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Cytochrome *c* Is Involved in Fas-mediated Apoptosis of Prostatic Carcinoma Cell Lines[1]

Andreas Gewies, Oskar W. Rokhlin, and Michael B. Cohen[2]

Departments of Pathology [A. G., O. W. R., M. B. C.] and Urology [M. B. C.], University of Iowa, Iowa City, Iowa 52242, and Veterans Affairs Medical Center, Iowa City, Iowa 52240 [M. B. C.]

ABSTRACT

We have shown previously that the pathways leading to Fas-mediated apoptosis in prostatic carcinoma cell lines are intact, because apoptosis can be triggered either by Fas ligation alone in the Fas-sensitive cell lines PC3 and ALVA31 or by rendering the Fas-resistant cell lines DU145 and JCA1 Fas-sensitive by combined treatment with anti-Fas monoclonal antibody and cycloheximide (O. W. Rokhlin *et al.*, Cancer Res., *57:* 1758–1768, 1997). In this study, we demonstrate that two of the early events after Fas ligation are the release of cytochrome *c* from the mitochondria and activation of caspase-9. We also found that Bid is processed after Fas ligation and thus might activate the mitochondria-dependent apoptotic cascade. In a cell-free system, cytochrome *c* induced caspase-3-like activity in cytoplasmic extracts from all four cell lines studied, although differences in the level of enzymatic activity were observed. Western blot analysis revealed that caspase-7 is activated by cytochrome *c* at the same level in all extracts, whereas expression and activation of caspase-3 varied considerably. Cytochrome *c*-activated extracts displayed different abilities in the induction of apoptotic features in isolated nuclei such as morphological changes and DNA fragmentation. However, differences in nuclear apoptotic activity induced by cytochrome *c* did not correlate with the level of caspase-3 like activity in the different extracts. These results suggest that the mitochondrial pathway is involved in Fas-mediated apoptosis in prostatic carcinoma cell lines and that, in addition to caspase-7 and caspase-3, there are other factors that confer nuclear apoptotic activity.

INTRODUCTION

The apoptotic process can be initiated by several different stimuli, *e.g.*, growth factor withdrawal (1), DNA damage (2), dysregulation of the cell cycle (3), or ligation of death receptors (4). These different apoptotic stimuli induce diverse early signaling events (induction phase), which then converge by activating a common central biochemical pathway that is responsible for the execution of apoptosis. Mitochondria appear to integrate different proapoptotic pathways and are probably a key regulator of apoptosis (5). Several different apoptotic stimuli have been reported to induce the release of cyto c[3] from the mitochondria into the cytosol (6), which results in the formation of the "apoptosome," a dATP-dependent complex between cyto c, Apaf-1, and procaspase-9 (7). Activated caspase-9 is released from the apoptosome and subsequently initiates a caspase cascade involving the executioner caspases caspase-3, caspase-6, and caspase-7 (8, 9), which are believed to directly cause many of the observed biochemical and morphological changes by cleaving specific substrates such as nuclear lamins, gelsolin, or DFF45 and others (10). Bcl-2 and Bcl-X_L have been reported to block cyto c release and thus prevent apoptosis (11, 12). Recently, another mitochondrial apoptosis-inducing factor has been characterized (13), which is released from the mitochondria upon alteration of the mitochondrial inner transmembrane potential (5).

The Fas (CD95) receptor is a type I transmembrane protein and belongs to the tumor necrosis factor receptor family (4, 14). Fas was identified as a cell surface receptor that mediates cell death after ligation with agonistic anti-Fas antibodies (15, 16). Because functional Fas is expressed on the surface of diverse cancer cells, it potentially provides an approach for the rapid and irreversible killing of tumor cells, although methods of proper targeting of the therapeutic Fas ligand specifically to the Fas receptors on the tumor cells still have to be devised to prevent deleterious side effects (17). Some of the pathways leading to Fas-mediated apoptosis have been characterized in detail. Engagement of Fas results in the formation of the DISC, a complex of Fas, Fas-associating protein with death domain, and pro-caspase-8 (4). Activated caspase-8 is released from the DISC (18) and has been shown to directly activate the executioner caspases (19). At the same time, caspase-8 has been reported to cleave Bid, a proapoptotic member of the Bcl-2 family, which then induces cyto c release, thus forming a link between Fas-mediated apoptosis and the mitochondrial pathway (20). In certain cell types, the direct activation of downstream caspases by the DISC appears to be sufficient for the execution of Fas-mediated apoptosis because Bcl-2 does not protect against Fas killing in these cell types (21–23). However, in other cell systems, Bcl-2 or Bcl-X_L was reported to protect against Fas-mediated apoptosis (12, 24–27). Thus, depending on the cell type studied, Fas-mediated apoptosis can be dependent or independent on the mitochondrial pathway, and sometimes the mitochondrial pathway at least appears to contribute to Fas-mediated apoptosis by amplifying the effects of caspase-8 on activation of downstream caspases (6, 28, 29).

