The Journal of Immunology

RESEARCH ARTICLE | DECEMBER 15 2001

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J Immunol (2001) 167 (12): 6736–6744.
https://doi.org/10.4049/jimmunol.167.12.6736

Differential Secretion of Fas Ligand- or APO2 Ligand/TNF-Related Apoptosis-Inducing Ligand-Carrying Microvesicles During Activation-Induced Death of Human T Cells[1]

Inmaculada Monleón,[2]* María José Martínez-Lorenzo,[2†] Luis Monteagudo,[‡] Pilar Lasierra,[†] Marta Taulés,[§] María Iturralde,* Andrés Piñeiro,* Luis Larrad,[†] María Angeles Alava,* Javier Naval,* and Alberto Anel[3]*

Preformed Fas ligand (FasL) and APO2 ligand (APO2L)/TNF-related apoptosis-inducing ligand (TRAIL) are stored in the cytoplasm of the human Jurkat T cell line and of normal human T cell blasts. The rapid release of these molecules in their bioactive form is involved in activation-induced cell death. In this study, we show by confocal microscopy that FasL and APO2L/TRAIL are mainly localized in lysosomal-like compartments in these cells. We show also by immunoelectron microscopy that FasL and APO2L/TRAIL are stored inside cytoplasmic compartments ~500 nm in diameter, with characteristics of multivesicular bodies. Most of these compartments share FasL and APO2L/TRAIL, although exclusive APO2L/TRAIL labeling can be also observed in separate compartments. Upon PHA activation, the mobilization of these compartments toward the plasma membrane is evident, resulting in the secretion of the internal microvesicles loaded with FasL and APO2L/TRAIL. In the case of activation with anti-CD59 mAb, the secretion of microvesicles labeled preferentially with APO2L/TRAIL predominates. These data provide the basis of a new and efficient mechanism for the rapid induction of autocrine or paracrine cell death during immune regulation and could modify the interpretation of the role of FasL and APO2L/TRAIL as effector mechanisms in physiological and pathological situations. ***The Journal of Immunology*, 2001, 167: 6736–6744.**

After a cellular immune response has taken place, most activated T cells should be eliminated by a process called activation-induced cell death (AICD)[4] to prevent potential autoimmune damage (1). Interaction of Fas (APO_1/CD95) receptor with its natural ligand, Fas ligand (FasL), mediates AICD of T cell hybridomas (2–4) and mature normal T lymphocytes (5, 6), playing a key role in regulation of peripheral tolerance and lymphocyte homeostasis (7, 8). TNF and TNF receptors have been also implicated in mature T cell AICD (9).

APO2 ligand (APO2L), also known as TNF-related apoptosis-inducing ligand (TRAIL) is another member of the TNF family, which induces cell death in a Fas-independent fashion (10, 11). Several receptors for APO2L/TRAIL, which belong to the TNF receptor family, have been cloned. Some of them transduce death signals, whereas others act as "decoy" receptors (12). APO2L/TRAIL may also participate in AICD of Jurkat and normal human T cell blasts (13).

Although it was initially reported that the soluble form of FasL, generated through the metalloproteinase-mediated cleavage of the membrane protein, retained its cytotoxic potential (14, 15), later studies demonstrated that membrane-bound FasL is the functional form of the molecule, being the toxic activity of the cleaved soluble form much lower (16–18). In fact, it has been proposed that FasL proteolytic processing is a mechanism of functional down-regulation (17).

In a previous work, we characterized that bioactive FasL and APO2L/TRAIL were rapidly released to the supernatant of human T cells undergoing AICD in the form of whole, nonproteolyzed proteins, associated with a particulate, ultracentrifugable fraction. This fraction was characterized by scanning electron microscopy as microvesicles 100–200 nm in diameter (19). More recently, it has been observed that Fas is present in trimeric form at the surface of T cells and that the induction of cell death is dependent on higher-order multimerization of the receptors (20). This role would be perfectly fitted by FasL-expressing microvesicles.

Additionally, we have found that exclusive cross-linking of the nonpolymorphic, GPI-linked surface molecule CD59 was also able to induce AICD in overactivated human T cells (21). It was also found that the supernatants of anti-CD59 mAb-activated T cells were cytotoxic against Jurkat cells and that, in this case, cytotoxicity was mainly due to APO2L/TRAIL release, with almost no contribution of FasL (21). In the present work, we have characterized the mechanisms of secretion of FasL and APO2L/TRAIL during AICD processes.

*Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, [†]Servicio de Inmunología, Hospital Clínico Universitario, and [‡]Departamento de Anatomía, Embriología y Genética, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain; and [§]Serveis Científico-Tècnics, Facultat de Medicina, Universitat de Barcelona, Barcelona, Spain

Received for publication August 14, 2001. Accepted for publication October 10, 2001.

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[1] This work was supported by Grant 99/1250 from Fondo de Investigaciones Sanitarias, Spain, and Grant P24/2000 from Diputación General de Aragón/Fondo Social Europeo; I.M. was supported by a fellowship from Diputación General de Aragón.

[2] I.M. amd M.J.M.-L. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Alberto Anel, Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, E-50009 Spain. E-mail address: anel@posta.unizar.es

[4] Abbreviations used in this paper: AICD, activation-induced cell death; FasL, Fas ligand; APO2L, APO2 ligand; TRAIL, TNF-related apoptosis inducing ligand; MVB, multivesicular body; PLT, progressive lowering temperature.

0022-1767/01/$02.00

Materials and Methods

Cells and cell culture

The human T cell leukemia Jurkat (American Type Culture Collection, Manassas, VA; clone E6.1) was cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics (complete medium). Human PBMC were obtained from blood of healthy donors by Ficoll-Paque density centrifugation, as indicated elsewhere (13). Seven-day T cell blasts were generated as follows. PBMC (2×10^6 cells/ml) were stimulated during 1 day with 10 μg/ml PHA. Afterward, PHA was washed, and cells were resuspended in complete medium supplemented with IL-2 and cultured for 6 days with medium changes every 48 h.

Cytotoxicity assays

Sensitivity to AICD was tested by pulse stimulation with 50 μg/ml PHA for 5 min and incubation for 16 h, as indicated in Ref. 22. Cell viability was determined by a modification of the MTT reduction method (23), and cell death was determined by trypan blue staining. Toxicity of supernatants from cells pulse-stimulated with PHA was tested as indicated in Ref. 22. For CD59 stimulation, the anti-CD59 mAb VJ1/12.2, kindly provided by Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain), was immobilized in the bottom of the wells of 96-well plates. Wells were washed with PBS, cells (5×10^6 cells/ml) were added in fresh complete medium or in RPMI 1640, and supernatants were collected after different incubation times at 37°C. To analyze the involvement of secreted FasL or APO2L/TRAIL in the toxicity of supernatants from PHA- or CD59-stimulated cells, bioassays were also performed in the presence of 100 ng/ml of the anti-human Fas mAb SM1/23 (Bender MedSystems, Barcelona, Spain) or of the anti-APO2L/TRAIL mAb 5C2 to prevent FasL and APO2L/TRAIL binding to their receptors, respectively (13, 21, 22).

