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Endosomal compartment contributes to the propagation of CD95/Fas-mediated signals in type II cells

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Abstract

Participation of diverse organelles in the intracellular signalling that follows CD95/Fas receptor ligation encompasses a series of subcellular changes that are mandatory for, or even bolster, the apoptotic cascade. In the present study, we analysed the role of endocytosis in the propagation of cell death signalling 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 (Fas ligand)-induced endocytosis also leads to an early and directional ‘movement’ of endocytic vesicles towards the mitochondrial compartment. In turn, this cross-talk between endosomal and mitochondrial compartments was followed by the loss of the mitochondrial membrane potential and apoptosis execution. This cell remodelling 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 multidrug resistance (CEM VBL100). In these cells a reduced FasL (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 signalling leading to type II cell demise.

Keywords

apoptosis; CD95/Fas; endocytosis; mitochondrial membrane potential (MMP); multidrug resistance; type II cell

INTRODUCTION

As a general rule, upon ligand binding, surface receptors are able to transduce signals by interacting with intracellular signalling 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 programme 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 pathway is activated after ligation of death receptors by members of the TNF (tumour necrosis factor) family, including TNF, CD95L [FasL (Fas ligand)/Apo-1L] and TRAIL (TNF-related apoptosis-inducing ligand) and is associated with the activation of caspase 8 as apical caspase. The second pathway 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 (death-inducing signalling complex), whereas type II cells are more dependent on 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 cross-talk 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 signalling. 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 study, the propagation events that impact on intracellular membranes and their dynamic interactions during the early steps of Fas signalling in type II cells have been analysed. 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 the 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 signalling 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 cross-talk and propagation of death signals in type II cells.

EXPERIMENTAL

Reagents

Recombinant superFasLigand (FasL) was purchased from Apotech (Lausanne, Switzerland), whereas neutralizing anti-human Fas IgG1 mAb (monoclonal antibody) (clone ZB4) was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Fluorescent probes and

assays were purchased from Molecular Probes/Invitrogen (Eugene, OR, U.S.A.). Polyclonal anti-VDAC-1 (voltage-dependent anion channel-1), anti-LAMP-1 (lysosome-associated membrane protein-1) and anti-CD95/Fas (C-20) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), whereas polyclonal anti-Rab-5 anti-EEA1 (early endosome antigen 1) was purchased from BD/Pharmingen (Oxford, U.K.). Z-VAD-FMK [benzyloxycarbonyl-Val-Ala-DL-Asp fluoromethyl ketone; also known as Z-Val-Ala-DL-Asp-CH₂F (benzyloxycarbonyl-valylalanyl-DL-aspartylfluoromethane)] was from Alexis (San Diego, CA, U.S.A.) and monensin from Sigma (St. Louis, MO, U.S.A.). Other chemicals were from Sigma.

Cell cultures

Human lymphoblastoid T-cell line CEM and its multidrug-resistant counterpart, VBL100 cells, were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS (fetal calf serum), 50 units/ml penicillin and 50 µg/ml streptomycin, in a humidified atmosphere with 5% (v/v) CO₂ at 37°C. For all the experiments, 5×10^5 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 STS (staurosporine; Sigma) for different times (20 and 30 min and 1 and 3 h respectively). For experiments with inhibitors, cells were pretreated for 30 min with Z-VAD-FMK (50 µM) or monensin (10 µM). Cells treated with each drug (Z-VAD-FMK or monensin) alone were considered as controls. Negative controls for endocytosis assays were performed by incubating cells with 250 ng/ml of neutralizing anti-human Fas IgG1 mAb (clone ZB4).

TEM (transmission electron microscopy) studies

Human lymphoblastoid T-cells were fixed in 2.5% cacodylate-buffered (0.2 M, pH 7.2) glutaraldehyde for 20 min at room temperature (25°C) and post-fixed in 1% OsO₄ in cacodylate buffer for 1 h 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 80 kV.

Morphometric analyses

Quantitative evaluations have been performed by analysing at least 200 cells by TEM at the same magnification ($\times 4000$).

