for 30 min in 100 mM glycine. Exosom- or FCS-coated beads were washed three times in FACS wash (3% FCS and 0.1% NaN<sub>3</sub> in PBS) and resuspended in 500 μl FACS wash. In parallel, D1 cells were washed twice in FACS wash. Cells (10<sup>5</sup>) or 10 μl coated beads were incubated for 1 h with each primary Ab, followed when necessary by incubation in FITC-conjugated secondary Ab, washed, and analyzed on a FACS Calibur (Becton Dickinson, San Diego, CA).

Detection of apoptosis, as evidenced by annexin V binding to phosphatidylserine exposed at the cell surface, was performed by FACS using the Early Apoptosis Detection Kit (Kamiya Biomedical, Seattle, WA). At various times after UV irradiation, cells were flushed from the tissue culture dish, washed once, and resuspended in 500 μl binding buffer. Cells were incubated for 5 min in the dark with 0.25 μg/ml FITC-labeled annexin V and analyzed on a FACS Calibur (Becton Dickinson) immediately after addition of 0.25 μg/ml propidium iodide (PI).

**Subcellular fractionation**

Subcellular fractionation of D1 cells was performed as previously described (14), on a free flow electrophoresis (FFE) chamber (Dr. Werber, Ismaning, Germany). Fractions were collected, pooled pairwise, and analyzed for protein content (Bradford assay; Bio-Rad, Hercules, CA) and β-hexosaminidase activity (19). Fifteen pools of fractions, within 10 fractions of the peaks, were β-glucuronidase activity peak for further analysis. They were centrifuged at 100,000 × g for 1 h, and the pellets were resuspended in SDS-sample buffer with or without (for CD9 detection) 100 mM DTT and run on SDS-PAGE for Western blot analysis.

**Materials and Methods**

**Cells and exosome purification**

The splenoid murine DC line D1 (15) was cultured in complete medium: IMDM (Sigma, St. Quentin, France) supplemented with 10% endotoxin-free FCS (Valbiotech, ABCYS, Paris, France) and 30% conditioned medium from J558 (a GM-CSF-secreting plasmacytoma, provided by Dr. D. Gray, Hammersmith Hospital, London, U.K.) (16). Cells were split twice a week in 145-mm non-tissue culture-treated petri dishes (5 × 10<sup>5</sup> cells/dish).

Apoptosis was induced in D1 cells by UV treatment. On day 3 after passage, culture medium was replaced with 2 ml PBS, and cells in petri dishes were irradiated for 50 s with 2 mJ/cm<sup>2</sup>/s, using a 6 × 15W TFX-UV table (Vilber-Lourmat, Marne la Vallée, France). Control cells were treated identically, except for UV irradiation. Fresh medium was added, and cells further cultured for up to 24 h.

For exosome production, cells were cultured in complete medium depleted of contaminating vesicles and protein aggregates by overnight centrifugation at 110,000 × g (14). Supernatants were collected either 3 days after passage or 24 h after changing the medium of 3-day-old D1 cells culture. After UV treatment, exosome purification was performed as previously described (3) by three successive centrifugations at 300 × g (5 min), 1,200 × g (20 min), and 10,000 × g (30 min) to pellet cells and debris, followed by centrifugation for 1 h at 110,000 × g. For large scale preparations of exosomes (biochemical analysis), the 1,200 and 10,000 × g centrifugations were replaced by filtration on 0.22 μm to eliminate large debris. As assessed by electron microscopy (EM), Western blotting with known exosomal markers (14), and protein pattern on Coomassie blue-stained acrylamide gel, exosomes obtained this way are quantitatively and qualitatively similar to those obtained after successive centrifugations (P. Véron, unpublished observations).

**Protein identification by peptide mass mapping and tandem mass spectrometry**

After separation of 50 μg of exosomal proteins on 10 or 15% SDS-PAGE, the Coomassie-stained protein bands were excised from the gel, trypsin digested, and analyzed essentially as previously described (14, 17). Mass spectra of the peptide mixtures were acquired on a BiHex (Bruker-Franzen Analytik, Bremen, Germany) MALDI-TOF mass spectrometer equipped with a nitrogen laser for anion delayed extraction. The instrument was operated in the reflector mode. A mass list of peptides was obtained for each protein digest, and the appropriate software was used to identify the proteins (usually MS-FIT: http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm).