Immunoblotting

Detection of FasL in cell lysates or culture supernatants was performed with a rabbit polyclonal Ab (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) which recognizes the intracellular N-terminal region of FasL, the specificity of which was validated in previous studies (13, 24). The N20 Ab lot (D159) used in this study detected a major 40-kDa band in Jurkat and T cell blasts. Rabbit anti-human APO2L/TRAIL polyclonal Ab pAb#3, directed against the extracellular region of APO2L/TRAIL, recognizes a 41-kDa band in extracts from Jurkat and human T cell blasts (13) and was kindly provided by Dr. A. Ashkenazi (Genentech, South San Francisco, CA).

The presence of APO2L/TRAIL in supernatants from cells activated with anti-CD59 mAb was analyzed as follows. Cells (2×10^7) were resuspended in 200 μl RPMI 1640, and four 50-μl aliquots were placed on wells of a 96-well plate, in which the anti-CD59 mAb had been previously immobilized. After 3 h incubation at 37°C, the plates were briefly centrifuged, and 40-μl aliquots of the supernatant were carefully harvested from each well, mixed, and clarified by two sequential centrifugations at $18{,}000 \times g$ for 5 s each. Supernatants from cells incubated in the same conditions on wells coated with 1% BSA were used as controls. Finally, supernatants were mixed with 50 μl 4×SDS sample buffer and subjected to 12% SDS-PAGE and immunoblot with the pAb#3 anti-APO2L/TRAIL Ab.

Ultracentrifugation

Cells (2×10^8) were resuspended in 10 ml RPMI 1640 and stimulated with immobilized anti-CD59 mAb, obtaining their supernatants as described above (total volume, 8 ml). A 6-ml aliquot of this supernatant was subjected to ultracentrifugation at $100{,}000 \times g$ for 8 h. Supernatant from ultracentrifugation was recovered, the pellet was resuspended in 1.5 ml RPMI 1640, and the presence of APO2L/TRAIL was analyzed by immunoblotting. For toxicity experiments, the pellet was resuspended to 6 ml with RPMI 1640, and the toxicity of both fractions on nonactivated Jurkat cells was analyzed by the MTT assay and trypan blue staining.

Flow cytometry analysis of microvesicles

Labeling of microvesicles was performed by adding 1 μl of a 1-mg/ml solution of the FITC-labeled rat anti-human FasL mAb (H11; Bender MedSystems) and 1 μl of a 2.2-mg/ml solution of the mouse anti-human APO2L/TRAIL mAb 5C2 to 100 μl cell supernatants, obtained in the different conditions described, and incubation at 4°C for 1 h. Then, 1 μl of a 1-mg/ml solution of a PE-labeled goat anti-mouse IgG (Caltag, Barcelona, Spain) was added to the microvesicle suspension and incubated for 30 min at 4°C. Labeled microvesicle suspension was diluted to 1 ml with PBS, and fluorescence was analyzed by flow cytometry (EPICS XL-MCL; Beckman Coulter, Barcelona, Spain). Supernatants obtained in the same conditions and incubated with a FITC-labeled rat IgG and the secondary PE-labeled Ab were used as controls. Microvesicles were gated as previously described (19).

Confocal microscopy

The following Abs directed against human proteins were used for confocal microscopy: rabbit and mouse anti-lamp-1 Abs, kindly provided by Dr. J. P. Gorvel (Center d'Immunologie de Marseille-Luminy, Marseille-Luminy, France); H5C6 mouse anti-CD63 mAb from the National Institute of Child Health and Human Development; rabbit anti-CD63 Ab, kindly provided by Dr. M. Fernández-Borja (Netherlands Cancer Institute, Amsterdam, The Netherlands); 1B5 rat anti-hsc73 mAb from Stressgene (Madrid, Spain); rabbit anti-FasL Ab N20, lot D159, from Santa Cruz Biotechnology; and 5C2 mouse anti-APO2L/TRAIL mAb, kindly provided by Dr. A. Ashkenazi (Genentech). Secondary anti-rabbit, anti-mouse or anti-rat IgG, labeled with FITC, Cy3, or Texas Red, as convenient, were from Caltag.

The use of the N20 rabbit anti-FasL Ab for immunocytochemistry has been recently questioned (25). In this study, the G247-4 and NOK-1 mouse mAbs from PharMingen were validated (25). As indicated above, the N20 lot (D159) used in this study recognized a major 40-kDa band in Jurkat cells or human T cell blasts by immunoblot techniques, a result that was corroborated previously by us using the G247-4 mAb (13). Using N20 and G247-4, we demonstrated previously by immunoblot that fresh human PBMC did not express FasL (13). We have now shown by confocal microscopy that fresh human PBMC did not stain for FasL with this N20 lot or with G247-4 or Alf1–2. However, more recent N20 lots recognized by immunoblot many other bands in PBMC, Jurkat, or T cell blasts and did stain fresh human PBMC by immunocytochemistry techniques, in agreement with that reported in Ref. 25.

Control cells or cells activated with the different stimuli described were collected, washed with PBS, and fixed in a solution of 4% paraformaldehyde in PBS. Cell suspensions were then placed on round coverglasses previously treated with L-polylysine, which were sequentially incubated with dilutions of the primary Abs between 1/500 and 1/1000 in PBS with a 5% goat serum and 0.1% saponin, and dilutions of the secondary Abs between 1/100 and 1/500 in PBS with 0.1% saponin. After several washings, coverglasses were mounted on glass slides using Mowiol (Calbiochem, Madrid, Spain). Preparations were observed in a Zeiss 310 confocal microscope, analyzed using the LSM 3.95 software, and finally processed using the Adobe Photoshop 5.0 software. Single cells were observed in 10 successive focal planes, separated 1 μm, and adjusted from the bottom to the top of the cell. The pictures showing single cells correspond to the central part of the cell, normally the fifth/sixth focal plane. No labeling was observed when using the secondary Abs alone, whether in control cells or in cells activated with PHA or anti-CD59 mAb.

Immunoelectron microscopy

Cell pellets (50×10^6 cells) were fixed overnight in 4% paraformaldehyde in PBS at 4°C and subjected to the progressive lowering temperature (PLT) dehydration technique (26). The resulting pellets were infiltrated using Lowicryl K4 M, collected into gelatin capsules, and polymerized under UV light, as indicated in Ref. 26. Ultrathin sections (60 nm) were cut using a Leica Ultracut UCT (Leica, Barcelona, Spain) and collected onto Formvar-coated (Sigma-Aldrich, Madrid, Spain) gold grids. Grids were washed with PBS, and samples blocked by incubation in PBS with 1% BSA and 0.1% Tween 20 and incubated with 10 μg/ml anti-FasL N20 Ab (lot D159) and 15 μg/ml anti-APO2L 5C2 mAb in PBS with 1% BSA and 0.1% Tween 20 for 2 h at room temperature. After several washings, grids were incubated with a 1/30 dilution of goat anti-rabbit and anti-mouse IgGs (British BioCell, Cardiff, U.K.) coupled with colloidal gold particles 15 and 5 nm in diameter, respectively, for 1 h. Grids were extensively washed with water, contrasted with 2% uranyl acetate for 20 min and Reynolds solution for 3 min, and observed in a Jeol JEM 1010 transmission electronic microscope (Jeol, Barcelona, Spain) at 80 kV.