We have shown previously that in the human prostatic carcinoma cell lines PC3, ALVA31, DU145, and JCA1, the pathway(s) leading to Fas-mediated apoptosis is intact (30). PC3 and ALVA31 are sensitive to treatment with anti-Fas mAb, whereas DU145 and JCA1 are only sensitive under combined treatment with anti-Fas mAb and CHX. CHX is necessary to convert DU145 and JCA1 from Fas-resistant to Fas-sensitive because of a labile-dominant inhibitory protein(s) presumably acting at the apex of the apoptotic cascade (31, 32).

In this study, we performed experiments to determine whether the mitochondrial pathway is involved in Fas-mediated apoptosis of prostatic carcinoma cell lines. Our results indicate that activation of the Fas pathway in prostatic carcinoma cells induces a cascade that includes activation of caspase-8, Bid cleavage, cyto c release, and activation of caspase-9. Experiments using a cell-free system indicate that the apoptotic executioner events downstream from cyto c are intact in PC3, ALVA31, and JCA1.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions. The human prostatic carcinoma cell lines were cultured as described previously (30). The RPMI 1640 complete medium (10% FCS) was exchanged at least 2 h before cells were treated with anti-Fas mAb (IPO-4). If cells were additionally treated with the caspase inhibitor zVAD-fmk, the inhibitor was added 1 h before anti-Fas mAb was added.

Preparation of Cytosols for cyto c Release. Mitochondria-free cytosol for the detection of cyto c release was prepared as described (11). Briefly, cells were grown for different times in the presence of 0.5 μg/ml anti-Fas mAb (IPO-4), harvested, washed, and lysed in ice-cold buffer M [20 mM HEPES

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[2] To whom requests for reprints should be addressed, at Department of Pathology, 1117 ML, University of Iowa, Iowa City, IA 52242-1087. Phone: (319) 335-8232; Fax: (319) 335-8916; E-mail: michael-cohen@uiowa.edu.

[3] The abbreviations used are: cyto c, cytochrome *c*; DISC, death-inducing signaling complex; mAb, monoclonal antibody; CHX, cycloheximide; PMSF, phenylmethylsulfonyl fluoride; AMC, aminomethylcoumarin; Ac, acetyl; fmk, fluoromethylketone; z, benzyloxycarbonyl; VAD, Val-Ala-Asp; YVAD, Tyr-Val-Ala-Asp; DEVD, Asp-Glu-Val-Asp; VEID, Val-Glu-Ile-Asp; CPP32, 32-kDa cystein protease.

(pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 250 mM sucrose, 0.1 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin] at about 2×10^6 cells per 100 μl by homogenization in a small glass homogenizer with a Teflon pestle (50 strokes on ice). The homogenates were spun at 16,000 × *g* for 20 min at 4°C, and the supernatants were used for anti-cyto c Western blot analysis. As a proof for the loading of equal amounts of protein, we also performed blots using anti-actin mAb.

Western Blotting. Western blot detection of proteins was performed as described previously (30). Briefly, 10–20 μg of proteins were separated on 4–20% gradient SDS-PAGE and blotted to nitrocellulose membrane (Novex, San Diego, CA). Membranes were incubated with the corresponding monoclonal antibodies: anti-cyto c, anti-caspase-3 (Transduction Laboratories, San Diego, CA), anti-actin mAb (Sigma Chemical Co., St. Louis, MO), anti-caspase-8, anti-caspase-10 (Upstate, Lake Placid, NY), goat polyclonal anti-Bid (R&D Systems, Minneapolis, MN), and rabbit antibody to caspase-7 (kindly provided by Dr. Yuri Lazebnik, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Anti-caspase-9 antibodies were monoclonal or rabbit polyclonal (as indicated in the figure legends; PharMingen, San Diego, CA, or Oncogene, La Jolla, CA). The blots were incubated with a goat antimouse or goat antirabbit IgG conjugated with horseradish peroxidase, and immunoreactive bands were visualized by incubation of the membrane with SuperSignal chemiluminescence reagent (Pierce, Rockford, IL).

Preparation of Cytoplasmic Extracts. Cytoplasmic extracts for use in the cell-free system were prepared essentially as described (33). Briefly, cells were harvested by trypsinization; trypsinization was stopped by adding fetal bovine serum to a final concentration of 50%. Cells were washed once in complete RPMI 1640, twice in ice-cold PBS, once in KPM buffer [50 mM PIPES (pH 7.0), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10 μg/ml cytochalasin B, 0.1 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin]. The cell pellet was resuspended in about 1 volume of KPM buffer and lysed by three cycles of freezing and thawing in liquid nitrogen. The lysate was centrifuged at 16,000 × *g* for 20 min at 4°C. The supernatant (15–25 mg/ml protein) was stored at −70°C.