Cell death assays

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

MMP (mitochondrial membrane potential) in living cells

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

Assays of endocytosis

After 30 min treatment with FasL, cells were washed three times, resuspended in RPMI 1640 medium (with or without FCS) and then incubated for an additional 30 min with dextran-FITC (1 mg/ml, Sigma) at 37°C. After three washings, cells were resuspended in PBS and immediately analysed by using 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 styryl fluorescent dye FM1-43 [8,15,16]. Fluorescence changes were recorded in a spectrofluorimeter (PerkinElmer LC50) with excitation at 470 nm (5 nm bandwidth) and emission at 565–580 nm (10 nm bandwidth), using 5×10^5 cells/ml in a modified Ringer's solution [15] and 2–4 μ M FM1-43. Complementary measurements were carried out in a plate reader with the emission filter centred at 538 nm (Fluoroskan Ascent; Thermo, Basingstoke, U.K.). Alternatively, after 30 min treatment with FasL, cells were incubated for an additional 30 min with BSA-FITC (Sigma) or BSA-gold (40 nm, Fitzgerald Industries International, Concord, MA, U.S.A.) at 37°C. After washings, cells were prepared for immunofluorescence and TEM respectively.

Sucrose-gradient fractionation

Analysis of the distribution of EEA1, Rab5, LAMP-1, VDAC-1 and CD95/Fas was performed by isopycnic centrifugation on a continuous density gradient, as previously described [17]. Briefly, approx. 1.5×10^8 CEM cells or VBL100 cells, either untreated or treated with FasL (100 ng/ml for 30 min 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 Z-VAD-FMK, and a 1:100 (v/v) cocktail of protease inhibitors] containing 8.5% sucrose. Alternatively, cells were pretreated with 50 μ M Z-VAD-FMK 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 min centrifugation at 1000 *g* and the supernatant was loaded on top of 4 ml of a 10–40% continuous sucrose gradient and spun for 16 h in a Beckman TL-70 ultracentrifuge (swinging bucket rotor, SW60 Ti) at 40 000 rev./min. After centrifugation, 20 fractions were collected from the bottom of the tube. After evaluation of the protein concentration with a Bio-Rad assay, fractions were subjected to electrophoresis. The respective proteins were localized by Western blotting.

Immunofluorescence

At the end of the incubation with BSA-FITC, the control and FasL-treated cells were fixed with 3.7% (w/v) 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 1 h with an anti-mitochondria mAb (Chemicon International, Temecula, CA, U.S.A.). Then, cells were labelled with Alexa Fluor® 594-conjugated anti-mouse IgG (Molecular Probes, Leiden, The Netherlands) for 30 min 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 colour chilled 3CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and analysed by the OPTILAB (Graftek) software.

Immunoblotting

Cell lysates and subcellular samples were separated by SDS/polyacrylamide gel and immunoblotted as described previously [18].

Data analysis and statistics

All samples were analysed with a FACScan cytometer (BD Biosciences) equipped with a 488 nm argon laser. At least 20 000 events were acquired. Data were recorded and statistically analysed by a Macintosh computer using CellQuest software (Becton–Dickinson). Statistical analysis of endocytosis and apoptosis data and morphometric analysis was performed by using a Student's *t* test or one-way ANOVA by using the StatView program for Macintosh. All the data reported were verified in at least three different experiments and are reported as means $\pm$ S.D. Only *P* values of less than 0.01 were considered as statistically significant.