Cell pellets (50×10^6 cells) to be processed for ultrathin cryosectioning were fixed overnight in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in PBS at 4°C. After washing, the pellets were embedded in 10% gelatin in PBS and cut into small blocks to be cryoprotected with increasing concentrations of sucrose (from 0.5 M to 2.3 M) overnight at 4°C. The blocks were then frozen in liquid nitrogen, and ultrathin cryosections were made with a Leica EM-FCS ultracryomicrotome. The 70-nm sections were picked up from the knife with a droplet of 2.3 M sucrose in a platinum loop and placed on Formvar-coated gold grids that were floated onto 2% gelatin in PBS. The grids were then washed, and samples were blocked by incubation with 1% FCS in PBS for 15 min and incubated with 3 μg/ml anti-FasL N20 Ab (lot D159) and 10 μg/ml anti-APO2L 5C2 mAb

in PBS with 2% BSA for 1 h at room temperature. After washings with 0.1% BSA, 0.01 M glycine in PBS, grids were incubated with a 1/50 dilution of the secondary Abs indicated above for 45 min at room temperature. After extensive washing with water, samples were floated in a cool mixture of 2% methylcellulose and 0.1% uranyl acetate for 10 min, the excess fluid was removed, and the sections become embedded in the thin covering after drying in air.

Under the labeling conditions described for each technique, no labeling was observed when using the secondary Abs alone, whether in control cells or in cells activated with PHA or anti-CD59 mAb. Under these conditions, the labeling was specific, localized exclusively in the cell cytoplasm, with no nuclear or extracellular labeling.

Results

APO2L/TRAIL is released in a particulate fraction from CD59-triggered Jurkat cells and human T cell blasts

We previously showed that CD59 triggering on Jurkat or human T cell blasts induced AICD which was associated with the release of bioactive APO2L/TRAIL to the supernatant, with almost no contribution of secreted FasL (21). APO2L/TRAIL and FasL are secreted from these cells upon PHA triggering, associated with a particulate, ultracentrifugable fraction (19). To analyze the pathway of APO2L/TRAIL secretion upon CD59 triggering, we first performed immunoblot analysis of culture supernatants from Jurkat and normal human T cell blasts after anti-CD59 mAb treatment. As shown in Fig. 1*a*, and in agreement with previous observations (13), both Jurkat and T cell blasts expressed abundant APO2L/TRAIL, with a molecular mass of 41 kDa. These cells also expressed other APO2L/TRAIL molecular forms, namely 35-kDa and 24-kDa peptides, which correspond to the molecular mass of the nonglycosylated polypeptide and of the cleaved soluble form, respectively (10). Upon CD59 triggering, as described previously, the amount of APO2L/TRAIL associated with the cells decreased substantially, correlating with the appearance of a cytotoxic activity in the culture medium, which was prevented by anti-APO2L/TRAIL-blocking Abs (21). Immunoblot analysis of these media showed the presence of APO2L/TRAIL as the 41-kDa, mature form of the protein (see Fig. 1*a*, third lanes). The 24-kDa, cleaved soluble form was not detected in these media, even after overexposure of the blot. No FasL was detected by immunoblot in the same supernatants (data not shown).

The toxicity of supernatants obtained from anti-CD59 mAb-treated normal T cell blasts on Jurkat cells was almost completely prevented by the APO2L/TRAIL blocking Ab 5C2 (Fig. 1*b* and Ref. 21). The toxicity of these supernatants was no longer observed after their ultracentrifugation, while it was almost completely retained in the pelleted fraction. The toxicity associated with the pellet was substantially blocked by the 5C2 mAb. This correlated with the presence of the 41-kDa APO2L/TRAIL band in the ultracentrifugation pellet, but not in the ultracentrifugation supernatants (Fig. 1*c*). These data demonstrate that APO2L/TRAIL is mainly released in a particulate fraction from anti-CD59-triggered human T cells.

Characterization of the microvesicle populations secreted upon PHA or anti-CD59 triggering by flow cytometry

In our previous work, we developed a gating protocol to analyze the secreted microvesicle populations by flow cytometry (19). Using this protocol, we have now analyzed the FasL and APO2L/TRAIL labeling pattern of microvesicles secreted from Jurkat and human T cell blasts upon PHA or CD59 triggering. As shown in the central panels of Fig. 2, many of the microvesicles positive for fluorescence labeling and secreted after PHA triggering of Jurkat or human T cell blasts expressed FasL, but not APO2L/TRAIL, on their surface (60% for Jurkat and 43% for T cell blasts). Additionally, a significant subpopulation expressed both FasL and APO2L/TRAIL (22% for Jurkat and 8% for T cell blasts), whereas the population with exclusive APO2L/TRAIL labeling was in the minority (5% for Jurkat and 2% for T cell blasts). The microvesicles secreted after CD59 triggering had a different labeling pattern (Fig. 2, *lower panels*): the relative amount of the subpopulation expressing APO2L/TRAIL but not FasL increased (18% for Jurkat and 10% for T cell blasts), and also detectable was a subpopulation with expression of both FasL and APO2L/TRAIL (20% for Jurkat and 3% for T cell blasts). A population with exclusive FasL labeling, which was the main population after PHA activation, was not detected in this case. These results agree with the functional bioassays performed previously with supernatants from PHA or anti-CD59 mAb-stimulated cells and anti-Fas- and/or anti-APO2L/TRAIL-blocking Abs (13, 21).