When a protein could not be confidently identified from its peptide mass map, the trypsin digest was extracted with acetonitrile and a 5% formic acid solution. The digest solution and the extracts were then pooled, dried in a vacuum centrifuge, and desalted with ZipTip C18 (Millipore, Bedford, MA) before the nanospray tandem mass spectrometry (MS/MS) analysis (18). A Q-TOF instrument (Micromass, Manchester, U.K.) was used with a Z-Spray ion source operating in the nanospray mode. About 3–5 μl of the desalted sample was introduced into a needle (medium sample needle, PROTANA, Odense, Denmark) to run MS and MS/MS experiments. The capillary voltage was set at an average voltage of 1000 V, and the sample cone was set at 50 V. Glutathionepetide was used to calibrate the instrum
Alternatively, eight fractions corresponding to the β-hexosaminidase activity peak were pooled and centrifuged for 1 h at 10,000 × g, and the pellet was loaded on 12% SDS-PAGE for Coomassie blue staining and protein analysis by trypsin digestion and MALDI-TOF mass spectrometry.

Western blotting

The same amount of proteins, as measured by Bradford assay, from control and UV-treated cells and pellets of the successive centrifugations were separated on 12% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore), and incubated with specific Abs, followed by HRP-conjugated secondary Abs, detected using an enhanced chemiluminescence kit (Roche Diagnostics, Meylan, France).

Sucrose gradient

Floatation of vesicles released by apoptotic cells on a continuous sucrose gradient was performed as described for exosomes (3, 14). Fractions of the gradient (1 ml each) were diluted in 2 ml PBS, centrifuged for 1 h at 100,000 × g, separated on a 12% SDS gel, and stained with Coomassie brilliant blue.

Electron microscopy

For EM observation of whole mounts of exosomes or apoptotic vesicles, pellets obtained after 110,000 × g centrifugation were fixed in 2% paraformaldehyde, loaded on Formvar/carbon-coated EM grids, postfixed in 1% glutaraldehyde, and contrasted successively in 2% uranyl acetate, pH 7, and 2% methylcellulose/0.4% uranyl acetate, pH 4. Observations were made with a CM20 Twin Phillips electron microscope (Phillips Electronic Instruments, Mahway, NJ).

Results

Identification of new exosomal proteins

The proteins identified to date in DC-derived exosomes are the major components of these vesicles and consist mostly of membrane-associated proteins (4, 14). The proposed model for exosome biogenesis, however, predicts that a small amount of cytosol is trapped inside exosomes. To further define their molecular identity, 50 µg of exosomes from a growth factor-dependent DC line (14, 15) were loaded on either 10 or 15% SDS gels (Fig. 1). All bands obtained were subjected to trypsin digestion and peptide mass mapping by MALDI-TOF mass spectrometry as previously described (14, 17), followed by tandem mass spectrometry (MS/MS) when necessary (18). Bands A–H in Fig. 1 correspond to major exosomal proteins identified previously (14), whereas numbers correspond to newly identified proteins. Table I gives a summary of the proteins identified.

Some of the proteins found in this study had been found previously in exosomes (14). Mac-1 β-chain (also called CR3 β or CD18) had been coimmunoprecipitated with its α counterpart (also called CD11b) from radiolabeled exosomes (14). Western blotting for various heat shock proteins had shown the presence of hsp84 in exosomes (14).

Whereas many of the major exosomal proteins identified before are transmembrane or peripherally associated with membranes (14), most of the newly identified proteins are cytosolic: cytoskeleton and cytoskeleton-binding proteins (tubulin, actin, collapsin, profilin I, elongation factor-1α), membrane-associated proteins involved in intracellular transport (annexins I, II, IV, V, and VII; small GTPase family members or related proteins: rab7, rab11, rap1B, and rab GDP dissociation inhibitor), or cytosolic proteins involved in signal transduction (Gαi, Gαq, syntaxin, and 14-3-3) or in protein translation (elongation factor-1α and elongation initiation factor-4A). Importantly, several of the newly identified proteins are related to apoptosis, either as markers specifically released by cells undergoing apoptosis (histones H2A–H4), or as putative pro- or anti-apoptosis factors (respectively, AIP1/Alix, thioredoxin peroxidase II (TPXII), 14-3-3, and galec tin-3).

This analysis therefore provides an extended set of proteins specifically targeted to exosomes in DCs. A schematic representation of DC-derived exosomes, as observed in this and our previous study, is given in Fig. 2.

