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Caspase Inhibition in Apoptotic T Cells Triggers Necrotic Cell Death Depending on the Cell Type and the Proapoptotic Stimulus

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Abstract CD95 (Fas/Apo-1) triggers apoptotic cell death via a caspase-dependent pathway. Inhibition of caspase activation blocks proapoptotic signaling and thus, prevents execution of apoptosis. Besides induction of apoptotic cell death, CD95 has been reported to trigger necrotic cell death in susceptible cells. In this study, we investigated the interplay between apoptotic and necrotic cell death signaling in T cells. Using the agonistic CD95 antibody, 7C11, we found that caspase inhibition mediated by the pancaspase inhibitor, zVAD-fmk, prevented CD95-triggered cell death in Jurkat T cells but not in A3.01 T cells, although typical hallmarks of apoptosis, such as DNA fragmentation or caspase activation were blocked. Moreover, the caspase-independent cell death in A3.01 cells exhibited typical signs of necrosis as detected by a rapid loss of cell membrane integrity and could be prevented by treatment with the radical scavenger butylated hydroxyanisole (BHA). Similar to CD95-induced cell death, apoptosis triggered by the DNA topoisomerase inhibitors, camptothecin or etoposide was shifted to necrosis when capsase activation was inhibited. In contrast to this, ZVAD was fully protective when apoptosis was triggered by the serpase inhibitor, Nα-tosyl-phenyl-chloromethyl ketone (TPCK). TPCK was not protective when administered to anti-CD95/ZVAD-treated A3.01 cells, indicating that TPCK does not possess anti-necrotic activity but fails to activate the necrotic death pathway. Our findings show (a) that caspase inhibition does not always protect apoptotic T cells from dying but merely activates a caspase-independent mode of cell death that results in necrosis and (b) that the caspase-inhibitor-induced shift from apoptotic to necrotic cell death is dependent on the cell type and the proapoptotic stimulus. J. Cell. Biochem. 97: 1350–1361, 2006.

Key words: apoptosis; necrosis; ZVAD; caspase inhibition; T cells; A3.01; CD95

CD95 (Fas/Apo-1) is a member of the tumor necrosis factor superfamily and plays a major role in apoptosis induction involved in lymphocyte homeostasis and in the cytotoxic immune response [Nagata and Golstein, 1995]. Trimerization of CD95 by its natural ligand, CD95L, recruits the adaptor protein, Fas associated death domain (FADD) to the cytoplasmatic domain of the receptor, forming a death-inducing signaling complex (DISC) that leads to activation of the initiator caspase-8, which in turn activates downstream effector caspases that mediate and exercise the apoptotic signal [Kischkel et al., 1995]. Apoptotic cell death is characterized by specific hallmarks, such as loss of cellular membrane asymmetry, breakdown of mitochondrial membrane potential, release of cytochrome c, and degradation of nuclear DNA to 180 bp oligomeres [Nagata and Golstein, 1995]. The crucial role of caspases in apoptotic signaling is underlined by the fact that caspase inhibition blocks execution of the apoptotic program [Zhu et al., 1995].

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Besides induction of apoptosis, cell death can be mediated by necrosis. Necrotic cell death is independent of caspase activation and is characterized by swelling of the cells and organelles and is, therefore, sometimes referred to as "oncosis" [Majno and Joris, 1995; Fiers et al., 1999]. Whereas the term "apoptosis" implies a specific molecular mechanism underlying cell death, "necrosis" in the strict sense refers to changes secondary to cell death by any mechanism—including apoptosis [Majno and Joris, 1995]. However, here we use the term "necrosis" to describe a caspase-independent mode of cell death, as used previously by other groups [Holler et al., 2000].

There is accumulating evidence that CD95, besides induction of apoptosis, triggers other signaling activities. It has been reported that CD95 mediates a costimulatory role in cellular proliferation [Alderson et al., 1993] or activation of proinflammatory signaling cascades leading to activation of NF-κB and cytokine expression [Ashany et al., 1999; Rescigno et al., 2000]. Alternatively, CD95 may trigger caspase-independent cell death, here referred as necrosis. Induction of caspase-inhibitor-induced necrosis has been reported for several cell lines, including lymphocytes, fibroblasts, and neuronal cells [Vercammen et al., 1998b; Luschen et al., 2000; Sayers et al., 2000; Volbracht et al., 2001; Kim and Han, 2001; Hetz et al., 2002; Los et al., 2002] as well as for primary human T cells [Holler et al., 2000; Scheller et al., 2002], suggesting that the pronecrotic signaling activity of CD95 may have a physiological background.

In this study, we investigated CD95-induced necrosis in the human T cell line, A3.01 and characterized the interplay between the proapoptotic and the pronecrotic signaling cascades triggered by CD95.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents

The human T cell lines, A3.01 [Folks et al., 1985] and Jurkat E6-1 [Weiss et al., 1984] were cultured at 37° in 5% CO_2 atmosphere in RPMI 1640 (Invitrogen, Karlsruhe, Germany), containing 10% fetal calf serum (FCS), penicillin, and streptomycin. Experiments were performed in a 96-well flat-bottom plate (10^5 cells/well) in a total volume of 200 μl, if not indicated otherwise. The agonistic anti-CD95 mAb 7C11 (Coulter Immunotech, Hialeah, FL) was used at 200 ng/ml if not indicated otherwise. The pancaspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk, Bachem, Weil am Rhein, Germany), the apoptosis-inducing topoisomerase inhibitors, camptothecin and etoposide (Alexis Biochemicals, Germany), the serpase inhibitor, N^{α}-tosyl-phe chloromethyl ketone (TPCK, Calbiochem, Bad Soden, Germany), the MEK inhibitor, PD098059 (Calbiochem), and the proteasome inhibitor, ALLN (Calbiochem) were dissolved in DMSO (stock solutions: ZVAD: 100 mM, Camptothecin: 2 mM, Etoposide 100 mM, TPCK: 100 mM, PD098059: 50 mM, ALLN: 100 mM). Reagents were diluted with RPMI to yield the following final concentrations in the experiments (if not indicated otherwise): ZVAD: 100 μM, Camptothecin: 10 μM, Etoposide: 500 μM, TPCK: 100 μM, PD098059: 100 μM, ALLN: 100 μM. All samples of each experiment were adjusted to identical solvent (DMSO) concentrations.