The number of microvesicles present in the supernatants was also quantified by flow cytometry; the amount of microvesicles secreted after PHA activation was clearly higher than after CD59 triggering (2340 $\pm$ 150 vesicles/min vs 1720 $\pm$ 120 vesicles/min, obtained from 20×10^6 cells). In addition, we demonstrated by the Bradford method that the total amount of protein released from the

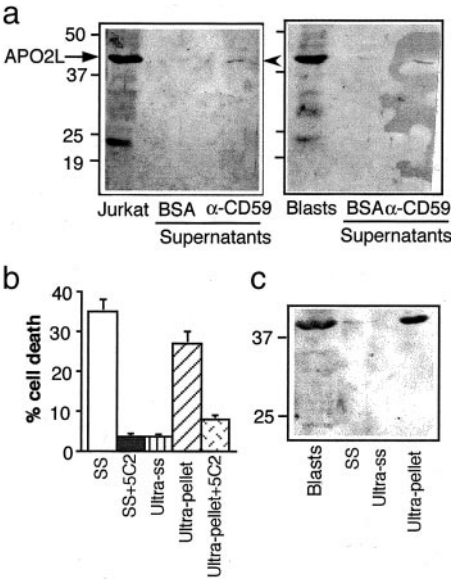

FIGURE 1. APO2L/TRAIL is released associated with a particulate fraction from CD59-triggered human T cells. *a,* Anti-APO2L/TRAIL immunoblot of 2×10^6 cells or of supernatants from 2×10^7 cells stimulated with immobilized anti-CD59 mAb VJ1/12.2 or placed on BSA-coated wells, as indicated, for 3 h. Results are representative of 10 experiments. *Left,* Jurkat cells and supernatants; *right,* 7-day human T cell blasts and supernatants. *b,* Six milliliters supernatants from 2×10^8 normal human T cell blasts stimulated with immobilized anti-CD59 mAb for 3 h were subjected to ultracentrifugation at 100,000 $\times$ *g* for 8 h. Ultracentrifugation supernatants were recovered (Ultra-ss), and the pellet was resuspended in 6 ml RPMI 1640 (Ultra-pellet). The toxicity of the initial supernatant, of the ultracentrifugation supernatant and of the ultracentrifugation pellet was assayed on nonactivated Jurkat cells in the presence or absence of the anti-APO2L/TRAIL-blocking mAb 5C2, as indicated. Cell death was determined by trypan blue staining, and the results shown correspond to the same supernatants analyzed by immunoblot. Similar results were obtained using the MTT reduction method or staining with annexin V-FITC. *c,* anti-APO2L/TRAIL immunoblot of 2×10^6 cells (Blasts) or of the same supernatant as in *b* before ultracentrifugation (SS), and of the supernatant (Ultra-ss) or of the pellet resuspended in 1.5 ml medium (Ultra-pellet) after their ultracentrifugation. *Left,* Position of the molecular mass markers. Arrowheads on the right of the gels show the position of APO2L/TRAIL. Values are representative of three experiments.

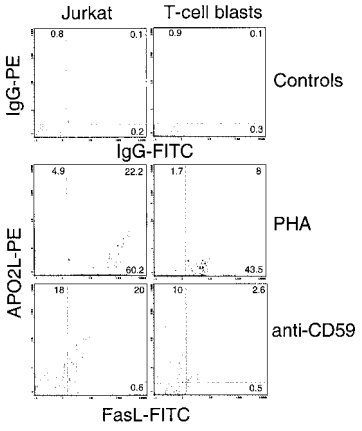

FIGURE 2. FasL and APO2L/TRAIL labeling of microvesicles secreted from human T cells. *Left*, Jurkat cell-derived microvesicles; *right*, 7-day T cell blast-derived microvesicles, as indicated. *Top*, Labeling of microvesicles obtained from cells stimulated with immobilized anti-CD59 mAb for 3 h, using FITC-labeled irrelevant rat IgG plus PE-labeled goat anti-mouse IgG; *middle*, labeling of microvesicles obtained from 1-h supernatants of cells pulse-stimulated with PHA, using FITC-labeled anti-FasL rat mAb H11 and anti-APO2L/TRAIL mouse mAb 5C2 plus PE-labeled goat anti-mouse IgG; *bottom*, labeling of microvesicles obtained from cells stimulated with immobilized anti-CD59 mAb for 3 h, as indicated above. Diagrams are representative of at least five experiments for each experimental condition.

cells after PHA activation was higher than after exclusive CD59 triggering (220 $\pm$ 40 μg vs 96 $\pm$ 18 μg, obtained from 20 $\times$ 10^6 cells).

Colocalization of FasL and APO2L/TRAIL with lysosomal/exosomal markers

The differential secretion of distinct microvesicle populations depending on the extracellular stimuli suggested that preformed FasL and APO2L/TRAIL could be stored in different intracellular compartments. Hence, we tried to characterize these compartments.

It is now known that some types of hemopoietic cells secrete a type of microvesicle called exosomes (27). Detailed studies have defined the fine structure of the exosome-containing compartments, identifying markers for the external membrane, the membrane of the exosomes, and the exosome interior (27, 28). To study whether the compartments containing FasL and APO2L/TRAIL are similar to the described secretory lysosomes, we have analyzed by confocal microscopy the colocalization of FasL and APO2L/TRAIL with different lysosomal/exosomal markers. Three markers with different distribution inside the secretory lysosome were chosen: lamp-1, associated with the lysosomal external membrane (29); CD63, a tetraspan protein that is enriched in the exosomal membrane (29); and hsc-73, a heat shock protein which is enriched in the lumen of the exosomes (30).

As shown in Fig. 3, FasL and APO2L/TRAIL labeling in Jurkat cells was exclusively cytoplasmic. Analysis of the labeling in sequential focal planes separated by 1 μm confirmed the cytoplasmic localization and the lack of plasma membrane labeling. This agreed with previous flow cytometry data showing the lack of plasma membrane expression of FasL and APO2L/TRAIL in Jurkat and human T cell blasts (19). As shown in Fig. 3, FasL colocalized with lamp-1 in Jurkat cells, especially in the brighter spots. However, the colocalization was never complete, leaving zones with lamp-1 or FasL labeling alone. In the case of CD63, the colocalization with FasL was almost complete, although zones remained with CD63