Endocytic origin of exosomes

Consistent with the proposed late endosomal origin of exosomes (3–5), several proteins identified in these vesicles are associated with endosomes and lysosomes: annexin II (20), Gα1α (18), hsc73 (21), MHC class II (22), MHC class I (4), and CD86 (23). This is not the case, however, for two of the major transmembrane proteins, CD9 and Mac-1, which have to date only been described intracellularly to exosomes.

To determine whether these two molecules are also present in endocytic compartments, endosomes and lysosomes of D1 cells were purified by FFE as previously described (14). As shown in Fig. 3A, membrane fractions deviated toward the anode of the electrophoresis chamber contain a very small fraction of the total proteins (fractions 30–35, proteins curve, lower panel). Most of the β-hexosaminidase (a lysosomal enzyme) activity (betaHex curve, lower panel), but no ER-resident proteins (14), are present in these fractions, which therefore represent an enriched population of endosomes and lysosomes.

Analysis of the FFE fractions by Western blot (Fig. 3A, upper panels) showed that MHC class II and CD9 are present in the endocytic fractions. The presence in these fractions of Mac-1, for which no Ab working in Western blotting is available, was revealed by SDS-PAGE of pooled lysosomal fractions (Fig. 3B), and peptide mass mapping of two bands running at 175 and 90 kDa, which corresponded, respectively, to the α- and β-chains of
<table>
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<th>Matching Peptides</th>
<th>Sequence Coverage (%)</th>
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a Identification method: MS: MALDI-TOF peptide mass fingerprinting; MS/MS: sequences obtained by tandem mass spectrometry (number of peptides sequenced).
b Accession number in SwissProt except where noted by footnotes (d and e).
c Bovine proteins from FCS.
d Accession number in NCBInr.
e Accession number in TrEMBL.
Mac-1. Another major exosome protein, milk fat globule-EGF factor 8 (MFG-E8)/lactadherin, was also found in purified endocytic compartments by peptide mass mapping (not shown).

Conversely, if exosomes are derived from endocytic compartments, certain membrane proteins expressed at the cell surface should be absent from exosomes. To examine surface expression of membrane proteins in cells and exosomes, we set up a FACS assay for exosomes. Exosomes were covalently linked to 3- to 4-μm aldehyde-activated latex beads, and the presence of membrane proteins was revealed by immunofluorescence. As expected from our proteomic analysis, MHC class I and II, CD86, CD9, and Mac-1 can be readily detected using this assay, as efficiently as on live cells (Fig. 4A). Importantly, Lamp-2, a lysosomal resident not expressed at the plasma membrane, was also readily detected on exosomes (Fig. 4, B and C). Furthermore, abundant plasma membrane proteins, such as type I (data not shown) and types II/III Fc receptor (FcγR II/III), were not detected in exosomes (Fig. 4, B and C). Enrichment of Lamp2 and absence of FcγR II/III in exosomes were also confirmed by Western blot (Fig. 4C). Consistent with their absence in exosomes, FcγR II/III were not detected in endocytic compartments (Fig. 3A, upper panel).

These results show a clear correlation between the presence in exosomes and the endocytic localization of various membrane proteins. In contrast, other membrane proteins that accumulate in endocytic compartments in immature DC (i.e., the vacuolar ATPase and the nonpolymorphic MHC class II molecule H-2M; data not shown) are not found in exosomes. Exosomes therefore bear a unique pattern of proteins related to apoptosis. histones are released by apoptotic cells as chromatin fragments (24), AIP-1/Alix, a protein of unknown function, interacts with the proapoptosis protein ALG-2 (25, 26), and TPxII (27, 28), 14-3-3 (29), and galectin-3 (30) can protect cells from apoptosis. Since apoptotic cells are known to release membrane microvesicles in vitro, it was important to determine whether exosome production is somehow related to apoptosis.

To induce apoptosis, D1 cells were submitted to UV irradiation and then cultured for up to 24 h in fresh culture medium. At different times after irradiation, cells were collected, stained with FITC-labeled annexin V, an early marker of apoptosis, and PI, a DNA intercalating compound that only stains cells with permeabilized membrane (i.e., necrotic cells). FACS analysis (Fig. 5) shows that between 3 and 6 h after UV treatment, cells transiently enter an early apoptosis stage characterized by annexin V staining and low PI staining (lower right quadrant in Fig. 5 contains 4% of cells at 3 h, 10% at 6 h, and 18% at 9 h). At later time points, cells undergo secondary necrosis, characterized by both annexin V and PI staining (upper right quadrant contains 32% of cells at 3 h, 58% at 6 h, 71% at 9 h, and 97% at 24 h).

