CD95 (Fas/APO-1) induces an increased phosphatidylserine synthesis that precedes its externalization during programmed cell death

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Abstract CD95 (Fas, APO-1)-induced programmed cell death (apoptosis) in T cell lines is accompanied by a rapid flip-flop of phosphatidylserine (PtdSer). Externalization of this phospholipid has been previously recognized as one of the early detectable events of cells undergoing apoptosis. We show here that CD95 induces a rapid (detectable at time <15 min), strong (2.5-fold) but transitory neosynthesis of PtdSer in the Jurkat cell line that precedes its externalization. PtdSer decarboxylation, a mitochondrial specific process, was strongly inhibited by CD95 suggesting that changes in mitochondrial activity take place in the early events of Fas-induced apoptosis and participate in the increased PtdSer synthesis observed. In cells undergoing apoptosis, newly synthesized PtdSer first exposed at the cell surface was in part shed with CD95-induced plasma membrane vesicles, a process that likely explains the transitory effect observed.

Key words: Apoptosis; Programmed cell death; Fas; CD95; Phosphatidylserine; Annexin V; T lymphocyte; Jurkat

1. Introduction

Phosphatidylserine (PtdSer) is synthesized by the serine base exchange enzyme system (serine-BEES) in the endoplasmic reticulum. This enzymatic system catalyzes a Ca^{2+}-dependent exchange of the polar head group of phospholipids (either choline or ethanolamine) by a serine [1,2]. Newly synthesized PtdSer migrates either to the internal leaflet of the plasma membrane or to the mitochondria where it is decarboxylated into phosphatidylethanolamine (PtdEtn).

Apoptosis is a general phenomenon that occurs in many cell types. It is characterized by membrane blebbing and vesiculation, chromosomal condensation, cleavage of DNA and subsequent cell death [3,4]. Binding of agonistic anti-Fas antibody to Fas induces apoptosis in Fas-bearing cells and kills them within hours. The mechanism used by the Fas antigen to kill cells involves the activation of a family of caspases recruited in the death-inducing signaling complex through FADD/MORT-1 and the Fas death domain [5,6]. Since the work of Fadok and collaborators [7] it has been clearly established that cross linking the Fas antigen induces the flip-flop of PtdSer. This anionic phospholipid is located at the internal leaflet of the plasma membrane. During the apoptotic process, PtdSer is translocated to the outer leaflet where it is easily detected using the annexin V-FITC binding properties [8,9]. It has been proposed that exposure of PtdSer at the cell surface is used for the recognition by phagocytes of cells undergoing apoptosis [10]. In addition, it has been shown that changes in mitochondrial activity (generally monitored through modification of the mitochondrial transmembrane potential) are one of the earlier events in the apoptotic process [11,12].

In the present report, we have examined the effect of CD95 on PtdSer metabolism and we show that CD95 induces an early and transitory synthesis of PtdSer together with an inhibition of PtdSer decarboxylation. The increase of PtdSer synthesis was detected within 15 min and thus clearly precedes its appearance at the cell surface (detected at time >60 min). Newly synthesized PtdSer was partly recovered on the external leaflet of the plasma membrane and in CD95-induced shed vesicles.

2. Materials and methods

2.1. Cells

Jurkat cells, either the clone D we have previously described [13] or clone JE6.1 (ATCC TIB 152), were cultured in RPMI 1640 (Gibco, Cergy-Pontoise, France) supplemented with 5% fetal calf serum (Biowhitaker, Fontenay, France), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate.

2.2. Materials

Purified (CH-11, IgM and ZB4, IgG) and phycoerythrin-conjugated (UB2) CD95 monoclonal antibodies were purchased from Immunotech (Marseille, France). L129 mouse IgM directed against CD99 [16] was produced in our laboratory and used as negative control. L-[3-^{3}H]Serine (0.74–1.48 TBq/mmol), [*methyl*-^{3}H]choline chloride (2.8–3.1 TBq/mmol) and [1-^{3}H]ethan-1-ol-amine hydrochloride (0.18–1.1 TBq/mmol) were from Amersham, UK.