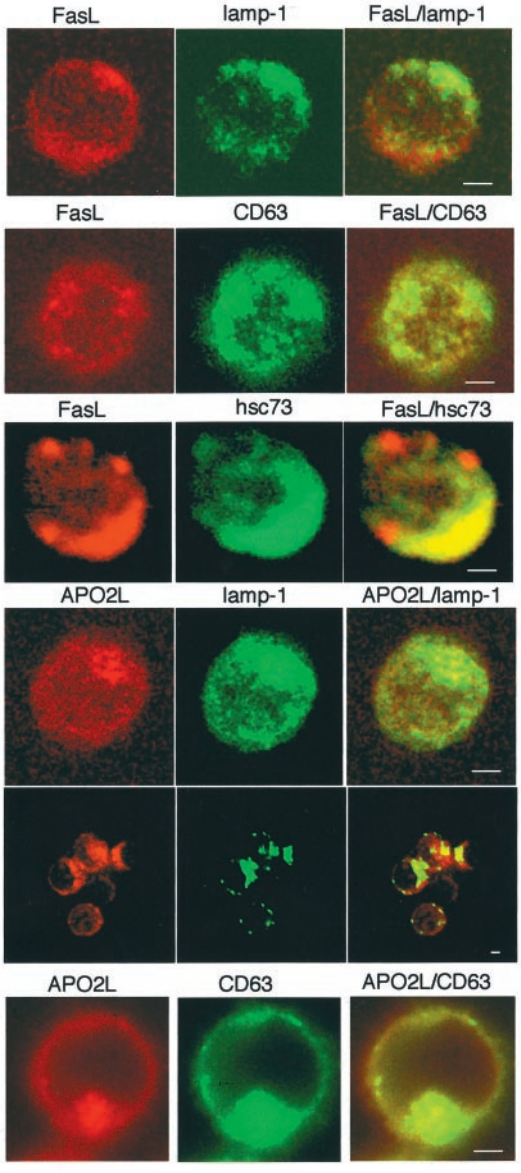

FIGURE 3. Intracellular localization of FasL and APO2L/TRAIL in Jurkat T cells. FasL was located by using the N20 rabbit Ab (lot D159) and Cy3-conjugated anti-rabbit Ab, and APO2L/TRAIL was located using the 5C2 mouse mAb and Cy3 or Texas Red-conjugated anti-mouse Ab (red fluorescence, *left*). Lamp-1 was located using mouse or rabbit Abs and FITC-conjugated anti-mouse or anti-rabbit Abs, respectively; CD63 was located using the H5C6 mouse mAb or rabbit Abs and FITC-conjugated anti-mouse or anti-rabbit Abs, respectively; hsc73 was located using the 1B5 rat mAb and FITC-conjugated anti-rat Ab (green fluorescence, *middle*). FasL or APO2L/TRAIL staining images were superimposed with lamp-1-, CD63- or hsc-73-staining ones, as indicated, to show overlapping signals as yellow fluorescence (*right*). Bar, 2 μm.

labeling alone. FaL also colocalized with hsc-73, although some bright spots with FasL labeling alone could be repetitively observed. The degree of APO2L/TRAIL colocalization with lamp-1 was similar to that of FasL, leaving also zones with lamp-1 or APO2L/TRAIL labeling alone. Again, the colocalization with CD63 seemed more extensive than with lamp-1, although zones remained of CD63 labeling alone. Similar results were obtained with human T cell blasts (data not shown).

Colocalization of FasL with APO2L/TRAIL in Jurkat cells and T cell blasts: differential effect of PHA or anti-CD59 mAb triggering

The data obtained in the previous section do not explain the differential secretion of FasL and APO2L/TRAIL, because their distribution with lysosomal/exosomal markers is indistinguishable. Hence, we analyzed by confocal microscopy the degree of colocalization between FasL and APO2L/TRAIL in these cells, before and after PHA or anti-CD59 mAb triggering. Most, but not all, of the FasL and APO2L/TRAIL labeling colocalized in nonactivated Jurkat cells (Fig. 4*a*, *upper panels*). Some spots with FasL or APO2L/TRAIL labeling alone were distinguishable. We have previously observed, both by immunofluorescence and by immunoblot, that PHA or anti-CD59 mAb activation of Jurkat cells or T cell blasts resulted in the drastic reduction of the amount of FasL and APO2L/TRAIL associated with the cells, correlating with the appearance of cytotoxic activity in the supernatant (13, 21, 22). In the case of PHA stimulation, this was observed after 15 min activation; whereas in the case of CD59 triggering, this process seems to be slower, with a detectable decrease at 1 h. Hence, for the present studies we used a 5-min activation time with PHA and 15 min for anti-CD59 mAb treatment. As shown in Fig. 4*a* (*middle upper panels*), PHA activation of Jurkat cells induced changes in cell shape and a partial accumulation of both FasL and APO2L/TRAIL labeling close to the plasma membrane. However, their degree of colocalization was maintained with respect to that observed in control cells. Anti-CD59 mAb triggering of Jurkat cells induced a massive mobilization of APO2L/TRAIL labeling to bright spots close to the plasma membrane, and the colocalization with FasL was reduced in some cells (Fig. 4*a*, *middle lower panels*). In some cases, this preferential mobilization of APO2L/TRAIL was so acute that the colocalization with FasL was almost completely lost (Fig. 4*a*, *lower panels*). Anti-CD59 mAb triggering induced small changes in the FasL labeling pattern.

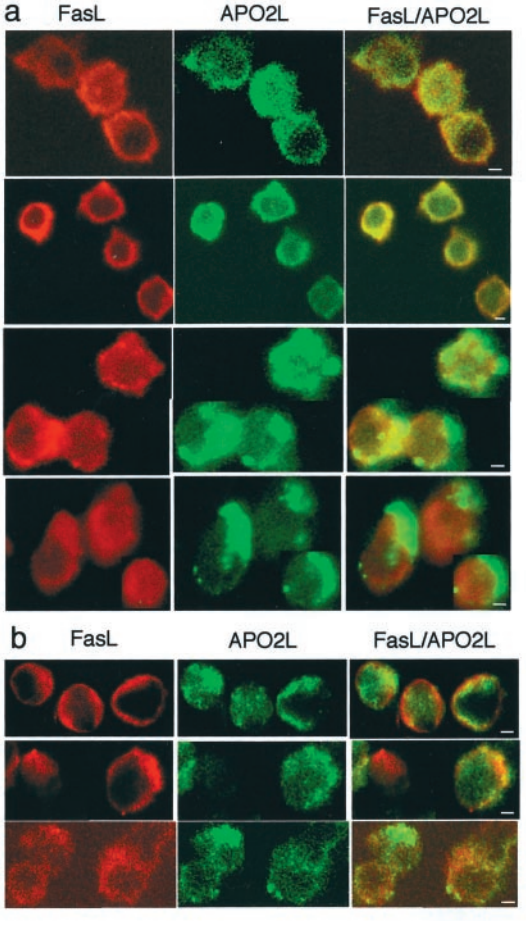

FIGURE 4. Intracellular localization of FasL and APO2L/TRAIL in control or activated human T cells. FasL was located using the N20 rabbit Ab (lot D159) and Cy3-conjugated anti-rabbit Ab (red fluorescence, *left*), and APO2L/TRAIL was located by using the 5C2 mouse mAb and FITC-conjugated anti-mouse Ab (green fluorescence, *middle*). FasL and APO2L/TRAIL staining images were superimposed, as indicated, to show overlapping signals as yellow fluorescence (*right*). *a, Upper panels,* control Jurkat cells; *upper middle panels,* Jurkat cells stimulated with 50 μg/ml PHA for 5 min; *lower middle* and *lower panels,* Jurkat cells stimulated with immobilized anti-CD59 mAb for 15 min. *b, Upper panels,* control T cell blasts; *middle panels,* T cell blasts stimulated with 50 μg/ml PHA for 5 min; *lower panels,* T cell blasts stimulated with immobilized anti-CD59 mAb for 15 min. Bar, 2 μm.

The degree of colocalization between FasL and APO2L/TRAIL in control T cell blasts was high, although not as extensive as in Jurkat cells (Fig. 4, compare *upper panels* of *a* and *b*). Upon PHA activation, the partial colocalization was maintained, with some accumulation of both FasL and APO2L/TRAIL labeling close to the plasma membrane (Fig. 4*b*, *middle panels*). After CD59 triggering, the mobilization of APO2L/TRAIL was more extensive than that of FasL, with accumulation of APO2L/TRAIL labeling in bright spots close to the plasma membrane (Fig. 4*b*, *lower panels*).

