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CAMPTOTHECIN INDUCES THE TRANSIT OF FasL TRIMERS TO THE CELL SURFACE IN APOPTOTIC HEp-2 CELLS

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Abstract: Fas ligand (L) is a membrane protein from the tumor necrosis factor (TNF) family. It induces apoptosis upon contact with its Fas/CD95/APO1 receptor. Trimerization of FasL on the surface of effector cells is essential in the binding of the Fas trimer of the target cells. The receptor then recruits an adaptor and caspase-like proteins which lead apoptosis. This paper reports on the fate of FasL in HEp-2 cells committed to apoptosis by induction with campthotecin. Our main results demonstrated that in non-apoptotic cells, FasL aggregates in the cytoplasm forming trimers of 120 kDa. Apoptosis increases the trimeric FasL species, but also induces its dissociation into monomers of 35 kDa. In conclusion, camptothecin appears to perturb the Fas and FasL segregation in the cytoplasm by promoting the transit of FasL to the cell surface, thus fostering a process of autocrine or paracrine apoptosis. FasL is trimerized prior to Fas/FasL complex formation, and after apoptosis, FasL undergoes an intense turnover.

Key words: FasL, Apoptosis, Oligomerization, Camptothecin, TUNEL

INTRODUCTION

The receptors of the TNF family transduce cellular death signals upon contact with their respective ligands. Fas/CD95, the most relevant of these receptors, is a homotrimeric transmembrane protein bearing a cytoplasmic domain that binds

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the Fas-adaptor protein FADD, which in turn induces a succession of events that lead to apoptosis [1].
The Fas ligand (FasL) is a 36 kDa protein which is expressed by a variety of effector cells and which induces apoptosis of Fas-sensitive cells after contact with its receptor [2]. The FasL/Fas complex constitutes a major signalling pathway that is triggered by chemotherapeutic agents [3-6]. Camptothecin and other anticancer drugs such as doxorubicin, methotrexate and bleomycin up-regulate the expression of CD95 on the cell membrane and induce the autocrine or paracrine expression of FasL, activating FasL/Fas-dependent apoptosis [7].
FasL is expressed by the lymphocytes and immunoprivileged tissues such as those of the testis and eyes. Other cells such as thyrocytes and epithelial cells should also express the FasL/Fas pair; however, these cells do not undergo autocrine or paracrine apoptosis. The physical segregation of Fas and FasL to different cellular compartments nullifies the risk of spontaneous cell death [8-13]. Nevertheless, some chemotherapeutic agents including camptothecin foster fratricide apoptosis [6].
The formation of the FasL/Fas complex is preceded by trimerization of FasL. In this process, the auto-assembly of FasL forms three clefts at the interfaces of the monomers, and then the trimeric Fas receptor molecules bind diagonally along these clefts on the FasL trimer [14].
Several types of cell contain both Fas and FasL. Under particular stimulatory conditions, these cells can rapidly undergo autocrine apoptosis. In these types of cell, spontaneous cell death is avoided by keeping Fas and FasL in separate cytoplasmic compartments. Moreover, apoptosis specifically requires the FasL/Fas complex, which can only form after the trimerization of FasL. At this point, it is unclear whether FasL trimer assembly occurs during translocation to the effector cell's surface, or if FasL is stored as a trimer in the cytoplasm. Thus, is important to address the condition of the FasL before and after apoptosis, since FasL and Fas form supramolecular clusters easily. Due to the therapeutic implications, it is interesting to define whether campthotecin induces FasL aggregation as a pre-requisite of paracrine or autocrine apoptosis; this issue is of importance in tumor sensitivity to DNA Topoisomerase I poisons. The purpose of this study was to investigate the fate of FasL after chemically induced apoptosis.

MATERIAL AND METHODS

Cell cultures

Epithelial HEp-2 cells from human epithelial larynx carcinoma were obtained from ATTC and grown in DMEM medium supplemented with 10% fetal calf serum (Gibco, BRL Gaithersburg MD). To induce apoptosis, the cells were plated in polystyrene dishes containing cover slips at a density of $5x10^6$/ml, and incubated at 37ºC in 5% CO_2. Cells growing in logarithmic phase ($1x10^6$) were

incubated for 24 h with 20 μM camptothecin (Sigma. St Louis, MO) [15]. The control cells were cultured without camptothecin.

