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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 (collin, profilin I, and elongation factor 1α) and intracellular membrane transport and signaling factors (such as several annexins, rab 7 and 11, rap1B, and syntenis). 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, β, 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-
tumor immune responses in vivo and induce the rejection of established tumors (4). The mechanism of action of exosomes in vivo is poorly understood. Exosomes could stimulate T cells directly, through the MHC-peptide complexes they harbor, or they could be captured by other professional APC, which could then use peptide-loaded MHC molecules, Ags, or peptides present in exosomes to stimulate T cells.

To define the identity and the modes of action of DC-derived exosomes, we recently undertook an analysis of their protein composition (14). Using trypsin digestion and peptide mapping by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, we identified nine major protein components of exosomes. Potentially interesting exosomal components thus identified are hsc70, a heat shock protein with potent immune stimulatory activity, and several membrane-associated proteins with affinity for ligands on other cell membranes that may target exosomes to their effector cells.

Here we have identified a new set of 21 proteins specifically enriched in exosomes, thus establishing an extensive molecular map of DC-derived exosomes. Exosomal proteins include molecules initially described in the endocytic pathway, at the plasma membrane, or in the cytosol, but not in mitochondria, Golgi apparatus, or the ER. Interestingly, four identified proteins play a role in apoptosis. These findings led us to re-examine the possible relationship between exosomes and the plasma membrane, and between exosomes and membranes produced by DCs undergoing apoptosis (apoptotic microvesicles). By direct comparison of the biochemical composition of exosomes, plasma membrane, endocytic compartments, and microvesicles released by apoptotic cells, we provide new evidence of the biochemical similarities between exosomes and endocytic compartments and of the distinct nature of exosomes and membrane microvesicles released by cells undergoing apoptosis.

Materials and Methods

Cells and exosome purification

The spleen-derived 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 × 10^5) 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^2/s, using a 6 passage, culture medium was replaced with 2 ml PBS, and cells in petri dishes were flushed from the tissue culture apparatus, or the ER. Interestingly, four identified proteins play a role in apoptosis. These findings led us to re-examine the possible relationship between exosomes and the plasma membrane, and between exosomes and membranes produced by DCs undergoing apoptosis (apoptotic microvesicles). By direct comparison of the biochemical composition of exosomes, plasma membrane, endocytic compartments, and microvesicles released by apoptotic cells, we provide new evidence of the biochemical similarities between exosomes and endocytic compartments and of the distinct nature of exosomes and membrane microvesicles released by cells undergoing apoptosis.

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 Biflex (Bruker-Franzen Analytik, Bremen, Germany) MALDI-TOF mass spectrometer equipped with a nitrogen laser. 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 working 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: Pepfrag (http://prowl1.rockefeller.edu/prowl/pepfragch.html), peptide search (http://www.mann.emb-lheidelberg.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-C2 (AF6-88.5.1 clone), I-A^b (AF6-120.1 clone), CD11c (HL3), and the corresponding FITC-conjugated isotype-matched controls rat IgG2b, mouse IgG2a, and hamster IgG (all from PharMingen, San Diego, CA), and rat anti-mouse FcγR type II/III (2.4G2 clone), followed by FITC-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA); for FACS analysis: Western blotting: rat anti-mouse CD9 (KMCD9 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 antisera anti-mouse MHC class II α-chain C terminus (14), anti- FcγR type II/III (provided by Dr. C. Santes, Institut National de la Santé et de la Recherche Médicale, Unit 255, Paris, France), and anti- AIP1/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 5 min in 100 mM glycine. Exosome- or FCS-coated beads were washed three times in FACS wash (3% FCS and 0.1% NaNO3 in PBS) and resuspended in 500 μl FACS wash. In parallel, D1 cells were washed twice in FACS wash. Cells (10^7) 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-hexosaminidase activity-irradiated 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.
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 Philips 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, cofilin, 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 (Gα, α, syntelin, 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 galectin-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), Gα,α (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 at the cell surface.

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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<tr>
<th>Band (Fig. 1)</th>
<th>Protein Name</th>
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<th>Molecular Mass (kDa)</th>
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\( ^{\text{a}} \) Identification method: MS: MALDI-TOF peptide mass fingerprinting; MS/MS: sequences obtained by tandem mass spectrometry (number of peptides sequenced).

\( ^{\text{b}} \) Accession number in SwissProt except where noted by footnotes (\( ^{\text{d}} \) and \( ^{\text{e}} \)).

\( ^{\text{c}} \) Bovine proteins from FCS.

\( ^{\text{d}} \) Accession number in NCBInr.

\( ^{\text{e}} \) Accession number in TrEMBL.
important to determine whether exosome production is somehow
associated with apoptosis. However, proteins known to be
involved in apoptosis, such as lipid raft proteins ALG-2 (25, 26),
and TPxII (27, 28), 14-3-3 (29), and Alix, a protein of unknown
function, interacts with the proapoptotic protein Bax (30), which is
released by apoptotic cells as chromatin fragments (24), AIP-1/
Alix, is also present in exosomes. Histones are released from
nuclei and accumulate in endo-lysosomes and other endocytic
compartments in immature DC (i.e., the vacuolar ATPase
complex) (31). However, certain membrane proteins expressed
together with histones are not found in exosomes. Exosomes
therefore bear a distinct membrane orientation (Fig. 3) that
must be absent from endosome-derived vesicles. This
orientation is consistent with the current model for the biogenesis
of these vesicles (3, 5).