FasL and APO2L/TRAIL localization and secretion upon PHA or anti-CD59 triggering visualized by immunoelectron microscopy

To study FasL and APO2L/TRAIL localization in more detail, we analyzed by immunoelectron microscopy samples obtained in the same conditions as those analyzed by confocal microscopy.

We first used the technique of PLT sample dehydration and embedding in Lowicryl K4 M followed by immunolabeling. Detection of immunocomplexes was performed by using secondary Abs coupled to colloidal gold particles of different sizes (15 nm for FasL and 5 nm for APO2L/TRAIL). The labeling obtained by this technique was intense and specific, localized exclusively in the cytoplasm. An overview of FasL labeling is shown in Fig. 5*a*. Cytoplasmic labeling was sometimes diffuse, but the most characteristic feature was its accumulation in rounded compartments with a size between 300 and 500 nm (Fig. 5*a*, arrows). A higher magnification view confirmed the presence of FasL and APO2L/TRAIL in these compartments (Fig. 5*b*). The amount of FasL labeling was always higher than that of APO2L/TRAIL, and APO2L/TRAIL labeling was observed inside the compartment delimited by the intense FasL labeling. However, it was repeatedly observed the presence of zones of APO2L/TRAIL labeling alone in close proximity to the main compartment (Fig. 5*b*, arrow). After treatment with PHA for 5 min, cell morphology changed, and the emission of pseudopodia was frequently observed (Fig. 5*c*). In a representative image shown in Fig. 5*c*, an accumulation of FasL labeling at the edge of a pseudopod is observed, together with some secreted FasL in the outside of the cell. A higher magnification of this image shows that this compartment, which is especially dense to the electron beam, is in close contact with the plasma membrane, and that both FasL and APO2L/TRAIL are being released associated to small structures of 50 - 100 nm in diameter (Fig. 5*d*). CD59 triggering also affected the cell morphology, although to a lesser extent than PHA treatment, and in this case the most characteristic feature was the accumulation of APO2L/TRAIL labeling, with few FasL labeling, in electron-dense compartments close to the plasma membrane (see the representative Fig. 5*e*). This was not observed in any case upon PHA

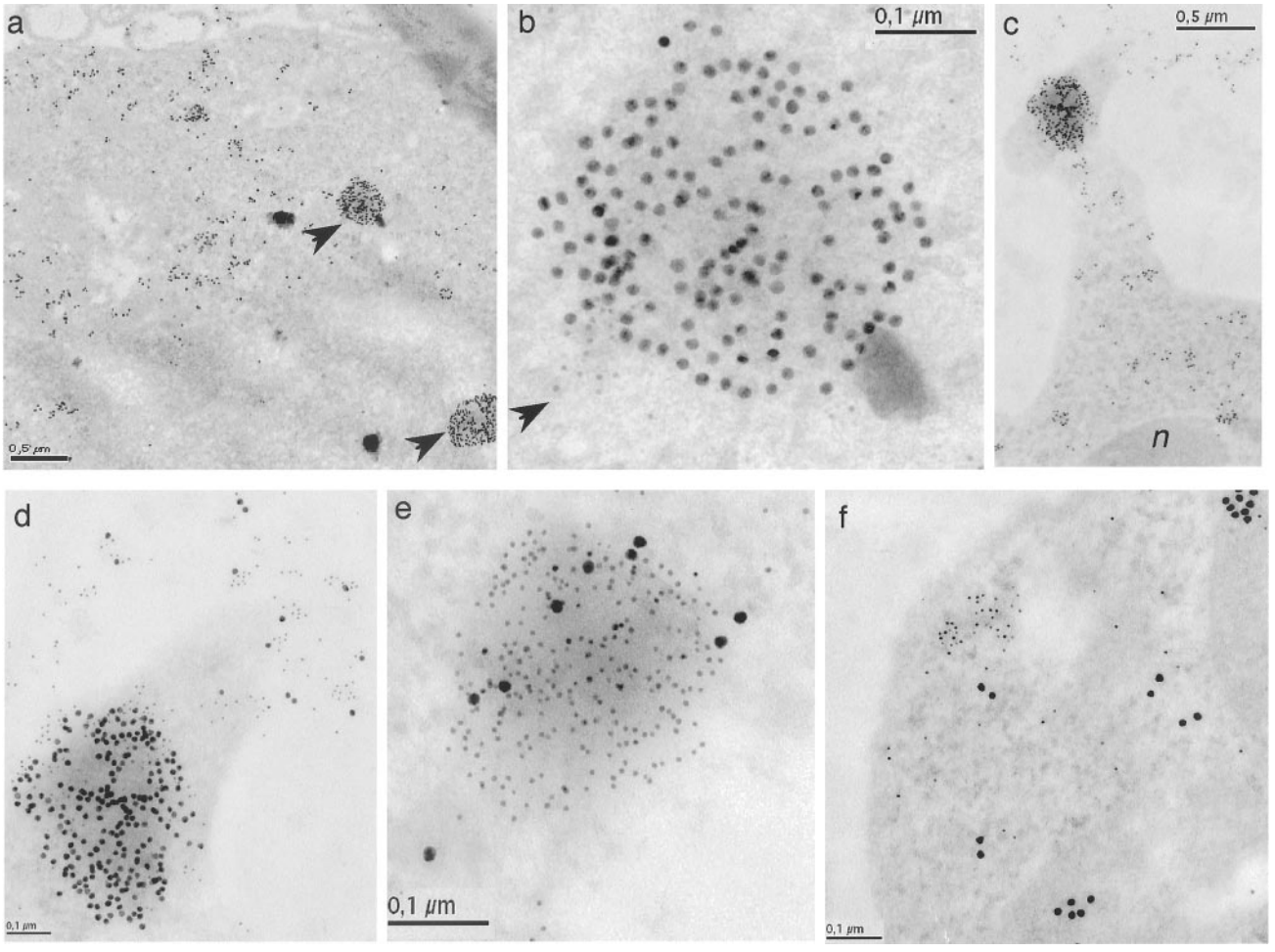

FIGURE 5. Ultrastructural localization of FasL and APO2L/TRAIL in human T cells (Lowicryl inclusion). FasL was located using the N20 rabbit Ab (lot D159) and anti-rabbit Ab conjugated with colloidal gold particles 15 nm in diameter, whereas APO2L/TRAIL was located using the 5C2 mouse mAb and anti-mouse Ab conjugated with colloidal gold particles 5 nm in diameter. *a*, Jurkat control cells. Arrows mark FasL labeling accumulation in round intracytoplasmic structures; *b*, Jurkat control cells. Arrow marks exclusive APO2L/TRAIL labeling accumulation; *c* and *d*, human T cell blasts activated with 50 μg/ml PHA for 5 min. *n*, nucleus; *e* and *f*, Jurkat cells stimulated with immobilized anti-CD59 mAb for 15 min.

activation. In some instances, it could be also observed compartments with APO2L/TRAIL-labeled microvesicles in its interior close to fusion with the plasma membrane (Fig. 5*f*).