Twenty-four hours after UV irradiation, culture supernatants were collected, centrifuged successively at 300 × g, 1,200 × g, and 10,000 × g to pellet cells and large debris, and finally at 110,000 × g to collect small vesicles. The amount of membrane material collected in the successive pellets after centrifugation of supernatants from UV-treated cells was usually 2–4 times larger than what was collected from control cells (Fig. 6A). Thirty or 10 μg of proteins from whole cells or from the three successive pellets (1,200, 10,000, and 110,000 × g) were loaded on 10% SDS-PAGE and subjected to Western blotting using Abs specific for MHC class II, a major exosomal protein, or for the apoptosis-related protein AIP-1/Alix. As expected, MHC class II molecules are abundant in exosomes (i.e., 110,000 × g pellet from control cells; Fig. 6B), but are hardly detected in the 1,200 and 10,000 × g pellets of control cells. Apoptotic cells, on the other hand (Fig. 6B, +UV), release abundant MHC class II molecules associated to both low speed (1,200 and 10,000 × g) and high speed (110,000 × g) pellets. Confirming our proteomic analysis, we detected AIP-1/Alix by Western blot in the 110,000 × g pellet from both control and UV-treated cells, where it is roughly 3 times more concentrated than in the cells (Fig. 6B). Alx is also present in the 1,200 × g pellet from UV-treated cells, but not from control cells (Fig. 6B, 1,200 × g). This pellet probably represents large membrane blebs released by apoptotic cells, whereas the 110,000 × g pellet contains smaller vesicles.

The protein composition of lysed cells, or the 110,000 × g pellet purified from control or UV-treated cells, was analyzed by SDS-PAGE and Coomassie blue staining. Fig. 6C shows that the protein patterns of the control and apoptotic microvesicles are distinct;
whereas the 110,000 × g pellet produced by non-UV-treated cells contains the typical exosomal major proteins (bands B–G, Fig. 6C), the same pellet from apoptotic cells also contains major proteins that are absent from exosomes: a protein strongly expressed at the cell surface, type II/III FcγR, is absent from exosomes; conversely, a lysosomal protein, Lamp2, weakly expressed at the cell surface, is present on exosomes. Fig. 6C, however, shows that they are present at hardly detectable levels when 30 μg of total proteins from control exosomes are run on SDS-PAGE (50 μg had been used for the analysis in Table I). This suggests that exosome preparations may contain some material coming from the few cells undergoing spontaneous apoptosis in the culture, but that this material is scarce compared with exosomes produced by live cells. In contrast, vesicles produced by apoptotic cells contain some regular exosomes, most likely produced by the cells before they enter apoptosis, plus a large proportion of histone-containing material. Interestingly, as shown in Fig. 6D, membranes secreted by apoptotic cells contain two different populations of vesicles characterized by different densities on a sucrose gradient. Histones are associated to membranes that float at a density of 1.24–1.28 g/ml (bands 1, 3, and 4 in Fig. 6D), whereas most other proteins are associated with membranes floating at a density of 1.18 g/ml. This latter density is slightly higher than the usual exosomal density (1.15 g/ml) (3, 12, 14). Finally, observation by EM of the material obtained showed that apoptotic vesicles (Fig. 7B) are much larger and denser and do not present the characteristic cup shape of exosomes (Fig. 7A), making them easily distinguishable.

In conclusion, exosomes and apoptotic vesicles represent distinct populations of secreted membranes, differing in their modes of production and in their protein compositions.

Discussion

Secretion of membranes by cells of the immune system represents an ill-defined biological process. Both the modes of biogenesis and the potential physiological role of secreted membranes are as yet unclear. In an attempt to better understand the function in the immune response of a particular population of secreted membrane vesicles called exosomes, we have undertaken an extensive analysis of their protein composition. We thus established the first

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**FIGURE 3.** Comparison of the protein composition of exosomes and endocytic compartments. Endocytic compartments of D1 cells were purified by FFE as previously described (14). A, Membrane fractions deviated toward the anode (fractions 30–35), which represent a very small fraction of the total membrane proteins (proteins curve), contain lysosomal enzymes (β-hexosaminidase, betaHex curve) and MHC class II molecules (known to be retained in lysosomal compartments in immature DCs). CD9 is present in these fractions, but FcγRII/III is not and is also absent from exosomes (see Fig. 4). B, Anode-deviated fractions from D1 cell fractionation by FFE were pooled and run on a 10% SDS-gel (pool). The two major bands running around 175 and 90 kDa were analyzed by trypsin digestion and peptide mass mapping and were identified as Mac-1 α- and β-chains respectively.