Apoptotic features

Morphological changes characteristic for apoptosis were detected using the Wright and Giemsa staining method. DNA fragmentation was determined by agarose gel electrophoresis. The DNA was extracted with DNAzol, according to the manufacturer's instructions (Gibco, BRL. Gaithersburg MD). The DNA was dried, dissolved in 8 mM NaOH, electrophoresed in 1.5% agarose, and stained with ethidium bromide.

Immunofluorescence

Chromatin changes were monitored in the cellular monolayers as follows: HEp-2 cells were fixed for 2 minutes in pure acetone, permeabilized with 0.01% Triton X-100/phosphate buffer saline (PBS), and then covered with a 1:100 dilution of an anti-DNA autoantibody from a systemic lupus erythematosus patient. The expression of FasL was detected after incubation for 2 h with a 1:500 dilution of a fluorescein-labelled monoclonal anti-FasL (clone CD95L abm. Research Diagnostics Inc, Flanders NJ); the slides were washed and mounted, and examined under a confocal scanning microscope LSM (Axiovert 200M, Carl Zeiss, Göttingen Germany). Combinations of fluorescein filters with excitations of 450-490 nm and rodamine filters with emissions of 515-565 nm were used; the objectives were LCI "Plan-Neofluar", and the image processing was done with a Zeiss LSM Image examiner.

TdT-mediated dUTP nick end labelling (TUNEL)

A TUNEL analysis was done according to the manufacturer's instructions (Roche Molecular Biochemicals. Penzberg, Germany). Nuclear stripping was performed on a cell monolayer by immersing the slides for 5 minutes in 10 mM Tris-HCl, pH 8.0, followed by 15 minutes in 20 μg/ml proteinase K dissolved in the same buffer, and finally washing with PBS. Elongation of the DNA fragments was performed by incubation for 60 minutes at 37ºC with 75 μl of the reaction mixture [DDW, 10XTdT buffer (30 mM Tris base, 140 mM sodium cacodylate, pH 7.2, 1 mM cobalt chloride, 1 mM DTT); 10% of the final volume], fluorescein-11-dUTP (0.5 mg dissolved in 1 ml of 10 mM Tris-HCl, pH 7.0), and TdT enzyme (0.3 enzyme units/μl). The reaction was terminated by adding the stop solution (300 mM NaCl, 30 mM sodium citrate, pH 8.0) [16]. Finally, the slides were washed in PBS and evaluated using confocal microscopy.

Protein extraction

Apoptotic and non-apoptotic cells were harvested with a scrapper and spun at 1200 rpm for 10 minutes. The cellular pellets were resuspended in denaturing lysis buffer (1% SDS, 0.25 M EDTA, 1 mM Tris, pH 7.5, 5 mM PMSF; adjusted to 1 ml H_2O). Soluble antigens were extracted by sonication: 5 pulses of 30

seconds at 50%. The lysates were centrifuged at 12000 rpm/10 minutes and the supernatants were immediately used for analysis. The protein concentration was spectrophotometrically measured at 280 nm.

SDS-PAGE and Western blot.
Cellular extracts were submitted to 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, as described by Laemmli and Favre [17]; non-reducing conditions were also applied in the absence of bis-mercaptoethanol or DTT in the sample buffer. The unstained gels were blotted onto nitro-cellulose membranes using the procedure described by Towbin *et al.* [18]. Non-specific active sites were blocked with 1% non-fat milk. The blotted proteins were probed at room temperature for 1 h with monoclonal anti-FasL antibodies diluted 1:1000 in phosphate saline buffer (PBS). Bound antibodies were detected with rabbit anti-mouse IgG-peroxidase (Sigma. St Louis, MO). Immunoreactive bands were visualized with an ECL chemiluminescent kit (Amersham Pharmacia Biotech. Piscataway, NJ). The bands were recorded using an image analysis system; with this tool, the molecular weights at the immunoreactive bands could be determined by comparison with standards of known molecular mass (BioRad, Richmond. CA).