2.3. Cytofluorimetry

Cell analysis by cytofluorimetry was performed with a FACStar Plus cell sorter (Becton Dickinson, Mountain View, CA, USA). Apoalert Annexin V apoptosis kits were from Clontech Laboratories (Palo Alto, CA, USA); they were used exactly as described by the manufacturer. CD95-induced apoptosis was also studied by cytofluorimetry with the use of the DNA marker, Hoechst 33342 [14].

2.4. Phospholipid synthesis

Jurkat cells (2×10^6) were maintained in 0.5 ml of a buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 2.5 mM glucose, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$ at 37°C in the presence of either 4 μCi of L-[3-^{3}H]serine, [methyl-^{3}H]choline chloride or [1-^{3}H]ethan-1-ol-amine hydrochloride and effectors (see concentrations in the figure legends). After an incubation period varying from 0 to 2 h the cells were rapidly sedimented in an Eppendorf centrifuge, the supernatants were either discarded or further analyzed after centrifugation (13 000 rpm, 15 min). The cellular phospholipids were extracted using chloroform/methanol according to Bligh and Dyer [15]. This two-step extraction procedure allowed the determination of ^{3}H-labeled products

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Abbreviations: PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; Serine-BEES, serine base exchange enzyme system

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incorporated into the cells by measuring a 25-μl sample of the chloroform/methanol extract. Then the addition of chloroform and water allowed the separation of the organic and aqueous phases. The lipid extracts (organic phases) were analyzed by thin layer chromatography on LK6D chromatography plates (Whatman) in a solvent system composed of chloroform/methanol/acetic acid/water (75/45/12/3). Authentic phospholipid standards (Sigma) were run in parallel and detected with iodide vapors. Derivatization with trinitrobenzene sulfonate (TNBS) was performed exactly as previously described [16]. Radioactivity in lipid spots was determined using an automatic linear radiochromatography analyzer, Tracemaster 20 (Berthold), equipped with an 8-mm window and the integration software supplied by the manufacturer.

2.5. Analysis of DNA fragmentation

Analysis of DNA fragmentation was performed by agarose gel electrophoresis. Briefly, pelleted cells were lysed in 0.2% Triton X-100 in 10 mM Tris-HCl/1 mM EDTA, pH 7.5 and the cell lysates were treated with proteinase K and RNase centrifuged at $15\,000\times g$ for 15 min. The final extracts were resolved by electrophoresis on agarose gels impregnated with ethidium bromide and were visualized under UV light.

2.6. Measurements of changes in cytosolic Ca^{2+}

The assay of cytosolic Ca^{2+} was performed using Indo-1 (Calbiochem). Cells (5×10^6/ml) were loaded by using 5 μM Indo-1 at 37°C in the dark for 1 h and then washed and resuspended in medium. The analyses were performed on a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson) fitted with two argon lasers, one tuned to 488 nm and the other to UV. The fluorescence intensity at 480 nm corresponding to the free Indo-1 concentration as well as the fluorescence at 400 nm corresponding to the complex Ca^{2+}-Indo-1 was measured. Calculation of the ratio of fluorescence at 400 nm to fluorescence at 480 nm allows the evaluation of changes in cytosolic free Ca^{2+} concentration independently of the cell size and the intracellular Indo-1 concentration. The flow rate was set at 1000 cells/s and the mean ratio of 3000 cells was noted every 30 s. Damaged cells and debris were gated out according to the dual scatter dot plot from the blue laser.

2.7. Measurements of membrane potential

Membrane potential was estimated by measuring the fluorescence of Jurkat cells incubated with the carbocyanine-based probe, DiOC5(3) (Molecular Probes, Interchim, Paris). Cells were allowed to react with the probe in 1 ml of the appropriate medium for 15 min and then the fluorescence was analyzed by cytofluorimetry (FACStar Plus, Becton Dickinson). Calibration experiments were done with Jurkat cells treated with gramicidin as previously described [17].