**FIGURE 4.** Direct comparison of exosomes with the plasma membrane, using FACS analysis. A and B. An assay to analyze exosomal proteins by FACS was performed. Exosomes (Exos) were coated on beads, then stained with FITC-coupled Abs specific for various membrane-associated proteins (bold line), or the corresponding isotype-matched negative control (thin line). Nonpermeabilized cells (Cells) were stained in parallel with the same Abs. A. All the proteins known to be present in exosomes (4, 14) are detected by FACS both at the cell surface and in exosomes, confirming the validity of this assay. B, FACS analysis was used to detect the presence of other membrane proteins on exosomes: a protein strongly expressed at the cell surface, type II/III FcγR, is absent from exosomes; conversely, a lysosomal protein, Lamp2, weakly expressed at the cell surface, is present on exosomes. C, Enrichment of Lamp2 and absence of FcγRII/III on exosomes were confirmed by Western blot. The quality of the exosome preparation was confirmed by hybridization with an anti-MHC class II Ab.
extensive protein map of DC-derived exosomes. Together with our
functional analysis published previously, the results presented here
define exosomes as a bona fide cellular compartment, characterized
by a unique molecular composition and mediating a specific
biological function.

Unexpectedly, our proteomic analysis revealed a novel category
eyes of exosomal proteins, composed of several molecules implicated in
apoptosis. This observation led us to explore possible structural
relationships between exosomes and apoptotic blebs. Indeed, both
exosomes from DCs (4) and apoptotic blebs and bodies from
monocytes (10) have been shown to induce immune responses
mediated by DCs. Consistent with previous observations showing
that apoptotic cells release membrane particles (7), we obtained
larger amounts of membrane-bound material from dying than from
healthy cells. UV-treated cells release particularly abundant large
membrane particles, probably corresponding to apoptotic corpses.
In addition, we also purified, from apoptotic cell supernatants,
smaller membrane particles that pellet only at high speed, like
exosomes from healthy cells. These small vesicles from apoptotic
cells are distinct from exosomes, since they contain very abundant
histones associated with membranes floating at a high sucrose density
(1.24–1.28 g/ml), and they are very heterogeneous in size and
morphology when observed by EM. In exosomal preparations from
healthy, non-UV-treated cells, some large dense vesicles can occasionally be observed by EM, and some histones can be
detected, but they represent a very minor subset of vesicles, most
likely resulting from the small number of apoptotic cells present in
the culture. Exosomes, as defined by their protein composition (no ER-
or nuclear-resident proteins), their density on sucrose gradient
(1.15–1.18 g/ml), and their cup-shaped morphology in EM, are
therefore a defined subcellular compartment released by living
DCs as a physiological process.

Therefor, exosomes and apoptotic blebs are different in nature.
It is most likely that the small amount of histones found in exo-
some preparations come from some apoptotic material present in
the preparations. This is probably also true for the nuclear and
Golgi-associated protein, the transcription factor tumor suscepti-
bility protein (32), and the translation initiation factor elongation
initiation factor-4A, which is normally associated with ribosomes.
The other apoptosis-related proteins identified in exosomes, how-
ever, are most likely real exosome components. AIP-1/Alix is a
Previously reported by some of us (J. Garin) to be present in mac
somes. Importantly, several proteins identified in exosomes were
polymerization (42), which may, in turn, induce membrane invag-
ation/invagination-coupled process may also be involved in the

Our results also strongly support the previous model of exosome
biogenesis in the endocytic pathway. Indeed, most exosomal com-
ponents have been previously shown, or are shown herein, to be
present in or associated with endosomes and lysosomes. This is
ture for membrane proteins, such as tetraspanins (Ref. 2 and this
study), Lamp2, MHC molecules (22), or Mac-1 (this study), and
for cytosolic proteins, such as hsc73 (21), syntenin (33), rab7 (34),
rab11 (35), rap1B (36), and several annexins (37). Both actin and
and/or functional relationships between the endocytic pathway and
the apoptotic process, which remain to be defined.