Oligonucleotides
The oligonucleotides used for PCR amplification were: FasL forward 5'-CAA GTC CAA CTC AAG GTC CAT GCC-3' and backward 5'-CAG AGA GAG CTC AGA TAC GTT TGA C-3' [14]; Fas forward 5'-GGT GGG TTA CAC TGG TTT ACA-3', and backward 5'-GTG CTA CTC CTA ACT GTG AC-3' [19]; Caspase 3 forward 5'-TCC AGT CGG AGG CCA GAT CTG AG-3', and backward 5'-CTG AAG CCT GCC TCC CGG GAT GA-3' [SNP000005036]; DFF40 forward 5'-CTC TGG GGT ACT CGT TGG AT-3', and backward 5'-ACT GCT GTT CAG ATC CGC GT-3' [20]; and G3PDH forward 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC -3', and backward 5'-CAT GTA GGC CAT GAG GTC CAC CAC -3' (Clontech, Palo Alto, CA).

Reverse-transcription/polymerase chain reaction (RT-PCR)
Total RNA was extracted with TRIzol according to the manufacturer's instructions (Gibco, BRL. Gaithersburg, MD). RNA concentration was determined by measuring the optical density at 260 nm. For cDNA synthesis, 250 ng of the total RNA was incubated with 200 μM dNTP and 0.7 μM of the backward primer, mixed with 5 U/20 μl of rTth/DNA polymerase (SuperScript™ One-Step, Invitrogen Life Technology Ltd., Carlsbad CA). The reverse transcription was performed at 70ºC for 10 min; the reaction was stopped by cooling on an ice bed. After reverse transcription, amplification of the FasL, Fas, Caspase 3, DFF40 and G3PDH cDNAs was carried out by PCR via the addition of 0.15 μM of the forward primer. The reaction tubes containing 50 μl of the sample mixture were amplified in a thermocycler (Perkin Elmer, GeneAmp PCR system 2400), using 30 cycles under the following conditions:

94ºC for 2 min, 48ºC for 2 min and 72ºC for 1.4 min. At the end of the PCR reaction, the samples were electrophoresed in 0.8% agarose containing 0.5 mg/ml of ethidium bromide. The PCR products were observed under UV light [21]. Band migration and density were documented using a BioRad image analysis system, and pro-apoptotic transcript levels were determined by comparison with the G3PDH densitometry. Differences between the samples were determined using the ANOVA test (Number Cruncher Statistical Systems NCSS program).

RESULTS

Campthothecin induces apoptosis

Based on previous reports of topoisomerase inhibitors inducing programmed cell death, camptothecin was used to induce apoptosis in HEp-2 cells. These cells were chosen because they rarely undergo autocrine apoptosis in the absence of particular stimuli.

The effect of camptothecin was evident in all the cells after 24 h, as demonstrated by the Wright and Giemsa staining; chromatin condensation, blebs and apoptotic bodies were evident in the cells treated with this Topo-poison (Fig. 1B). DNA fragmentation was shown as strand breaks (~300 bp fragments) as shown in Fig. 2. Control chromatin stained by a human anti-DNA autoantibody exhibited intact homogeneous nuclear localization; this was disrupted by apoptosis: the DNA fragments were scattered along the cell body, in the cell surface and into the apoptotic bodies (Fig. 2C).

TUNEL

The percentage of apoptotic cells in the control cultures was less than 1% in 100 fields. All the cells incubated with campthotecin showed an apoptotic result 24 h later (Fig. 1D). To differentiate the true green tag of apoptotic cells from background incorporation, cells were counterstained with 0.5% propidium iodide, giving the non-apoptotic nuclei develop a red stain (Fig. 1C).

FasL is redistributed during apoptosis

Fas and FasL are widely expressed in epithelial cells and can be detected by immunofluorescence. Confocal microscopy allowed us to determine the distribution of FasL in normal cells: the ligand was absent at the cell surface, but appeared as fine granules scattered through the cytoplasm. 24 hours after incubation with camptothecin, the FasL content was significantly increased in the cytoplasm and along the cell surface (Fig. 3).