Isolation of Nuclei. Nuclei were prepared essentially as described previously (34). Briefly, 10 μM cytochalasin B was added to adherent growing prostate carcinoma cells, and incubation was continued for 30 min (for preparation of Jurkat nuclei this step was omitted). Cells were harvested, washed twice with PBS and once with Nuclei Buffer [10 mM PIPES (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 10 μg/ml cytochalasin B, 0.1 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin], and gently lysed with a Dounce homogenizer, and the homogenate was layered over 30% sucrose in Nuclei Buffer and pelleted by centrifugation at 800 × *g* for 10 min. For the preparation of radioactive-labeled nuclei, ALVA31 cells were harvested, and 5×10^6 cells were reseeded in 162-cm^2 tissue culture flasks in complete RPMI 1640 + 2 μCi/ml [^{3}H]thymidine. After 24 h of incubation, labeled cells were harvested, and nuclei were prepared as described above. The degree of labeling was about 1 cpm/nucleus.

Caspase Enzymatic Assays. To measure caspase activity, cytoplasmic extracts were diluted to a protein concentration of 2.5 mg/ml with Dilution Buffer containing an ATP-regeneration system [10 mM HEPES (pH 7.0), 5 mM EGTA, 50 mM NaCl, 2 mM MgCl, 1 mM DTT, 2 mM ATP, 10 mM phosphocreatine, and 50 μg/ml creatine kinase] and activated with 5 μM cyto c (Sigma) and 1 mM dATP (Promega Corp., Madison, WI) in a total volume of 15 μl. After incubation at 37°C for 45 min, the extracts were incubated for 30 min at room temperature in 200 μl Assay Buffer [50 mM PIPES-KOH (pH 7.2), 0.1 mM EDTA, and 10% glycerol] with 20 μM fluorescent substrates: Ac-DEVD-AMC (CPP32 subfamily substrate), Ac-YVAD-AMC (interleukin-1β-converting enzyme subfamily substrate), and Ac-VEID-AMC (caspase-6 substrate; all from Calbiochem, San Diego, CA). Fluorescence was measured with a FL600 fluorimeter (Bio-Tek Instruments, Inc., Burlington, VT).

Radioactive DNA Fragmentation Assay. Radioactive nuclei were prepared as described above. Prior to use in the cell-free system, 5×10^4 nuclei were distributed in 0.5 ml microcentrifuge tubes and were washed once in Dilution Buffer. The nuclei were incubated in cytoplasmic extracts (7.5 mg/ml) in the presence or absence of 10 μM cyto c and 1 mM dATP in a total volume of 10 μl for 4 h at 37°C (650 nuclei/μg protein). After incubation, the DNA of the nuclei was harvested on a glass fiber membrane, and the retained radioactivity was measured by scintillation counting. Experiments were run in triplicate or pentuplicate for each condition. The percentage of DNA fragmentation was calculated as follows: [(cpm of nuclei in pure extracts) − (cpm of nuclei in extracts + cyto c/dATP)/(cpm of nuclei in pure extracts)] × 100.

4′6-Diamidino-2-phenylindole Staining and DNA Laddering. A total of 3×10^5 nuclei from frozen stocks (1.5 μl of 2×10^8 nuclei/ml) was incubated for 4 h in cytoplasmic extracts at a protein concentration of 7.5 mg/ml in a total volume of 50 μl (800 nuclei/μg protein) in the presence or absence of 10 μM cyto c and 1 mM dATP. If zVAD-fmk was used, it was added to the extracts first, and then cyto c and dATP were added. After incubation, a sample of 5×10^4 nuclei was fixed in 4% PBS-buffered paraformaldehyde solution in the presence of 2 μg/ml 4′6-diamidino-2-phenylindole (Sigma) and observed using a fluorescence microscope (BH Series; Olympus, New Hyde Park, NY) at excitation wavelength 350 nm. DNA was prepared by incubation of the remaining nuclei in Lysis Buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.2% SDS, and 0.5 mg/ml proteinase K] overnight at 37°C, and precipitation of the DNA by adding 0.1 volume of 3 M NaOAc and 2 volumes of ice-cold ethanol. The dried pellet was dissolved in 20 μl of TE [50 mM Tris-HCl (pH 8.0), 1 mM EDTA], and RNase A was added at 0.1 mg/ml. After incubation for 1 h at 37°C, the DNA was analyzed on a 1.5% agarose gel (at 4 V/cm) containing 0.5 μg/ml ethidium bromide.

RESULTS

We have shown previously that PC3 and ALVA31 were sensitive to Fas-mediated apoptosis, whereas DU145 and JCA1 were resistant. We estimated the response of these cell lines to treatment with anti-Fas agonistic mAb by different methods including proliferation assay, quantitative DNA fragmentation assay, DNA laddering, and staining with Annexin V. We performed time course experiments investigating the cell death from 2 h of treatment up to 48 h, and these studies have shown that the differences between cell lines are qualitative: 90–95% of PC3 and ALVA31 were killed after 48 h of treatment, whereas DU145 and JCA1 were completely resistant (30). We have also shown that CHX converted phenotypes of DU145 and JCA1 from Fas-resistant to Fas-sensitive. Simultaneous treatment of resistant cell lines with CHX and anti-Fas mAb induced DEVDase activity and activated both caspase-8 and caspase-7 (31). In this study, we further investigate whether the mitochondrial pathway is involved in Fas-mediated apoptosis of prostatic carcinoma cell lines.