Although the labeling using the PLT/Lowicryl K4 M method was good, the membrane ultrastructure of the compartments cannot be visualized by this technique. To alleviate this problem, we also used immunogold labeling of cryosections. In unstimulated cells, the accumulation of FasL labeling was observed in membranous compartments of a size between 400 and 600 nm with features of a multivesicular body (MVB; Fig. 6, *a* and *b*). Most of the labeling was localized on the internal membranes, rather than in the external membrane of the compartment, in agreement with that reported by Mincheva-Nilsson et al. (31) in decidual human $\gamma\delta$ T cells. Some APO2L/TRAIL labeling was observed in the same compartments that exhibited intense FasL labeling (Fig. 6*a*, small arrowhead), but it was also observed in membranous, smaller structures close to the larger MVB, and with exclusive APO2L/TRAIL labeling (Fig. 6*a*, large arrowhead). In fact, APO2L/TRAIL labeling was frequently localized outside the MVB with predominant FasL labeling (Fig. 6*b*, arrowheads). These compartments were localized into the cytoplasm of unstimulated cells, sometimes close to the nucleus (see Fig. 6*a*), but upon PHA activation, the presence of similar MVB close to the plasma membrane could be observed (Fig. 6*c*). In the lower right part of this image, a superposition between MVB membranes and the plasma membrane can be observed. This MVB contains both FasL and APO2L/TRAIL (arrowheads) labeling on their internal membranes. Once the apposition of membranes has culminated, the internal microvesicles seem to be secreted intact to the extracellular medium, as shown in Fig. 6*d*. Upon CD59 triggering, the change in cell shape and MVB mobilization was less pronounced, but in this case, and distinct to PHA triggering, the secretion of microvesicles with exclusive APO2L/TRAIL labeling could be observed (Fig. 6*e*), which in some cases aggregate (Fig. 6*f*).

Discussion

The present studies indicate that the mechanism of FasL and APO2L/TRAIL release from overactivated T cells, which are sensitive to AICD, is similar to exosome secretion from APC (27). This suggests that the exosomal release of bioactive proteins could be a generalized mechanism of immune regulation and function. The exosomes described in APC are 60- to 100-nm microvesicles expressing high amounts of Ag-loaded MHC-II, which are secreted by professional APC, such as B and dendritic cells (27). Exosomes are stored in a post-Golgi, prelysosomal compartment with the structure of a MVB. Exosomes contain membrane proteins correctly oriented, suggesting that the internal vesicles are formed by inward vesiculation of the limiting membrane of the

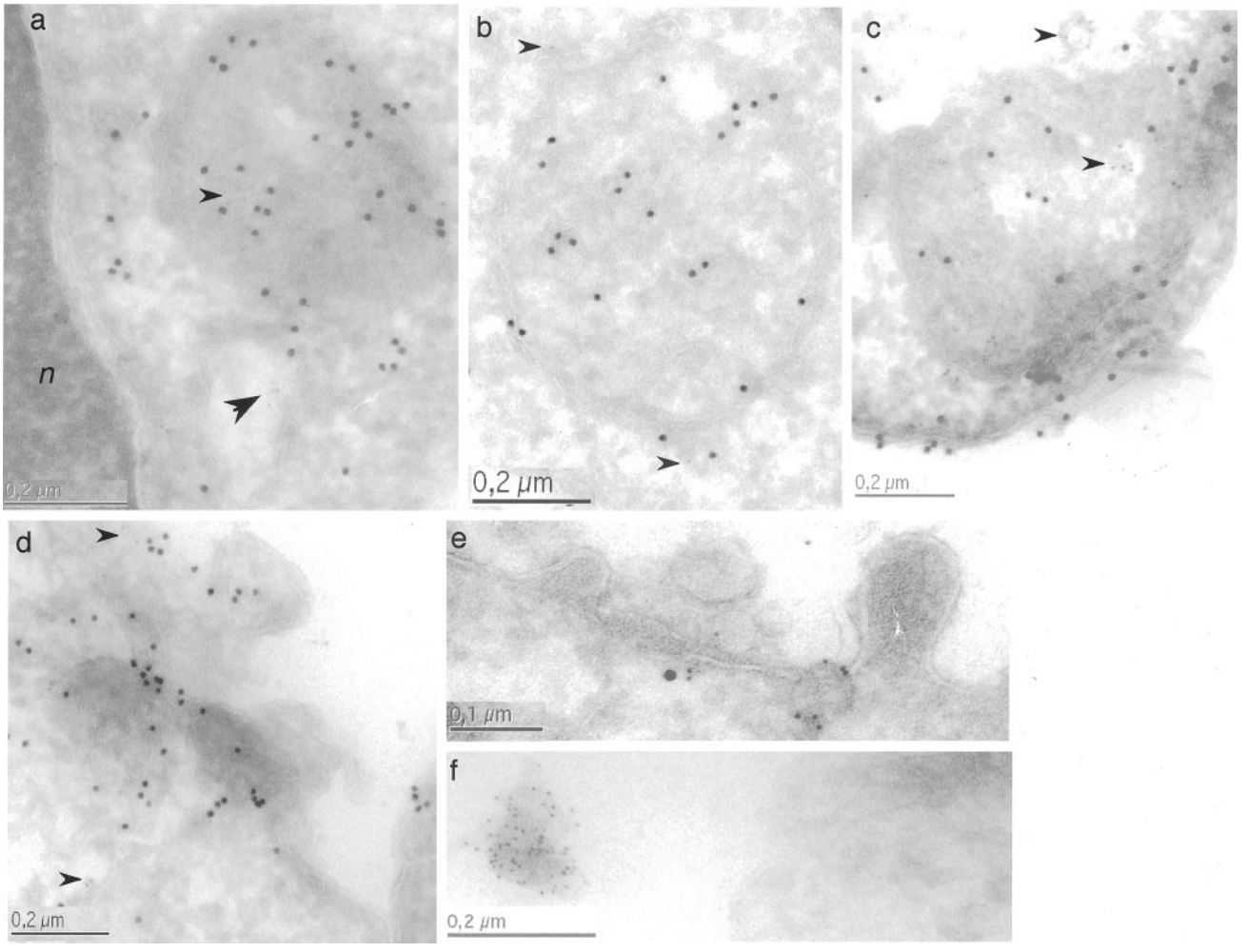

FIGURE 6. Ultrastructural localization of FasL and APO2L/TRAIL in human T cells (ultracryomicrotomy). FasL (15-nm dots) and APO2L/TRAIL (5-nm dots) were located as indicated in Fig. 5. *a*, Control T cell blasts. Large arrowhead marks a membranous structure with exclusive APO2L/TRAIL labeling. Small arrowhead marks APO2L/TRAIL labeling inside a MVB with FasL labeling. *n*, nucleus. *b*, Control T cell blasts. Arrowheads mark APO2L/TRAIL labeling. *c* and *d*, T cell blasts stimulated with 50 μg/ml PHA for 5 min. Arrowheads mark APO2L/TRAIL labeling. *e* and *f*, T cell blasts stimulated with immobilized anti-CD59 mAb for 15 min.