Another category of exosome-associated proteins are those in-
olved in membrane traffic. Annexins bind to intracellular mem-
branes and are generally involved in intracellular membrane fusion
(46). Association of annexins with exosomes could be a conse-
quence of the presence of phosphatidylserine in these vesicles (P.
Véron, unpublished observation). The small GTP-binding protein
rab7 associates with endosomes upon GTP binding, and the cycle
between GDP-bound cytosolic and GTP-bound membrane-associated
forms of rab proteins is regulated by the GDP-dissociating
inhibitor rabGDI (47). Association of rab7 to late endosomes is
necessary for fusion with lysosomes (48). Rap1B is also a late
endosome-associated GTP-binding protein (36), but its role and
the compartments it regulates are not yet known (49). All these
proteins could be involved in budding of vesicles from the external
membrane of the multivesicular endosome to form the exosomes
and/or in fusion of these compartments with the plasma membrane
that result in exosome secretion. Interestingly, two cytosolic pro-
teins found in exosomes have been described in the extracellular
environment: galectin-3, which modulates cell interaction with
laminin (50, 51), and annexin II (52). These proteins do not bear
a signal sequence responsible for secretion through the constitutive
pathway; it would therefore be interesting to determine whether
exosomes represent an unconventional secretion pathway for some
proteins (53).

Besides proteins potentially implicated in the process of exo-
some formation, we have also evidenced many proteins that may
be involved in the biological functions of exosomes. Several pro-
teins exposed at the surface of exosomes bind ligands on other
cytosolic and membrane-associated protein binding to the pro-
apoptosis factor ALG-2 in the presence of Ca^{2+} (25, 26). We
confirmed here by Western blot (Fig. 6B) that AIP-1/Alix is abun-
dant in exosomes, and, most importantly, that, even if it is also
present in vesicles produced by apoptotic cells, it is not as enriched
therein. The presence of Alix in exosomes could be related to its
reported association with internal membranes (26) and phagosomes
(18). It will be interesting to analyze precisely the intracel-
ular compartments with which AIP-1/Alix associates and their
relation to late endosomes and lysosomes from which exosomes
originate. AIP-1/Alix is homologous to yeast and fungus genes
participating in signal transduction pathways (25, 26); it may
therefore have other functions, unrelated to its binding to the pro-
apoptotic molecule ALG-2, important for exosome’s biology. The
other proteins found in exosomes and related to apoptosis have
antiapoptotic activities. TPxII (27, 28) and galectin-3 (30) protect
cells against oxidative damages, and 14-3-3 inhibits the proapop-
tosis effect of the protein Bad (29). The presence in exosomes of
these apoptosis-related proteins suggests unexpected structural
and/or functional relationships between the endocytic pathway and
the apoptotic process, which remain to be defined.

Table II. Identification of proteins released by apoptotic cells, based on
MALDI-TOF peptide mass fingerprinting

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^a Accession number in SwissProt except where indicated by footnotes (b and c).
^b Accession number in TrEMBL.
^c Accession number in NCBI nr.
membranes: adhesion molecules ICAM-1 and -2 for Mac-1 (54), integrins αβ2 and αβ5 for MFG-E8/lactadherin (55, 56), and an EGF-like growth factor receptor for CD9 (57, 58). CD9 also has an essential role in sperm-oocyte docking and/or fusion during fecundation (59, 60). These proteins could be involved in exosomes targeting, docking, and/or fusion with other cells. Exosomes could thus represent a new way of communication, i.e., exchange of antigenic information, between cells in the immune system. This idea is consistent with recent reports showing that exchange of membranes bearing MHC-peptide complexes occurs between APCs (61) or between APCs and T cells (62). It could also account for older observations that described shedding of membrane vesicles from spleen (63) or tumor cells (64), giving rise to antigenic material, or soluble MHC molecules in the serum of transplant patients (65).

Thus, besides direct cell-cell contact and the secretion of soluble proteins, exosomes could represent an additional means of communication between cells of the immune system. Exosomes could deliver integrated signals through different surface receptors on target cells and, if exosomes fuse with acceptor cells, they could also transfer membrane and cytosolic proteins between different cells. In vivo, exosomes have been evidenced in tosnil B follicles (13) or in serum (our unpublished observations). The cellular source of these exosomes, however, is probably heterogeneous, and formal demonstration that DCs secrete exosomes in vivo awaits further analyses.

Finally, although a physiological role for exosomes has yet to be demonstrated, their use in tumor immunotherapy is currently being implemented. This study should also allow to improve exosome-based immunotherapy strategies and help in defining new vaccination strategies.

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References