Involvement of Bid, cyto c, and Caspase-9 in Fas-mediated Apoptosis of Prostatic Carcinoma Cell Lines. To determine whether the cyto c pathway is involved in Fas-mediated apoptosis in prostatic carcinoma cell lines, PC3, DU145, and ALVA31 were treated with anti-Fas mAb for different times, and cytosol was prepared by gentle lysis. Western blots revealed increasing amounts of cyto c in the cytosols of PC3 and ALVA31 during anti-Fas treatment, whereas in DU145 an increase of cyto c was observed only under combined treatment with anti-Fas mAb and CHX (Fig. 1*A*). The increase of cyto c in the cytosols of PC3 and ALVA31 was already observed after 2 h of anti-Fas treatment when morphologically no cell death could be detected. For PC3 we also demonstrate that the pan-caspase-inhibitor zVAD-fmk prevents cyto c release into the cytosol (Fig. 1*A*). Additionally, we detected activation of caspase-9 in anti-Fas-treated PC3 and ALVA31, as judged by the decrease of the proenzyme band (Fig. 1*B*). In DU145, the level of pro-caspase-9 decreased only after combined treatment with anti-Fas and CHX.

Bid, a proapoptotic Bcl-2 family member, was identified recently as a cytosolic protein that triggers cyto c release from the mitochondria after proteolytic processing by caspase-8 (35, 36). In this report, we show that caspase-8 is activated and Bid is processed after Fas ligation in prostatic cancer cell lines (Fig. 2). Note that Bid processing was prevented in the presence of the caspase inhibitor zVAD-fmk, as shown for PC3 (Fig. 2*B*).

Thus, in prostatic carcinoma cell lines, ligation of the Fas receptor results in the release of cyto c into the cytosol and in activation of caspase-9. Bid apparently links activated caspase-8 with the events at the mitochondria that result in the release of cyto c into the cytosol. These data suggest that the mitochondrial apoptotic pathway is involved in Fas-mediated apoptosis in prostatic carcinoma cells.

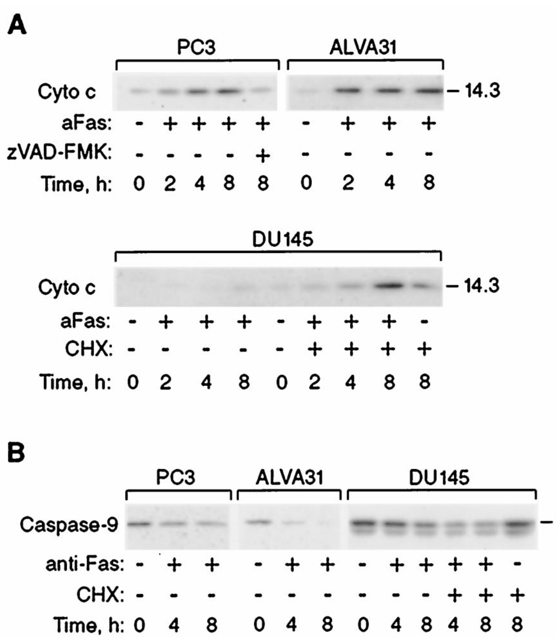

Fig. 1. cyto c is released into the cytosol, and caspase-9 is activated after anti-Fas treatment. The cell lines PC3 and ALVA31 were treated with anti-Fas mAb (IPO-4, 1 μg/ml) for different times, and cytosols were prepared as described in "Materials and Methods." DU145 was treated with anti-Fas mAb alone or in parallel with anti-Fas mAb and CHX (25 μg/ml). In PC3, we additionally examined the effect of zVAD-fmk on cyto c release after anti-Fas treatment. Equal amounts (15 μg) of cytosolic protein were separated by SDS-PAGE, and Western blots with anti-cyto c mAb (*A*) and anti-caspase-9 mAb (*B*) were performed as described in "Materials and Methods." In PC3 and ALVA31, monoclonal anti-caspase-9 antibodies were used; in DU145, rabbit polyclonal anti-caspase-9 antibodies were used (both from PharMingen). The anti-caspase-9 antibodies recognized only the proenzyme at M_r 46,000; decrease of this band indicates activation of caspase-9.

cyto c Induces Caspase Activity in Cytoplasmic Extracts. Because anti-Fas treatment triggers cyto c release into the cytosol, we analyzed the effect of cyto c on cytoplasmic extracts from PC3, DU145, ALVA31, and JCA1. After incubation of cytoplasmic extracts with 5 μM cyto c and 1 mM dATP at 37°C for 45 min, we detected caspase-3-like activity in activated extracts from all four cell lines when using Ac-DEVD-AMC as substrate (Fig. 3). Extracts from JCA1 displayed levels of DEVDase activity comparable with those from Jurkat, whereas extracts from PC3, ALVA31, and DU145 displayed lower activity. cyto c-activated extracts also displayed activity with Ac-VEID-AMC, a selective substrate for caspase-6 (37), which was about three times lower than DEVDase activity, and we did not detect any activity with the caspase-1 substrate Ac-YVAD-AMC (data not shown). Thus, we found caspase activity characteristic for members of the CPP32 subfamily, but activity specific for caspases of the interleukin-1β-converting enzyme subfamily was not detected.