ELISA

Cells were cultured in a 96-well flat-bottom plate (10^5 cells/well) in a total volume of 200 μl. To determine concentration of TNF-α, 50 μl of the supernatants were analyzed by ELISA according to the instructions of the manufacturer (OptEia, BD-Biosciences, Heidelberg, Germany).

DNA Fragmentation Assay

Cells were cultured in a 24-well plate (10^6 cells/well). For analysis of apoptotic DNA-fragmentation, cells were washed with PBS and lysed in PBS containing 1% (w/v) Triton X-100, 200 mM NaCl, and 20 mM EDTA. Cellular debris was removed and DNA was precipitated with 2-propanole. DNA was analyzed with agarose (1.5%) gel electrophoresis.

Chromium Release Assay

Cells were labeled with $Na_2{}^{51}CrO_4$ for 60 min and used for cytotoxicity assays. As a positive control, cells were lysed with 1% (w/v) Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany). Cell-free supernatants were harvested after 7 h and radioactivity was determined in a gamma counter (Compugamma 1282, Long Island Scientific).

Western Blot

Cells were cultured in a 24-well plate (10^6 cells/well). To determine caspase-8 cleavage,

cells were washed with PBS and lysed with Laemmli buffer at 99°C. Proteins were analyzed by Western blot using a PVDF membrane (Millipore Corporation, Bedford), a polyclonal rabbit anti-caspase-8 (BD-Biosciences), and a horseradish peroxidase (HRP)-conjugated swine anti-rabbit serum (DAKO, Hamburg, Germany). HRP activity was detected with an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Freiburg, Germany) using an X-ray photofilm (Fuji Photofilm Europe Gmbh, Düsseldorf, Germany).

Flow Cytometry

For detection of plasma membrane alterations, cells were stained with Annexin-V-FITC (BD Biosciences, Heidelberg, Germany) and counterstained with 7-AAD (BD Biosciences) according to the instructions of the manufacturer. Cells were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Markers were set according to medium-treated cells. Annexin-V-positive/7-AAD-negative events usually indicate apoptotic cells. 7-AAD-positive or double-positive events indicate plasma membrane leakage and are typical for necrotic cells or late apoptotic cells. Double negative events are scored as living cells. For detection of caspase-3 activation, cells were fixed with 4% formalin for 20 min and permeabilized with PBS, containing 0.5% saponin and 5% BSA. Cells were stained with a phycoerythrin (PE)-labeled polyclonal rabbit anti-caspase-8 serum (BD-Biosciences) and analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson).

RESULTS

We have previously reported that caspase inhibition induces a switch from CD95-induced apoptosis to proinflammatory signaling in primary T cells [Scheller et al., 2002]. This switch was accompanied by activation of NF-κB and expression of proinflammatory cytokines, such as TNF-α. In addition, apoptosis inhibition resulted in rapid onset of necrosis. This CD95-induced necrosis in primary T cells has also been reported by other groups [Holler et al., 2000], demonstrating that the physiologic signaling repertoire of primary lymphocytes allows for execution of this alternative death pathway.

The human T cell line, A3.01, is an excellent model to study the alternative CD95-signaling events observed in primary human T cells, since A3.01 T cells not only undergo CD95-induced necrosis when caspase activity is inhibited but similarly activate proinflammatory signaling cascades leading to cytokine production comparable to what is observed in primary T cells [Scheller et al., 2002]. In contrast to what has been found in primary T cells and A3.01 cells, Jurkat T cells are protected from CD95-induced cell death when caspase activity is inhibited (Fig. 1). CD95 stimulation with the agonistic anti-CD95 mAb 7C11 resulted in rapid onset of cell death in A3.01 and Jurkat T cells as detected by chromium release assay (Fig. 1A). The pancaspase inhibitor, zVAD-fmk inhibited CD95-induced DNA fragmentation both in Jurkat and A3.01 T cells (Fig. 1B) and prevented cell death in Jurkat cells (Fig. 1A). However, zVAD-fmk failed to rescue A3.01 cells from CD95-induced cell death, suggesting that the observed cell death is independent of caspase activity. To confirm caspase inhibition by zVAD-fmk in A3.01 T cells, we examined activation of caspase-8, the most CD95-proximal caspase, by Western-Blot analysis. As depicted in Figure 1D, caspase-8 was activated following CD95-stimulation, whereas no cleavage was detectable in the presence of zVAD-fmk. Despite the lack of caspase-8 activity, cells exhibited loss of membrane integrity as analyzed by Annexin-V/7-AAD flow cytometry (Fig. 1C). Cells treated for 7 h with mAb 7C11 alone were positive for Annexin-V-staining but negative for 7-AAD, a characteristic of cells undergoing apoptosis. In contrast, anti-CD95/ZVAD-treated cells were double positive for Annexin-V/7-AAD staining, indicating that these cells died by necrosis. Taken together, the data presented in Figure 1 indicates that caspase inhibition induces a switch from apoptotic to necrotic cell death in CD95-stimulated A3.01 T cells but not in Jurkat T cells. Moreover, since caspase-8 activation was inhibited by zVAD, CD95-induced necrosis is mediated by a signaling event upstream of or in parallel to caspase-8 activation, an early event in CD95-induced signaling.

Interestingly, despite the lack of any detectable caspase-8 activation, 20% of the cells treated with anti-CD95/ZVAD were annexin-V-positive but negative for the counterstain 7-AAD (Fig. 1C and also Fig. 2A,C, Fig. 3D, Fig. 4, and Fig. 5B,D). Since caspase-dependent