Two FasL cellular fractions were detected

Since FasL forms supramolecular clusters easily detected under non-reducing conditions, some gels were preformed in the absence of bis-mercaptoethanol and/or DTT; under this condition, the Western blot analysis yielded the following results. The control cells exhibited an ~120 kDa immunoreactive band

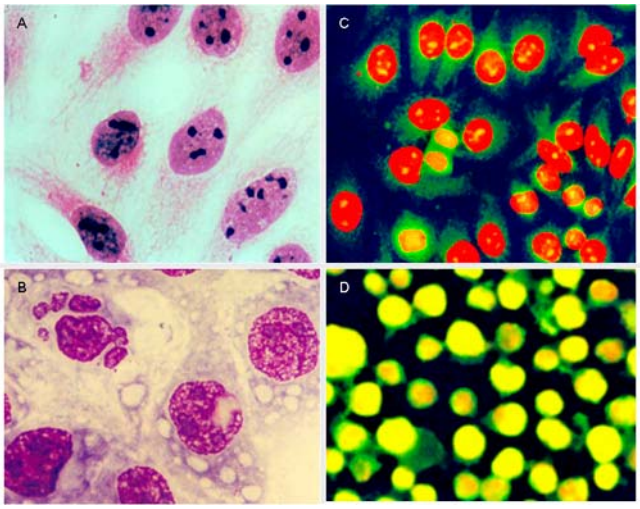

Fig. 1. Camptothecin's effect on HEp-2 cells. A - Control cells stained with Wright. B - Changes characteristic of apoptosis, such as blebbing and apoptotic bodies, were detected in cells treated with camptothecin. C - Negative TUNEL assays in control cells: nuclei are stained red by the propidium iodide contrast. D - Apoptotic cells are green tagged, indicating the incorporation of the fluorescent nick end labelling into the apoptotic DNA fragments.

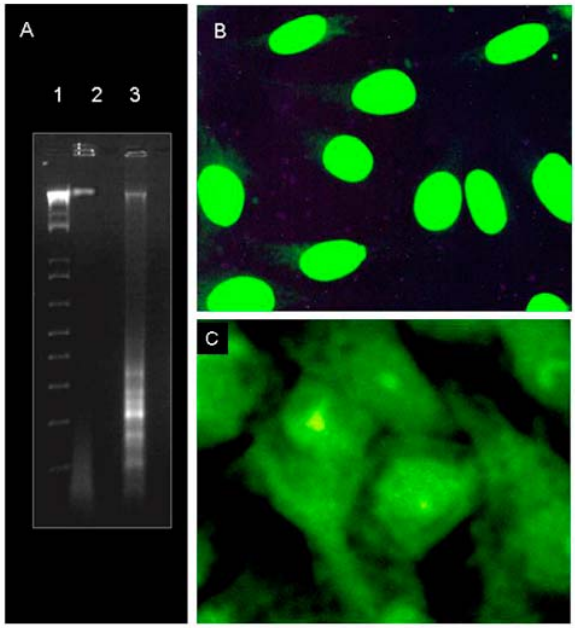

Fig. 2. Camptothecin and DNA fragmentation. A - Agarose gel electrophoresis. Line 1. 1 kb DNA leader. Line 2. DNA extract from control cells. Line 3. Nuclear extracts from apoptotic cells. B - Immunofluorescence of Hep-2 cells. An anti-DNA autoantibody decorated the nuclei of non-apoptotic cells in a homogeneous fluorescent distribution. C - Camptothecin-treated cells exhibit DNA fragment redistribution into apoptotic bodies, as is characteristic of apoptosis.