3. Results

3.1. CD95 induces an early and transitory PtdSer synthesis

We first tested the ability of CD95 (CH-11, IgM) to modify phospholipid synthesis in Jurkat cells. Dose-response curves and kinetics of [^{3}H]serine incorporation into PtdSer were studied in parallel. In our first experiments, the incorporation of [^{3}H]serine into PtdSer was studied by introducing simultaneously both CD95 and [^{3}H]serine into the cell culture. In these conditions, it appeared that CD95 induced PtdSer synthesis with an EC_{50} between 0.25 and 0.5 μg/ml. The incorporation of [^{3}H]serine into PtdSer was doubled when studied at time 15 min. Increasing the incubation time in the presence of CD95 to 60 min or 120 min resulted in a lower PtdSer synthesis (Fig. 1A). To confirm this unexpected result, cells were first incubated with CD95 for different time periods varying from 0 to 120 min and then the incorporation of [^{3}H]serine into PtdSer was monitored at time 15 and 30 min. Using these experimental conditions, it appeared clearly that CD95-induced PtdSer synthesis decreased with incubation time (Fig. 2). The CD95-induced PtdSer synthesis was 43 ± 7%, 30 ± 5% and 22 ± 8% after 30, 60 and 90 min respectively. After 120 min of treatment with 0.25 μg CD95, the amount of PtdSer synthesized was lower than in the controls.

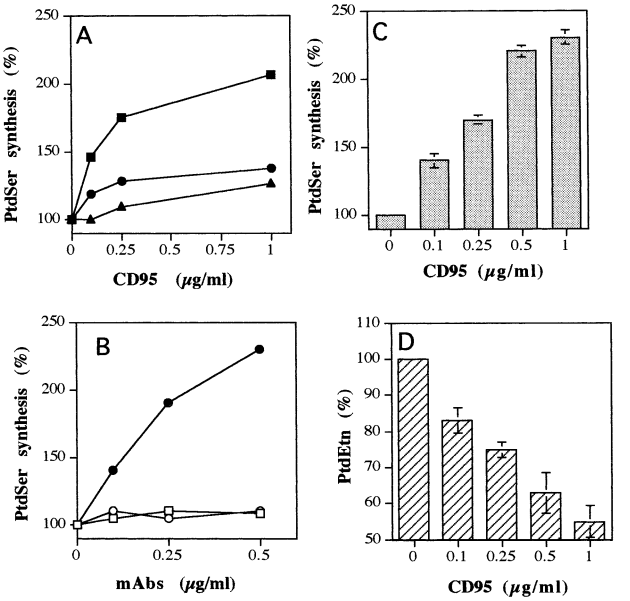

Fig. 1. Effect of CD95 mAb (CH-11) on the incorporation of [^{3}H]serine into phosphatidylserine (PtdSer) in Jurkat cells. 5×10^6/ml cells were incubated for different periods of time at 37°C in the absence or presence of CD95 mAbs with 4 μCi/ml [^{3}H]serine. Phospholipids were extracted, analyzed by thin layer chromatography and quantified with a radiochromatography scanner. A: Dose-response curves obtained after 15 min (■), 60 min (●) or 90 min (▲) after CD95 treatment. The results are expressed in % versus control untreated cells. B: The agonistic CD95 mAb (CH-11) (●) was compared to ZB-4 (□), another CD95 mAb unable to induce apoptosis in Jurkat cells, and to L129 (○), an irrelevant mouse IgM directed agaisnt CD99. C: The CD95 concentration-dependent increase in PtdSer synthesis 15 min after CD95 treatment of cells. D: The amount of PtdSer decarboxylated to PtdEtn 15 min after treatment of the cells with different concentrations of CD95 (CH-11). For panels C and D, each bar corresponds to the mean value ± S.D. from triplicate points and is representative of several experiments.