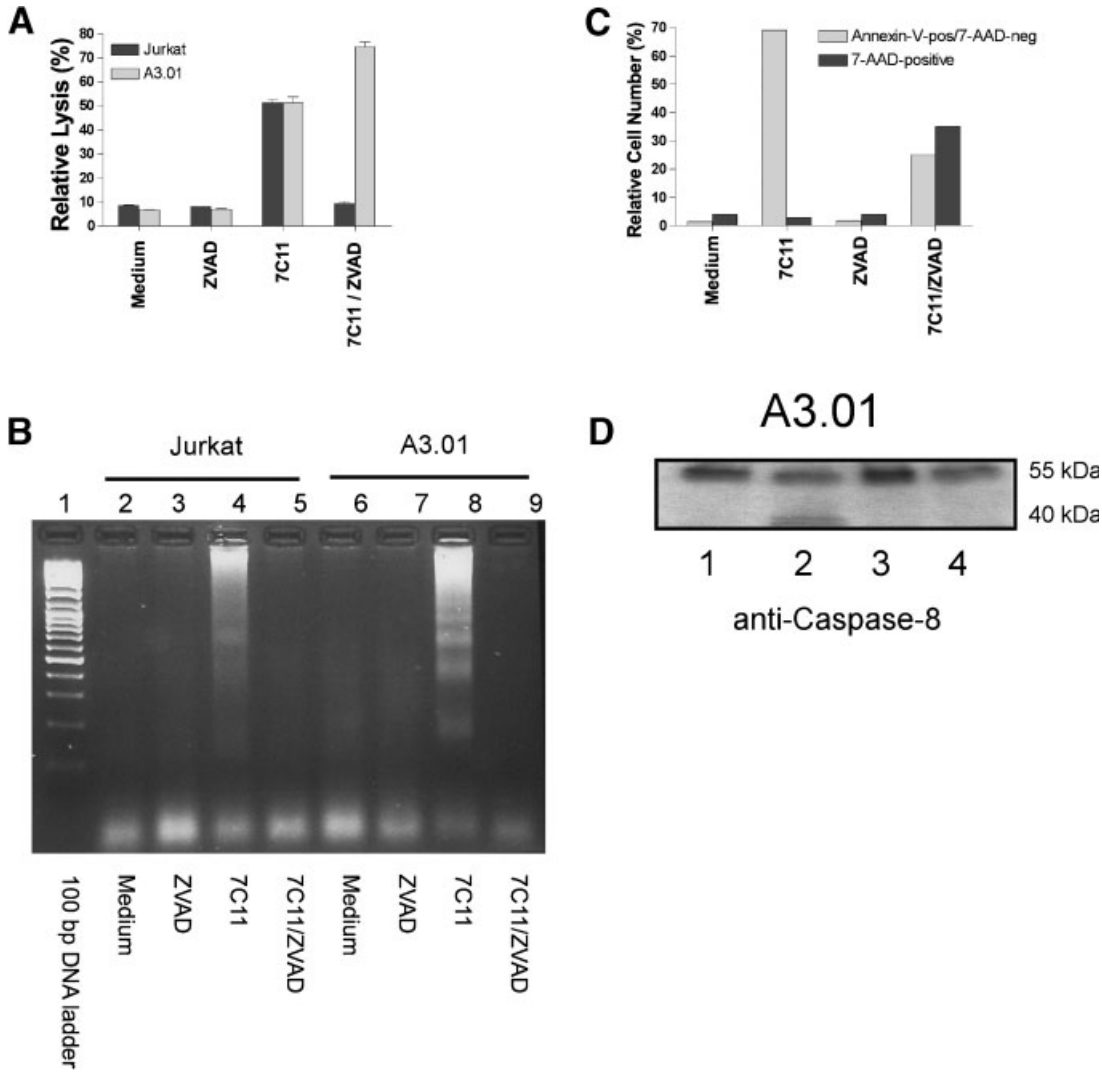

Fig. 1. Comparison of CD95-induced cell death in A3.01 and Jurkat E6-1 T cells. **A**: Cells were labeled with ^{51}Cr and were incubated for 7 h with medium alone, with the caspase inhibitor, zVAD-fmk (100 μM), with the anti-CD95 mAb 7C11 (200 ng/ml), or with both. Radioactivity released into the medium (supernatant) was measured in a gamma counter. Triplicate analysis, data represent means ± SEM. **B**: Analysis of apoptotic DNA fragmentation. **Lane 1**: 100 bp ladder, **lane 2**: Jurkat (medium), **lane 3**: Jurkat (ZVAD), **lane 4**: Jurkat (7C11), **lane 5**: Jurkat (7C11/ZVAD), **lane 6**: A3.01 (medium), **lane 7**: A3.01 (ZVAD), **lane 8**: A3.01 (7C11), **lane 9**: A3.01 (7C11/ZVAD). **C**: Human A3.01 T cells were incubated for 7 h with medium alone or in the presence of the anti-CD95 mAb 7C11 (200 ng/ml), the caspase inhibitor, zVAD-fmk (100 μM) or both. Cells were stained with Annexin-V-FITC/7-AAD and analyzed by flow cytometry. **D**: Cells derived from the experiment presented in (C) were analyzed by anti-caspase-8 Western blot.

apoptosis in these cells was excluded by the caspase-8-Western blot (Fig. 1D, lane 4), cell death in these cells cannot be attributed to apoptosis but rather was a result of necrotic mechanisms. The fact that this cell population was negative for 7-AAD staining reveals that membrane integrity of these cells was still intact. Apparently, at least in this cell population, CD95-incuced necrosis involves phosphatidylserine flipflop in the absence of caspase activation, which is usually only observed in apoptotic cells. The reason for this unexpected finding remains to be elucidated but it could be attributed to a caspase-independent inactivation of the enzyme scramblase [Verhoven et al., 1999] as well as a result of an inactivation of the enzymes floppase and translocase due to the depletion of cellular ATP in CD95/ZVAD-induced necrosis [Los et al., 2002].

Similar to the situation observed in the chromium release assay presented in Figure 1, Jurkat cells, in contrast to A3.01 T cells, did not

