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ENDOSOMAL COMPARTMENT CONTRIBUTES TO THE PROPAGATION OF CD95/FAS-MEDIATED SIGNALS IN TYPE II CELLS

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SYNOPSIS

Participation of diverse organelles to the intracellular signaling that follows CD95/Fas receptor ligation encompasses a series of subcellular changes that are mandatory for, or even bolster, the apoptotic cascade. In this work we analyzed the role of endocytosis in the propagation of cell death signaling after CD95/Fas engagement in type II cells (CEM cells). We show that this receptor-ligand interaction triggers endocytosis independently of any caspase activation. This FasL-induced endocytosis also leads to an early and directional “movement” of endocytic vesicles towards mitochondrial compartment. In turn, this crosstalk between endosomal and mitochondrial compartments was followed by the loss of the mitochondrial membrane potential and apoptosis execution. This cell remodeling was absent in receptor-independent cell death, such as that induced by the mitochondriotropic drug staurosporine, and in a CEM cell line selected for its multi-drug resistance (CEM VBL100). In these cells a reduced Fas ligand-induced endocytosis and a reduced organelle cross-talk corresponded to a reduced apoptosis. Altogether these findings suggest a key role of endocytosis in the propagation and amplification of the CD95/Fas-activated signaling leading to type II cell demise.

INTRODUCTION

As a general rule, upon ligand binding, surface receptors are able to transduce signals by interacting with intracellular signaling molecules. For many receptors the interaction with their ligands induces receptor clustering, followed by down-regulation of activated surface receptors through endocytosis. Moreover, receptor internalization can target activated receptors to the early endosomes of the endocytic compartment contributing to the propagation and regulation of intracellular signals [1,2]. Receptor binding also represents an important signal able to trigger the complex program that leads to cell death by apoptosis. Induction of apoptosis occurs by two main pathways: the extrinsic pathway and the intrinsic one, or mitochondrial pathway. The first is activated after ligation of death receptors by members of the TNF family, including TNF, CD95L (FasL/Apo-1L), and TNF-related apoptosis-inducing ligand (TRAIL) and is associated with the activation of caspase-8 as apical caspase. The second is activated after perturbation of mitochondria resulting from cellular stress, growth factor withdrawal or cytotoxic insults. However, these two pathways can be strictly intertwined. In fact, CD95-mediated apoptosis is transduced through two general modes [3,4]: type I cells exhibit rapid receptor internalization and form large amounts of DISC, while type II cells are more dependent upon the mitochondrial amplification pathway and exhibit quantitatively less and slower DISC assembly. Fittingly, type I cells undergo apoptosis without involving mitochondria whereas in type II cells a crosstalk between receptor-mediated and mitochondria-mediated pathways results in apoptosis execution. Along these lines, the requirement for receptor internalization has recently been demonstrated for CD95/Fas-induced apoptosis of type I cells [2,5]. Indeed, recent evidence suggests that recruitment of DISC components to the activated CD95/Fas receptors occurs in part after internalization into the endosomal compartment [2]. Furthermore, the observation that the blockade of CD95/Fas internalization impairs apoptosis has suggested that CD95 receptor endocytosis could play a key role in transmitting death signals, at least in type I cells [2,5].

On the other hand, in type II cells, e.g. in CD4⁺ T cells, mitochondria may also integrate death receptor signaling. It has been demonstrated that the truncated form of Bid molecule, could link the two pathways [6]. However, it is now increasingly appreciated that vacuolar changes are often enhanced following activation of surface receptors in different forms of cell death [1,2,7,8]. In the present work the propagation events that impact on intracellular membranes and their dynamic interactions during the early steps of Fas signaling in type II cells have been analyzed. We found that CD95 ligation, besides triggering caspase-mediated CD95 receptor internalization [2], also triggers the activation of extensive endocytosis which is caspase independent and a directional “movement” of early and late endosomes towards mitochondrial compartment. This could also contribute, in type II cells, to those mitochondrial changes that can lead to cell death execution. Importantly, we also evaluated Fas-triggered endocytosis in a T cell line well characterized for its extrinsic resistance, i.e. a multidrug-resistant cell line. We found that this endocytosis-mediated propagation signaling is defective in these cells. We thus argue that, as for other organelles [9-11], extensive “activation” of the endocytic pathway could play a key role in the organelle crosstalk and propagation of death signals in type II cells.

EXPERIMENTAL

Reagents. Recombinant superFasLigand (FasL) was purchased from Apotech (Lausanne, Switzerland) while neutralizing anti-human Fas IgG1 mAb (clone ZB4) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Fluorescent probes and assays were purchased from Molecular Probes/Invitrogen (Eugene, OR, USA). Polyclonal anti-VDAC-1, anti-LAMP-1 and anti-CD95/Fas (C-20) antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) while polyclonal anti-Rab-5 anti-EEA-1 were purchased from BD/Pharmingen (Oxford, UK). zVAD from Alexis (San Diego, CA, USA) and monensin from Sigma (St Louis, MO, USA). Other chemicals were from Sigma.

Cell cultures. Human lymphoblastoid T cell line CEM and its multidrug-resistant counterpart, VBL100 cells were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin, in a humidified atmosphere with 5% (v/v) CO₂ at 37 °C. For all experiments 5x10⁵ cells/ml were seeded and treated as specified (see below).

Treatments. Apoptosis was induced by treating cells with 100 ng/ml recombinant FasL or with 2 µM staurosporine (STS, Sigma) for different times (20 and 30 minutes, 1 hour and 3 hours). For experiments with inhibitors, cells were pre-treated for 30 minutes with: zVAD (50 µM); or monensin (10 µM). Cells treated with each drug (zVAD or monensin) alone were considered as controls. Negative controls for endocytosis assay were performed by incubating cells with 250 ng/ml of neutralizing anti-human Fas IgG1 mAb (clone ZB4).

TEM studies. Human lymphoblastoid T cells were fixed in 2.5% cacodylate-buffered (0.2 M, pH 7.2) glutaraldehyde for 20 minutes at room temperature and post-fixed in 1% OsO₄ in cacodylate buffer for 1 hour at room temperature and processed as stated elsewhere [12]. Serial ultrathin sections were collected on 200-mesh grids and then counterstained with uranyl acetate and lead citrate. Samples were observed with a Philips 208 electron microscope at 80kV.

Morphometric analyses. Quantitative evaluations have been performed by analyzing at least 200 cells by TEM at the same magnification (4,000x).

Cell-death assays. Quantitative evaluation of apoptosis was performed by flow cytometry after double staining using FITC-conjugated annexin V/propidium iodide (PI) apoptosis detection kit (Eppendorf, Milan, Italy), which allows discrimination between early apoptotic, late apoptotic and necrotic cell.

Mitochondrial membrane potential in living cells. The mitochondrial membrane potential (MMP) of controls and treated cells was studied by using 5-5',6-6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1, Molecular Probes) probe. In line with this method, living cells were stained with 10 µM of JC-1, as previously described [13]. Tetramethylrhodamine ester (1 µM, TMRM, Molecular Probes, red fluorescence) was also used to confirm data obtained by JC-1.

Assays of endocytosis. After 30 minutes treatment with FasL, cells were washed three times, resuspended in RPMI medium (with or without FCS) and then incubated for additional 30 minutes with dextran-FITC (1 mg/ml, Sigma) at 37 °C. After three washings, cells were resuspended in PBS and immediately analyzed by a cytometer. To distinguish the ingested dextran particles from those attached on the cell surface we used fluorescence quenching with Trypan blue as previously described [14]. Endocytosis was also measured by following the fluorescence increase of FM1-43 [8,15,16]. Fluorescence changes were recorded in a spectrofluorimeter (Perkin-Elmer LC50) with excitation at 470 nm (5 nm bandwidth) and emission at 565-580 nm (10 nm bandwidth), using 5x10⁵ cells/ml in a modified Ringer solution [15] and 2-4 µM FM1-43. Complementary measurements were carried out in a plate reader with emission filter centered at 538 nm (Fluoroskan Ascent, Thermo, Basingstoke, UK). Alternatively, after 30 minutes treatment with FasL, cells were incubated for additional 30 minutes with BSA-FITC (Sigma) or BSA-gold (40nm, Fitzgerald Industries International Inc., Concord, MA, USA) at 37 °C. After washings cells were prepared for immunofluorescence and TEM, respectively.