organelle (27). Upon activation, the external membrane of this compartment can fuse with the plasma membrane, leaving a portion of the small internal vesicles to be secreted intact. A similar structure is shared, at least in part, by CTL granules, which have been defined as secretory lysosomes (32). In fact, preformed FasL was previously localized in the same granules as perforin and granzymes in a $CD8^+$ human CTL clone, in a human NK cell line (33), and in decidual human $\gamma\delta$ T cells (31). The data obtained in our work indicate that both FasL and APO2L/TRAIL are mainly associated with lysosomal-like compartments in Jurkat cells and in human T cell blasts (Fig. 3). Confocal microscopy (Fig. 4) and immunoelectron microscopy analysis (Figs. 5 and 6) revealed that most FasL and APO2L/TRAIL are stored in the interior of the same MVB in these cells, and PHA activation induces the release of microvesicles containing both FasL and APO2L/TRAIL, whereas CD59 triggering results in the specific release of APO2L/TRAIL-containing microvesicles. The existence of membrane compartments with exclusive APO2L/TRAIL labeling close to MVB containing both FasL and APO2L/TRAIL (Figs. 5 and 6) offers the first clue to understand the differential secretion of FasL or APO2L/TRAIL upon different surface triggering. The physical proximity of these compartments and even the contact between their membranes could suggest that they are the result of the maturation of a common post-Golgi compartment. Heterogeneity in the granule population is a known feature in neutrophils, where four distinct types of granules have been characterized (34). The model for the generation of these distinct granule populations is in agreement with maturation from a common post-Golgi compartment (34). In our cellular models, a simple explanation for the specific release of APO2L/TRAIL-enriched microvesicles upon CD59 engagement would be that CD59 provides a weaker activation signal than PHA/TCR, which mobilizes preferentially the APO2L/TRAIL-enriched microvesicles. A strongest activation signal would result in the mobilization of all the microvesicle populations available for secretion. This is in agreement with the detection of fewer microvesicles and total protein in the supernatant of the cells after anti-CD59 mAb than after PHA activation. The differential sorting of FasL and APO2L, belonging to the same family of death messengers, should be ascribed to differences in their intracytoplasmic domains. In fact, the cytoplasmic tails of these proteins are very different: whereas that of human FasL has 80 amino acid residues (35), human APO2L/TRAIL cytoplasmic tail has only 14 amino acid residues (10) with no homology in their sequences. Bossi et al. (36) have mapped the polyproline sequences of the FasL cytoplasmic tail as the domain responsible for sorting to lysosomal-like compartments in human hemopoietic

cells. These polyproline sequences are absent in the APO2L/TRAIL cytoplasmic tail. Typically, the sorting of membrane proteins to lysosomal-like compartments is dependent on the interaction of the AP3 adaptor with dileucine or tyrosine motifs, in a clathrin-independent mechanism (37). Although no tyrosine residue is present in the APO2L/TRAIL cytoplasmic tail, a Leu-Gly sequence is localized immediately after its transmembrane domain. Clearly, more studies are needed to characterize both the sorting and the signal transduction pathways that trigger the differential release of both molecules.

FasL follows the constitutive secretory pathway and is expressed at the plasma membrane in several cell types, like HeLa and Sertoli cells (33, 36) and activated murine CTL clones (38, 39), whereas in other cell types, like RBL cells and human T and NK cells (present data and Refs. 33 and 36), it is sorted toward lysosomal-like compartments and stored until an additional stimulus occurs. This differential sorting may be dependent on the expression of a still unidentified adaptor containing Src homology 3 domains which, through interaction with FasL polyproline sequences, would direct the protein toward secretory lysosomes (36). Once those cells receive a degranulation stimulus, FasL and/or APO2L/TRAIL would be expressed at the plasma membrane if they were localized in the external membrane of the storage MVB, as has been shown for FasL in RBL cells (33). FasL and/or APO2L/TRAIL would be rather secreted on the surface of microvesicles if they were initially localized mainly in the internal vesicles of the MVB structures, as seems to be the case in the cells analyzed in the present study. The extent of internal vesicle localization of these proteins would depend on the extent of inward vesiculation of the external membrane of the compartment, which could be different depending on the cell type or animal species analyzed.

Our findings in normal human T cell blasts could have physiological significance. The secretion of these death messengers on the surface of microvesicles guarantees their cytotoxic potential, because they retain their multimerization ability. High order multimerization of death receptors, which are expressed in the form of trimers on the T cell surface, has been shown to be the crucial event in apoptosis induction (40, 41). In this sense, it has been recently described that soluble FasL retains its proapoptotic potential by interaction with extracellular matrix proteins such as fibronectin (42). That bioactive death ligands can be released in microvesicles is a fact that must be considered to understand the precise role of these death messengers in diverse physiological (T cell effector function, AICD regulation, immune privilege) and pathological situations (autoimmune diseases, tumor counterattack). The potential apoptotic ability of FasL and APO2L/TRAIL would depend not only on the balance between cell-associated and proteolyzed forms of the proteins but also on the capacity of a given cell to secrete them associated with microvesicles. The balance between these three situations would be different depending on the cell type studied and its activation state. For example, in relation with the tumor counterattack hypothesis (43), a tumor expressing FasL and/or APO2L/TRAIL on their surface or secreting FasL- and/or APO2L/TRAIL-containing microvesicles would exert a potent apoptotic activity against T cells, whereas if these proteins were produced by the tumor mainly in their proteolyzed form, their proapoptotic activity would be negligible. However, it has also been reported that soluble FasL could increase neutrophil infiltration in a tumoral area, leading finally to acute rejection of the tumor by the immune system (44, 45), indicating that the in vivo outcome is more complex. In addition, the possible therapeutic application of viral particles engineered to express death ligands, imitating the physiological microvesicles described here, is considered (46).

Acknowledgments

We thank Dr. Avi Ashkenazi (Genentech) for anti-APO2L/TRAIL Abs and support; Dr. Francisco Sánchez-Madrid (Hospital de la Princesa) for anti-CD59 mAb; Dr. Mar Fernández-Borja (Netherlands Cancer Institute) for rabbit anti-CD63 mAb; Dr. Jean Pierre Gorvel (Center d'Immunologie de Marseille-Luminy) for anti-lamp-1 Abs; Dr. Nuria Cortadellas (Serveis Científico-Tecniques, Universidad de Barcelona) for Lowicryl sample preparation; and Dr. Carlos Enrich and Dr. Rosa Aligué (Departamento de Biología Celular, Universidad de Barcelona) for electronic microscopy and support.

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