RESULTS

CD95/Fas ligation and endocytosis

In line with previous studies hypothesizing a role for endocytosis of CD95/Fas in triggering of apoptosis 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 after increased endocytosis [15,16]. Treatment with FasL rapidly elevated FM1-43 fluorescence, indicating endocytic uptake, which intensified with increasing FasL concentration within 1 h of addition and then progressively decayed (Figure 1A). This time course analysis indicated that Fas triggering led to an early increase in 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 in endocytosis, the FM1-43 uptake being not significantly different from that observed in untreated control cells (Figure 1A). Actually, the FasL-induced changes in FM1-43 fluorescence were in the same direction as those obtained with ionomycin, an established inducer of rapid endocytosis (Figure 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 analysed after FasL administration: (i) CD95/Fas surface expression and (ii) endocytosis. These control analyses, performed on living cells, showed that treatment with FasL for 30 min did not induce any significant decrease in CD95 surface expression (Figure 1C, black line), but a very small decline in CD95 surface expression level with respect to untreated cells (Figure 1C, light-grey shaded area) was detected only starting from 1 h after FasL administration (Figure 1C, mid-grey line). With regards to 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 1(D), which displays the means $\pm$ S.D. obtained from four different experiments. It can be observed that: (i) CD95/Fas triggering significantly stimulated the endocytosis of FITC–dextran beads within 30 min of FasL administration (Figure 1D, left panel); and that (ii) after serum deprivation the uptake of FITC–dextran beads in FasL-triggered cells was significantly increased (Figure 1D, right panel). In addition, we also found that FasL-induced endocytosis was caspase-independent, being unaffected by pretreating the cells with the pan-caspase inhibitor Z-VAD-FMK (Figure 1D) either in the presence (white bars) or absence (black bars) 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 (Figure 1D) pretreatment significantly inhibited FasL-induced uptake of FITC–dextran beads, both in the presence (white bars) and absence (black bars) of FCS. Importantly, we also observed that cell treatment with (i) anti-human Fas IgG1 neutralizing antibody (clone ZB4), which binds Fas receptor without triggering apoptosis (results not shown), and (ii) STS, a drug that induces apoptosis via a receptor-independent mechanism, i.e. via the mitochondrial pathway

(Figure 1D), did not influence the endocytotic rate, as evaluated by dextran beads internalization.

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

Altogether, these results 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 under our experimental conditions in CEM cells. In particular, we found that, as expected in type II cells, the pan-caspase inhibitor Z-VAD-FMK 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 (Figures 2A and 2B). In particular, the analysis of both annexin V-positive cells and propidium iodide-positive cells clearly indicated that monensin was ineffective against STS-induced apoptosis. A representative experiment is shown in Figure 2(A).

Redistribution 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 analysed by means of TEM. As expected, with respect to control cells, we observed an increased presence of membranous endocytic structures in the cytoplasm of FasL-treated cells (Figure 3A). However, more importantly, some of these endocytic vesicles were detected in close proximity to the mitochondria (Figures 3B and 3C). The quantitative evaluation of this phenomenon, performed by morphometric analysis (see the 'Experimental' section), revealed that the frequency of vacuoles in close proximity to mitochondria was significantly much higher in FasL-treated cells than in control untreated cells (Figure 3D). Moreover, it is worth noting that these cytoplasmic changes occurred early (30 min) after FasL-induced CD95 triggering in cells that do not yet display ultrastructural signs of apoptosis and increased with time (up to 1 h). After this time, namely after 3 h, typical signs of apoptosis were detectable (results not shown). To further study this phenomenon, we decided to evaluate BSA endocytosis under apoptotic stimulation. The results obtained by both IEM (immunoelectron microscopy) (Figures 4A–4D) and fluorescence analyses (Figures 4E–4F) clearly indicated that BSA endocytosis associated with CD95/Fas triggering can eventually 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 analyse in detail the subcellular distribution of endosomes in our system, i.e. after CD95/Fas triggering. A series of experiments was thus performed to analyse by Western blotting three different membrane markers of the endocytic pathway: EEA1, Rab5 and LAMP-1. We initially focused on EEA1, a membrane-tethering protein that normally resides in the cytosol and is recruited to endosomal membranes by interaction with phosphoinositol 3-phosphate, the

distinctive phospholipid of early endosomes [21,22], by means of cell subfractionation after a continuous 10–40% sucrose density gradient, as stated elsewhere [17]. EEA1 was normally distributed in the low-density fractions in untreated cells (Figure 5A), but when cells were treated with FasL, a minor redistribution of this molecule towards the VDAC-1-enriched fractions, corresponding to mitochondria-enriched fractions (Figure 5D), was detected. Interestingly, this redistribution of EEA1 was caspase-independent, since it was still evident after treatment with Z-VAD-FMK (Figure 5A). More intriguingly, monensin pretreatment (Figure 5A) prevented the Fas-mediated redistribution of EEA1 towards mitochondria-rich fractions.