Sucrose-gradient fractionation. Analysis of the distribution of EEA-1, Rab-5, LAMP-1, VDAC-1 and CD95/Fas was performed by isopycnic centrifugation on a continuous density gradient, as previously described [17]. Briefly, about 1.5×10^8 CEM cells or VBL100 cells, either untreated or treated with FasL (100 ng/ml for 30 minutes at 37°C) were harvested in PBS and resuspended in 0.3 ml of buffer A (3 mM imidazole pH 7.4, 1 mM EDTA, 50 μ M zVAD, 1:100 v.v, cocktail of protease inhibitors) containing 8.5% sucrose. Alternatively, cells were pre-treated with 50 μ M zVAD or monensin and then treated with FasL as above. The cell suspension was mechanically disrupted by Dounce homogenization (20 strokes). Nuclei were removed by 10 minutes centrifugation at 1,000xg and the supernatant was loaded on top of 4 ml of a 10%-40% continuous sucrose gradient and spun for 16 hours in a Beckman TL-70 ultracentrifuge (swinging bucket rotor, SW60 Ti) at 40,000 rpm. After centrifugation, 20 fractions were collected from the bottom of the tube. After evaluation of the protein concentration with a BioRad assay, fractions were subjected to electrophoresis. The respective proteins were localized by Western blotting.

Immunofluorescence. At the end of the incubation time with BSA-FITC, control and FasL-treated cells were fixed with 3.7% paraformaldehyde in PBS for 30 min at room temperature; washed in the same buffer and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min. After washings, samples were incubated at 4°C for 1h with an anti-mitochondria monoclonal antibody (Chemicon International, Inc. Temecula, CA, USA). Then, cells were labeled with Alexa Fluor 594-conjugated anti-mouse IgG (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37 °C in the dark. After washings, all samples were mounted with glycerol:PBS (2:1) and observed with a Nikon Microphot fluorescence microscope. Images were captured by a color chilled 3CCD camera (Hamamatsu, Japan) and analyzed by the OPTILAB (Graftek, France) software.

Immunoblotting. Cell lysates and subcellular samples were separated by SDS-polyacrylamide gel in paraformaldehyde electrophoresis and immunoblotted as described previously [18].

Data analysis and statistics. All samples were analyzed with a FACScan cytometer (BD) equipped with a 488 argon laser. At least 20,000 events were acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest Software. Statistical analysis of endocytosis and apoptosis data and morphometric analysis was performed by using Student's *t*-test or one-way variance analysis by using Statview program for Macintosh. All data reported were verified in at least three different experiments and reported as mean \pm SD. Only *p* values of less than 0.01 were considered as statistically significant.

RESULTS

CD95/Fas ligation and endocytosis. In line with literature hypothesizing a role for endocytosis of CD95/Fas in apoptosis triggering in type I cells [2], we first evaluated this phenomenon in our experimental setting, i.e. in the type II T cell line CEM. Biochemical analyses were first undertaken by monitoring the fluorescence changes of FM1-43, a membrane probe that reversibly distributes into intracellular membranes following an increased endocytosis [15,16]. Treatment with FasL rapidly elevated FM1-43 fluorescence, indicating endocytic uptake, which intensified with increasing FasL concentration within 1 hour of addition and then progressively decayed (**Fig. 1A**). This time-course analysis indicated that Fas triggering led to an early increase of endocytic activity. By contrast, incubation of CEM cells with anti-human Fas IgG1 neutralizing antibody (clone ZB4), which is known to bind CD95/Fas but without activating CD95/Fas and triggering apoptosis [2], did not induce any increase of endocytosis, the FM1-43 uptake not being significantly different from that observed in untreated control cells (**Fig. 1A**). Actually, the FasL-induced changes in FM1-43 fluorescence were in the same direction of those obtained with ionomycin, an established inducer of rapid endocytosis (**Fig. 1B**) [8,15]. Complementary studies were also performed by flow cytometry to quantitatively evaluate Fas-L induced endocytosis in this T cell line. Two different points have been analyzed following FasL administration: i) CD95/Fas surface expression and ii) endocytosis. These control analyses, performed by on living cells, showed that treatment with FasL for 30 minutes did not induce any significant decrease of CD95 surface expression (**Fig. 1C**, black histogram), but a very small decline of CD95 surface expression level with respect to untreated cells (**Fig. 1C**, full grey histogram) was detected only starting from 1 hour after FasL administration (**Fig. 1C**, empty grey histogram). As concerns the second point, a quantitative evaluation of the uptake of a dextran tracer was used to evaluate endocytic activity [19]. Our results are summarized in **Figure 1D**, which displays the mean values \pm SD obtained from four different experiments. It can be observed that: i) CD95/Fas triggering significantly stimulated the endocytosis of FITC-dextran beads within 30 minutes from FasL administration (**Fig. 1D**, left panel) and that ii) after serum deprivation the uptake of FITC-dextran beads in FasL-triggered cells was significantly increased (**Fig. 1D**, right panel). In addition, we also found that FasL-induced endocytosis was caspase-independent, being unaffected by pre-treating cells with the pan-caspase inhibitor zVAD (**Fig. 1D**) either in the presence (white columns) or absence (black columns) of FCS. Moreover, we also used monensin, a lysosomotropic agent and an Na^+ ionophore, which alters endosomal pH by inhibiting vacuolar proton pump and consequently inhibiting intracellular traffic and receptor recycling at the cell surface [20]. As expected, monensin (**Fig. 1D**) pre-treatment significantly inhibited FasL-induced uptake of FITC-dextran beads, both in the presence (white columns) and absence (black columns) of FCS. Importantly, we also observed that cell treatment with i) anti-human Fas IgG1 neutralizing antibody (clone ZB4), which bind Fas receptor without triggering apoptosis (not shown) and ii) staurosporine, a drug that induces apoptosis via a receptor-independent mechanism, i.e. via mitochondrial pathway (**Fig. 1D**), did not influence the endocytotic rate, as evaluated by dextran beads internalization.

In **Figures 1E** and **1F** results obtained in a representative flow cytometry experiment carried out in the presence of FCS (**Fig. 1E**) or in the absence of FCS (withdrawal of growth factors, **Fig. 1F**) are shown. The addition of Trypan blue to quench the surface absorption of fluorescent dextran had little effect on cell fluorescence (panels **E2-E5** vs **E6-E9** and **F2-F5** vs **F6-F9**), indicating that the majority of the beads were endocytosed inside the cells.

Altogether these data seem to indicate that in type II cells CD95/Fas ligation stimulates the endocytic pathway on the whole, independently of the caspase-dependent internalization and recycling of activated CD95/Fas receptor. This is in accord with recent studies indicating that the fast CD95/Fas internalization, and consequent DISC formation and caspase 8 activation, is restricted to type I cells only [2].

Monensin inhibits FasL-induced but not STS-induced apoptosis. On the basis of the above results, we evaluated by means of flow cytometry analysis the occurrence of apoptosis in our experimental conditions in CEM cells. In particular, we found that, as expected in type II cells, pan-caspase inhibitor zVAD potently hindered apoptosis induced by both FasL and STS (both known as caspase-dependent cell death inducers). Moreover, according to the results reported in Figure 1, we also found that monensin administration was capable as well of inhibiting apoptosis in FasL-treated cells only (**Fig. 2 A, B**). In particular, the analysis of both annexin V positive cells as well as PI positive cells clearly indicated that monensin was ineffective against STS-induced apoptosis. A representative experiment is shown in **Fig. 2A**.

Re-distribution of endosomal proteins after CD95/Fas-ligation. On the basis of the above results, the effects of CD95 ligation on the endocytic compartment of type II cells were analyzed by means of transmission electron microscopy. As expected, with respect to control cells, we observed an increased presence of membranous endocytic structures in the cytoplasm of FasL-treated cells (**Fig. 3A**). However, more importantly, some of these endocytic vesicles were detected in close proximity of the mitochondria (**Fig. 3B, C**). The quantitative evaluation of this phenomenon, performed by morphometric analysis (see Experimental Procedures), revealed that the frequency of vacuoles in close proximity of mitochondria was significantly much higher in FasL-treated cells than in control untreated cells (**Fig. 3D**). Moreover, it is worth noting that these cytoplasmic changes occurred early (30 minutes) after FasL-induced CD95 triggering in cells that do not yet display ultrastructural signs of apoptosis and increased with time (up to 1 hour). After this time, namely after 3 hours, typical signs of apoptosis were detectable (not shown). To further study this phenomenon, we decided to evaluate BSA endocytosis under apoptotic stimulation. The results obtained by both immunoelectron microscopy (Fig. 4 A-D) and fluorescence analyses (Fig. 4E-F), clearly indicated that BSA endocytosis associated with CD95Fas triggering can finally result in the presence of BSA in the mitochondria.

