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Glutamine deprivation-mediated cell shrinkage induces ligand-independent CD95 receptor signaling and apoptosis

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Abstract

Cell shrinkage and loss of cell viability by apoptosis have been examined in cultured CD95(Fas/Apo-1)-expressing leukemia-derived CEM and HL-60 cells subjected to acute deprivation of glutamine, a major compatible osmolyte engaged in cell volume control. Glutamine deprivation-mediated cell shrinkage promoted a ligand-independent activation of the CD95-mediated apoptotic pathway. Cell transfection with plasmids expressing FADD-DN or v-Flip viral proteins pointed to a functional clustering of CD95 receptors at the cell surface with activation of the 'extrinsic pathway' caspase cascade. Accordingly, cell shrinkage did not induce apoptosis in CD95 receptor-negative lymphoma L1210 cells. Replacement of glutamine with surrogate compatible osmolytes counteracted cell volume decrement and protected the CD95-expressing cells from apoptosis. A glutamine deprivation-dependent cell shrinkage with activation of the CD95-mediated pathway was also observed when asparaginase was added to the medium. Asparagine depletion had no role in this process. The cell-size shrinkage-dependent apoptosis induced by glutamine restriction in CD95-expressing leukemic cells may therefore be of clinical relevance in amidohydrolase enzyme therapies. *Cell Death and Differentiation* (2001) **8,** 1004–1013.

Keywords: cell-shrinkage; CD95 (Fas/Apo-1) receptor; apoptosis; glutamine; asparaginase

Abbreviations: ALL, acute lymphoblastic leukemia; CD95L, CD95 ligand; DISC, death-inducing signaling complex; FADD, Fas-associated death domain protein; FADD-DN, dominant negative FADD; GFP, green fluorescent protein; OMG, 3-*o*-methyl-*D*-glucose; TNFR, tumor necrosis factor receptor; z-DEVD, Asp-Glu-Val-Asp; z-IETD, Iso-Glu-Thr-Asp

Introduction

L-Glutamine, the most abundant free amino acid of the human body, has a central role in the energy metabolism of many tissues,[1–5] is a growth-limiting amino acid for several cell types including lymphocytes[6–9] and leukemia cell lines,[10,11] engages in cell volume control as compatible osmolyte[12,13] and serves as a precursor of neurotransmitters.[14] Previous studies with cultured human leukemia/lymphoma cell lines (CEM, HL-60, U937, Namalwa) indicated that glutamine restriction induces loss of cell viability by apoptosis.[15,16] When these cells encountered a medium-glutamine concentration lower than 0.3–0.4 mM for at least 24 h ceased multiplying and entered the apoptotic pathway, unless glutamine was reinstated to values compatible with the resumption of proliferation.[16] Lymphoblastic leukemia-derived CEM $CD4^+$-enriched cells, clone 13, used in the present study, express the MYC oncoprotein[17] and are highly susceptible to agonistic anti-CD95 antibody-induced apoptosis (C Fumarola and GG Guidotti, unpublished observations), a death pathway sensitized by c-*myc* overexpression.[18,19] CD95 (Fas/Apo-1) is a death-promoting receptor that belongs to the tumor necrosis factor receptor (TNFR) family. Surface clustering of CD95 induced by natural ligands (CD95L) or by agonistic antibodies is required for the transduction of the apoptotic signal.[20,21] The death pathway involves oligomerization of the initiator caspase-8 and its proximity-induced autoproteolytic activation via recruitment of adapter FADD molecules in a death-inducing signaling complex.[22] Active caspase-8 cleaves a number of proteins including procaspase-3, which results in its activation with completion of the cell death program in type I cells,[23] and Bid,[24,25] a Bcl-2 family member that leads to amplification of the caspase cascade in type II cells.[23] Assembly of CD95 receptors with activation of the apoptotic pathway independently of CD95 ligand-receptor interaction has been induced in several mammalian cell types by ultraviolet (UV) light exposure,[26,27] in murine hepatocytes by toxic bile salts[28] and in colon carcinoma cells by some anticancer drugs.[29] In H1299 (p53-null) lung carcinoma cells, UV irradiation has been shown to promote apoptosis by ligand independent aggregation of TNFR-1.[30]

Here we report that in cultured CEM cells clone 13, and in other cells (CEM-CCRF and HL-60) expressing a functional CD95-dependent signaling mechanism, cell shrinkage associated with glutamine deprivation promotes a ligand-independent activation of surface CD95 receptors. Cell shrinkage presumably arises from the decrement of intracellular osmolytes and release of osmotically obliged water. The surface receptor assembly launches the extrinsic death pathway by recruitment of FADD adapter molecules and caspase-8 autoproteolytic activation. A comparable induction of the CD95-dependent caspase

cascade that triggers apoptosis is also observed when glutamine concentration in the medium of cultured CEM and HL-60 cells is lowered to exhaustion by adding *E. coli* asparaginase (L-asparagine amidohydrolase), an enzyme preparation that has glutaminase activity. Activation of apoptotic pathways seems to be fundamental in chemotherapeutic strategies whereby anti-oncogenic agents effect their responses. In the combined therapy of childhood acute lymphoblastic leukemia (ALL) the administration of amidohydrolase enzymes as asparaginase or glutaminase-asparaginase from bacterial sources[31–35] is associated with a marked reduction of glutamine concentration in body fluids.[36–38] The cell-size shrinkage-dependent apoptosis induced by glutamine depletion in CD95-expressing leukemic cells may therefore be relevant to the antineoplastic activity and to the sites of toxicity in the therapy with amidohydrolase enzymes.

Results

Glutamine deprivation activates the CD95 signaling pathway and promotes apoptosis

The treatment of CEM cells with an agonistic IgM anti-CD95 antibody (CH-11) resulted in a massive apoptosis. As monitored by DNA fragmentation, apoptosis was prevented by specific inhibitors of caspase-8 (z-IETD) and caspase-3 (z-DEVD) (Figure 1a), to indicate that these cells possess a functional CD95 signaling mechanism. When incubated in a glutamine-free medium, CEM cells entered an apoptotic pathway as shown by rapid activation of caspase-8 and caspase-3 (Figure 1b), DNA fragmentation (Figure 1c) and morphology (Figure 1d). In the early intervals (up to 6 h of incubation) Annexin V-FITC-positive cells excluded propidium iodide. DNA fragmentation was completely prevented by inhibitors of caspase-8 and caspase-3 and similar results were obtained in the CD95-expressing HL-60 and CEM-CCRF cells (Figure 1e). These results suggest that the apoptotic death promoted by glutamine deprivation is associated with the induction of the CD95-dependent caspase cascade. In all cell models used, glutamine deprivation-dependent caspase activation and cell death were asynchronous, but consistent events. Under the same conditions, glutamine deprivation did not induce apoptosis in CD95-negative murine lymphoma L1210 cells (see Figure 5).