Next, we investigated the distribution of the membrane marker Rab5, specific for early/recycling endosomes [22,23]. Rab5 redistribution partially matched the change in the EEA1 fractionation profile (Figure 5B). In fact, in the absence of FasL treatment, Rab5 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 Rab5 normally localizes on early as well as recycling endosomes [24]. Conversely, cell treatment with FasL narrowed the bimodal distribution of Rab5 across density gradients and increased its levels in mitochondria-rich fractions (Figure 5B). Again, the FasL-induced change in Rab5 distribution was virtually unaffected by treatment with the pan-caspase inhibitor Z-VAD-FMK, but was prevented by pretreatment with monensin (Figure 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 (Figure 5C). This shift in LAMP-1 distribution remained clearly evident in the presence of Z-VAD-FMK, but did not occur after pretreatment with monensin (Figure 5C).

As a control, the distribution pattern of CD95/Fas in the same fractions was analysed in the presence or in the absence of FasL-induced endocytosis (Figure 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 redistribution of this molecule towards mitochondria-rich fractions.

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

Monensin hinders the loss of MMP

Considering the key role of mitochondria in apoptosis execution in type II cells, further analyses were also conducted to evaluate the MMP, known to decrease in cells undergoing apoptosis and associated with the release of apoptogenic factors [4-6] (Figure 6). The typical loss of MMP was detectable in both FasL- and STS-treated cells (Figure 6A). In both instances, MMP loss was significantly hindered by Z-VAD-FMK, 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 Figure 6A a representative experiment is shown). The same results were found by using an alternative probe (TMRM) as shown in Figure 6(B). In fact, the percentage of cells with depolarized mitochondria was significantly reduced by monensin pretreatment in FasL-treated cells only (Figure 6C). Altogether, these findings suggest that monensin, perturbing the endocytic pathway, also perturbs apoptosis execution and reduces MMP loss.

Resistance to Fas-mediated apoptosis is associated with decreased endocytosis

To analyse further the mechanisms underlying the role of endocytosis in propagating FasL-mediated signalling, we decided to evaluate the endocytotic process triggered by FasL in the multidrug-resistant counterpart of CEM cells, the VBL100 cells [25]. Induction of an MDR (multidrug resistance) phenotype is referred as to cells that are able to extrude unrelated drugs via cell surface ATP-dependent pumps, for example by P-glycoprotein. It has been generally accepted that MDR is normally associated with 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 wild-type 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 analysed 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 wild-type and VBL CEM cells was comparable (Figure 7A); and (ii) the basal level of endocytosis was significantly lower in VBL100 cells with respect to wild-type CEM cells (compare the black bars in Figure 7C with the black bars in Figure 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 wild-type CEM cells (compare the white bars in Figure 7C with the white bars in Figure 7B); (iv) Z-VAD-FMK was ineffective in preventing FasL-induced endocytosis; (v) although the FasL-triggered endocytosis is very low compared with that of wild-type cells, monensin was however able to significantly prevent the endocytic phenomenon also in VBL100 cells. Finally, according to the results obtained in wild-type CEM cells, (v) STS did not influence the endocytic activity in VBL100 drug-resistant CEM cell variant (Figure 7C). These results are in accordance with apoptosis (Figure 7D) and MMP (Figure 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 with wild-type CEM cells (Figure 7D). Accordingly, in VBL100 cells, FasL was less effective in inducing MMP loss than STS (Figure 7E).

In light of these results, it was mandatory to evaluate the possible presence of endocytic components, i.e. EEA1, Rab5 and LAMP-1, in cell subfractionation after a continuous 10–40% sucrose density in VBL100 cells too. Western-blot analyses (Figure 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 remodelling of the endosomal compartment after FasL administration (as observed in wild-type CEM cells) and that, concomitantly, they are also less susceptible to FasL-induced apoptosis.

DISCUSSION

In the present study, 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 study 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 an MDR-resistant variant of CEM cells, i.e. VBL100 cells, which 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 can be attributed neither to a reduced expression of CD95 nor to a defect in the apoptotic machinery, VBL100 cells being sensitive to STS-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 on 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 demonstrated 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 (epidermal growth factor) 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 mitochondria-enriched cell subfractions (see below) [8]. How can this cross-talk between endosomal and mitochondrial compartments contribute to apoptosis execution? The idea that various subcellular organelles can interact with 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 ER (endoplasmic reticulum) 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 remodelling and intermixing and is implicated in the pathophysiology of several neurodegenerative and cardiovascular diseases [10]. In the same vein, lysosomal organelles have been analysed in different experimental settings, precisely devoted to the understanding of how certain lysosomal cysteinyl proteases, e.g. cathepsins, can contribute to the cross-talk between endolysosomes and mitochondria during apoptosis [33]. More recently, the possible implications of the Golgi apparatus remodelling in the execution of apoptosis have been analysed in detail. It was found that also this organelle also participates in 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 remodelling seems to represent a very early event in the propagation of FasL-mediated signalling.