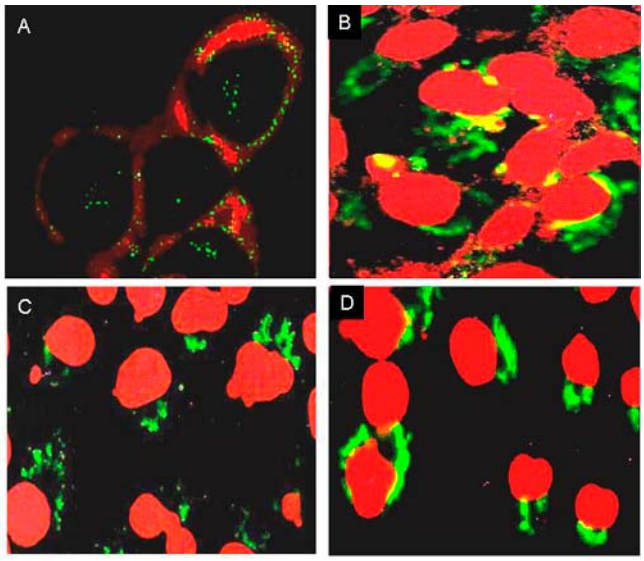

Fig. 3. FasL redistributed during apoptosis as shown by indirect immunofluorescence (green) in cells counterstained by propidium iodide. A - FasL is located faintly in the cytoplasm of control HEp-2 cells. B - FasL expression augmented significantly in the cytoplasm and cell membrane of cells treated with camptothecin. C - Treatment with cycloheximide induces the translocation of FasL to the cell membrane. D - Fas L expression on the cell surface of cells treated with a mixture of camptothecin and cycloheximide.

as the main molecular species; this finding was unexpected, as the molecular mass of FasL is around 35-40 kDa. Surprisingly, the apoptotic cells exhibited an ~10 fold increase of the 120 kDa band; interestingly, additional immunoreactive bands of 70 kDa and the ~35 kDa monomeric species of FasL were also detected. This initial finding suggest that the 120 kDa band corresponded to an FasL aggregate; in consequence, the following simple experiment was carried out to test whether the 120 kDa band corresponded to a true trimer. The non-apoptotic cell extracts containing the 120 kDa band were electrophoresed under reducing conditions, and then blotted and probed with anti-FasL; interestingly, the 120 kDa band collapsed into the 35 kDa band. This result demonstrated that the bis-mercaptoethanol induced FasL disaggregation and suggested that the 120 kDa band may correspond to a true FasL trimer (Fig. 4).

Based on our previous finding that the 35 kDa band represented the main form of FasL during apoptosis, we questioned whether this 35 kDa band was the result of *de novo* synthesis rather than of the disassembly of FasL. We inhibited protein synthesis with 25 μg cycloheximide (Sigma. St Louis, MO); at this concentration, the drug also induces apoptosis. After a 2-h incubation with

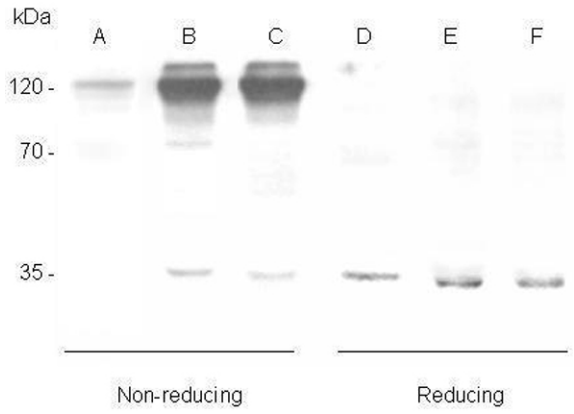

Fig 4. FasL aggregates detected by Western blot in non-reducing and reducing conditions. A - A trimeric form of 120 kDa was mainly detected in the control cells. B - Camptothecin increases the 120 kDa trimer, but simultaneously disassembles the FasL trimer into 35 kDa monomers. C - Cell extract obtained after a 2-h incubation with cycloheximide and after extensive washings and incubation with campothecin; the 35 kDa band remained as an immunoreactive species. D - Control cell extract electrophoresed under reducing conditions; the 120 kDa trimer collapsed into a 35 kDa monomer**.** E - Cells treated with camptothecin and cell extracts resolved under reducing conditions**.** F - Cells incubated for 2 h with cycloheximide and after extensive washings and incubation with campothecin; extracts resolved under reducing conditions.