Glutamine deprivation induces apoptosis independently of CD95L-CD95 interaction

Total expression of CD95 in CEM cells, as quantitated by photometric immunoassay, was not affected by a 24-h glutamine deprivation (not shown) and, within this period of time, cytofluorimetric analysis showed that this treatment did not change its surface expression (Figure 2a). The treatment of CEM cells with neutralizing anti-CD95L (NOK-1) and anti-CD95 (SM1/23) antibodies that block CD95L-mediated apoptosis failed to protect them from glutamine deprivation-induced cell death (Figure 2c,d). Western blot analysis (using two different primary anti-CD95L antibodies, N-20 and clone 33) showed that the CD95 ligand protein was expressed in CEM cells but, as shown in Figure 2b, its level was not affected by glutamine deprivation. Flow cytometry of CEM cells stained for CD95L indicated that the ligand was not on the cell surface and that glutamine deprivation did not promote its surface delivery from intracellular stores. However, on reaching the surface, the extracellular domain of CD95L is likely to be cleaved by a metalloproteinase[39] producing a soluble form and a membrane-bound fragment that might be re-internalized. Experiments were therefore performed in the presence of a metalloproteinase inhibitor, but again flow cytometry did not detect CD95L on the surface of cells incubated in the presence or absence of glutamine. To further exclude that a rapid recycling of CD95L may elude cytofluorimetric detection, while retaining agonistic activity, CEM cells were incubated in glutamine-containing or glutamine-free media supplemented with the metalloproteinase inhibitor and apoptosis was assessed by DNA fragmentation and cell morphology. In these experiments the inhibition of metalloproteinase was not associated with induction of apoptosis in control cells or with enhancement of it in cells incubated in the absence of glutamine (Figure 2e). In separate experiments performed in the absence of the metalloproteinase inhibitor, soluble CD95L was undetectable in media of CEM cells incubated in the presence or absence of glutamine. Taken together, these results argue against a role of CD95L–CD95 interaction in the glutamine deprivation-induced apoptotic cell death.

Glutamine deprivation is associated with cell shrinkage and surface contraction

Cell volume decreased rapidly when CEM cells were transferred from a glutamine-containing into a glutamine-free RPMI 1640 (supplemented with glutamine-free FCS). This cell shrinkage presumably followed the significant decrement of intracellular osmolytes, as indicated by a lowered glutamine/glutamate and NPS (ninhydrin positive substances[40]) cell content (results not shown), coupled with a release of osmotically obliged water. As estimated by measurements of cell water content based on transmembrane distribution of labelled 3-*o*-methyl-*D*-glucose (OMG), the mean cell volume decreased of approximately 15% within 60 min of incubation and shrinkage increased progressively up to 12 h when cell volume reached about 60% of its initial value (Figure 3a). Measurements of cell geometry parameters (area and diameter) by computer-assisted image analysis (see Materials and Methods) allowed to estimate the average cell surface contraction induced by glutamine deprivation (13% of the initial value after 6 h of incubation) and to extrapolate a cell volume decrease close to 20% that confirmed the CEM cells shrinkage detected by OMG distribution. The glutamine deprivation-induced decrements in cell volume are greater when estimated from OMG distribution that measures water content (about 80% of the cell mass in our CEM clone) than when extrapolated from surface image measurements that apply to the entire cell mass. Afer proper correction for the quantity being measured, this discrepancy attenuates, suggesting that glutamine deprivation does not significantly affect the macromolecular mass of the cell. These results indicate that glutamine deprivation is followed by a loss of intracellular