We found that CD95/Fas-triggered apoptosis was significantly prevented by the inhibition of endosomal acidification by monensin, a univalent ion-selective ionophore [20]. Importantly, this compound also inhibited the shift of EEA1, Rab5 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 Z-VAD-FMK, 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 in endocytic activity.

Some further points should be considered in this context. One deals with the fact that the increase in endocytic compartment activity has been found in receptor-mediated cell death only. In fact, inhibition of endocytosis by monensin did not affect the execution of apoptosis induced by the mitochondriotropic drug STS. Hence Fas ligation, by stimulating endocytosis, could affect membrane traffic and generate a 'controlled' wave of endocytic vesicles that, inducing organelle cross-talk, might contribute to introducing those mitochondrial conditions favourable to the execution of apoptosis (e.g. changes in membrane potential and release of apoptogenic factors).

These hypotheses are also supported by the data obtained by using the MDR counterpart of CEM cells (VBL100 cells) as a 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 the CEM VBL100 cell line with respect to its wild-type 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 cross-talk 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

DISC	death-inducing signalling complex
EEA1	early endosome antigen 1
ER	endoplasmic reticulum
FasL	Fas ligand
FCS	fetal calf serum
LAMP-1	lysosome-associated membrane protein-1
mAb	monoclonal antibody
MDR	multidrug resistance
MMP	mitochondrial membrane potential
STS	staurosporine
TEM	transmission electron microscopy

TMRM	tetramethylrhodamine ester
TNF	tumour necrosis factor
VDAC-1	voltage-dependent anion channel-1
Z-VAD-FMK	benzyloxycarbonyl-Val-Ala-DL-Asp fluoromethyl ketone

REFERENCES

1. Di Fiore PP, De Camilli P. Endocytosis and signaling, an inseparable partnership. *Cell*. 2001; 106:1–4. [PubMed: 11461694]
2. Lee KH, Feig C, Tchikov V, Schickel R, Hallas C, Schutze S, Peter ME, Chan AC. The role of receptor internalization in CD95 signaling. *EMBO J*. 2006; 25:1009–1023. [PubMed: 16498403]
3. Algeciras-Schimmich A, Pietras EM, Barnhart BC, Legembre P, Vijayan S, Holbeck SL, Peter ME. Two CD95 tumor classes with different sensitivities to antitumor drugs. *Proc. Natl. Acad. Sci. U.S.A.* 2003; 100:11445–11450. [PubMed: 14504390]
4. Barnhart BC, Alappat EC, Peter ME. The CD95 type I/type II model. *Semin. Immunol.* 2003; 15:185–193. [PubMed: 14563117]
5. Austin CD, Lawrence DA, Peden AA, Varfolomeev EE, Totpal K, De Maziere AM, Klumperman J, Arnott D, Pham V, Scheller RH, Ashkenazi A. Death-receptor activation halts clathrin-dependent endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103:10283–10288. [PubMed: 16801533]
6. Yin XM. Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. *Gene*. 2006; 369:7–19. [PubMed: 16446060]
7. Jaattela M, Tschopp J. Caspase-independent cell death in T lymphocytes. *Nat. Immunol.* 2003; 4:416–423. [PubMed: 12719731]
8. Fomina AF, Deerinck TJ, Ellisman MH, Cahalan MD. Regulation of membrane trafficking and subcellular organization of endocytic compartments revealed with FM1-43 in resting and activated human T cells. *Exp. Cell Res.* 2003; 291:150–166. [PubMed: 14597416]
9. Faitova J, Krekac D, Hrstka R, Vojtesek B. Endoplasmic reticulum stress and apoptosis. *Cell. Mol. Biol. Lett.* 2006; 11:488–505. [PubMed: 16977377]
10. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* 2006; 7:880–885. [PubMed: 16953201]
11. Ouasti S, Matarrese P, Paddon R, Khosravi-Far R, Sorice M, Tinari A, Malorni W, Degli Esposti M. Death receptor ligation triggers membrane scrambling between Golgi and mitochondria. *Cell Death Differ.* 2007; 14:453–461. [PubMed: 17008914]
12. Matarrese P, Tinari A, Mormone E, Bianco GA, Toscano MA, Ascione B, Rabinovich GA, Malorni W. Galectin-1 sensitizes resting human T lymphocytes to Fas (CD95)-mediated cell death via mitochondrial hyperpolarization, budding, and fission. *J. Biol. Chem.* 2005; 280:6969–6985. [PubMed: 15556941]
13. Cossarizza A, Franceschi C, Monti D, Salvioli S, Bellesia E, Rivabene R, Biondo L, Rainaldi G, Tinari A, Malorni W. Protective effect of *N*-acetylcysteine in tumor necrosis factor- α -induced apoptosis in U937 cells: the role of mitochondria. *Exp. Cell Res.* 1995; 220:232–240. [PubMed: 7664840]
14. Hed J, Hallden G, Johansson SG, Larsson P. The use of fluorescence quenching in flow cytofluorometry to measure the attachment and ingestion phases in phagocytosis in peripheral blood without prior cell separation. *J. Immunol. Methods.* 1987; 101:119–125. [PubMed: 3112235]
15. Zweifach A. FM1-43 reports plasma membrane phospholipid scrambling in T-lymphocytes. *Biochem. J.* 2000; 349:255–260. [PubMed: 10861236]
16. Kawasaki Y, Saito T, Shiota-Someya Y, Ikegami Y, Komano H, Lee MH, Froelich CJ, Shinohara N, Takayama H. Cell death-associated translocation of plasma membrane components induced by CTL. *J. Immunol.* 2000; 164:4641–4648. [PubMed: 10779768]