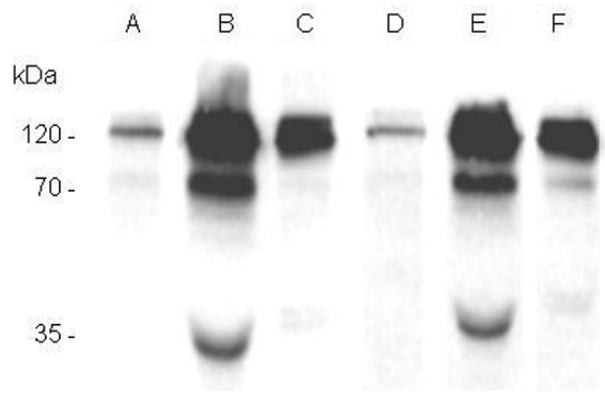

Fig. 5. Effect of Ac-DEVD-CMK on FasL trimers. SDS-PAGE in non-reducing conditions/Western blot. A - The trimeric form of 120 kDa was detected in the control cells. B - Camptothecin increases the level of the 120 kDa trimer and simultaneously disassembles it into 70 kDa dimers and 35 kDa monomers. C - FasL remained in a trimeric form of 120 kDa in cultures treated with Ac-DEVD-CMK and then pulsed with campthotecin. D - Control cells. E - Cycloheximide treatment disassembles the 120 kDa trimer into 70 kDa dimers and 35 kDa species. F - The FasL remained as a 120 kDa trimer in HEp-2 cells treated with Ac-DEVD-CMK followed by cycloheximide.

cycloheximide and after extensive washings and incubation with campothecin, the 35 kDa band remained as an immunoreactive species (Fig. 4). These findings suggest that the 35 kDa band is not the result of *de novo* synthesis. Interestingly, when cells were exclusively incubated with cycloheximide, this drug produced similar effects to camptothecin as detected using immunoflorescence and Western blotting analysis.

Finally, to demonstrate that the 35 kDa monomer was indeed produced by apoptosis, cell cultures were treated with 30 nM Ac-DEVD-CMK (Calbiochem. San Diego, CA) followed by treatment with camptothecin and/or cycloheximide. As expected, the cultures treated with this caspase inhibitor became refractory to camptothecin- or cycloheximide-induced apoptosis; furthermore, FasL remained in the trimeric form of 120 kDa (Fig. 5).

Camptothecin augments the transcription of FasL

The pro-apoptotic molecules FasL, Fas/CD95, Caspase 3 and DFF40 were overdriven by camptothecin treatment, as demonstrated using the RT-PCR assay. The cell cultures treated with this drug yielded higher values than untreated cell cultures. Similar results were obtained in three replicas of each experiment (Fig. 6).

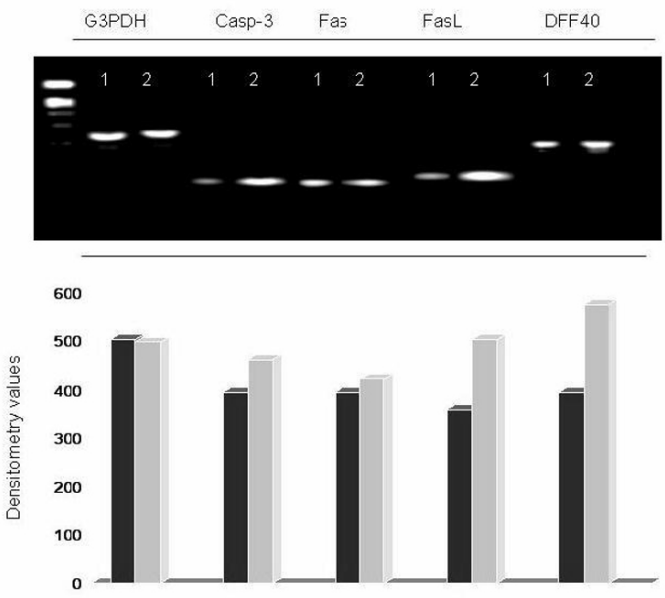

Fig. 6. Apoptosis increases the transcription of FasL, the Fas/CD95 receptor, Caspase 3 and DFF40 as compared to housekeeping gene G3PDH. Densitometric analysis of RT-PCR generated bands. Line 1 corresponds to control cells and line 2 to apoptotic cells.

DISCUSSION

Our study characterized the supramolecular forms of FasL during chemically induced apoptosis; this is of medical importance in the understanding of the molecular mechanisms of cellular sensitivity and resistance to camptothecin derivatives in cancer. We analyzed the fate of FasL before and after apoptosis. The main results of this investigation show that: 1. FasL is present in the cytoplasm of living HEp-2 cells; 2. FasL is translocated to the cell surface after the induction of apoptosis; 3. FasL is assembled and stored in the cytoplasmic compartments of non-apoptotic cells as a trimer of ~120 kDa; and 4. the level of trimeric FasL increases in ten-fold and is disassembled after apoptotic signalling is triggered, resulting in monomers of ~35 kDa.