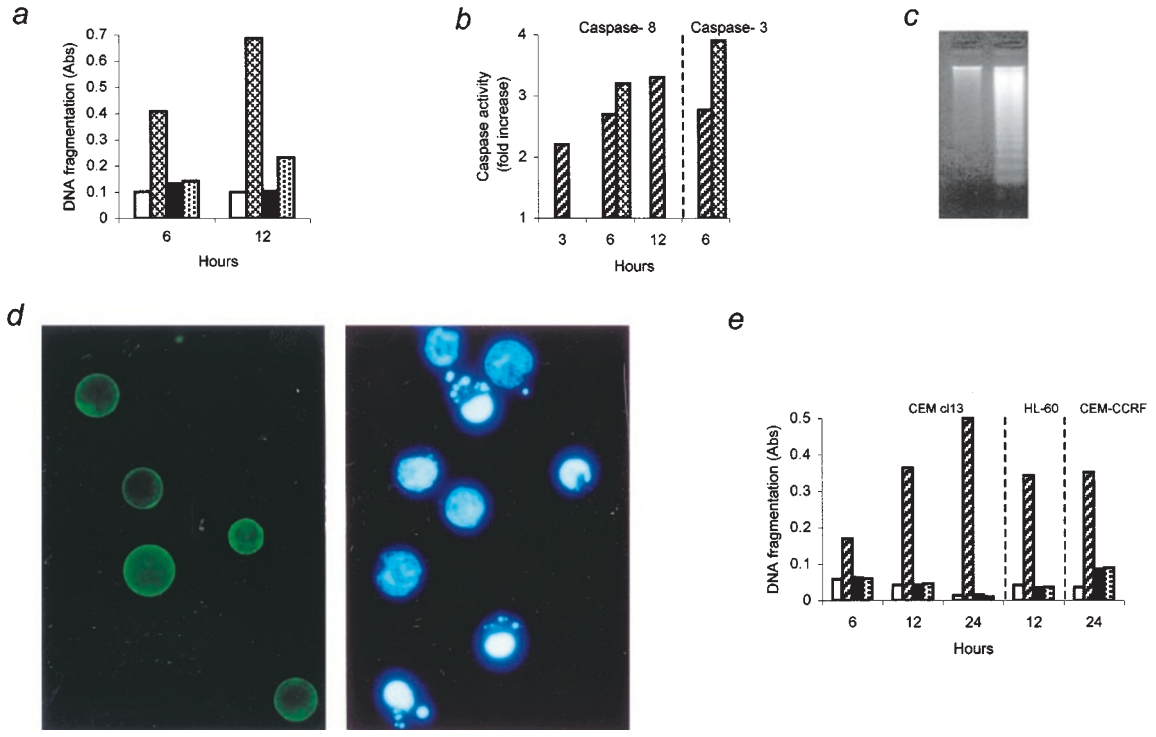

Figure 1 Apoptosis induced by anti-CD95 antibodies and by glutamine deprivation is prevented by caspase-8 and caspase-3 inhibitors. Cells (CEM clone 13, **a–d**; CEM clone 13, CEM-CCRF and HL-60 cells, **e**) were incubated in 2 mM glutamine-containing (controls) or in glutamine-free RPMI 1640 supplemented with 10% dialyzed FCS. (**a**) DNA fragmentation (photometric enzyme immunoassay) in control cells (□) and in control cells treated with CH-11 anti-CD95 antibody (0.5 μg/ml) in the absence (▨) or in the presence of caspase inhibitors (z-IETD, 40 μM, ■; z-DEVD, 40 μM, ▩); (**b**) caspase activity of control cells treated with CH-11 anti-CD95 antibody (0.5 μg/ml, ▨) or incubated in glutamine-free medium (▨); data are expressed as fold activation relative to control cells; (**c**) DNA laddering; low molecular-mass DNA extracted from control cells (left) or from cells incubated for 24 h in glutamine-free medium (right), and resolved in agarose gel electrophoresis; (**d**) morphology (fluoresence microscopy); cells incubated in glutamine-free medium for 6 h and stained with Annexin V-FITC (left) or for 24 h and stained with Hoechst 33342 (right). (**e**) DNA fragmentation (photometric enzyme immunoassay) in control cells (□) and in cells incubated in glutamine-free medium in the absence (▨) and in the presence of z-IETD (40 μM, ■) or z-DEVD (40 μM, ▩). Data in **a**, **b** and **e** are from a representative experiment. Each experiment, repeated at least four times, yielded similar results

water that, in turn, accounts for the decrease in cell volume and for cell surface contraction. Comparable decrements in cell size (OMG) distribution were also observed in HL-60 (26%), CCRF-CEM (18%) and L1210 cells (38%) incubated for 6 h in glutamine-free FCS-supplemented RPMI 1640.

Cell shrinkage induces the CD95-dependent apoptotic pathway

Cell shrinkage induced by glutamine deprivation was not prevented in CEM cells treated with the caspase-8 inhibitor z-IETD at concentrations that abolished apoptosis (Figure 3b,c), indicating that the cell volume decreased independently of caspase-8 activation. In these experiments cell shrinkage preceded the activation of caspase-8 (barely detectable before 2–3 h of incubation) and the fragmentation of DNA (not shown). A cell shrinkage occurring upstream of caspase-8 activation has been also induced in CEM cells by hyperosmotic stress (Fumarola, La Monica and Guidotti, unpublished results) and recently reported for Jurkat T cells treated with agonistic anti-CD95 antibodies.[41] The replacement of L-glutamine in the culture medium with 10 mM betaine, a surrogate compatible organic osmolyte,[12] fully counteracted the cell volume decrement at 3 h and allowed CEM cells to retain more than 85% of their initial volume at 6 h (Figure 4a). Under these conditions the cells were significantly protected from apoptosis (Figure 4b,c). Similarly, a remarkable inhibition of cell shrinkage and a comparable protection from apoptosis were obtained when L-glutamine was replaced by 6 mM D-glutamine or by 5 mM 2-methylaminoisobutyric acid, a nonmetabolizable amino acid analog[42] (not shown). These results imply that the cell volume decrement drives the activation of the CD95-signaling death pathway. However, an apoptotic volume decrease (AVD) associated with the progression of apoptosis and promoted by the activation of K^+ and Cl^- channels[41,43–45] cannot be excluded. When all the amino acids were withdrawn from the culture medium a marked decrement in the cell volume ensued that was associated with caspase-8 activation and DNA fragmentation, whereas the presence of glutamine (2 mM) in the medium deprived of all other amino acids prevented cell shrinkage, caspase-8 activation and DNA fragmentation (Figure 4d–f), and let CEM cells to retain viability for at least 20 h. As reported above, glutamine deprivation caused a marked decrement in cell size also in mouse lymphoma L1210 cells (Figure 5a). However, in these CD95-negative cells the volume decrement was not followed by caspase-8 activation or DNA fragmentation (Figure 5b,c),

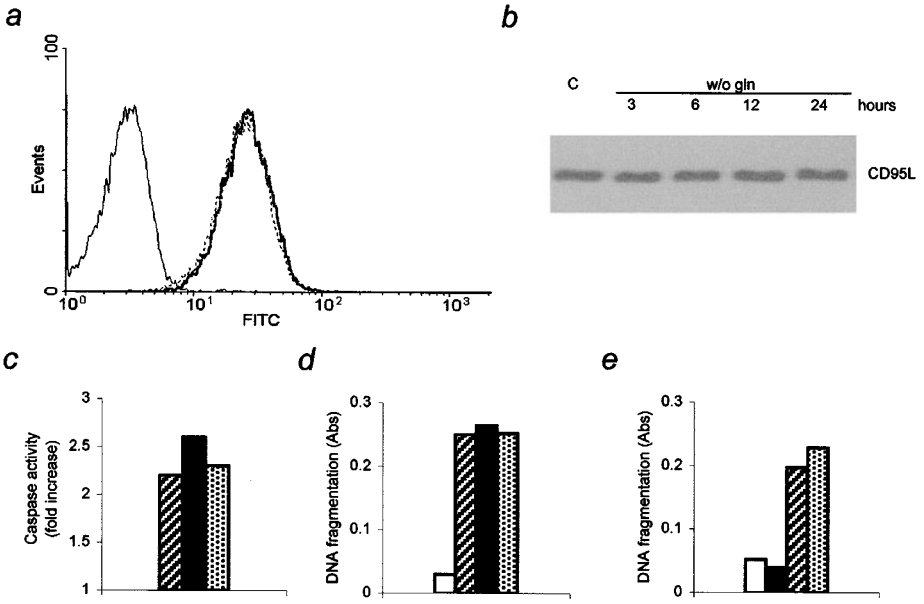

Figure 2 Apoptosis induced by glutamine deprivation is independent of CD95 ligand/CD95 interaction. CEM cells were incubated in glutamine-containing or in glutamine-free medium. (**a**) Surface expression of CD95 was determined by flow cytometry with the FITC-conjugated SM1/23 anti-CD95 antibody: left, isotype-matched control antibody; right, positive monoclonal antibody in cells incubated for 6 h in the presence (continuous line) and absence of glutamine (dashed line); (**b**) total expression of CD95L was estimated by Western blotting (using clone 33 monoclonal mouse anti-CD95L antibody) at the indicated time intervals; (**c**) caspase-8 activity (fold activation relative to control cells) and (**d**) DNA fragmentation (photometric enzyme immunoassay) were determined in cells incubated for 6 h in glutamine-containing (□) and in glutamine-free medium in the absence (▨) and in the presence of neutralizing anti-CD95 SM1/23 antibody (10 μg/ml, ■) or anti-CD95L NOK-1 antibody (10 μg/ml, ▩); (**e**) CEM cells were incubated in glutamine-containing (controls, □) or in glutamine-free medium (deprived cells, ▨) with KB8301 metalloproteinase inhibitor (10 μM) added to control (■) and deprived cells (▩). Data in **a**–**e** are from a representative experiment. Each experiment, repeated at least three times, yielded similar results