cyto c-activated Extracts from PC3, JCA1, and ALVA31, but not from DU145, Induce Apoptotic Events in Isolated Nuclei. Subsequently, we examined whether the cyto c-induced caspase activity in the cytoplasmic extracts can mediate apoptotic events in isolated cell nuclei. When isolated nuclei were incubated in cyto c-activated cytoplasmic extracts from ALVA31 and JCA1, almost all nuclei displayed peripheral chromatin condensation and apoptotic bodies, whereas activated PC3 extracts induced apoptotic features in many but not all nuclei. Incubation with DU145 extracts did not result in significant morphological changes of the nuclei. DNA fragmentation was quantified by a radioactive DNA fragmentation assay using prelabeled nuclei (Fig. 4). Activated ALVA31 extracts induced strong DNA fragmentation (60%) in nuclei, whereas PC3 and JCA1 extracts induced only 20–30% DNA fragmentation. cyto c-activated DU145 extracts did not induce any significant DNA fragmentation. To determine whether the inability of DU145 extracts to induce apoptosis in isolated nuclei was specific for the stimulation of apoptotic activity by cyto c, we also stimulated cytoplasmic extracts by the addition of active recombinant caspase-8. Extracts from PC3, JCA1, and ALVA31, but not extract from DU145, induced morphological changes and DNA fragmentation in nuclei when active recombinant caspase-8 was added to the cell-free system (data not shown). In summary, stimulation of cytoplasmic extracts from ALVA31, PC3,

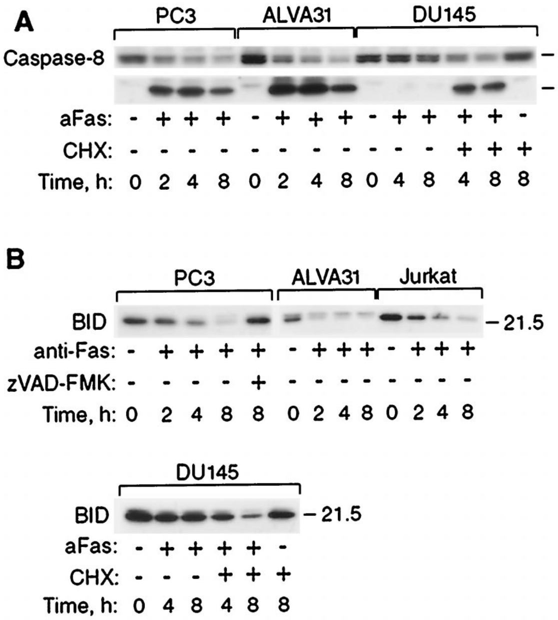

Fig. 2. Caspase-8 is activated and Bid is processed after anti-Fas treatment. The cell lines PC3 and ALVA31 were treated with anti-Fas mAb (IPO-4; 1 μg/ml) for different times, and cytosols were prepared as described in "Materials and Methods." DU145 was treated with anti-Fas mAb alone or in parallel with anti-Fas mAb and CHX (25 μg/ml). In PC3, we additionally examined the effect of zVAD-fmk on the processing of Bid after anti-Fas treatment. Equal amounts (15 μg) of cytosolic protein were separated by SDS-PAGE, and Western blots with anti-caspase-8 (*A*) and anti-Bid (*B*) polyclonal antibody were performed as described in "Materials and Methods." The anti-Bid antibodies only recognized the full-size Bid at M_r ~23,000 in PC3 and ALVA31, often together with a slightly higher running side-band. For this reason, we also show a Bid blot with extracts from anti-Fas-treated Jurkat cells as a positive control. A decreasing p23 Bid band indicates processing of Bid and supposedly the generation of active Bid.

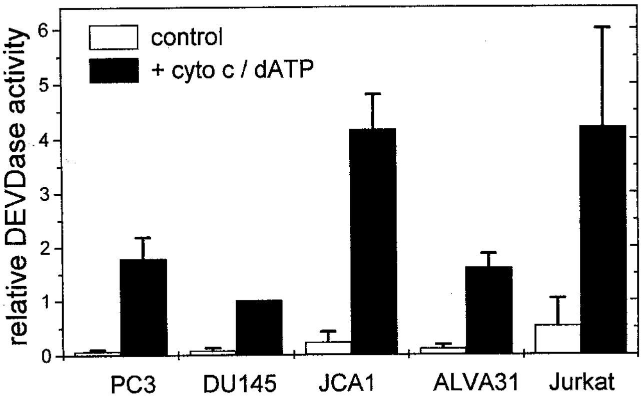

Fig. 3. Cyto c/dATP induces DEVDase activity in cytoplasmic extracts. Cytoplasmic extracts were prepared from untreated prostatic carcinoma cells and incubated for 45 min in the absence (control) or presence of cyto c/dATP (5 μM/1 mM) at 37°C. After incubation, caspase activity was determined with caspase-3-like fluorogenic substrate (Ac-DEVD-AMC). The values were normalized to the value obtained for activated DU145 extracts. Values shown are the means of four experiments; *bars,* SD. DEVDase activity for Jurkat extracts is shown for comparison.