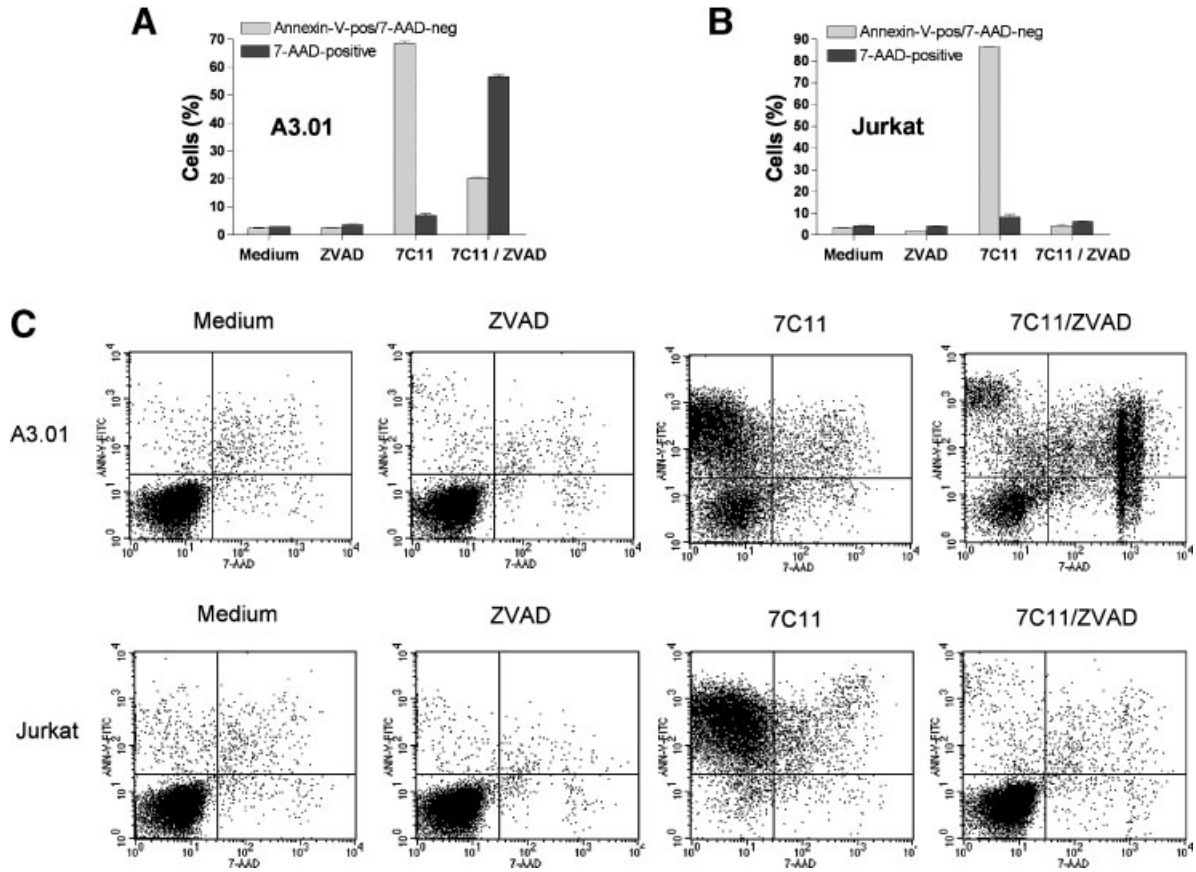

Fig. 2. Comparison of CD95-induced membrane alterations in A3.01 and Jurkat E6-1 T cells. Cells were incubated for 7 h with medium alone, with the anti-CD95 mAb 7C11 (200 ng/ml), with the caspase inhibitor, zVAD-fmk (100 μM), or with both. Cells were stained with Annexin-V-FITC/7-AAD and analyzed by flow cytometry. Triplicate analysis, data represent means ± SEM. **A**: Human A3.01 T lymphoblasts. **B**: Human Jurkat E6-1 T lymphoblasts. **C**: Representative pictures of FACS analysis derived from the experiments presented in (A) and (B).

show a switch from apoptosis to necrosis in the presence of caspase inhibition, but were protected from CD95-induced cell death by ZVAD (Fig. 2). Inhibition of CD95-induced cell death in Jurkat T cells by ZVAD has been similarly observed by other groups [Boise and Thompson, 1997; Wilson et al., 1999; Hetz et al., 2002; Wilson and Browning, 2002]. These findings are in contrast to what has been reported by Holler and colleagues, who found caspase-independent cell death also in Jurkat T cells [Holler et al., 2000]. The reason for this discrepancy is unclear but it could be attributed to the different proapoptotic stimuli used. Whereas we used the agonistic CD95-antibody 7C11 to trigger CD95-activation, Holler and colleagues used a FLAG-tagged Fas ligand whose activity was further enhanced by a crosslinking antibody. Moreover, Holler et al. used prolonged incubation times (14–16 h) compared to 7 h as presented in this manuscript, indicating that strong stimuli in combination with prolonged incubation times may be needed to observe CD95-induced necrosis in Jurkat cells.

CD95-induced necrosis in A3.01 T cells is accompanied by expression of TNF-α (Fig. 3A) as we already published earlier [Scheller et al., 2002]. We could further demonstrate that CD95/ZVAD-induced TNF-α production is vulnerable to MAP-kinase inhibitors [Scheller et al., 2002]. To assess whether CD95/ZVAD-induced necrosis is similarly dependent on activation of MAP kinases, we tested the effect of the MEK inhibitor, PD098059. As depicted in Figure 3C, MEK-inhibition did not reduce the amount of CD95/ZVAD-induced cell death, nor did it influence the proportions of annexin-V/7-AAD staining results (Fig. 3D). Therefore, MEK is not involved in CD95-induced necrosis. Interestingly, in the absence