On the basis of these morphological observations, which could imply a directional movement of some endocytic vesicles towards mitochondria, we decided to analyze in detail the subcellular distribution of endosomes in our system, i.e. after CD95/Fas triggering. A series of experiments was thus performed to analyze by Western blotting three different membrane markers of the endocytic pathway: EEA1, Rab5 and LAMP-1. We initially focused on EEA-1, a membrane-tethering protein that normally resides in the cytosol and is recruited to endosomal membranes by interaction with phosphoinositol-3-phosphate (PI3P), the distinctive phospholipid of early endosomes [21,22], by means of cell subfractionation following a continuous 10-40% sucrose density gradient, as stated elsewhere [17]. EEA-1 was normally distributed in the low-density fractions in untreated cells (**Fig. 5A**) but, when cells were treated with FasL, a minor re-distribution of this molecule towards the VDAC-1 enriched fractions, corresponding to mitochondria enriched fractions (**Fig. 5D**), was detected. Interestingly, this re-distribution of EEA-1 was caspase independent, since it was still evident after treatment with zVAD (**Fig. 5A**). More intriguingly, monensin pre-treatment (**Fig. 5A**) prevented the Fas-mediated redistribution of EEA-1 towards mitochondria-rich fractions.

Next, we investigated the distribution of the membrane marker Rab-5, specific for early/recycling endosomes [22,23]. Rab-5 re-distribution partially matched the change of EEA-1 fractionation profile (**Fig. 5B**). In fact, in the absence of FasL treatment, Rab-5 protein displayed a bimodal distribution. Significant levels of the protein were detectable in both low- and high-density fractions. This is consistent with the notion that Rab-5 normally localizes on early as well as recycling endosomes [24]. Conversely, cell treatment with FasL narrowed the bimodal distribution of Rab-5 across density gradients and increased its levels in mitochondria-rich fractions (**Fig. 5B**). Again, the FasL-induced change in Rab-5 distribution was virtually unaffected by treatment with the pan-caspase inhibitor zVAD, but was prevented by pre-treatment with monensin (**Fig. 5B**).

The analysis of a late-endosome protein marker such as LAMP-1 was also carried out under the same experimental conditions as above. LAMP-1, which normally was concentrated in the high-

density fractions in untreated cells, moved to fractions with lower density 30 min after FasL treatment (**Fig. 5C**). This shift in LAMP-1 distribution remained well evident in the presence of zVAD, but did not occur after pre-treatment with monensin (**Fig. 5C**).

As a control, the distribution pattern of CD95/Fas in the same fractions was analyzed in the presence or in the absence of Fas ligand-induced endocytosis (**Fig. 5E**). As expected, in untreated cells CD95/Fas was detected in the low density fractions. After CD95/Fas triggered endocytosis [2], the receptor was still detected in low density fractions, where early endosomes are also localized, without a detectable re-distribution of this molecule towards mitochondrial rich fractions.

Altogether these findings seem to indicate that the increased endocytosis occurring after CD95 ligation in type II cells (**Fig. 1**) was paralleled by a directional movement of early and late endosomes towards the mitochondria-enriched compartment.

Monensin hinders the loss of MMP. In consideration of the key role of mitochondria in apoptosis execution in type II cells, further analyses were also conducted to evaluate the mitochondrial membrane potential (MMP), known to decrease in cells undergoing apoptosis and associated with the release of apoptogenic factors [4-6] (**Fig. 6**). The typical loss of MMP was detectable in both FasL- and STS-treated cells (**Fig. 6A**). In both instances, MMP loss was significantly hindered by zVAD but monensin blocked only FasL-caused loss of MMP. In fact, endosomal compartment perturbation by monensin differently influenced MMP after FasL and STS treatments: it potently protected mitochondria from MMP loss in FasL-treated cells but it was ineffective in STS treated cells (in **Fig. 6A** a representative experiment is shown). The same results were found by using an alternative probe (TMRM) as shown in **Figure 6B**. In fact, the percentage of cells with depolarized mitochondria was significantly reduced by monensin pre-treatment in FasL-treated cells only (**Fig. 6C**). Altogether, these findings suggest that monensin, perturbing the endocytic pathway also perturbs apoptosis execution and reduces the MMP loss.

Resistance to Fas-mediated apoptosis is associated with decreased endocytosis. To further analyze the mechanisms underlying the role of endocytosis in propagating FasL-mediated signaling, we decided to evaluate the endocytotic process triggered by FasL in the multi-drug resistant counterpart of CEM cells, the VBL100 cells [25]. Induction of a multidrug resistant (MDR) phenotype is referred as to cells that are able to extrude unrelated drugs via cell surface ATP-dependent pumps, e.g. by P-glycoprotein. It has been generally accepted that multi-drug resistance is normally associated to resistance to anticancer drug-induced injury and death, which essentially depends on this acquired ability to extrude drugs. It was also shown that MDR cultured cell lines display, with respect to their *wt* counterparts, a different susceptibility to receptor-mediated and mitochondria-mediated apoptosis [25,26]. With this in mind, we investigated the features of FasL-induced endocytic activity in VBL100 multidrug-resistant CEM cells.

Initially, we analyzed in VBL100 cells i) the CD95 expression pattern in order to assess the suitability of the model system and ii) the endocytic phenomenon in cells treated with FasL or STS in comparison with untreated control cells. We found that: i) the expression at the cell surface of CD95 in *wt* and VBL CEM cells was comparable (**Fig. 7A**); ii) the *basal* level of endocytosis was significantly lower in VBL100 cells with respect to *wt* CEM cells (compare black columns in **Fig. 7C** with those in **Fig. 7B**). This occurred either in the presence or absence of FCS (note that different scales were used in order to show differences within the same cell system). More importantly, iii) FasL treatment induced a significantly lower endocytosis in VBL100 cells with respect to *wt* CEM cells (compare white columns in **Fig. 7C** with those in **Fig. 7B**); iv) zVAD was ineffective in preventing FasL-induced endocytosis whereas v) although the FasL-triggered endocytosis is very low if compared to that of *wt* cells, monensin was however able to significantly prevent the endocytic phenomenon also in VBL100 cells. Finally, according to the results obtained in *wt* CEM cells, v) STS did not influence the endocytic activity in VBL100 drug resistant CEM cells variant (**Fig. 7C**). These results are in accordance with apoptosis (**Fig. 7D**) and MMP (**Fig. 7E**) data. In fact, VBL100 cells showed a significantly lower susceptibility to FasL-induced

apoptosis and a significantly higher susceptibility to STS-induced apoptosis compared to *wt* CEM cells (**Fig. 7D**). Accordingly, in VBL 100 cells, FasL was less effective in inducing MMP loss than STS (**Fig. 7E**).

In light of these results, it was mandatory to evaluate the possible presence of endocytic components, i.e. EEA-1, Rab-5 and LAMP-1, in cell subfractionation following a continuous 10-40% sucrose density in VBL100 cells too. Western blot analyses (**Fig. 8**) clearly indicated that no redistribution of early and late endosomal markers might be detected in mitochondria enriched fractions from FasL-treated VBL100 cells.

Altogether these results indicate that VBL100 cells do not display an increased activity and remodeling of the endosomal compartment following FasL administration (as observed in *wt* CEM cells) and that, concomitantly, they are also less susceptible to FasL-induced apoptosis.

DISCUSSION

In the present work we show that in type II CEM cells the interaction of death receptor CD95/Fas with its physiological ligand, FasL, triggers and enhances endocytosis before the occurrence of apoptosis and independently of caspase activation. In particular, FasL administration seems to trigger a directional “movement” of some early and late endosomes towards mitochondria, the main activator of apoptosis execution in type II cells. The main point of the present work deals thus with the contribution of the endosomal compartment to the mitochondria-mediated apoptotic execution, as previously suggested for other subcellular organelles [9-11]. This is also suggested by the fact that in a MDR-resistant variant of CEM cells, i.e. VBL100 cells, that are significantly less susceptible to Fas-induced apoptosis, the endocytic compartment was not involved in the apoptosis execution after CD95 activation by FasL. In fact, the limited susceptibility to FasL observed in these cells cannot be attributed neither to a reduced expression of CD95, nor to a defect in apoptotic machinery, being VBL100 cells sensitive to staurosporine-induced cell death.