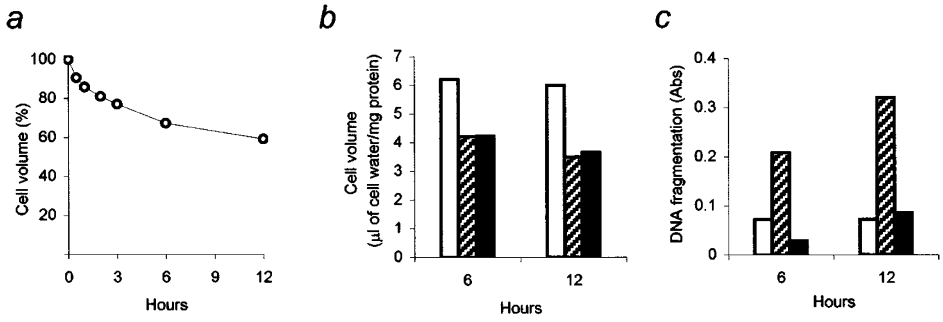

Figure 3 Glutamine deprivation promotes cell shrinkage upstream of caspase-8 activation. (**a**) CEM cells were incubated in glutamine-containing (controls) or in glutamine-free RPMI supplemented with 10% dialyzed FCS and cell volume was estimated at the indicated time intervals by measurements of the steady-state distribution of 3-*o*-methyl-D-[1-^{3}H]glucose (^{3}H-OMG) added 30–40 min before the assay; data, expressed as per cent of the control value, are means of experiments. CEM cells were incubated in glutamine containing (□) or in glutamine-free medium in the absence (▨) and in the presence of 40 μM z-IETD (■): (**b**) cell volume estimated by transmembrane ^{3}H-OMG distribution and expressed as μl of cell water/mg protein, and (**c**) DNA fragmentation determined by photometric enzyme immunoassay. Data in **b** and **c** are from a representative experiment. Each experiment, repeated at least three times, yielded similar results

suggesting that a functional membrane receptor-implemented death pathway was involved in the induction of the shrinkage-dependent apoptosis in CD95-bearing cells.

Cell shrinkage induces functional clustering of CD95 at the cell membrane

In type II CEM cells the formation of the death-inducing signaling complex (DISC) is strongly reduced, but also in these cells FADD and caspase-8 seem to initiate the death signal at the CD95 receptor level[23] (with mitochondria as subsequent amplifiers[21]). Figure 6a,b show the cell shrinkage-induced activation of caspase-8 (cleavage products in Western blotting) and the inhibition of apoptosis in CEM cells transfected with a dominant negative form of FADD.[46] To rule out that the apoptotic pathway promoted by CD95 receptor clustering at the cell surface was reinforced (or superseded) by a direct downstream oligomerization and autoactivation of caspase-8, CEM cells were cotransfected with the pEGFP-C1 vector along with plasmids expressing the v-Flip viral proteins

MC159 or E8[47,48] and incubated in the presence and absence of glutamine. The expression of protein MC159 that binds to FADD[48] and disrupts normal formation of the death-inducing signaling complex inhibited the glutamine deprivation-induced apoptosis (Figure 6c), implying that a direct autoactivation of caspase-8 does not take place and confirming that the cell shrinkage-dependent activation of the apoptotic pathway starts upstream. The expression of protein E8 that binds to the caspase-8 prodomain[48] and hinders its recruitment to the adaptor FADD also inhibited glutamine deprivation-induced apoptosis almost completely (Figure 6c), implying that an initial caspase-8 transactivation at the receptor level is required to propagate the caspase cascade. In CEM cells incubated in a glutamine-containing medium, the treatment with IgG divalent anti-CD95 antibodies (SM1/1, APO-1-3) did not affect the rate of proliferation for at least 24 h (Figure 6d) and was not accompanied by a detectable apoptotic effect (Figure 6e). In contrast, when CEM cells were incubated in the absence of glutamine, the treatment with divalent anti-CD95 antibodies suppressed proliferation, decreased cell viability (Figure 6d) and enhanced the apoptotic activity associated with glutamine deprivation toward levels observed in cells treated with agonistic IgM multivalent anti-CD95 antibodies (Figure 6f). These results suggest that the cell shrinkage promoted by glutamine deprivation increases close proximity of CD95 receptors (or receptor oligomers) to a membrane