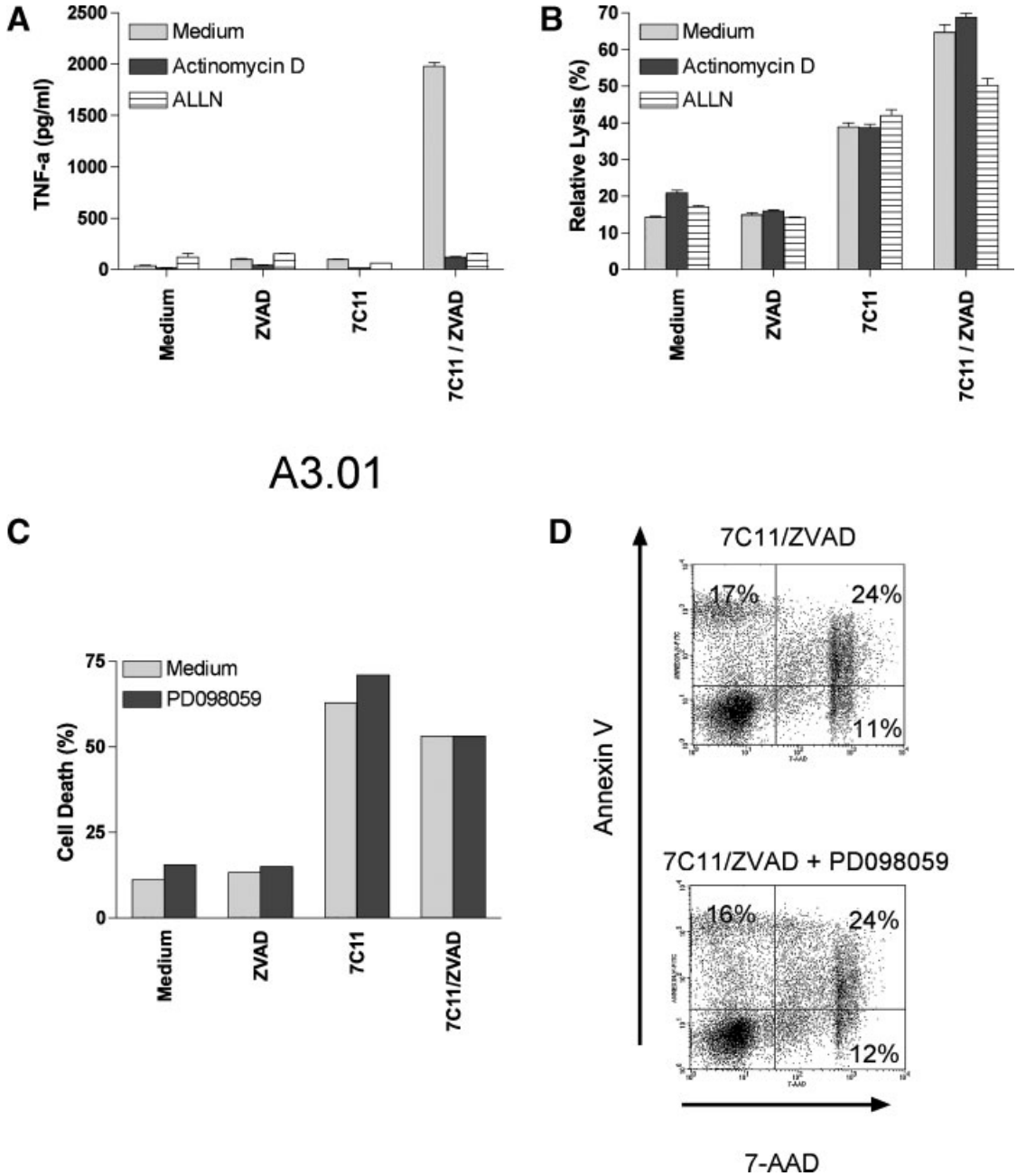

Fig. 3. CD95-induced necrosis is independent of TNF-α. **A**: A3.01 T cells were incubated for 7 h with medium alone, with the caspase inhibitor, zVAD-fmk (100 μM), with the anti-CD95 mAb 7C11 (200 ng/ml), or with both in the presence or absence of actinomycin D (10 μg/ml) or ALLN (100 μM), respectively. Supernatants were analyzed by TNF-α-ELISA. Triplicate analysis, data represent means ± SEM. **B**: A3.01 cells were labeled with ^{51}Cr and treated as described in (A). Radioactivity released into the medium (supernatant) was measured in a gamma counter. Triplicate analysis, data represent means ± SEM. **C**: A3.01 cells were treated with 7C11 and ZVAD as described in (A) in the presence or absence of the MEK inhibitor, PD098059 (100 μM) and stained with Annexin-V/7AAD and analyzed by flow cytometry. Annexin-V-positive events and 7-AAD-positive events were scored as dead cells and were summed up as ''Cell Death.'' **D**: Pictures of FACS analysis derived from the experiments presented in (C).

of ZVAD, PD098059 slightly enhanced CD95-induced cell death (Fig. 3C), suggesting that MEK activation counteracts the proapoptotic activity of CD95. Similar observations were being made by Wilson and colleagues who studied the effects of MEK inhibitors on CD95-induced apoptosis in Jurkat T cells [Wilson et al., 1999].

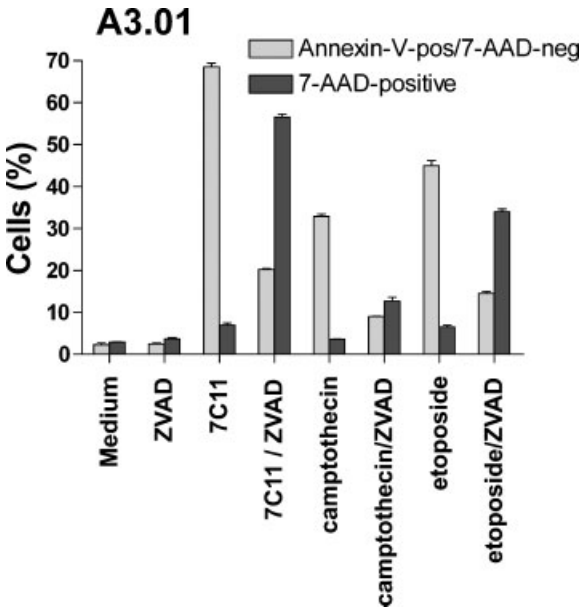

Fig. 4. DNA topoisomerase inhibitors in the presence of ZVAD trigger necrosis. A3.01 T cells were incubated for 24 h with medium alone, zVAD-fmk (100 μM), 7C11 (200 ng/ml), 7C11/zVAD (200 ng/ml, 100 μM), camptothecin (10 μM), camptothecin/zVAD (10 μM, 100 μM), etoposide (500 μM), or etoposide/zVAD (500 μM, 100 μM). Cells were stained with Annexin-V-FITC/7-AAD and analyzed by flow cytometry.

A caspase inhibitor-induced switch from apoptosis to necrosis has been described for activation through other members of the TNF-receptor family, in particular for TNF-R1, which is activated by TNF-α [Vercammen et al., 1998a]. This observation is noteworthy, as CD95-stimulation in the presence of ZVAD triggers the production of TNF-α. To address the question whether CD95/ZVAD-induced necrosis is directly mediated by CD95 signaling or whether it is mediated by CD95/ZVAD-induced TNF-α production, we blocked cytokine expression with actinomycin D or the proteasome inhibitor, ALLN. As depicted in Figure 3A, TNF-α expression was suppressed to baseline levels in the presence of actinomycin D or ALLN, whereas cellular lysis remained unaffected (Fig. 3B). Thus, CD95-induced necrosis is not related to TNF-α production of the cells.