The early increase of PtdSer synthesis induced by CD95 was observed in the absence of changes in the total uptake of [^{3}H]serine by the cells (see Fig. 4). CD95 treatments longer

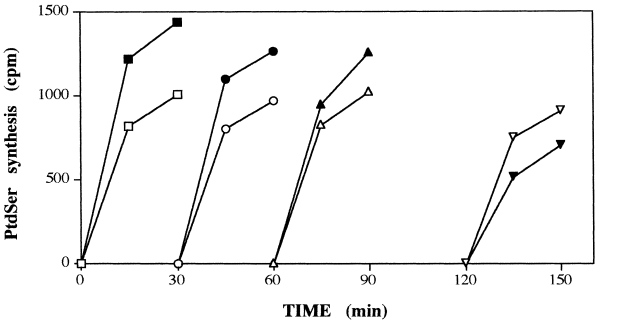

Fig. 2. Effect of preincubation with CD95 mAb (CH-11) on PtdSer synthesis. Cells were left untreated (open symbols) or incubated at time 0 with 250 ng of CD95 mAb (closed symbols) then at time 0 (■, □), 30 (●, ○), 60 (▲, △) and 120 min (▼, ▽) [^{3}H]serine was added and PtdSer synthesis was evaluated after an additional incubation time of 15 or 30 min.

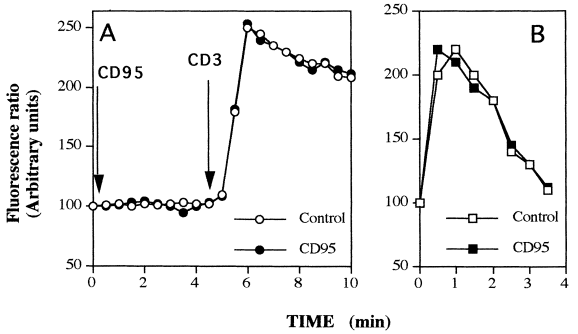

Fig. 3. Lack of effect of CD95 on Ca^{2+} movements. Jurkat cells loaded with Indo-1 were analyzed as described in Section 2. A: Control cells (○) and 1 μg/ml CD95 (CH-11)-treated cells (●). CD95 was added at time 0 and the Indo-1 fluorescence ratio corresponding to Ca^{2+} was monitored for 5 min. Then at time 5 min, CD3 mAb 2 μg/ml was added and the fluorescence ratio was monitored for an additional 5 min period. B: Untreated cells (□) and cells pretreated for 15 min with 1 μg/ml CD95 (■) were placed in 2 mM EGTA-containing medium then immediately treated with 10^{-6} M ionomycin in order to evaluate the Ca^{2+} content of the intracellular stores. The figure represents one experiment representative of several independent measurements.

than 60 min resulted in a decreased [^{3}H]serine uptake by the cells as a consequence of cell depolarization (see below). Measurements of the incorporation of [^{3}H]choline or [^{3}H]ethanolamine into PtdCho or PtdEtn respectively after 15 or 30 min treatment with CD95 showed no significant modifications (not shown). Identical results were obtained with two Jurkat clones, J D and J E6.1 that differ by the presence (J D) or absence (J E6.1) of the tyrosine protein kinase p72syk [18].

3.2. PtdSer synthesis is generated only by agonistic mAbs

The enhancing effect of CD95 on PtdSer synthesis was further confirmed by comparing the effects of two CD95 mAbs CH-11 (IgM) and ZB-4 (IgG1), the first capable of inducing apoptosis while the second is not. Fig. 1B shows that only CH-11 is able to markedly increase Ptdser synthesis.

3.3. CD95 does not modify Ca^{2+} movements

Since the serine base exchange enzyme is a Ca^{2+}-dependent enzyme located in the endoplasmic reticulum and since changes in PtdSer metabolism generally occurred in parallel with changes in Ca^{2+} mobilization we tested whether CD95 is able to induce changes in $[Ca^{2+}]_i$ or to modify CD3-induced Ca^{2+} movements. This was not the case (Fig. 3A). In addition, the Ca^{2+} content of the endoplasmic reticulum in control and CD95-treated cells was assessed using ionomycin in EGTA-containing media. As shown in Fig. 3B, no modification was found suggesting that the CD95-induced PtdSer synthesis we observed was not related to changes in intracellular Ca^{2+}.