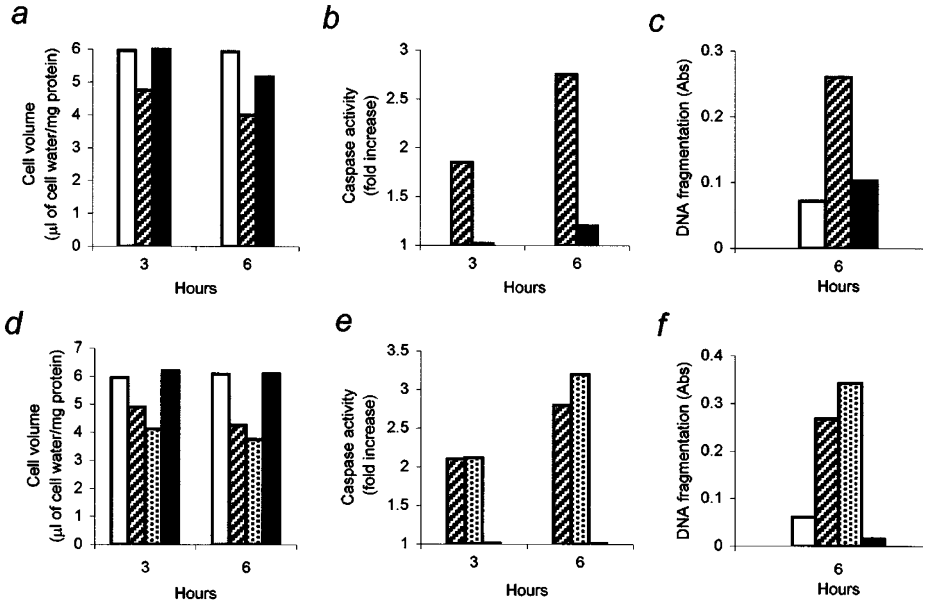

Figure 4 Glutamine deprivation-induced cell shrinkage and activation of the CD95-dependent pathway are restrained by surrogate compatible osmolytes. Glutamine supplementation prevents cell shrinkage and caspase-8 activation promoted by amino acid withdrawal. CEM cells were incubated for 3–6 h in glutamine-containing (□) or in glutamine free medium in the absence (▨) and in the presence of 10 mM betaine (■); (**a**) cell volume (μl of cell water/mg protein), estimated by transmembrane ^{3}H-OMG distribution; (**b**) caspase activity, expressed as fold activation relative to control cells and (**c**) DNA fragmentation, determined by photometric enzyme immunoassay. CEM cells were incubated in reconstituted complete- (□), glutamine-free- (▨) or amino acid-free-RPMI in the absence (▦) and in the presence of 2 mM glutamine (■); (**d**) cell volume (μl of cell water/mg protein), estimated by transmembrane ^{3}H-OMG distribution; (**e**) caspase activity, expressed as fold activation relative to control cells; (**f**) DNA fragmentation, determined by photometric enzyme immunoassay. Data in **a–f** are from a representative experiment. Each experiment, repeated at least three times, yielded similar results

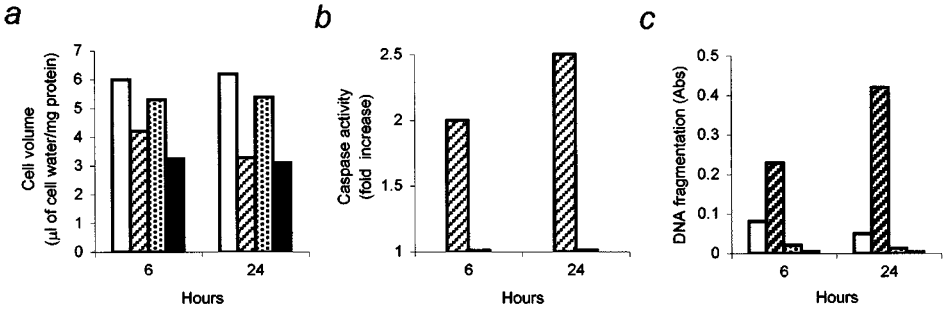

Figure 5 Glutamine deprivation-induced cell shrinkage fails to promote apoptosis in CD95-negative cells. Murine CD95-negative L1210 cells and CD95-expressing CEM cells were incubated for 6–24 h in glutamine-containing (CEM □; L1210 ▦) and in glutamine-free medium (CEM ▨; L1210 ■); (**a**) cell volume (μl of cell water/mg protein), estimated by transmembrane ^{3}H-OMG distribution; (**b**) caspase activity, expressed as fold activation relative to control cells and (**c**) DNA fragmentation, determined by photometric enzyme immunoassay. Data in **a–c** are from a representative experiment. Each experiment, repeated at least three times, yielded similar results

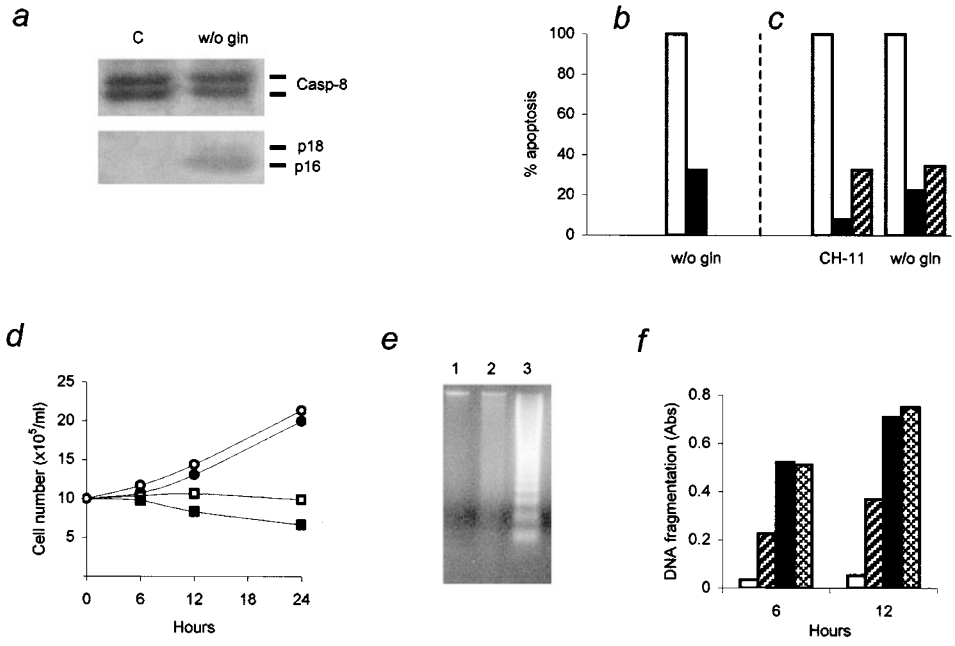

Figure 6 Expression of FADD-DN and v-Flip viral proteins (MC159, E8) inhibits the glutamine deprivation-induced activation of the CD95-dependent pathway. Cell shrinkage promoted by glutamine-deprivation is associated with a gain of agonistic function of divalent IgG anti-CD95 antibodies. (**a**) CEM cells were incubated for 6 h in the presence (**C**) and absence of glutamine (w/o gln) and lysate proteins were analyzed by Western blotting; the migration position of full length caspase-8 (two isoforms, Casp-8), the active subunit p18 and its further processing product p16 are indicated. (**b**) CEM cells were transfected with the plasmids pcDNA3,1-GFPΔFADD (■) or with pEGFP-C1 (□) as control (see Materials and Methods) and incubated for 16 h in glutamine-containing or in glutamine-free medium (w/o gln). Apoptosis was quantitated by fluorescence microscopy on GFP-positive cells stained with Hoechst 33342; data are expressed as per cent values relative to pEGFP-C1-transfected cells after correction for dead cells counted in plasmid-transfected cells incubated in control medium. (**c**) CEM cells were cotransfected with the plasmids: pCI-neo (□), pCI-MC159 (■) or pCI-E8 (▨) along with pEGFP-C1 (see Materials and Methods) and incubated for 16 h in glutamine containing FCS-supplemented RPMI 1640 in the presence of 0.1 μg/ml of anti-CD95 antibody (CH-11) or in glutamine-free medium (w/o gln). Apoptosis was quantitated by fluorescence microscopy on GFP-positive cells stained with Hoechst 33342; data are expressed as per cent values relative to pCI-neo-transfected cells after correction for dead cells counted in plasmid-transfected cells incubated in control medium. (**d**) CEM cells, seeded at the initial density of 1×10^6 cells/ml, were incubated in glutamine-containing medium in the absence (○) or in the presence of 0.5 μg/ml divalent IgG_{2a} SM1/1 anti-CD95 antibody (●) and in glutamine-free medium in the absence (□) or in the presence of the same anti-CD95 antibody (■); cell survival at the indicated time intervals was assessed by Trypan blue exclusion. (**e**) Low molecular-mass DNA was extracted from cells incubated for 24 h in glutamine-containing medium in the absence (lane 1, controls) and in the presence of 0.5 μg/ml IgG_{2a} SM1/1 anti-CD95 antibody (lane 2) or in glutamine-free medium (lane 3), and resolved in agarose gel electrophoresis. (**f**) Cells were incubated for 6–12 h in glutamine-containing (□) or in glutamine-free medium in the absence (▨) and in the presence of 0.5 μg/ml IgG_{2a} SM1/1 anti-CD95 antibody (■) or in glutamine-containing medium supplemented with 0.5 μg/ml IgM CH-11 anti-CD95 antibody (▩); DNA fragmentation was evaluated by photometric enzyme immunoassay. Data in **b–d** and **f** are from a representative experiment. Each experiment, repeated at least four times, yielded similar results