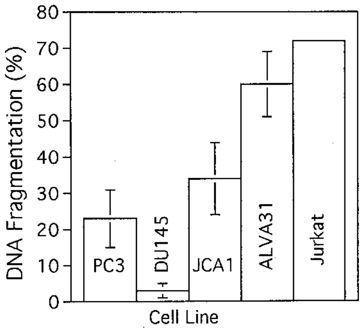

Fig. 4. cyto c-activated extracts induce DNA fragmentation in isolated nuclei. Prelabeled ALVA31 nuclei were isolated and reconstituted in the cell-free system by incubating 50,000 nuclei in cytoplasmic cell extracts (75 μg of protein) with or without cyto c/dATP for 3–4 h at 37°C. After incubation, the nuclei were harvested through a glass fiber membrane, and retained DNA was quantified by scintillation counting. Three to five identical reactions were always run in parallel to account for experimental variations of the input number of nuclei. By comparison of the values obtained in stimulated and nonstimulated extracts, the percentage of nuclear fragmentation was calculated. The presented values in this diagram are the means of eight (PC3), seven (DU145), two (JCA1), and six (ALVA31) independent experiments; *bars,* SD. The fragmentation activity of activated Jurkat extracts as a positive reference control was only determined once for comparison.

and JCA1 with cyto c or active caspase-8 induced nuclear apoptotic activity, whereas DU145 extracts were inactive.

No Evidence Was Found for an Inhibitory Factor in DU145 Extracts. As shown, cyto c-activated extracts from the Fas-resistant cell line DU145 did not induce nuclear apoptotic events in the cell-free system. We performed experiments to determine whether the presence of an inhibitory factor might be responsible for this lack of activity. We added extract from DU145 to cyto c-activated extracts from PC3 and ALVA31. The ability of PC3 and ALVA31 extracts to induce nuclear morphological changes and DNA fragmentation after cyto c stimulation was not inhibited by the addition of DU145 cytoplasmic extract, but as expected, 1 μM of the CPP32 subfamily peptide-inhibitor zDEVD-fmk inhibited morphological changes and DNA laddering (Fig. 5).

In the presence of CHX, DU145 becomes sensitive to Fas-mediated apoptosis (30). Thus, we examined whether extracts from CHX-treated DU145 cells can induce DNA fragmentation upon cyto c stimulation. Extracts from CHX-treated DU145 did not induce DNA fragmentation (data not shown). Thus, there is no evidence for the existence of an inhibitory factor(s) in DU145 extracts that might be responsible for the inactivity of DU145 extracts in the cell-free system.

Activation Status of Caspases in cyto c-stimulated Extracts. cyto c treatment induced caspase activity in the cell extracts from all four cell lines, although there were differences in the level of activity (Fig. 3). Furthermore, remarkable differences in the nuclear apoptotic activity were also detected in the cytoplasmic extracts from the four different cell lines (Fig. 4) which, however, did not correlate with the differences in caspase activity. Therefore, the activation status of individual caspases was determined. Western blotting revealed that caspase-9 was activated in all extracts when cyto c and dATP were added (Fig. 6). We detected comparable expression and activation of caspase-7 in all activated extracts (Fig. 7*A*). In contrast, levels of pro-caspase-3 and its activated p17 subunit varied considerably in extracts from the different cell lines (Fig. 7*B*). High levels of the p17 subunit were detected in JCA1 extracts, moderate levels in PC3 and in ALVA31 extracts, and DU145 extracts displayed the lowest level of active caspase-3.

We could not detect activation of caspase-8 and caspase-10 in the extracts, but we have evidence for the activation of caspase-6, as judged by the decrease of the corresponding proenzyme bands after cyto c stimulation (data not shown).

DISCUSSION

cyto c is released from the mitochondrial intermembrane space into the cytosol after the induction of apoptosis by many different stimuli (5, 38). Fas ligation has also been found to result in cyto c release (29, 39), although not in all reports (40–42). Most studies have used hematopoietic cell lines to examine the involvement and mechanisms of cyto c release in the apoptotic process, whereas there are few reports concerning cyto c release during apoptosis in epithelial cells. For prostatic carcinoma cell lines, cyto c release has been detected in response to stimuli such as staurosporine (43), phosphatidylinositol 3′-kinase inhibitors (44), anticancer drugs, and tumor necrosis factor-α (45). However, it also has been reported that certain drugs induce apoptosis in PC3 but do not trigger cyto c release, suggesting that cyto c release is an inducer-dependent phenomenon (45).