All in all these results are in line with the increased endocytotic activity observed by other authors in type I cells. Those findings, however, were focused to the CD95 receptor internalization that follows the interaction of CD95 with its ligand in type I cells, which underwent apoptosis depending on the efficient endocytosis of the activated receptor [2]. In type I cells the CD95 triggering induces typical receptor internalization resulting in the formation of large amounts of DISC and leading to caspase activation and apoptosis without any involvement of mitochondria. In our experimental model, i.e., type II CEM cells, the surprising evidence is provided here of a FasL-induced endocytosis. This was well evidenced by a dramatic increase in the uptake of FM1-43, a dye usually employed to evaluate endocytosis [8,15], as well as in the uptake of dextran beads. Given the close relationship between the uptake of dextran beads and (macro)pinocytosis [27], our results additionally indicated that triggering of CD95 by FasL stimulates a pinocytic type of endocytosis reminiscent of that previously detected after EGF ligation to its receptor [28]. Furthermore, the increased endosomal compartment activity, irrespective of the receptor internalization and caspase activation, was associated with a directional movement of these endosomes towards the mitochondrial-enriched cell subfractions (see below) [8]. How can this crosstalk between endosomal and mitochondrial compartments contribute to apoptosis execution? The idea that various subcellular organelles can interact each other and modify the fate of the cell has recently been hypothesized [2,29,30]. Some research points to a directional scrambling of molecules in order to ensure the cell undergoing apoptosis with a series of organelle changes that contributes, or even triggers, apoptosis execution. For instance, the importance of endoplasmic reticulum (ER) in the apoptotic cascade has recently been investigated [9,31]. Under ER stress, ER transmembrane receptors initiate the unfolded protein response [32]. If the adaptive response fails, apoptotic cell death ensues. This response is associated with organelle remodeling and intermixing and is implicated in the pathophysiology of several neurodegenerative and cardiovascular diseases [10]. In the same vein, lysosomal organelles have been analyzed in different experimental settings, precisely devoted to the understanding of how certain lysosomal cysteinyl proteases, e.g. cathepsins, can contribute to the crosstalk between endolysosomes and mitochondria during apoptosis [33]. More recently, the possible implications of the Golgi apparatus remodeling in the apoptosis execution has also been analyzed in detail. It was found that also this organelle participates to the complex framework of subcellular intermixing activities that lead to CD95/Fas-triggered apoptosis [11]. The results reported here seem to suggest that a further actor could participate in this complex scenario: the endosomal compartment. In fact, the endosomal remodeling seems to represent a very early event in the propagation of FasL-mediated signaling.

We found that CD95/Fas-triggered apoptosis was significantly prevented by the inhibition of endosomal acidification by monensin, a monovalent ion-selective ionophore [20]. Importantly, this compound also inhibited the shift of EEA-1, Rab-5 and LAMP-1 endosomal markers to subcellular

fractions with lower density, corresponding to mitochondria enriched fractions. Moreover, the endocytic activity stimulated by FasL occurred independently of any activation of caspase cascade, as demonstrated by using the pan caspase inhibitor zVAD, and anti-Fas neutralizing antibody, which binds receptor without triggering cell death. Thus, FasL treatment seems to induce two distinct subcellular processes: i) the caspase-dependent internalization of ligated receptors, as previously suggested elsewhere [34] and ii) the caspase-independent increase of endocytic activity. Some further points should be considered in this context. One deals with the fact that the increase of endocytic compartment activity has been found in receptor-mediated cell death only. In fact, endocytosis hindering by monensin did not affect the apoptosis execution induced by the mitochondriotropic drug staurosporine. Hence, Fas ligation, by stimulating endocytosis, could affect membrane traffic and generate a “controlled” wave of endocytic vesicles that, inducing organelle crosstalk, might contribute to instruct those mitochondrial conditions favorable to apoptosis execution (e.g., changes in membrane potential, release of apoptogenic factors, etc).

These hypotheses are also supported by the data obtained by using the multidrug-resistant (MDR) counterpart of CEM cells (VBL100 cells) as model system [25,35]. This subclone of CEM cells induced to acquire a resistant phenotype, i.e. expressing the so-called P-glycoprotein at the cell surface, exhibited a limited susceptibility to Fas-mediated cell death [25] that was paralleled by a reduced endocytic activity, i.e. by a reduced Fas-induced organelle cross talk. In fact, the directional protein traffic from the endocytic compartment to the mitochondria was significantly decreased in CEM VBL 100 cell line with respect to its *wt* counterpart.

In line with recent papers suggesting a key role of ER stress and organelle scrambling in apoptosis induction [11,36], we can hypothesize that a sort of endosomal-stress can occur in the very early phases of receptor-mediated apoptosis in type II cells. This may contribute to the complex organelle crosstalk aimed at the propagation of the signals to appropriate downstream effectors (e.g., to mitochondria) that finally lead to cell demise.

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Abbreviations used are: BSA, bovine serum albumin, EEA-1, early endosomal autoantigen-1; LAMP-1, lysosome-associated membrane glycoprotein; TNF, Tumor necrosis factor; DISC, death inducing signaling complex; TEM, transmission electron microscopy; IEM, immuno-electron microscopy; FasL, Fas ligand; zVAD, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; FITC, fluorescence isothiocyanate; PI3P, phosphatidylinositol-3-phosphate; STS, staurosporine; MMP, mitochondrial membrane potential; RIP1, receptor induced kinase 1; MDR, multidrug resistance; HPA, Helix pomatia agglutinin; ER, endoplasmic reticulum; EGF, epidermal growth factor.

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FIGURE LEGENDS

Figure 1. CD95/Fas ligation and endocytosis. (A) Spectrofluorimetric measurements of endocytosis obtained by following the uptake of FM1-43 in CEM cells suspended in Ringer's buffer without (control) and with FasL. Its final concentration ($\mu\text{g/ml}$) and pre-incubation times are indicated. As additional control we used neutralizing anti-human Fas IgG1 mAb, clone ZB4 (black line). (B) Fluorescence of FM1-43 was monitored in a plate reader under the same conditions as in (A). Mean values of quintuplicate wells were obtained after 20 minutes incubation with FasL (0.5 $\mu\text{g/ml}$) or 1 μM ionomycin (an inducer of rapid endocytosis). (C) Flow cytometry analysis of CD95 surface expression in living cells (selected by PI exclusion test) treated with FasL for 30 min or 1 hour in comparison with untreated cells. Numbers represent median fluorescence. Note that a small decrease of CD95 expression was visible only after 1 FasL treatment. (D-F) *Modulation of FasL-induced endocytosis.* (D) Flow cytometry analysis of endocytosis performed in the presence (white columns) or absence (black columns) of FCS. Values reported represent the mean \pm SD of the percentage of cells that ingested dextran-FITC obtained from four different experiments. Note that: i) the same trend was detectable in the presence or in the absence of FCS; ii) a significant increase of dextran uptake was detectable in cells under FCS withdrawal (note different scales used); iii) either zVAD, or monensin given alone did not modify *per se* dextran uptake but, iv) whereas pre-treatment with zVAD did not modify the FasL-induced dextran uptake, monensin pre-treatment significantly impaired the process. (E, F) Quantitative flow cytometry analysis of endocytosis by dextran-FITC uptake evaluation obtained in a representative experiment performed in the presence (E) or absence (F) of FCS. Numbers represent the percentage of cells that ingested dextran-FITC. Negative controls (auto-fluorescence) are reported in panels E1 and F1. The addition of Trypan blue to quench the surface absorption of fluorescent dextran had little effect on cell fluorescence (panels E2-E5 vs E6-E9 and F2-F5 vs F6-F9), indicating that the majority of the beads were endocytosed inside the cells. Note that zVAD was ineffective either in FCS supplemented cells or in cells under FCS starvation (panels E3 vs E4 and F3 vs F4) whereas monensin strongly reduced the percentage of dextran loaded cells (panels E3 vs E5 and F3 vs F5).

Figure 2. Analysis of cell apoptosis. Flow cytometry analysis after double staining with annexin V/propidium iodide. Untreated control cells (left panels), cells exposed to FasL (middle panels) or STS (right panels) alone (upper panels) or after zVAD (middle) or monensin (bottom) pre-treatments are shown as dot plots from a representative experiment in (A). Numbers represent the percentage of annexin V single positive (early apoptosis, upper right quadrant) or annexin V/PI double positive (late apoptosis, lower right quadrant) cells. (B) Results obtained from four independent experiments are reported as mean \pm SD. Note that: i) zVAD significantly prevents both STS- and FasL-induced apoptosis whereas ii) monensin prevents FasL-induced cell death only.

Figure 3. Morphological evidence of endocytosis. (A-C) Transmission electron micrographs of CEM cells treated with FasL for 30 minutes (A) and for 1 hour (B). Note the endocytic vesicles in A (arrows) and the presence of vesicles in close proximity of mitochondria in (B). The micrograph (C) shows a higher magnification of the boxed area in (B). Note the membranous vesicle in close contact with the mitochondrion. (D) Morphometric and statistical analysis of the percentage of cells showing one or more vesicles close to mitochondria 30 minutes and 1 hour after FasL administration, as evaluated by TEM observation. (*) Represents $p < 0.01$ vs untreated cells. Magnification: A and C: 28000x.