density compatible with their spontaneous (or divalent anti-CD95 antibody-mediated) functional multimerization. Retained cell viability and lack of apoptosis in CD95-negative L1210 cells induced to shrink by glutamine deprivation agree with this interpretation (see Figure 5).

Amidohydrolase-enzyme treatments

Amidohydrolase enzymes purified from a variety of bacterial sources include asparaginases and glutaminases that catalyze the hydrolysis of asparagine to aspartic acid and of glutamine to glutamic acid.[31–33,49,50] Conditions of glutamine deprivation associated with effects comparable to those described above (cell shrinkage, activation of the CD95 pathway followed by apoptotic cell death) were generated by supplementation of the culture medium (RPMI 1640 containing 2 mM glutamine and 10% dialyzed FCS, pH 7.4) with asparaginase (L-asparagine amidohydrolase from *E. coli*; pH range of activity, 5–9) at concentration of 2 U/ml. This treatment lowered sharply the concentration of glutamine in the medium (Figure 7a) and, within 24 h, caused massive apoptosis in cultured CEM (Figure 7b) and HL60 cells, but not in CD95-negative L1210 cells (not shown). At 2 U/ml glutaminase (L-glutamine amidohydrolase from *E. coli*, pH optimum 4.9) lowered glutamine concentration even faster than it did at pH values below 5.8 that were toxic for the cells. In addition to glutamine, asparaginase from *E. coli* depletes the medium of asparagine (being in fact catalytically more specific for asparagine than for glutamine). To determine whether the deprivation of asparagine had a role in the induction of the apoptotic process CEM cells were incubated in an asparagine-free RPMI 1640. As shown in Figure 7c–f asparagine depletion was not associated with cell shrinkage, caspase activation, DNA fragmentation and did not affect cell proliferation for at least 24 h.

Discussion

Physical perturbations of the membrane by UV light or by osmotic shock have been reported to induce clustering of cell surface receptors for growth factors and cytokines.[51] Recent observations suggest that surface CD95 is pre-

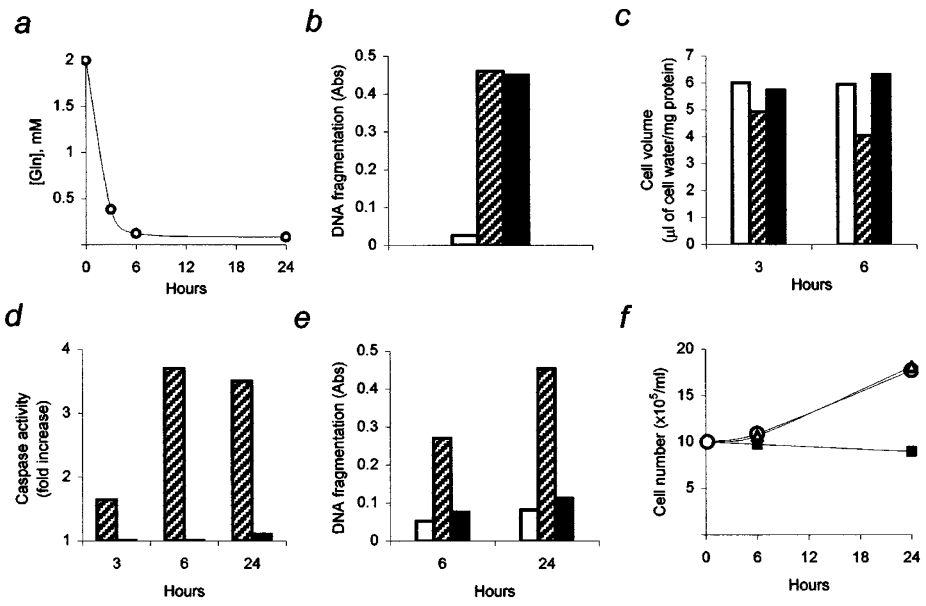

Figure 7 Asparaginase lowers glutamine concentration in cell culture medium and induces a massive CD95-mediated apoptosis in a process not affected by asparagine-depletion. (**a**) Changes in glutamine concentration of the culture medium (RPMI 1640 containing 2 mM glutamine and 10% dialyzed FCS, pH 7.4) supplemented with 2 U/ml asparaginase (L-asparagine amidohydrolase); glutamine concentration was determined by spectrophotometric enzyme assay. (**b**) CEM cells were incubated for 24 h in glutamine containing RPMI 1640 in the absence (□) and in the presence of 2 U/ml asparaginase (■) or in glutamine-free medium (▨); DNA fragmentation was evaluated by photometric enzyme immunoassay. CEM cells were incubated for 3–24 h in reconstituted glutamine-containing (controls, □) and glutamine-free RPMI 1640 medium (▨) or in a reconstituted glutamine-containing, asparagine-free medium (■) and assayed for : (**c**) cell volume (μl of cell water/mg protein) estimated by transmembrane ^{3}H-OMG distribution, (**d**) caspase activity, expressed as fold activation relative to control cells and (**e**) DNA fragmentation, determined by photometric enzyme immunoassay. (**f**) Viability and proliferation of CEM cells incubated in glutamine-containing (○), glutamine-containing, asparagine-free (△) and glutamine-free (■) medium were assessed by cell count using Trypan blue exclusion. Data in panels **a–f** are from a representative experiment. Each experiment, repeated at least three times, yielded similar results