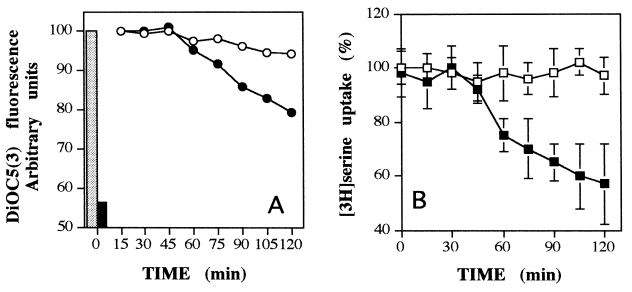

Fig. 4. CD95 induces a late plasma membrane depolarization and a decreased uptake of [^{3}H]serine. Jurkat cells (A) were left untreated (○) or treated with CD95 (0.5 μg/ml) (●) for different periods of time and the membrane potential was measured by cytofluorimetry with the DiOC5(3) probe. The bars on the left of the figure show the polarization status of the cells at time 0 and after 15 min of treatment with 1 μM gramicidin. B: The total uptake of [^{3}H]serine by the cells was measured as a function of time. Each point is the mean ± S.D. of triplicate determinations from a typical experiment.

3.4. CD95 inhibits PtdSer decarboxylation

It is well known that newly synthesized PtdSer migrates either to the internal leaflet of the plasma membrane or to the mitochondria where it is decarboxylated to PtdEtn. Since early changes of mitochondrial activity occur during CD95-induced apoptosis we examined the fate of PtdSer. Monitoring the formation of PtdEtn through decarboxylation of PtdSer indicated (Fig. 1D) that the decarboxylation process was strongly diminished.

3.5. CD95 induces cell depolarization

In a previous paper [17] we have shown that PtdSer synthesis was highly regulated by changes in the membrane potential. In fact, a depolarization of the cells reduced the transport of serine through the plasma membrane resulting in a strong reduction of PtdSer synthesis. In order to see whether a change in membrane potential occurred in CD95-treated cells we first measured the membrane potential with the DiOC5(3)

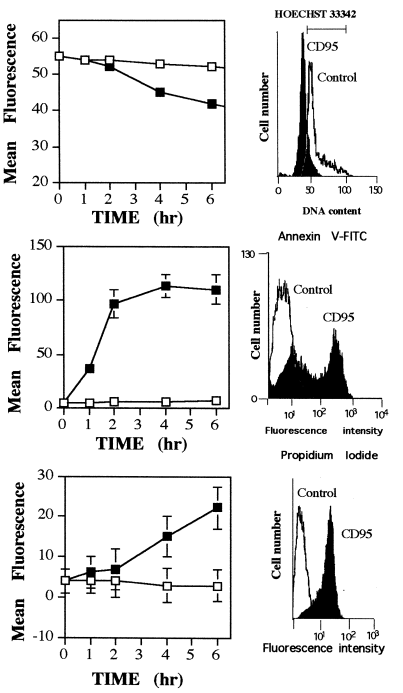

Fig. 5. Evaluation of CD95-induced apoptosis by cytofluorimetry. Cells left untreated (□) or treated with 500 ng CD95 (CH-11) (■) were labeled either with Hoechst 33342 (top panels), annexin V-FITC (middle panels) or propidium iodide (lower panels). Results represent a typical experiment. Each point is the mean ± S.D. of triplicates. When not appearent, the S.D. value was within the point. On the right is represented the FACS analysis at 2 h for Hoechst 33342 and annexin V-FITC and 6 h for propidium iodide.