17. Stockinger W, Sailer B, Strasser V, Recheis B, Fasching D, Kahr L, Schneider WJ, Nimpf J. The PX-domain protein SNX17 interacts with members of the LDL receptor family and modulates endocytosis of the LDL receptor. *EMBO J.* 2002; 21:4259–4267. [PubMed: 12169628]
18. Degli Esposti M, Cristea IM, Gaskell SJ, Nakao Y, Dive C. Proapoptotic Bid binds to monolysocardiolipin, a new molecular connection between mitochondrial membranes and cell death. *Cell Death Differ.* 2003; 10:300–1309.
19. Nilsson C, Kagedal K, Johansson U, Ollinger K. Analysis of cytosolic and lysosomal pH in apoptotic cells by flow cytometry. *Methods Cell Sci.* 2003; 25:185–194. [PubMed: 15801164]
20. Simonsen A, Wurmser AE, Emr SD, Stenmark H. The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* 2001; 13:485–492. [PubMed: 11454456]
21. Rink J, Ghigo E, Kalaidzidis Y, Zerial M. Rab conversion as a mechanism of progression from early to late endosomes. *Cell.* 2005; 122:735–749. [PubMed: 16143105]
22. Schnatwinkel C, Christoforidis S, Lindsay MR, Uttenweiler-Joseph S, Wilm M, Parton RG, Zerial M. The Rab5 effector Rabankyrin-5 regulates and coordinates different endocytic mechanisms. *PLoS Biol.* 2004; 2:E261. [PubMed: 15328530]
23. Gorvel JP, Chavrier P, Zerial M, Gruenberg J. Rab5 controls early endosome fusion *in vitro*. *Cell.* 1991; 64:915–925. [PubMed: 1900457]
24. Dinter A, Berger EG. Golgi-disturbing agents. *Histochem. Cell Biol.* 1998; 109:571–590. [PubMed: 9681636]
25. Matarrese P, Testa U, Cauda R, Vella S, Gambardella L, Malorni W. Expression of P-170 glycoprotein sensitizes lymphoblastoid CEM cells to mitochondria-mediated apoptosis. *Biochem. J.* 2001; 355:587–595. [PubMed: 11311119]
26. Calcabrini A, Arancia G, Marra M, Crateri P, Befani O, Martone A, Agostinelli E. Enzymatic oxidation products of spermine induce greater cytotoxic effects on human multidrug-resistant colon carcinoma cells (LoVo) than on their wild-type counterparts. *Int. J. Cancer.* 2002; 99:43–52. [PubMed: 11948490]
27. Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature.* 2003; 422:37–44. [PubMed: 12621426]
28. Hamasaki M, Araki N, Hatae T. Association of early endosomal autoantigen 1 with macropinocytosis in EGF-stimulated A431 cells. *Anat. Rec. A. Discov. Mol. Cell. Evol. Biol.* 2004; 277:298–306. [PubMed: 15052657]
29. Goldenthal MJ, Marin-Garcia J. Mitochondrial signaling pathways: a receiver/integrator organelle. *Mol. Cell. Biochem.* 2004; 262:1–16. [PubMed: 15532704]
30. Le Bras M, Rouy I, Brenner C. The modulation of inter-organelle cross-talk to control apoptosis. *Med. Chem.* 2006; 2:1–12. [PubMed: 16787350]
31. Csordas G, Renken C, Varnai P, Walter L, Weaver D, Buttle KF, Balla T, Mannella CA, Hajnoczky G. Structural and functional features and significance of the physical linkage between ER and mitochondria. *J. Cell Biol.* 2006; 174:915–21. [PubMed: 16982799]
32. Lai E, Teodoro T, Volchuk A. Endoplasmic reticulum stress: signaling the unfolded protein response. *Physiology.* 2007; 22:193–201. [PubMed: 17557940]
33. Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.* 2001; 3:E255–E263. [PubMed: 11715037]
34. Algeciras-Schimmich A, Shen L, Barnhart BC, Murmann AE, Burkhardt JK, Peter ME. Molecular ordering of the initial signaling events of CD95. *Mol. Cell. Biol.* 2002; 22:207–220. [PubMed: 11739735]
35. Malorni W, Rainaldi G, Tritarelli E, Rivabene R, Cianfriglia M, Lehnert M, Donelli G, Peschle C, Testa U. Tumor necrosis factor alpha is a powerful apoptotic inducer in lymphoid leukemic cells expressing the P-170 glycoprotein. *Int. J. Cancer.* 1996; 67:238–247. [PubMed: 8760594]
36. Shiraishi H, Okamoto H, Yoshimura A, Yoshida H. ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1. *J. Cell Sci.* 2006; 119:3958–3966. [PubMed: 16954146]