assembled into functionally neutral trimers by self-association domains mapping to the extracellular region of the receptor[52,53] and that a high local density of death domains at the membrane (by CD95/FADD interaction) is required to send the cell death signal.[20] Here we show that the CD95-dependent signaling mechanism is activated by the physical perturbation associated with the glutamine deprivation-induced cell shrinkage and that this activation promotes the apoptotic death in CEM and HL-60 cells. Experiments with FADD-DN-, MC159- and E8-transfected cells suggest that the activation of the CD95-implemented pathway starts at the cell surface possibly by CD95-receptor multimerization that promotes the initial recruitment of FADD and caspase-8 required to propagate the caspase cascade. An increased propensity of oligomeric CD95 receptors to aggregate (bringing together intracellular death domains separated in the basal state) or a stabilization of interacting receptor subunits may result from the reduced tension of the cell surface that is likely to occur in cells induced to shrink and from the associated dehydration that may contribute local threshold concentrations for receptor functional activation. Consistent with this proposition are the gain-of-(agonistic)-function exhibited by divalent anti-CD95 antibodies in CEM cells incubated in the absence of glutamine and the resistance to the shrinkage-induced apoptosis of CD95-negative mouse L1210 cells, in which a transient expression of CD95 has been reported to confer sensitivity to killing by anti-CD95 agonistic antibodies.[54]

The induction of the CD95 pathway by cell shrinkage is not a CEM cell-line-specific phenomenon since it has been also observed in HL-60 cells and suggested for Jurkat[41] and U937 cells.[44] If this cell shrinkage-dependent process occurs in other CD95-expressing cell types, CD95 could be considered as a cell size-detecting pro-apoptotic sensor that activates a death pathway when a whatever induced persistent decrement in cell volume becomes incompatible with cell survival. Apparently, the persistence of the cell volume decrement is a necessary requirement for the activation of the apoptotic pathway, since cells displaying RVI (regulatory volume increase) that recover rapidly their initial volume were found to be resistant against apoptosis promoted by hyperosmotic stress.[55] These observations underlie a cell-specific impact of shrinkage in CD95-induced apoptosis, likely attributable to clustering and activation of receptors at the cell surface. The barely detectable surface expression of TNFR-1 in CEM cells clone 13 (C Fumarola and GG Guidotti, unpublished observations) and the resistance of L1210 cells (that express both TNFR p60 and p80 on the surface[56]) to glutamine deprivation-induced cell death suggest that TNF receptors are not primarily involved in the shrinkage-dependent activation of the apoptotic pathway. However, our results do not rule out the possibility that glutamine deprivation might also promote aggregation/activation of other death domain-containing receptors known to relay apoptotic signals via FADD.[57,58] Recent observations

suggest that shrinkage might have an even deeper significance in cell death induced by a variety of apoptotic stimuli, being an early prerequisite that promotes, *per se*, the execution of the downstream events of apoptosis.[44] If so, further mechanisms by which cell volume loss could be identified by cellular sensors and transduced into apoptotic signals must be envisaged.[45]

Checkpoints play a significant role in chemotherapeutic strategies to eliminate cancer cells and a variety of agents kill cancer cells by activating checkpoint-mediated apoptosis pathways.[59] Asparaginase has been found to induce cell cycle arrest and apoptosis in murine lymphoma/leukemia cell lines.[60,61] The contribution of the asparaginase-induced glutamine deprivation to the anti-leukemic therapy may result from a shrinkage-dependent targeting of a cell size-control checkpoint with activation of a CD95-mediated death pathway. In most cases of acute lymphoblastic leukemia the cells constitutively express surface CD95 receptors, but functional studies indicated that they are rather resistant to apoptosis upon CD95-triggering by anti-CD95 antibodies.[62] The asparaginase treatment, by promoting glutamine depletion that favors functional CD95 clustering, may enhance the cytotoxic activity of the associated anticancer drugs in the combined therapy of ALL and account for direct anti-tumor effects in patients with lymphoblastic leukemia-bearing type-II Myc-sensitized cells (like CEM) in which CD95-induced cell death is enhanced by cytochrome *c* release from mitochondria.[19] However, amidohydrolase therapy often has severe side effects[34] and, despite its relative cytotoxic selectivity toward malignant cells, disorders of clinical relevance may result from a temporary insufficient supply of glutamine in specific organs or compartments with metabolic derangements, including induction of apoptosis.[34,63,64] Moreover, the decreased extracellular glutamine concentration in the central nervous system[65] may seriously impair the function of the recently characterized GlnT transporter ($Km \cong 0.5$ mM) in glutaminergic neurons[14] that depends on glutamine as precursor of the neurotransmitter glutamate.

Materials and Methods

Cells and culture conditions

CEM cells clone 13 (lymphoblastic leukemia $CD4^+$-enriched cells) from our collection,[16] CEM-CCRF, HL-60, and murine L1210 cell lines purchased from the American Type Culture Collection (Rockville, MD, USA) were maintained in 10% FCS-supplemented RPMI 1640 at 37°C in an atmosphere of 5% CO_2 in air. Cultures were split 24 h before the experiments.[16] All measurements were made on subcultures (0.5-1×10^6 cells/ml) seeded into 25 cm^2 flasks and incubated for 0.5–24 h in 2 mM glutamine-containing or glutamine-free RPMI 1640 (or reconstituted RPMI, see Results) supplemented with 10% dialyzed FCS. When used, caspase inhibitors were added to the medium at the beginning of cell incubation.

Antibodies, enzymes and reagents

Monoclonal anti-CD95 IgM antibody CH-11 and mouse FITC-conjugated IgG isotype were obtained from Immunotech, Coulter Co. (Miami, FL, USA). Monoclonal anti-CD95 IgG antibodies SM1/1, APO-1-3, SM1/23 and FITC-conjugated SM1/23 were purchased from Bender MedSystems (Vienna, Austria). Monoclonal mouse anti-CD95L antibody NOK-1 was provided by PharMingen (San Diego, CA, USA). Polyclonal rabbit anti-CD95L antibody N-20, polyclonal goat anti-caspase-8 antibody p20 and HRP-conjugated donkey anti-goat secondary antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Monoclonal mouse anti-CD95L antibody clone 33, HRP-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse secondary antibodies were from Transduction Laboratories (Lexington, KY, USA). Sheep anti-mouse Ig-fluorescein $F(ab')_2$ fragment was provided by Roche (Mannheim, Germany). Caspase inhibitors z-IETD-FMK and z-DEVD-FMK were purchased from Enzyme Systems Products (Livermore, CA, USA). Metalloproteinase inhibitor KB8301 was obtained from PharMingen. 3-*o*-methyl-D-[1-^{3}H]glucose (4.5 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). L-Asparagine amidohydrolase, L-glutamine amidohydrolase, glutamine/glutamate determination kit and all other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Detection of apoptosis