Figure 4. BSA endocytosis after CD95/Fas ligation. Immunogold labeling of BSA in CEM cells 30 minutes after treatment with FasL (pre-embedding technique). Note the presence of gold particles in endosomal vesicles (A), in vesicles in close proximity of mitochondria (B), on the mitochondrial

membrane (C. arrow) and in the mitochondria (D, arrows). Fluorescence microscopy analysis also shows that, in comparison to control cells (E), yellow staining indicating BSA (red)-mitochondria (green) overlay is appreciable after CD95/Fas ligation (F). Arrows indicate cells included in the insets.

Figure 5. *Re-distribution of endosomal proteins after CD95/Fas-ligation.* CEM cells, either untreated or treated with FasL (100 ng/ml for 30 minutes at 37°C) were lysed in lysis buffer and post-nuclear supernatant prepared and loaded on top of a 10-40% continuous sucrose gradient. Alternatively, cells were pre-treated with zVAD or monensin and then treated with Fas-L as above. After centrifugation, fractions were collected, and samples of each fraction were separated by SDS-PAGE and analyzed by Western blot. Fractions obtained after sucrose density gradient, either untreated or treated with FasL and alternatively, pre-treated with 50 μ M zVAD or 10 μ M monensin as above, were analyzed using a polyclonal antibodies to EEA-1 (A), Rab-5 (B) or LAMP-1 (C). As control, fractions obtained after sucrose density gradient from untreated and FasL-treated cells, were analyzed using a polyclonal anti-VDAC-1 (D) or a polyclonal anti-CD95/Fas (E). Note that LAMP-1 and, to a less extent, early endosome markers, but not CD95/Fas, re-distributed towards the VDAC-1 enriched fractions following CD95/Fas ligation. Such re-distribution was virtually undetectable in cells pre-treated with the pan-caspase inhibitor zVAD, but was prevented by pre-treatment with monensin.

Figure 6. *Analysis of mitochondrial membrane potential.* (A) Quantitative and qualitative biparametric flow cytometry analysis after staining with JC-1 of untreated control cells and cells exposed to FasL or STS given alone or after zVAD or monensin pre-treatments. (A) Dot plots from a representative experiment are shown. In the area under the dashed line, the numbers indicate the percentage of cells with depolarized mitochondria. (B) Semiquantitative flow cytometry analysis of MMP by using TMRM probe. Numbers represent the median values of fluorescence intensity histograms. (C) Results obtained from four independent experiments performed by using JC-1 probe are reported as mean \pm SD. Note that zVAD pre-treatment hinders both FasL- and STS-induced MMP loss whereas monensin significantly reduces FasL mitochondrial effects only.

Figure 7. *Resistance to Fas-mediated apoptosis is associated with decreased endocytosis in VBL100 cells.* (A) Flow cytometry analysis of CD95 surface expression in living cells (selected by PI exclusion test) treated with FasL for 30 min or 1 hour in comparison with untreated cells. Numbers represents median fluorescence. Note that a small decrease of CD95 expression was visible only after 1 FasL treatment. (B) Flow cytometry analysis of endocytosis performed in the presence or absence of FCS in wt CEM cells untreated or treated with FasL for 30 minutes to be compared with (C) VBL100 cells in different experimental conditions. Results, representing the percentage of cells that ingested dextran-FITC, are reported as mean \pm SD of the data obtained from four different experiments performed in the absence or presence of zVAD or monensin before treatment with FasL or STS. (D) *Analysis of apoptosis in VBL100 cells.* FACS analysis after double staining with annexin V/propidium iodide of untreated control cells, cells exposed to FasL or STS given alone or after zVAD or monensin pre-treatments. Results are reported as mean \pm SD from four independent experiments. (E) *Analysis of mitochondrial membrane potential in VBL100 cells.* Biparametric flow cytometry analysis after staining with JC-1 of untreated control cells and cells exposed to FasL or STS given alone or after zVAD or monensin pre-treatment. The percentage of cells with depolarized mitochondria is reported as mean \pm SD of the data obtained from four independent experiments.

Figure 8. *Re-distribution of endosomal proteins after CD95/Fas-ligation in VBL100 cell line.* Cells, either untreated or treated with FasL, were lysed in lysis buffer and postnuclear supernatant

prepared and loaded on top of a 10-40% continuous sucrose gradient as described under “Materials and Methods”. After centrifugation, fractions were collected, and samples of each fraction were separated by SDS-PAGE and analyzed by Western blot, using a monoclonal anti-EEA-1, a monoclonal anti-Rab-5, a monoclonal anti-LAMP-1, a polyclonal anti-VDAC-1. Note that EEA-1, Rab-5, LAMP-1 do not virtually modify their distribution following CD95/Fas ligation.