Figure 1. CD95/Fas ligand 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 an 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 min 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 the propidium iodide exclusion test) treated with FasL for 30 min or 1 h in comparison with untreated cells. Numbers represent median fluorescence. Note that a small decrease in CD95 expression was visible only after 1 h FasL treatment. (D, F) Modulation of FasL-induced endocytosis. (D) Flow-cytometry analysis of endocytosis performed in the presence (white bars) or absence (black bars) of FCS. The values reported represent the means $\pm$ S.D. 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 absence of FCS; (ii) a significant increase in dextran uptake was detectable in cells under FCS withdrawal (note different scales used); (iii) either Z-VAD-FMK or monensin given alone did not modify on its own dextran uptake; (iv) whereas pretreatment with Z-VAD-FMK did not modify the FasL-induced dextran uptake, monensin pretreatment 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 (autofluorescence) are reported in (E1) and (F1). The addition of Trypan Blue to quench the surface absorption of fluorescent dextran had little effect on cell fluorescence (E2-E5 versus E6-E9 and F2-F5 versus F6-F9), indicating that most of the beads were endocytosed inside the cells. Note that Z-VAD-FMK was ineffective either in FCS-supplemented cells or in cells under FCS starvation (E3

versus **E4** and **F3** versus **F4**), whereas monensin strongly reduced the percentage of dextran-loaded cells (**E3** versus **E5** and **F3** versus **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 Z-VAD-FMK (middle) or monensin (bottom) pretreatments 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/propidium iodide double positive (late apoptosis, lower right quadrant) cells. (B) Results obtained from four independent experiments are reported as means $\pm$ S.D. Note that: (i) Z-VAD-FMK 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 min (A) and for 1 h (B). Note the endocytic vesicles in (A) (arrows) and the presence of vesicles in close proximity to mitochondria in (B). (C) Higher magnification of the boxed area in (B). Note the membranous vesicle in close contact with the mitochondrion. (D) Morphometric and statistical analyses of the percentage of cells showing one or more vesicles close to mitochondria 30 min and 1 h after FasL administration, as evaluated by TEM observation. * $P < 0.01$ compared with untreated cells. Magnification: (A, C) $\times 28000$.