Comparison of exosomes and vesicles released by cells
undergoing apoptosis

Another striking observation from our proteomic analysis is that
exosomes contain proteins associated with apoptosis: histones are
released by apoptotic cells as chromatin fragments (24), AIP-1/
Alix, a protein of unknown function, interacts with the proapop-
tosis protein ALG-2 (25, 26), and TPxII (27, 28), 14-3-3 (29), and
galecian-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 perme-
able membranes (i.e., necrotic cells). FACs analysis (Fig. 5) shows
that between 3 and 6 h after UV treatment, cells transiently
undergo 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 pel-
lets (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 concen-
trated than in the cells (Fig. 6B). Alix 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 con-
tains 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;

FIGURE 2. Schematic representation of our current
model for their biogenesis (3, 5).
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 (bands 1–4, Fig. 6C). The four major bands running around 20 kDa (no. 1–4) in apoptotic 110,000 × g pellet were trypsin-digested and identified by peptide mass mapping (Table II); they correspond to four types of mammalian histones, known to be released as complexes with DNA (chromatin) by cells undergoing apoptosis (24, 31). Interestingly, these histones have been identified in the proteomic analysis of control exosomes (Table I). 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
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 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.

Therefore, exosomes and apoptotic blebs are different in nature. It is most likely that the small amount of histones found in exosome 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 susceptibility 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, however, are most likely real exosome components. AIP-1/Alix is a
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 abundant 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 intracellular 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 proapoptosis 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.

Our results also strongly support the previous model of exosome biogenesis in the endocytic pathway. Indeed, most exosomal compounds have been previously shown, or are shown herein, to be present in or associated with endosomes and lysosomes. This is true 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), synetin (33), rab7 (34), rab11 (35), rap1B (36), and several annexins (37). Both actin and tubulin interact with endosomes and/or lysosomes (38), and it is therefore not surprising that we also found several actin-binding proteins associated with exosomes: cofilin (39), profilin I (40), and elongation factor-1α (41). Interestingly, cofilin promotes actin depolymerization (42), which may, in turn, induce membrane invagination at the plasma membrane (43). A similar actin depolymerization/invagination-coupled process may also be involved in the formation of exosomes from the limiting membrane of late endosomes. Importantly, several proteins identified in exosomes were previously reported by some of us (J. Garin) to be present in macrophage phagosomes: G$_{i2\alpha}$, galectin 3, 14-3-3, Alix, synetin, rab7, rab11, rap1-B, annexin V, hsc70, hsp84, and MFG-E8/lactadherin (18).

Several proteins identified in exosomes play roles in different signal transduction pathways. The many isoforms of 14-3-3, four of which are present in exosomes, are ligands for various intracellular proteins, especially serine-phosphorylated transmembrane receptors or actors of signaling cascades (44). Synetin also functions as an adaptor molecule between transmembrane receptors and signaling pathways (45). The signal transduction factors that accumulate in exosomes are most likely involved either in endocytic transport through late endosomes or in the biogenesis of internal vesicles within multivesicular endosomes.

Another category of exosome-associated proteins are those involved in membrane traffic. Annexins bind to intracellular membranes and are generally involved in intracellular membrane fusion (46). Association of annexins with exosomes could be a consequence 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 proteins 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 exosome formation, we have also evidenced many proteins that may be involved in the biological functions of exosomes. Several proteins exposed at the surface of exosomes bind ligands on other

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

<table>
<thead>
<tr>
<th>Band (Fig. 6)</th>
<th>Protein Name</th>
<th>Accession Number*</th>
<th>Molecular Mass (kDa)</th>
<th>Matching Peptides</th>
<th>Sequence Coverage (%)</th>
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<td>4</td>
<td>Histone H4</td>
<td>90626c</td>
<td>11</td>
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* Accession number in SwissProt except where indicated by footnotes (b and c).
* Accession number in TrEMBL.
* Accession number in NCBI nr.

FIGURE 7. EM observation of membrane particles purified from control and apoptotic cells. The 110,000 $\times$ g pellet obtained from the supernatants of control (A) or apoptotic (B) D1 cells was fixed in 2% paraformaldehyde and processed as described in Materials and Methods for EM observation. A. Small 40- to 90-nm vesicles displaying a cup-shaped morphology characteristic of exosomes are observed in the supernatant of living cells. B. Larger membrane vesicles, denser to electrons and very heterogeneous in size are purified from the supernatant of dying cells. Scale bar = 1 µm.
membranes: adhesion molecules ICAM-1 and -2 for Mac-1 (54), integrins αβ, and αβ for MFG-E8/factadherin (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 exosome 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 tonsil 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