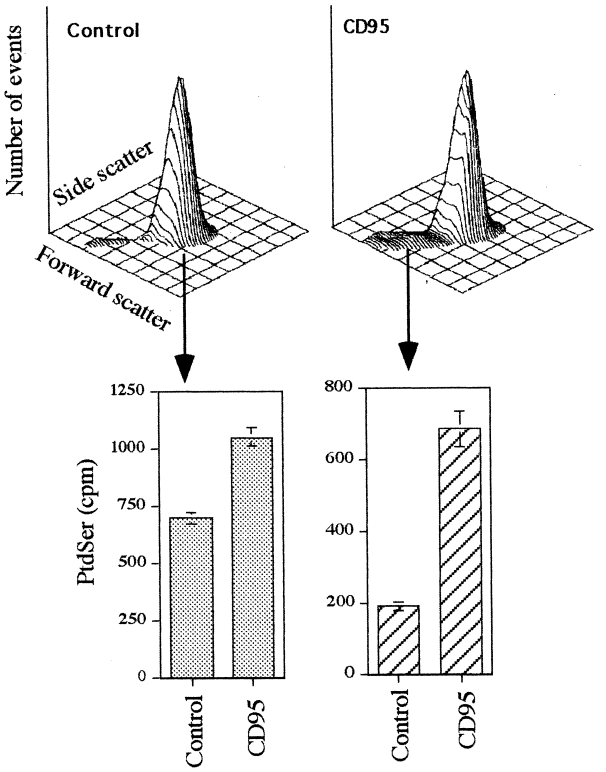

Fig. 6. Jurkat cells were maintained for 3 h without (controls) or with CD95 (1 μg/ml). A portion of the cells were analyzed by cytofluorimetry (top panels) to visualize the formation of membrane vesicles. The remaining cells were centrifuged and analyzed for their content in newly synthesized [^{3}H]PtdSer (left panel). The particles shed in the incubation medium were recovered by centrifugation and also analyzed for their content of [^{3}H]PtdSer (right panel). The values are the mean of triplicates from a representative experiment.

probe. As shown in Fig. 4A, CD95 caused a marked depolarization that started 45 min after CD95 addition. In parallel, we measured the uptake of [^{3}H]serine CD95-treated Jurkat cells. We found that CD95 induced a marked decrease of [^{3}H]serine uptake (Fig. 4B) that was fully compatible with the changes of membrane potential and the decreased PtdSer synthesis observed in cells treated for times greater than 60 min.

3.6. Time course of CD95-induced apoptosis

CD95-mediated apoptosis was monitored by cytofluorimetry using either Hoechst 33342 or the Apoalert apoptosis kit including labeling of cells with annexin V-FITC and propidium iodide. As shown in Fig. 5, CD95 induced significant changes in Hoechst 33342 DNA labeling within 2 h. Labeling of cells with annexin V-FITC was clearly evidenced 1 h after CD95 treatment while the cell permeability to propidium iodide remained unchanged before 2 h of treatment. Monitoring DNA fragmentation by electrophoresis in agarose gels (not shown) indicated that DNA fragmentation occurred 2 h after CD95 addition. A similar timing was observed with CD95 concentrations ranging from 250 ng/ml to 2 μg/ml (not shown).

3.7. Newly synthesized PtdSer is translocated to the cell surface

Since the CD95-induced rise in PtdSer synthesis occurred before annexin V-FITC labeling we tested whether newly synthesized [^{3}H]Ptdser could be translocated to the external face of the plasma membrane and thus could be derivatized with trinitrobenzene sulfonate (TNBS). The amount of TNBS-derivatized [^{3}H]serine was 348 ± 62 cpm/10^6 CD95-treated cells versus 42 ± 25 cpm/10^6 cells in controls. This indicated that during the 1 h incubation with CD95, about 40% of the newly synthesized PtdSer appeared at the cell surface.

3.8. PtdSer is released with membrane vesicles

We have shown above that long-term (>2 h) treatment of cells with CD95 resulted in an apparent decrease in PtdSer synthesis. Since it is well known that CD95 induces the shedding of membrane vesicles, we have on the one hand visualized the occurrence of membrane shedding by cytofluorimetry (Fig. 6) and on the other hand measured the possible presence of [^{3}H]PtdSer in the CD95-induced vesicles obtained after centrifugation of the supernatant of cells. As shown in Fig. 6 the CD95-induced plasma membrane vesicles obtained after 3 h of CD95 treatment clearly contained [^{3}H]PtdSer. This fact agrees well with the apparently decreased PtdSer synthesis (Fig. 1AFig. 2) observed after long-term treatment (>2 h) with CD95.