Figure 4. BSA endocytosis after CD95/Fas ligation

Immunogold labelling of BSA in CEM cells 30 min after treatment with FasL (pre-embedding technique). Note the presence of gold particles in endosomal vesicles (**A**), in vesicles in close proximity to mitochondria (**B**), on the mitochondrial membrane (**C**, arrow) and in the mitochondria (**D**, arrows). Fluorescence microscopy analysis also shows that, in comparison with 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. Redistribution of endosomal proteins after CD95/Fas ligation

CEM cells, either untreated or treated with FasL (100 ng/ml for 30 min at 37°C), were lysed in lysis buffer and post-nuclear supernatant was prepared and loaded on top of a continuous 10–40%-(w/v)-sucrose gradient. Alternatively, cells were pretreated with Z-VAD-FMK 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 analysed by Western blotting. Fractions obtained after sucrose-density-gradient centrifugation, either untreated or treated with FasL and alternatively pretreated with 50 μ M Z-VAD-FMK or 10 μ M monensin as above, were analysed using polyclonal antibodies directed to EEA1 (**A**), Rab5 (**B**) or LAMP-1 (**C**). As a control, fractions obtained after sucrose-density-gradient centrifugation from untreated and FasL-treated cells were analysed using a polyclonal anti-VDAC-1 (**D**) or a polyclonal anti-CD95/Fas (**E**). Note that LAMP-1 and, to a lesser extent, early endosome markers, but not CD95/Fas, redistributed towards the VDAC-1-enriched fractions after CD95/Fas ligation. Such a redistribution was virtually undetectable in cells pretreated with the pan-caspase inhibitor Z-VAD-FMK, but was prevented by pretreatment with monensin.

Figure 6. Analysis of MMP

(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 Z-VAD-FMK or monensin pretreatments. (A) Dot plots from a representative experiment are shown. In the area under the broken line, the numbers indicate the percentage of cells with depolarized mitochondria. (B) Semi-quantitative flow-cytometry analysis of MMP using the TMRM probe. Numbers represent the median values of fluorescence intensity histograms. (C) The results obtained from four independent experiments performed by using the JC-1 probe are reported as means $\pm$ S.D. Note that Z-VAD-FMK pretreatment 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 the propidium iodide exclusion test) treated with FasL for 30 min or 1 h in comparison with untreated cells. Numbers represent median fluorescence. Note that a small decrease in CD95 expression was visible only after 1 h FasL treatment. (B) Flow cytometry analysis of endocytosis performed in the presence or absence of FCS in wild-type CEM cells untreated or treated with FasL for 30 min compared with (C) VBL100 cells under different experimental conditions. Results, representing the percentage of cells that ingested dextran-FITC, are reported as means $\pm$ S.D. of the data obtained from four different experiments performed in the absence or presence of Z-VAD-FMK or monensin before treatment with FasL or STS. (D) Analysis of apoptosis in VBL100 cells. FACS analysis was performed after the double staining with annexin V/propidium iodide of untreated control cells, cells exposed to FasL or STS given alone or after Z-VAD-FMK or monensin pretreatments. Results are reported as means $\pm$ S.D. from four independent experiments. (E) Analysis of MMP in VBL100 cells. Biparametric flow-cytometry analysis was performed after the staining with JC-1 of untreated control cells and cells exposed to FasL or STS given alone or after Z-VAD-FMK or monensin pretreatment. The percentage of cells with depolarized mitochondria is reported as means $\pm$ S.D. of the data obtained from four independent experiments.

Figure 8. Redistribution of endosomal proteins after CD95/Fas ligation in the VBL100 cell line
Cells, either untreated or treated with FasL, were lysed in lysis buffer and post-nuclear supernatant was prepared and loaded on top of a 10–40% continuous sucrose gradient as described in the ‘Experimental’ section. After centrifugation, fractions were collected, and samples of each fraction were separated by SDS/PAGE and analysed by Western blotting, using a monoclonal anti-EEA1, a monoclonal anti-Rab-5, a monoclonal anti-LAMP-1 and a polyclonal anti-VDAC-1. Note that EEA1, Rab5 and LAMP-1 do not virtually modify their distribution after CD95/Fas ligation.