A caspase inhibitor-induced switch from apoptosis to necrosis has not only been reported for death receptor-triggered apoptosis such as CD95 stimulation [Vercammen et al., 1998b; Holler et al., 2000; Luschen et al., 2000; Sayers et al., 2000; Hetz et al., 2002; Scheller et al., 2002; Kemp et al., 2003], TNF-R1-stimulation [Vercammen et al., 1998a; Los et al., 2002; Kemp et al., 2003] or TRAIL-stimulation [Holler et al., 2000; Wilson and Browning, 2002; Kemp et al., 2003] but has similarly been described for other proapoptotic stimuli, such as tomatine adjuvant [Yang et al., 2004], cyanide [Prabhakaran et al., 2004], imatinib mesylate [Okada et al., 2004], or staphylococcus aureus alpha-toxine [Essmann et al., 2003]. We have screened additional inducers of apoptosis and found that apoptosis triggered by the DNA topoisomerase inhibitors, camptothecin and etoposide is similarly shifted towards necrosis when caspase activity is inhibited (Fig. 4). In contrast to this, apoptosis triggered by the serpase inhibitor, TPCK is not directed to necrosis (Fig. 5). Treatment of A3.01 cells with TPCK induced activation of caspase-3 (Fig. 5A,C) and phosphatidylserine flipflop (Fig. 5B,D), similar to the stimulation with the agonistic CD95 antibody 7C11. In contrast to CD95-induced apoptosis, TPCK-induced apoptosis was not shifted to necrosis by caspase inhibitors (Fig. 5B,D), indicating that the shift from apoptosis to necrosis is dependent on the proapoptotic stimulus. We questioned why TPCK, in contrast to other apoptosis inducers, does not induce necrotic cell death. The lack of detectable necrosis could either be attributed to a potential protective effect of TPCK that prevents onset of necrosis, or to the fact that TPCK triggers only one death signal (i.e., the proapototic signal) instead of two (i.e., the proapoptotic and the pronecrotic signal). To address this question, we coincubated A3.01 cells both with anti-CD95 and TPCK and analyzed the cell viability in the presence of ZVAD. As shown in Figure 5B, TPCK did not prevent CD95/ZVAD-induced necrosis but rather enhanced CD95/ZVAD-induced cell death, demonstrating that TPCK does not exert a protective activity to prevent CD95/ZVAD-induced necrosis. We suggest that TPCK stimulates apoptosis downstream of the signaling event at which the proapoptotic and pronecrotic cellular signaling pathways divide, whereas other cytotoxic stimuli, such as death receptor stimulation or other proapoptotic substances like camptothecin or etoposide, act upstream of this point.

Our results indicate that in the absence of caspase inhibitors the pronecrotic signal of CD95 remains cryptic, indicating that the proapoptotic signaling pathway negatively regulates the pronecrotic signal cascade. We

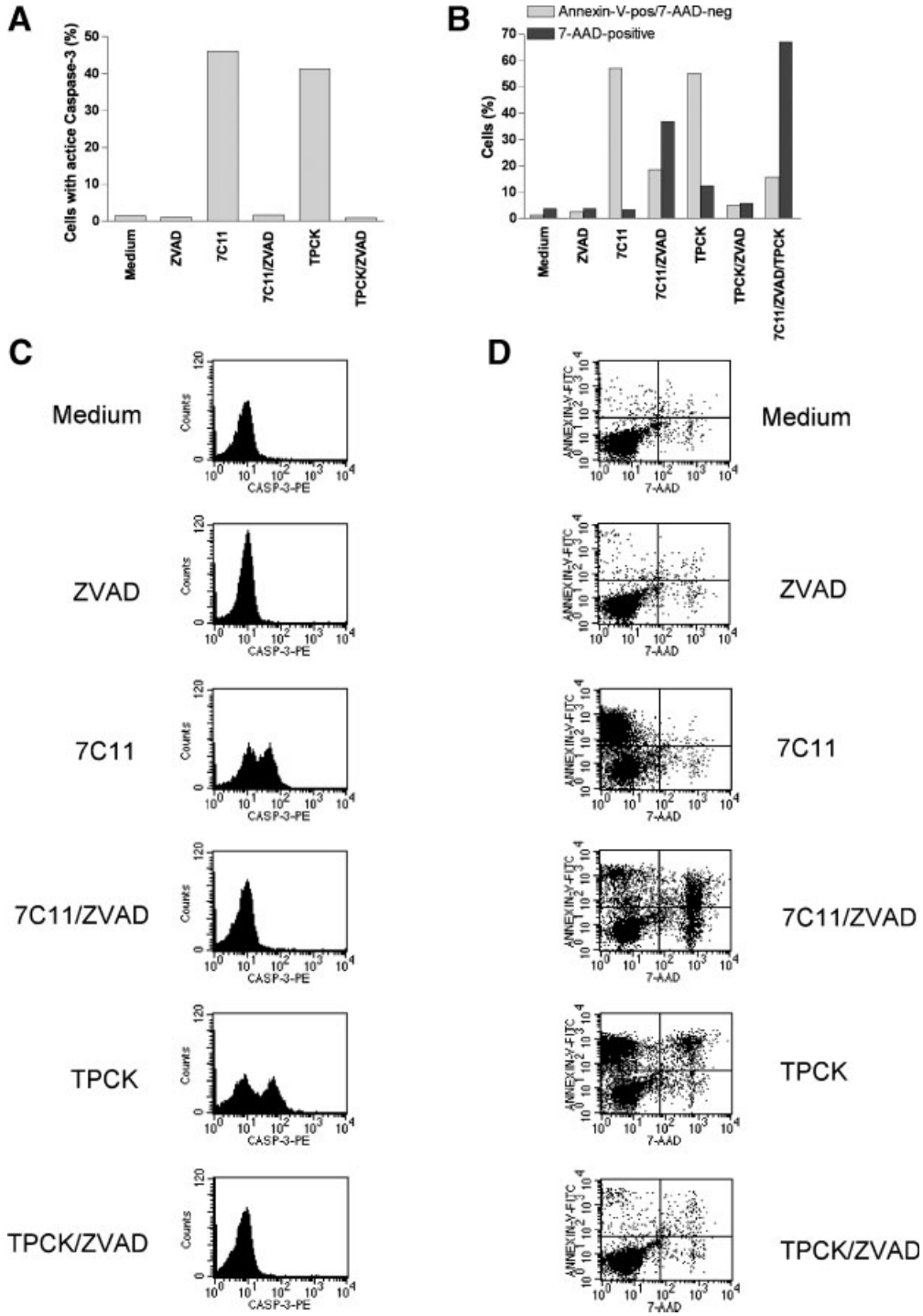

Fig. 5. The switch from apoptosis to necrosis is dependent on the proapototic stimulus. **A**: A3.01 T cells were incubated for 7 h with medium alone, the anti-CD95 mAb 7C11 (200 ng/ml), or the serpase inhibitor, TPCK (100 μM) in the presence or absence of the caspase inhibitor, zVAD-fmk (100 μM). A: Caspase-3 activation was analyzed by flow cytometry. **B**: Cells derived from the experiment presented in (A) were stained with Annexin-V-FITC/7-AAD and analyzed by flow cytometry. Annexin-V-FITC-positive/7-AAD-negative cells were scored as apoptotic cells, double positive cells were scored as necrotic cells and shown in percent. **C**, **D**: Pictures of FACS analysis derived from the experiments presented in (A) and (B).