Stage 2(a) POST-PRINT

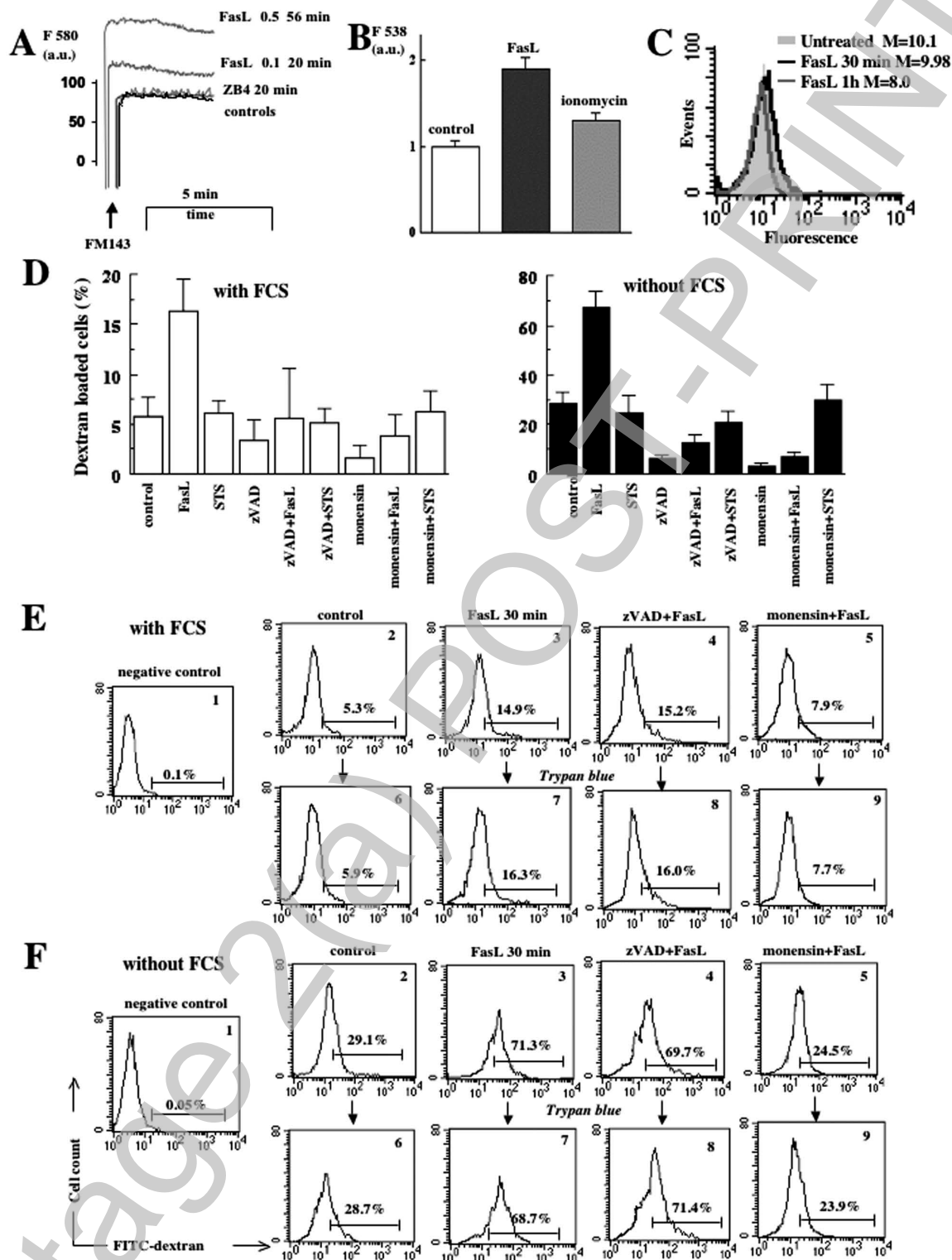


Figure 1

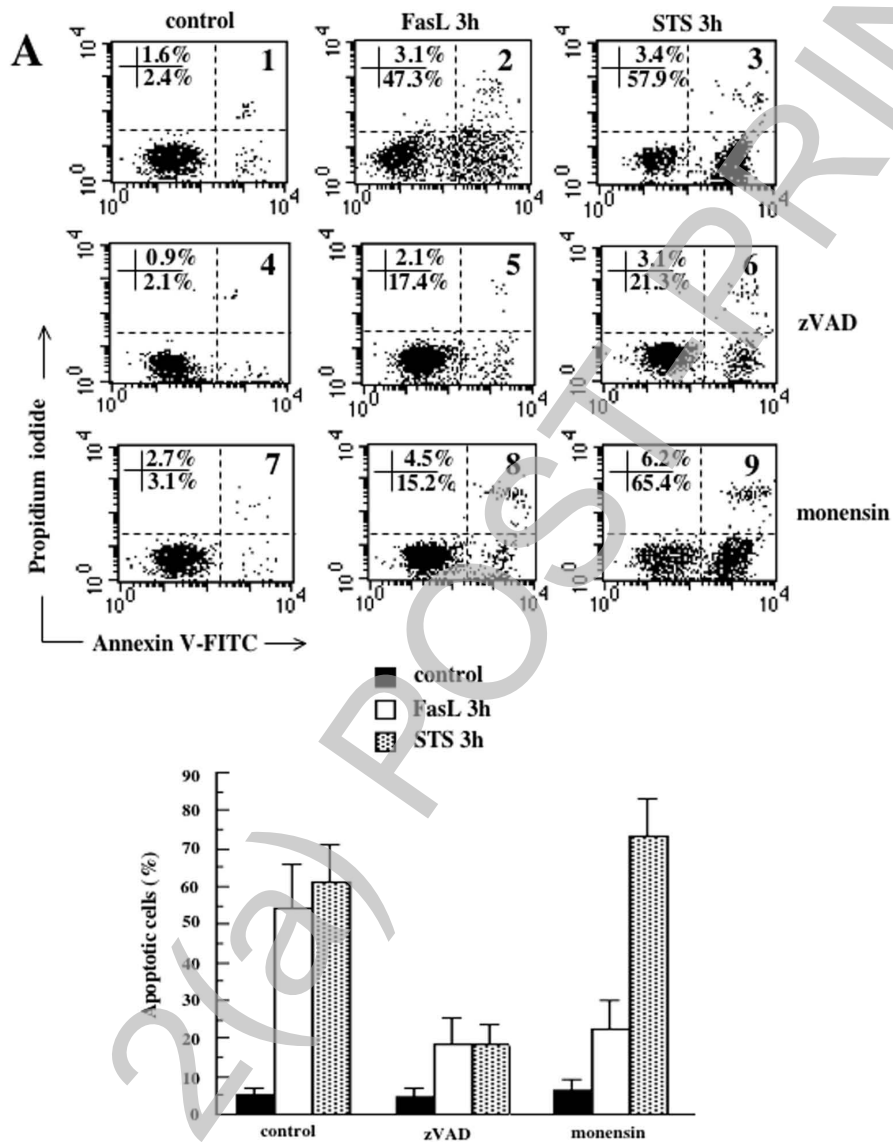


Figure 2

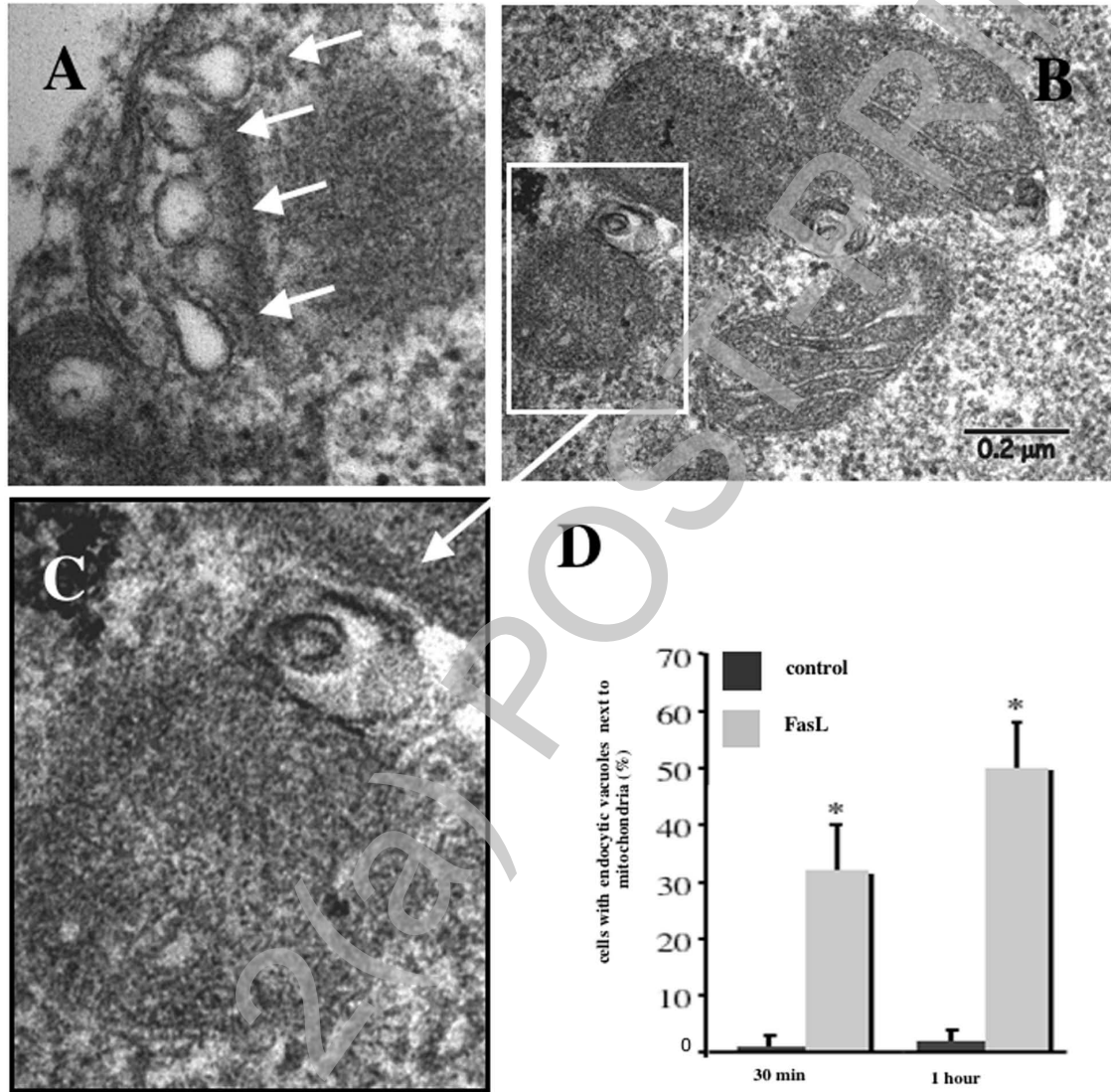


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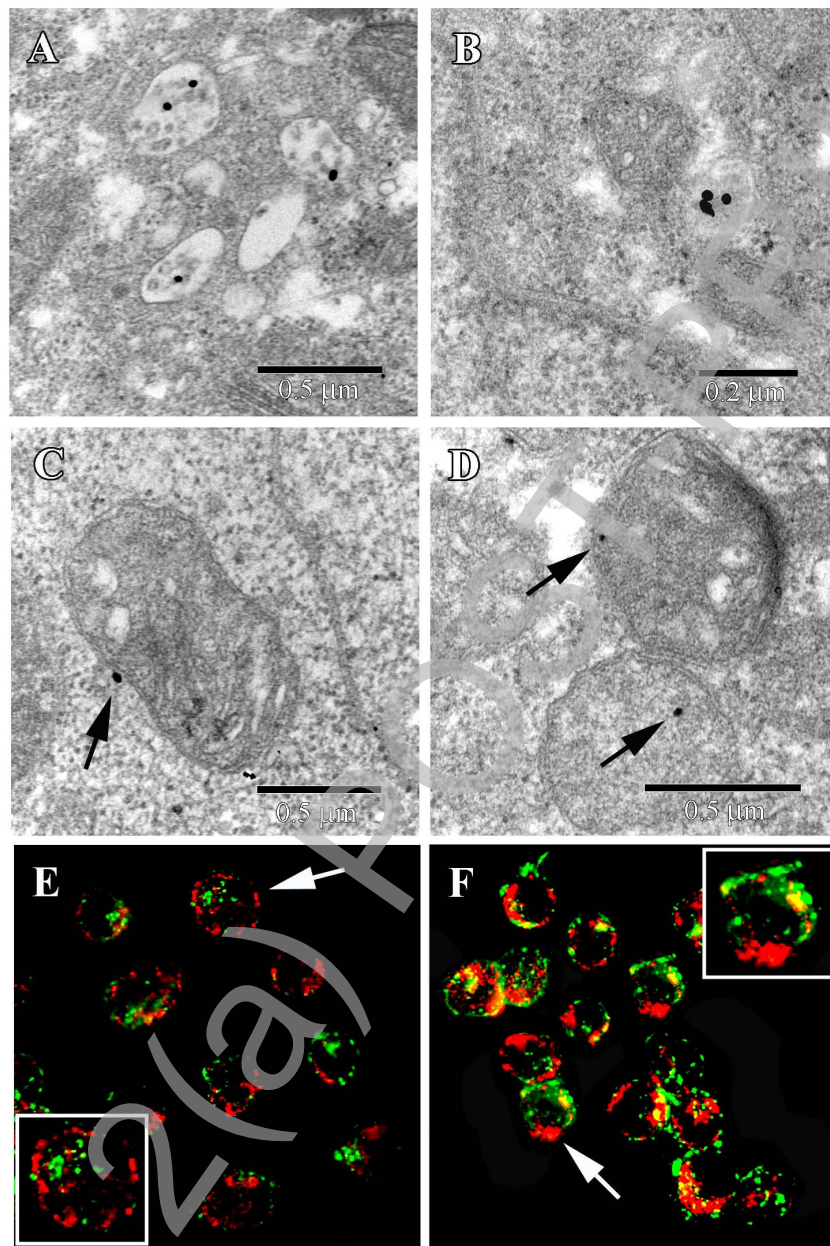