A variety of epithelial cells co-express Fas and FasL without suffering spontaneous apoptosis, for example the MDCK cells in which Fas and FasL are segregated into different cellular compartments [13]. In them, FasL is exclusively located in the cytoplasm and is segregated from the Fas receptor which is located on the cell membrane. Interestingly, in the early stages of apoptosis, FasL is translocated to the cell membrane, fostering autocrine or paracrine apoptosis. Our studies confirm the presence of FasL in the cytoplasm of the epithelial cell line HEp-2, which is immortal in normal culture conditions.

The Fas/CD95 receptor transduces death signals upon contact with its Fas ligand; the formation of the FasL/Fas complex requires previous trimerization of FasL, which is followed by the translocation of trimers to the cell surface of the effector cell. The presence of FasL on the cell membrane induces super-aggregation of the trimerized Fas receptor of the target cells; such a cross-link induces a lateral segregation within the plasma membrane into “caps”. FasL/Fas complex formation is followed by the binding of FADD to the death domain of Fas, then FADD recruits the caspase FADD-homologous IL-1β-converting enzyme-like protease (FLICE)/MACH/caspase 8, providing the connection of death receptors to caspases [14, 22, 23].

It is known that the trimeric structure of TNF family ligands is required for it to accomplish its biological activity; a premature or inadequate oligomerization of the FasL molecule result in a significant loss of activity [24]. The cross-linking of FasL takes place before apoptosis; such self-assembly of FasL has been artificially induced to understand the properties of self aggregation of this ligand [14]. The results of our current study demonstrate that FasL forms SDS-stable trimers which must be preassembled before the triggering of apoptosis; the trimers remain stored in cytoplasmic compartments, and are ready to be used during apoptosis. The FasL trimer increases during apoptosis as a consequence of campthotecin stimuli; simultaneously trimers suffer disassembly producing a transitory species of 70 kDa, and its final form of 35 kDa (monomers). Consistent with previous results, the lysates from non-apoptotic cells yield 120 kDa trimers, which were reduced to 35 kDa monomers by the bis-mercaptoethanol; this species was comparable to the FasL immunoreactive form

found under apoptosis. Self-aggregation of Fas into SDS-stable polymers is phenomenon previously described [25]. Nevertheless, in this study, the self-aggregation of FasL into SDS-stable trimers is important because it seems to occur naturally as pre-requisite for apoptosis. Such aggregates were described previously only under chemical induction [14]. The region responsible for self-assembly resides in a region of the FasL extracellular domain at a certain distance from the COOH terminus [26].

The kinetics of FasL disassembly are similar to those of the Fas receptor. In the latter, this occurs immediately after the down-signalling transduction is accomplished. We believe that the presence of 35 kDa FasL monomers in apoptotic cell extracts is the result of a true disassembling rather than of digestion by metalloproteinases, because these FasL truncated forms have a lower molecular mass ~26 kDa [23]. The presence of a ~70 kDa intermediate species (dimmers) in the apoptotic cells suggests an active process of disassembly. In addition, we believe that the possible *de novo* synthesis of FasL does not interfere with the disassembly, and the inhibition of protein synthesis does not affect the reduction of FasL into monomers of 35 kDa on Hep-2 cells under camptothecin treatment. Furthermore, a caspase inhibitor blocked apototosis and FasL disassembly, maintaining the trimeric form of 120 kDa. Our results are of biological importance because they indicate the effect of camptothecine derivatives as an inductor of paracrine or autocrine apoptosis in tumor cells, and emphasize the regulatory mechanisms of supramolecular aggregate FasL formation, which is important in cancer resistance.

In conclusion, camptothecin appears to perturb the Fas and FasL segregation in the cytoplasm by promoting the transit of FasL to the cell surface. This fosters a process of autocrine or paracrine apoptosis. FasL is trimerized prior to Fas/FasL complex formation, and during apoptosis, FasL suffers an intense turnover.

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