subsequently questioned whether the pronecrotic activity suppresses the proapoptotic activity of CD95. We used the radical scavenger butylated hydroxyanisole (BHA), which inhibits death receptor-induced necrosis [Vercammen et al., 1998b; Luschen et al., 2000]. BHA was protective for cells treated with the anti-CD95 mAb 7C11 and ZVAD, but enhanced toxicity for cells treated with 7C11 alone (Fig. 6), suggesting that BHA sensitized cells for CD95-triggered apoptosis. Analysis of membrane alterations in these cells by Annexin-V/7-AAD staining revealed that BHA-treatment induced a higher incidence of apoptotic cells at early time points (Fig. 7A,B). At later time points, double positive cells accumulated in anti-CD95/BHA-treated cells compared to treatment with anti-CD95 alone, indicating that BHA may accelerate CD95-induced apoptosis. Alternatively, the presence of BHA may disrupt cell membrane integrity specifically in cells undergoing apoptosis, compared to normal cells or to cells in which apoptosis execution is blocked by ZVAD.

DISCUSSION

CD95 triggers at least two different signaling pathways in A3.01 T lymphocytes that ultimately lead to cell death. One of them is the classical apoptosis pathway mediated by FADD and caspase-8, the other is independent of caspase activity and triggers necrosis. Since CD95-induced necrosis can be observed in the presence of the broad range caspase inhibitor,

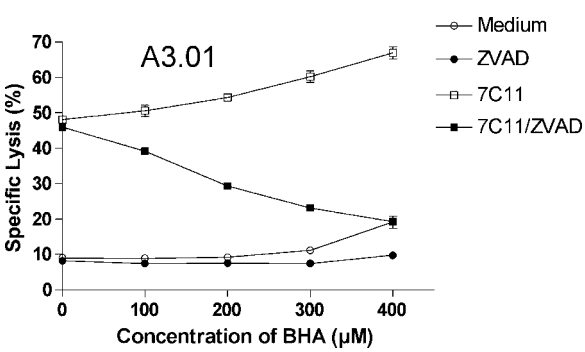

Fig. 6. BHA inhibits CD95-induced necrosis. A3.01 T cells were labeled with ^{51}Cr and were incubated for 7 h with medium alone, with the caspase inhibitor, zVAD-fmk (100 μM), with the anti-CD95 mAb 7C11 (200 ng/ml), or with both in the presence of the indicated BHA concentrations (0–400 μM). Radioactivity released into the medium (supernatant) was measured in a gamma counter. Triplicate analysis, data represent means ± SEM.

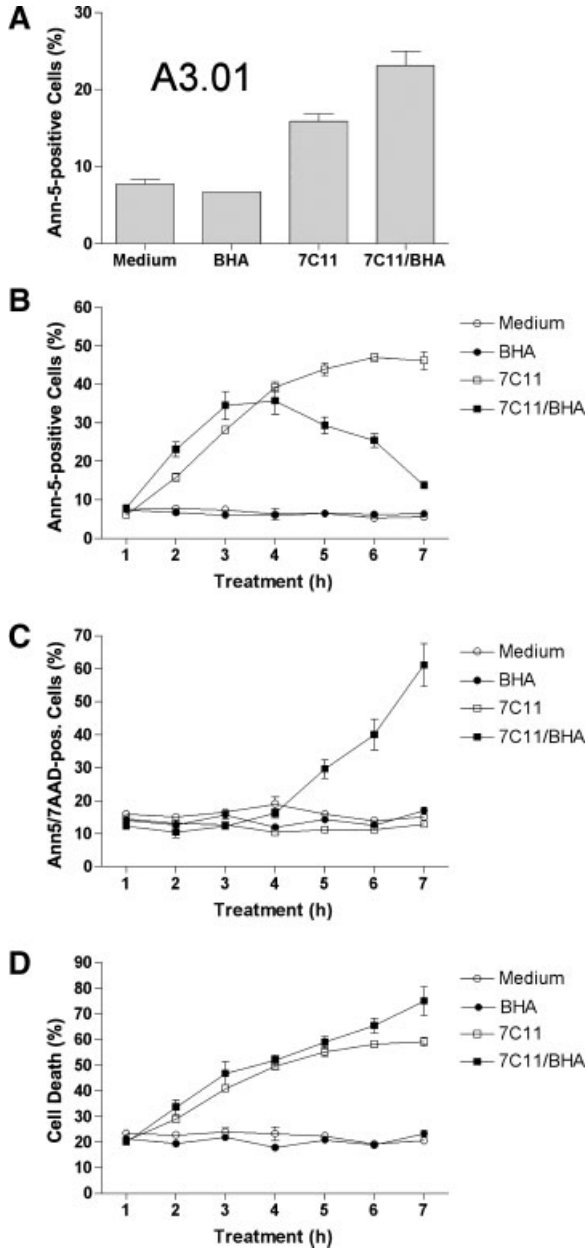

Fig. 7. BHA enhances CD95-induced apoptosis. A3.01 T cells were incubated for the indicated time with medium alone, with the free radical scavenger BHA (400 μM), with the anti-CD95 mAb 7C11 (200 ng/ml), or with both. Cells were stained with Annexin-V-FITC/7-AAD and analyzed by flow cytometry. Triplicate analysis, data represent means ± SEM. **A**: Analysis of early apoptotic cells (Annexin-V-positive/7-AAD-negative events) after 2 h of treatment (data taken from B). **B**: Analysis of early apoptotic cells (Annexin-V-positive/7-AAD-negative events). **C**: Analysis of late apoptotic or necrotic cells (Annexin-V-positive/7-AAD-positive events). **D**: Analysis of cell death (Annexin-V-positive/7-AAD-negative and Annexin-V-positive/7-AAD-positive events).

zVAD-fmk, death receptor-induced necrosis is mediated by a signaling event upstream of or adjacent to caspase-8. Proapoptotic stimuli that bypass death receptor signaling, such as the serpase inhibitor, TPCK, fail to induce necrosis when capsase activation is inhibited, indicating that necrosis induction is not an automatic event following caspase inhibition but requires specific events triggered by death receptor signaling. One likely candidate to mediate death receptor-induced necrosis is RIP, a kinase that assembles to the DISCs of all death receptors capable to trigger necrosis via FADD and that has been shown to play a vital role in CD95-induced necrosis in Jurkat T cells [Holler et al., 2000; Wilson and Browning, 2002; Kemp et al., 2003].