Figure 4

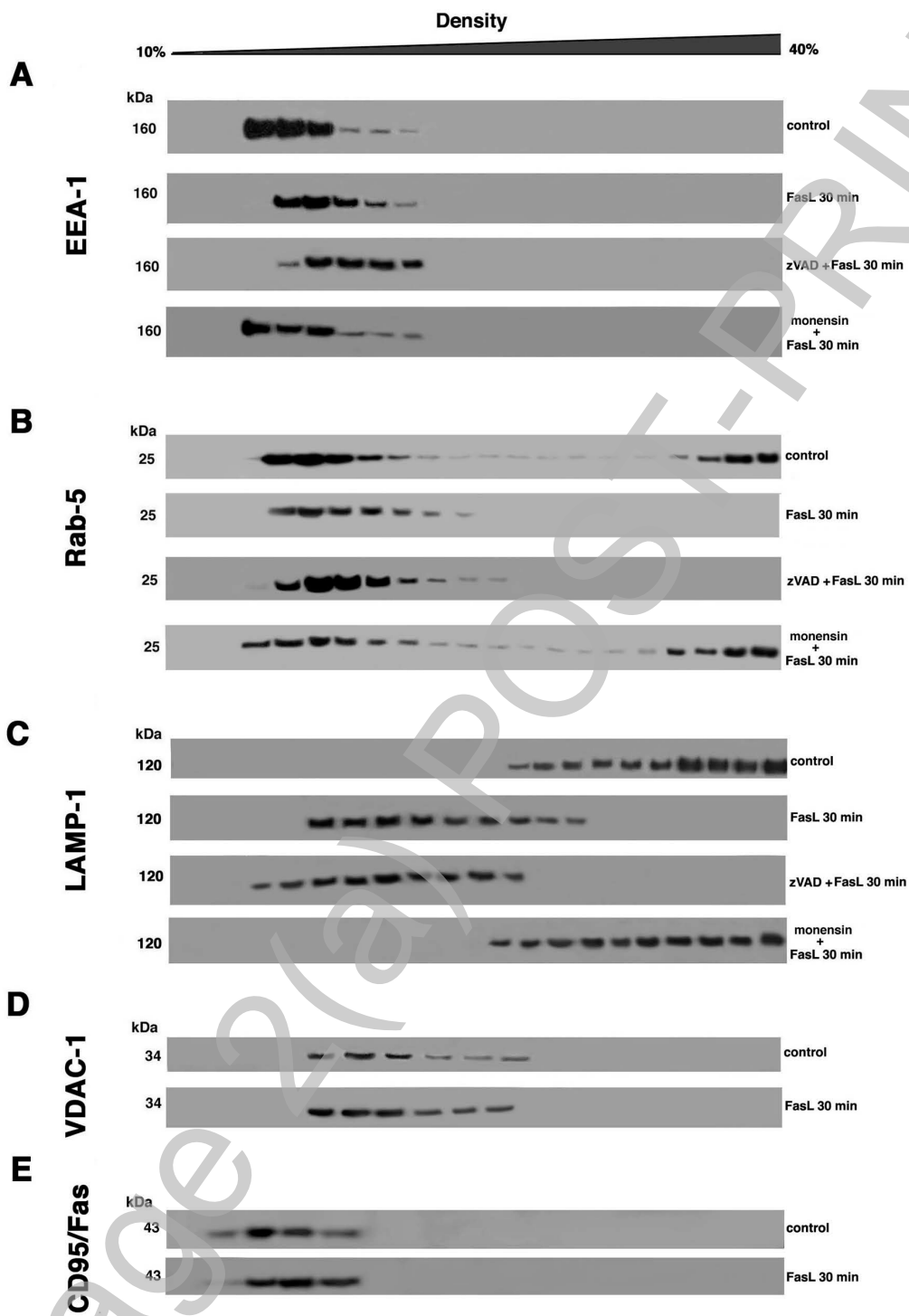


Figure 5

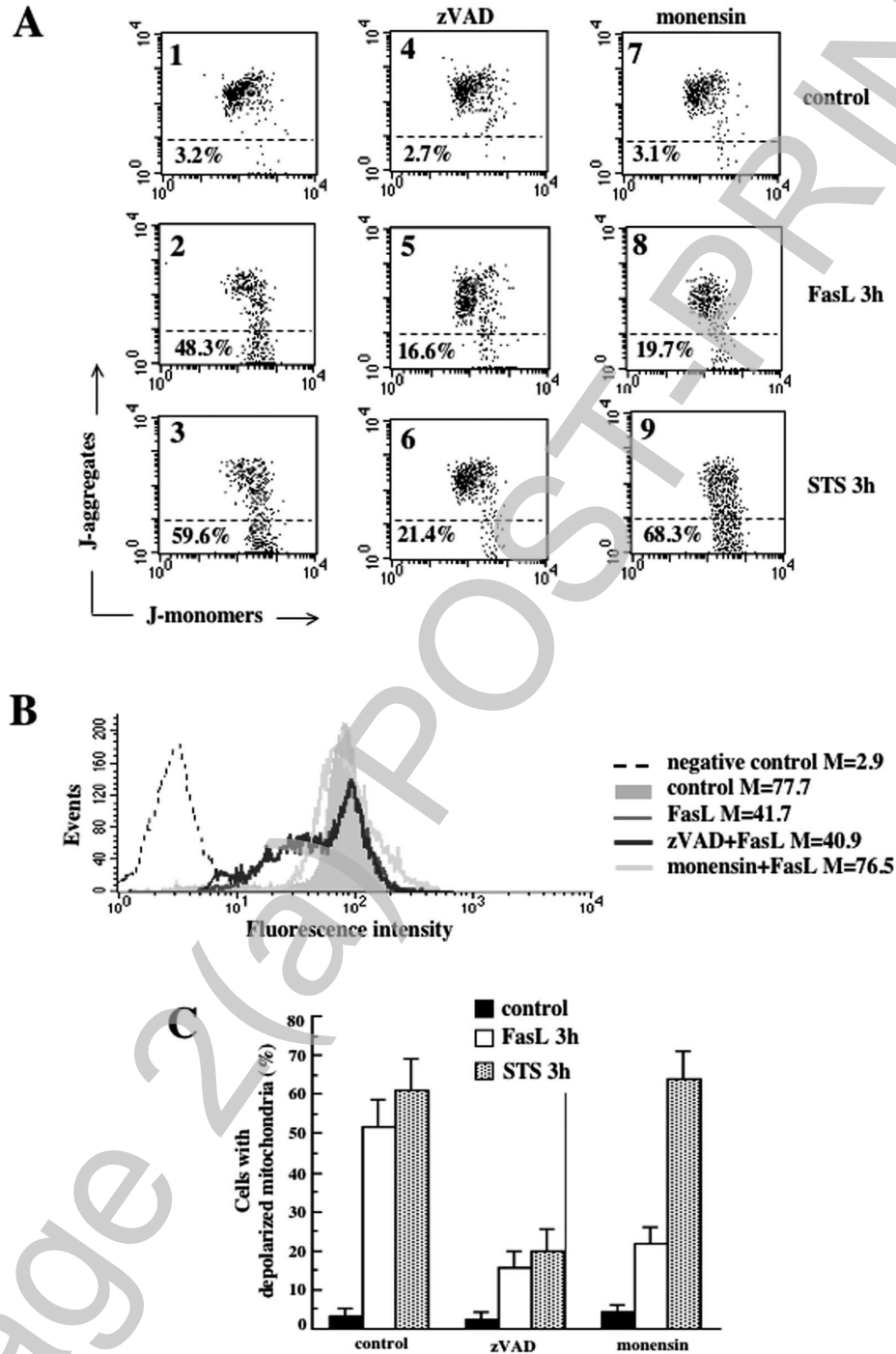