In this study, we examined whether cyto c is involved in Fas-mediated apoptosis of human prostatic carcinoma cell lines and investigated the cyto c signaling pathway in these cell lines. We found that after Fas ligation, cyto c is released into the cytosol in PC3 and ALVA31 (Fig. 1*A*). cyto c could be detected in the cytosol as early as 2 h after Fas ligation. We have shown previously that in the Fas-sensitive prostatic carcinoma cell lines PC3 and ALVA31, apoptotic features such as phosphatidylserine exposure and DNA fragmentation occur after at least 6 h of treatment with agonistic anti-Fas mAb (30). Additionally, we have demonstrated that in PC3 cells, activation of caspase-8 occurs between 1 and 2 h of anti-Fas treatment, and activation of caspase-7 occurs after 4 h of treatment (31). Detection of increased cyto c levels in the cytosol after 2 h of Fas ligation indicates that cyto c release is one of the early events in Fas-mediated apoptosis in prostatic carcinoma cell lines. We did not detect cytosolic cyto c increase in the Fas-resistant cell line DU145 after treatment with anti-Fas mAb, but cyto c release was observed after 4–8 h of combined treatment of DU145 with anti-Fas mAb and CHX (Fig. 1*A*). Because cyto c release in Fas-mediated apoptosis is considered to be mediated by activated caspase-8 (20, 36), this time course is consistent with our previous finding that caspase-8 is activated after only ~4 h of combined treatment with anti-Fas mAb and CHX (31). These results also support our hypothesis that the putative inhibitory factor(s), which is responsible for Fas resistance in DU145, acts at the apex of the cascade, presumably at the level of caspase-8 activation (31).

Recent studies of Fas-mediated apoptosis have implicated the cleavage of Bid by caspase-8, resulting in the translocation of the

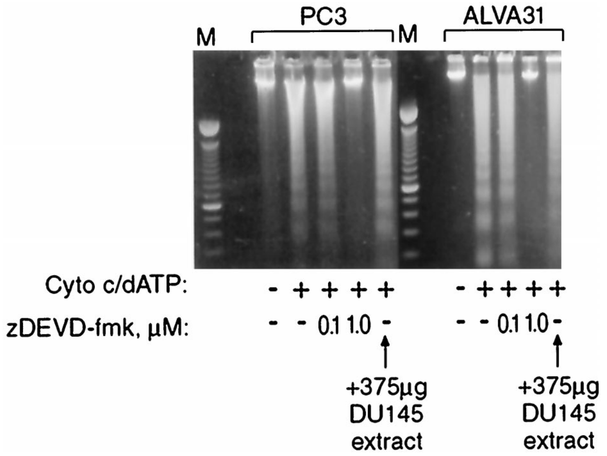

Fig. 5. DU145 extract does not inhibit DNA laddering activity of PC3 and ALVA31 extracts. Jurkat nuclei were incubated for 3–4 h at 37°C in cyto c-activated cytoplasmic extracts (375 μg protein) from PC3 or ALVA31 in the presence or absence of DU145 extract (375 μg protein) or in the presence or absence of zDEVD-fmk (0.1 and 1.0 μM). After incubation, genomic DNA was isolated from the nuclei and separated on 1.5% agarose gels. DNA was detected with ethidium bromide.

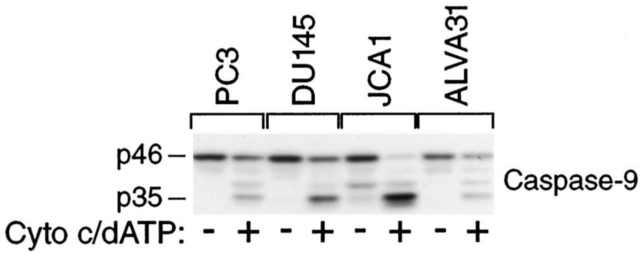

Fig. 6. Caspase-9 is activated by cyto c/dATP in all cytoplasmic extracts examined. Cytoplasmic extracts (75 μg protein) were incubated in the presence or absence of cyto c/dATP (5 μM/1 mM) at 37°C for 60 min. After incubation, equal amounts (20 μg) of total cytoplasmic protein were loaded onto SDS-PAGE (4–20%), and Western blots were performed using antibodies against caspase-9 (Oncogene). This antibody recognized the proenzyme band at M_r 46,000 and the active form at M_r 35,000.

truncated Bid to the mitochondria, where it induces the release of cyto c (20, 36). In this report, we demonstrate that Bid is processed after Fas-mediated activation of caspase-8 in PC3, DU145, and ALVA31. In PC3, Bid cleavage and cyto c release can be inhibited by the caspase inhibitor zVAD-fmk (Fig. 1*A* and Fig. 2*B*). The primary target of zVAD-fmk presumably is the inhibition of caspase-8 activation prior to any perturbation of mitochondria, resulting in the inhibition of all downstream biochemical effects including cyto c release (38). This result suggests that after Fas ligation and activation of caspase-8, Bid is cleaved by caspase-8, and the resulting truncated Bid triggers cyto c release from the mitochondria into the cytosol of prostatic carcinoma cell lines. However, it cannot be entirely ruled out that caspase-8 first activates downstream caspases such as caspase-7, which then induces cyto c release by activating cytosolic factors other than Bid, as was reported recently (29).

After cyto c release into the cytosol, caspase-9 is activated by the formation of the apoptosome (7), and by this the executioner phase of apoptosis is initiated, *e.g.,* by the activation of caspase-7, as has been reported previously in Fas-mediated apoptosis of prostatic carcinoma cell lines (31). In fact, we were able to detect the processing of caspase-9 after Fas ligation (Fig. 1*B*).