The mechanism for camptothecin- or etoposide-induced necrosis remains to be elucidated, since both substances mediate their cytotoxic activity bypassing the CD95L/CD95 system [McGahon et al., 1998]. It has been reported that etoposide triggers a multimerization of FADD independent of external CD95L/CD95 crosslink [Micheau et al., 1999; Wesselborg et al., 1999]. We hypothesize that this multimerization of FADD may in turn activate the apoptosis cascade downstream of CD95/FADD that can be switched to pronecrotic signaling following caspase-8 inhibition by ZVAD.

CD95/ZVAD-induced T cell death is blocked in the presence of the radical scavenger BHA. Previous publications have shown that death receptor-induced necrosis is accompanied by an increase in ROS and that BHA counteracts this oxidative stress [Vercammen et al., 1998b; Prabhakaran et al., 2004]. Interestingly, in the absence of ZVAD, BHA is not protective but rather enhances CD95-triggered cell death, suggesting that ROS formation may counteract execution of the proapoptotic signaling cascade. Alternatively, BHA may exert an intrinsic cytotoxic effect that synergizes with CD95-triggered cell death.

Under normal conditions, the CD95-induced proapoptotic signaling pathway is dominant and the pronecrotic activity remains cryptic. In the presence of caspase inhibition, however, the pronecrotic activity is being executed, resulting in necrotic cell death rates comparable to the classical apoptotic death cascade. Necrotic cell death following caspase-inhibited CD95-stimulation has also been reported for primary T cells [Holler et al., 2000; Scheller et al., 2002], indicating that the observed phenomenon may have a physiologic background. Naive T cells, for example, express the caspase inhibitory protein, FLIP that similarly induces a shift to alternative CD95 signaling [Tschopp et al., 1998; Kataoka et al., 2000]. The alternative signaling pathway triggered by CD95 is even more pronounced in dendritic cells, in which a caspase-independent signaling pathway can be observed also under normal conditions [Ashany et al., 1999; Rescigno et al., 2000].

Different cell lines exhibit varying sensitivity towards CD95-induced necrosis. Whereas A3.01 T cells were highly susceptible to caspase-independent cell death, Jurkat E6-1 cells were almost resistant. The molecular basis for this phenomenon remains to be elucidated. However, for prolonged incubation times combined with strong proapopototic stimuli, it has been reported that also Jurkat cells undergo CD95/ZVAD-induced necrosis [Holler et al., 2000], suggesting that the pronecrotic signaling activity may be less pronounced in this cell line compared to primary T cells or A3.01 T cells.

Caspase-dependent cell death (apoptosis) and caspase-independent cell death (necrosis) represent two tightly linked cellular death execution programs that may play a vital role in anti-cancer treatment. There is emerging evidence that both pathways are involved in cell death triggered by various cytostatics, such as imatinib mesylate or arsenic trioxide [McCafferty-Grad et al., 2003; Okada et al., 2004]. Also etoposide, which is shown in this manuscript to trigger both death pathways, plays an important role in cancer chemotherapy.

A dysregulation of apoptosis has been linked to several degenerative disorders, such as Morbus Alzheimer, Morbus Parkinson, ischemia, or AIDS, and apoptosis inhibition has been proposed as a therapeutic approach to inhibit abnormal cell death in vivo [Robertson et al., 2000; Tomaselli, 2001; O'Brien and Lee, 2004]. Our study indicates that apoptosis inhibition not necessarily prevents cell death but induces a shift from apoptotic to necrotic death, dependent on the proapoptotic stimulus. Whereas death receptor-induced apoptosis is shifted to necrosis, apoptosis induced by the serine protease inhibitor, TPCK is blocked without induction of necrotic damage. Thus, the success of in vivo applications of caspase inhibitors to prevent apoptosis may depend on the specific mechanism underlying cellular degradation.

In line with this view, recent publications indicate that in vivo inhibition of cellular degradation by caspase inhibitors may not be as effective as in vitro [Loetscher et al., 2001; Joly et al., 2004]. Moreover, transgenic mice expressing the viral caspase-inhibitory protein, CrmA neither develop T cell hyperplasia nor serum antibodies, compared to CD95 knockout mice [Watanabe-Fukunaga et al., 1992; Smith et al., 1996; Walsh et al., 1998], indicating that caspase inhibition does not abolish CD95-induced cell death in vivo.

We have shown that inhibition of the proapoptotic CD95-signaling pathway activates an alternative death pathway leading to necrotic cell death that remains cryptic in the absence of caspase inhibition. The molecular mechanism for this suppression is unclear. However, there is evidence that active caspases degrade many signaling proteins that could be involved in alternative death receptor signaling, including the kinase RIP [Martin et al., 1998; Stroh and Schulze-Osthoff, 1998; Lin et al., 1999] and Holler et al. [2000] have demonstrated that RIP is involved in CD95-induced necrosis. In this light, it remains to be elucidated whether CD95-induced necrosis represents an ancient death pathway that became obsolete after evolution of apoptosis or whether this alternative death pathway turns out to be a backup system to allow for execution of programmed cell death in cells that become resistant to the classical apoptosis pathway due to infection with viruses suppressing caspase activity [Aubert and Jerome, 2003].

Taken together, CD95 triggers at least two signaling pathways in T lymphocytes that induce cell death. Caspase inhibition does not protect CD95-activated T cells from undergoing cell death but rather changes the mode of death program from apoptosis to necrosis. Thus, prevention of death receptor-induced death requires both apoptosis and necrosis inhibition.

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