Figure 6

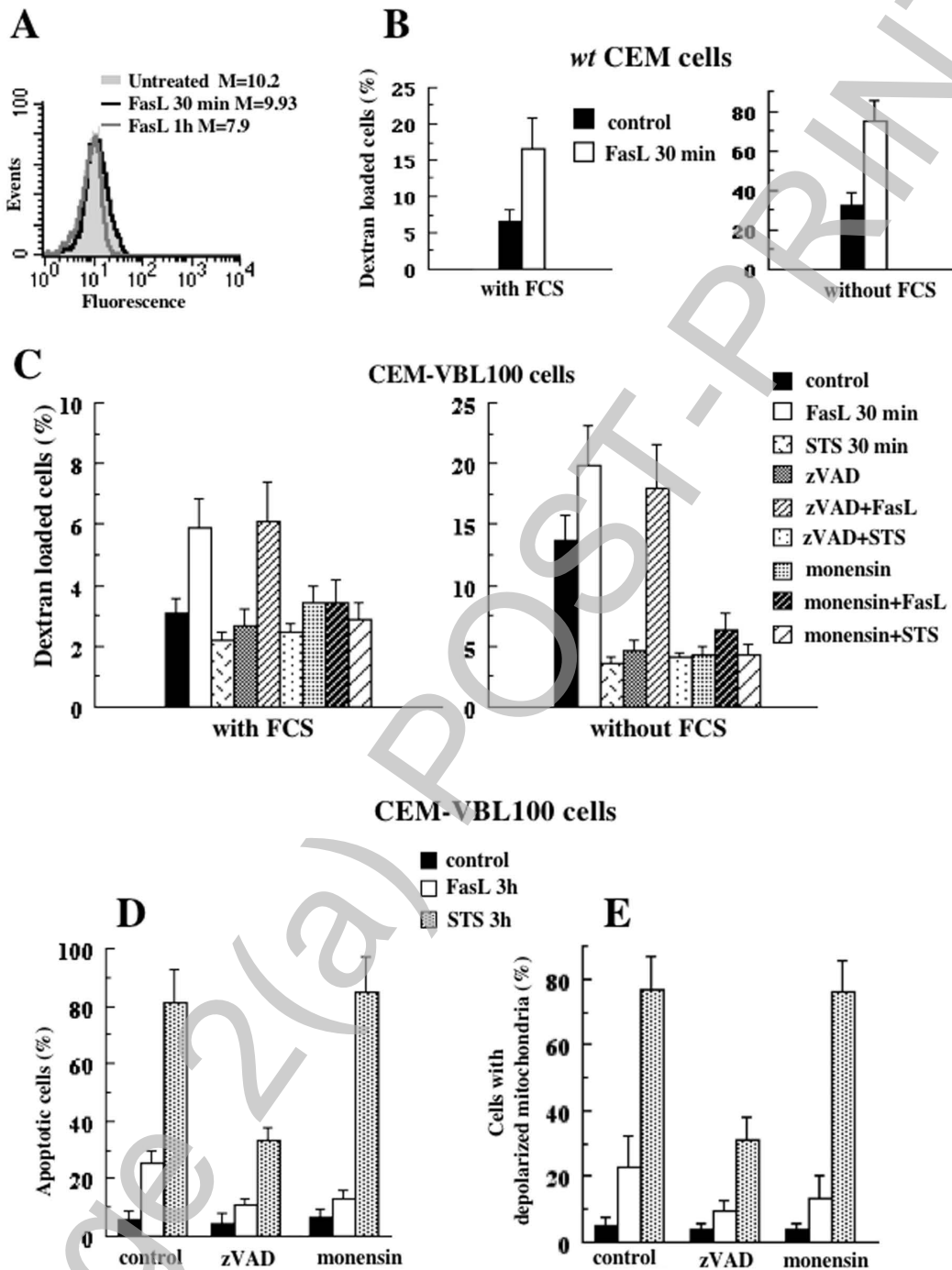
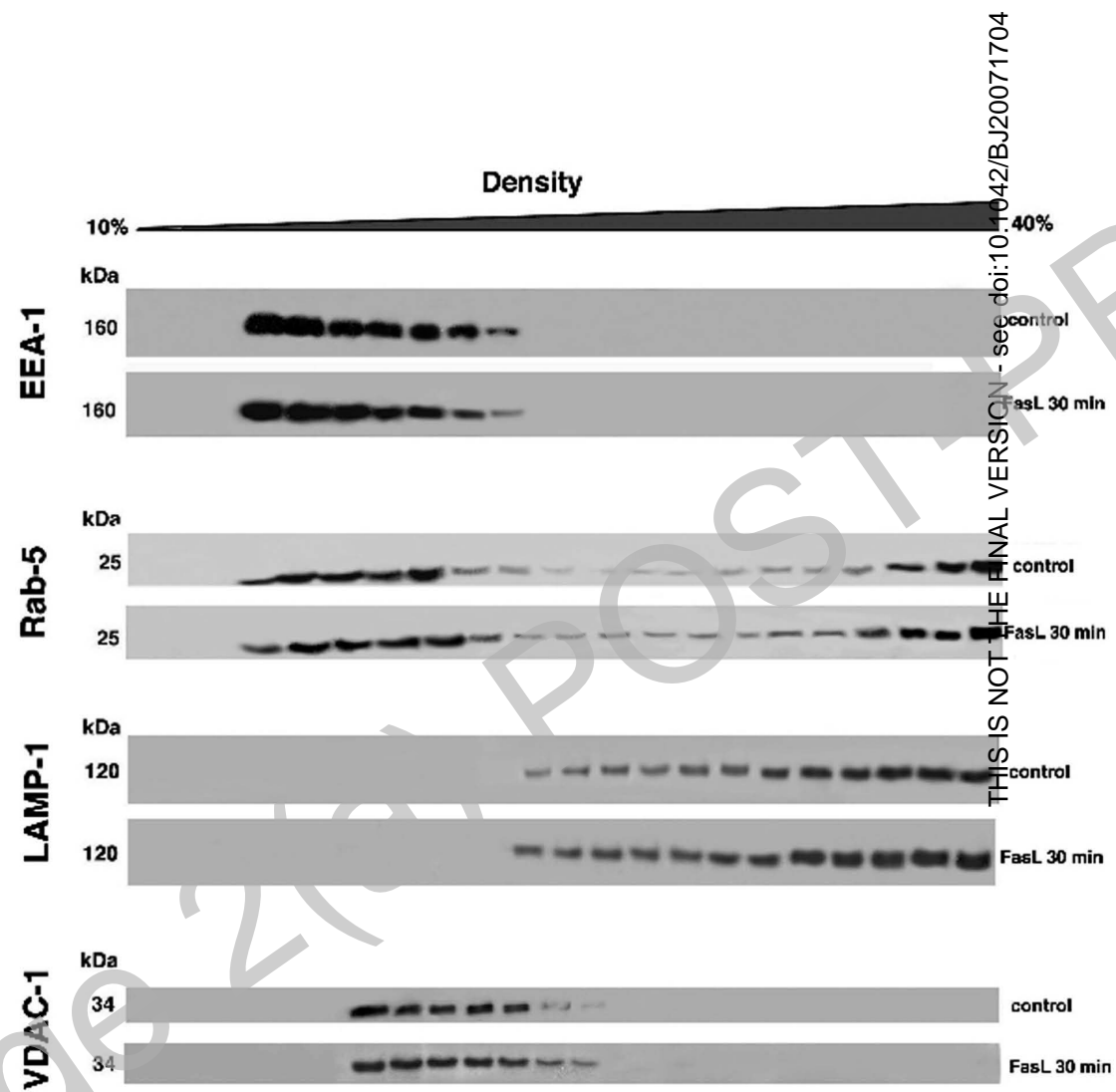


Figure 7

**Figure 8**