Apoptosis was assessed by: (a) morphology on stained (Hoechst 33342, propidium iodide) or unstained cells using light-, phase-contrast- and fluorescence-microscopy; (b) FITC-conjugated Annexin V assay (Bender MedSystems); (c) DNA fragmentation by gel electrophoresis analysis (DNA extracted with phenol-chloroform, resolved in 1.4% agarose gel and stained with ethidium bromide) and by photometric enzyme immunoassay (Cell Death Detection Elisa^{PLUS}, Roche); (d) caspase-8 and caspase-3 activity (Caspase Colorimetric Assay Kit, MBL Intern. Corp., Watertown, MA, USA) and inhibition by specific tetrapeptides (z-IETD-FMK for caspase-8 and z-DEVD-FMK for caspase-3); (e) caspase-8 activation (detection of cleavage products in Western blotting): 50 μg protein from CEM cell lysates were resolved by SDS–PAGE and transferred to nitrocellulose membranes; immunodetection was done using an enhanced chemiluminescence system (primary anti-caspase-8 antibody, 1 : 200 dilution, and HRP-conjugated secondary antibody, 1 : 2000 dilution).

CD95 ligand/CD95 expression and activity

Total expression of CD95 receptor in CEM cells was quantitated by a Fas/Apo-1 ELISA Kit according to the manufacturer's instructions (Calbiochem, La Jolla, CA, USA). CD95 expression on the cell surface was assayed by flow cytometry (Coulter EPICS XL-MCL, Miami, Florida, USA) using the monoclonal mouse FITC-conjugated SM1/23 anti-CD95 antibody and a mouse FITC-conjugated IgG as isotypic control. Total expression of CD95L was estimated by Western blotting. CEM cells were lysed and 50 μg protein from lysates were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Immunodetection was done using an enhanced chemiluminescence system (primary anti-CD95L antibodies, 1 : 1000 dilution, and HRP-conjugated secondary antibodies, 1 : 5000 dilution). Surface expression of CD95L was assayed by flow cytometry (Coulter EPICS XL-MCL). Cells were treated with 10 μM KB8301 (metalloproteinase inhibitor) added to the medium during the incubation and stained with the monoclonal mouse anti-CD95L NOK-1 primary antibody (10 μg/ml) followed by a sheep anti-mouse Ig-fluorescein $F(ab')_2$ fragment (1 : 100 dilution). In these experiments a PHA-stimulated T-cell clone, triggered for 5 and 10 min with ionomycin after addition of NOK-1 (a treatment that promotes the delivery of CD95L to the cell surface),[66] was used as a positive control. CD95L functional apoptotic activity was evaluated in CEM

cells incubated: (a) in glutamine-free RPMI supplemented with 10 μg/ml of the neutralizing anti-CD95L NOK-1 antibody or with the anti-CD95 SM1/23 antibody at a concentration (10 μg/ml) that suppressed completely the CD95-mediated apoptosis induced by a 0.5 μg/ml of the agonistic anti-CD95 IgM antibody CH-11 (in these experiments cells were always preincubated for 60 min in complete RPMI containing the neutralizing antibodies) and (b) in glutamine-containing and in glutamine-free RPMI in the presence of 10 μM KB8301 (metalloproteinase inhibitor). The concentration of soluble CD95L in the culture medium of CEM cells incubated in the presence and absence of glutamine was estimated by an ELISA kit provided by MBL Intern. Corp.

Cell volume

Cellular volumes were estimated by measurements of cell water based on the steady-state transmembrane distribution of 3-*o*-methyl-D-[1-^{3}H]glucose (OMG) as described previously.[12] Labeled OMG was added during the last 30–40 min of incubation to control or modified mediums (see Results) whose glucose concentration had been reduced to 0.5 mM. The cells were then quickly washed twice in ice-cold PBS containing 0.1 mM phloretin and extracted in ice-cold 10% trichloroacetic acid. Radioactivity was measured by liquid-scintillation counting in three independent determinations. The protein content of the pellets was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Previous experiments had shown that, under the conditions adopted, the tracer reached in/out equilibrium in less than 30 min and that its outward flow during the cell washing procedure in cold PBS was negligible and unaffected by the preceding conditions of incubation. Data are expressed as μl of cell water/mg protein. Geometry parameters (area, diameter) of the cells cultured in the presence or in the absence of glutamine were assessed directly during the incubation (at 37°C in air-5% CO_2) by computer-assisted image analysis (image-pro Plus Media Cybernetics, Silver Spring, MA, USA). The software provided measurements of area and diameter (max., min., ave.) for each cell in the population explored. At least 500 cells between 6 and 20 μm in diameter were recorded for each experimental condition.

Plasmids and transfection procedures

pCI-neo was purchased from Promega (Madison, WI, USA). pEGFP-C1 was obtained from Clontech Laboratories (Palo Alto, CA, USA). pCI-MC159 and pCI-E8 were kindly provided by Dr. Jeffrey Cohen (NIH, Bethesda, MD, USA). pcDNA3,1-GFPΔFADD was a generous gift of Drs. Harald Wajant and Frank Muehlenbeck (University of Stuttgart, Germany). CEM cells were transfected by a standard electroporation procedure. In brief, 5×10^6 cells in FCS-free RPMI 1640 were electroporated by a pulse of 250 V, 960 μF (Gene Pulser, Bio-Rad) with: (a) 15 μg of pcDNA3,1-GFPΔFADD or pEGFP-C1 as control, (b) pCI-neo, pCI-MC159 or pCI-E8 along with pEGFP-C1 at a 3:1 ratio (20 μg DNA total), and incubated for 24 h in FCS-supplemented RPMI 1640. Dead cells resulting from the transfection procedures were eliminated by magnetic cell sorting (Dead Cell Removal Kit, Miltenyi Biotech, Bergisch Gladbach, Germany). Viable cells were then incubated for 16 h in glutamine-containing or in glutamine-free FCS-supplemented RPMI 1640 medium. As controls, transfected cells were incubated in complete RPMI medium supplemented with 0.1 μg/ml of anti-CD95 IgM agonistic antibody (CH-11). Apoptosis was quantitated by fluorescence microscopy on GFP-positive cells stained with Hoechst 33342 dye. Cells were scored positive if they had a pyknotic and/or fragmented nucleus. Representative graphs are shown for experiments where at least 200 cells were scored.

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