4. Discussion

CD95-induced apoptosis involves intracellular signals transduced by the death domain of Fas [19–21]. The death domain interacts with FADD/MORT1 that couples Fas to a cascade of ICE-like proteases named caspases [22–25]. Following caspase activation, one of the earliest cellular changes described during the apoptotic process is a reduction of the mitochondrial transmembrane potential [11,12,25,26]. Disruption of mitochondrial transmembrane potential occurs before cells exhibit nuclear DNA fragmentation and exposure of PtdSer on the outer cell membrane leaflet. Inhibition of ICE-like proteases prevents Fas-triggered disruption of mitochondrial transmembrane potential and prevents apoptosis. Accordingly, reduction of mitochondrial potential is considered the earliest irreversible step of programmed cell death in lymphocytes [27–29].

In the present work, we have shown that triggering Fas with CD95 mAbs results in very early changes in PtdSer metabolism. CD95 induces an increased incorporation of [^{3}H]serine into PtdSer. This increased PtdSer synthesis is accompanied by a strong inhibition of PtdEtn formation through decarboxylation of PtdSer. Both the increased PtdSer and the reduction of its decarboxylation to PtdEtn were evidenced very early (15 min after CD95 treatment). The CD95-induced PtdSer synthesis precedes DNA fragmentation, Hoechst 33342 and propidium iodide labeling (all these events were detected at 2 h) as well as the exposure of PtdSer at the cell surface of cells (detected at 1 h) during programmed cell death (Fig. 5).

PtdSer synthesis takes place in the endoplasmic reticulum and results from an exchange of the polar head group of preexisting phospholipids (either PtdCho or PtdEtn) by a serine [1,2]. The exchange of the polar head group is catalyzed by the serine-BEES, a Ca^{2+}-dependent enzyme located in the endoplasmic reticulum. Generally, changes in the serine-BEES activity are correlated with changes in the Ca^{2+} content of the endoplasmic reticulum [13,30]. Because CD95 does not

induce changes in Ca^{2+} homeostasis (Fig. 3), an increased activity of the serine-BEES appeared unlikely to explain the CD95-induced increase in the incorporation of [^{3}H]serine into PtdSer, since, in parallel with the increased PtdSer, we showed a net decrease in the formation of PtdEtn through the decarboxylation of PtdSer. It appears likely that the arrest of the decarboxylation process participates at least in part in the accumulation of PtdSer. Whether disruption of the mitochondrial transmembrane potential caused by CD95 is the cause of the blockade of the decarboxylation process is not known. Nevertheless, the fact that PtdSer decarboxylation is a typical mitochondrial process suggests strongly that the early changes induced by CD95 in this cellular organite could be involved as a cause for the effect observed.

If a part of the PtdSer synthesized in the endoplasmic reticulum is routed to the mitochondria, another part is routed to the internal leaflet of the plasma membrane. Since the work by Fadok and collaborators [7] it has been known that CD95 induces a transfer of PtdSer from the internal to the external leaflet of the plasma membrane. It was thus of interest to see whether newly synthesized PtdSer was translocated to the external leaflet. For this purpose, PtdSer was derivatized with TNBS as previously described [16]. The results obtained indicated that a portion (40%) of [^{3}H]Ptdser was derivatizable with TNBS thus translocated to the external leaflet of the plasma membrane during the first hour following CD95 treatment. Longer treatments with CD95 induced the appearance of distinct protrusions of the plasma membrane (blebbing) followed by the shedding of membrane vesicles and cellular fragmentation. We have shown (Fig. 6) that membrane vesicles released into the incubation medium contained newly synthesized [^{3}H]PtdSer.

We have also shown (Fig. 4) that CD95 treatment resulted in a marked loss of membrane potential that started 45 min after addition of the mAb and increased with time up to 120 min, resulting in a decreased uptake of [^{3}H]serine and a concomitant decrease in PtdSer synthesis.

Both the blebbing and the membrane depolarization observed likely explain why treatment of cells with CD95 for times exceeding 1 h resulted in an apparent decrease of [^{3}H]PtdSer synthesis.

In conclusion, we have shown first that triggering the Fas antigen on Jurkat cells induces a very rapid and transitory increase in the incorporation of [^{3}H]serine into PtdSer. This increase appeared to be linked to an inhibition of PtdSer decarboxylation in the mitochondria. Secondly we have shown that newly synthesized tritium-labeled PtdSer is translocated to the external leaflet of the plasma membrane together with cold preexisting PtdSer.

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