Cell-free systems have been successfully applied previously in the dissection of biochemical mechanisms during the apoptotic process, such as the identification and characterization of the “apoptosome” (35), apoptosis-inducing factor (13), and the DNA fragmentation factor ICAD (inhibitor of caspase-activated DNase; Ref. 46). It has become clear that the apoptotic pathways acting in the cytoplasm function independently from the nucleus, and thus cell-free systems appear to be appropriate model systems that represent at least part of the apoptotic machinery and signaling mechanisms (47–49).

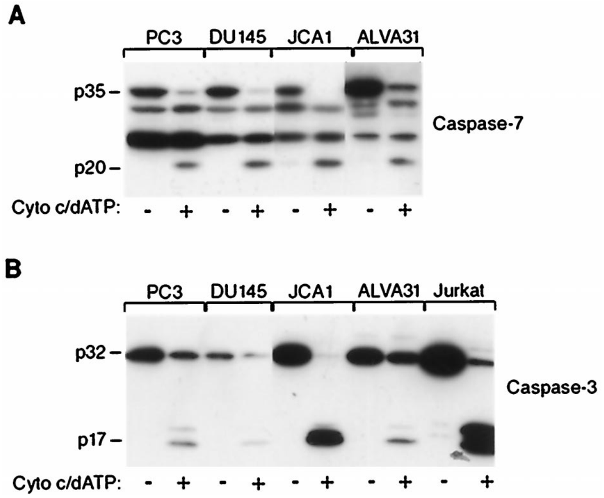

Fig. 7. Western blot analysis of caspase-3 and caspase-7 in cyto c-activated cytoplasmic extracts. Cytoplasmic extracts (75 μg protein) were incubated in the presence or absence of cyto c/dATP (5 μM/1 mM) at 37°C for 90 min. After incubation, equal amounts (20 μg) of total cytoplasmic protein were loaded onto SDS-PAGE (4–20%), and Western blots were performed using antibodies against caspase-7 (*A*; recognizes pro-caspase-7 at p35 kDa and active p20 subunit) and caspase-3 (*B*; recognizes pro-caspase-3 at p32 kDa and active p17 subunit). For the caspase-3 blot, Jurkat extracts were loaded for comparison.

We used a cell-free system to determine whether cyto c can trigger apoptotic activity in the cytosol of the prostatic cancer cell lines. Incubation of isolated nuclei together with cyto c-activated extracts from PC3, JCA1, and ALVA31 resulted in nuclear apoptotic features such as characteristic morphological changes and DNA fragmentation (Fig. 4). Thus, in these cell lines, the pathways leading from cyto c release to the execution of nuclear apoptosis appear to be intact. The degree of DNA fragmentation induced by activated extracts from the different cell lines varied considerably. Caspase-3-like activity was essential for the induction of DNA fragmentation in isolated nuclei because we found its complete inhibition by 1 μM zDEVD-fmk (Fig. 5). However, the level of caspase activity in a certain cell extract was not correlated with the degree of DNA fragmentation induced by this extract. For example, cyto c-activated JCA1 extracts exhibited a high level of active caspase-3 (Fig. 7*B*) and displayed high DEVDase activity (Fig. 3) but induced only moderate DNA fragmentation in nuclei (Fig. 4), whereas ALVA31 extracts induced strong DNA fragmentation activity but had much lower levels of active caspase-3. Thus, additional factors other than just the level of activated caspase-3 appear to influence the capacity of cytoplasmic extracts to induce DNA fragmentation in the cell-free system.

It should be mentioned that thus far we have been unable to detect activated caspase-3 in extracts from PC3 and DU145 treated with anti-Fas or anti-Fas/CHX in culture (31). However, we report here that cell-free activation of cytoplasmic extracts with cyto c (Fig. 7*B*) and also with active recombinant caspase-8 (not shown) does result in activated caspase-3. Because caspase activation in cell-free systems apparently recapitulates the selectivity observed in treated intact cells (49), our cell-free experiments might indicate that caspase-3 is activated during Fas-mediated apoptosis in PC3 and DU145 on a low level that can be detected under cell-free conditions but not in extracts from anti-Fas-treated cells because of a lack of detection sensitivity.

Consistent with previous studies (31, 43), the cell-free experiments show that caspase-7 is expressed at similar levels and is activated at a high level in all prostate carcinoma cells examined (Fig. 7*A*) and thus appears to be the dominant executioner caspase in these cell lines. However, high levels of active caspase-7 are obviously not sufficient to confer nuclear apoptotic activity because, for instance, stimulated DU145 extracts possess high levels of activated caspase-7 but do not induce oligonucleosomal DNA fragmentation in isolated nuclei.

The inability of cytoplasmic DU145 extracts to trigger nuclear apoptotic events in the cell-free system was shown to not be specific for a certain signaling pathway because neither cyto c nor caspase-8 was found to induce nuclear apoptotic events in cytoplasmic extracts from DU145. In contrast, caspase-8 induced strong nuclear apoptotic activity in extracts from PC3, JCA1, and ALVA31 (data not shown). Mixing experiments did not show any evidence for the presence of an inhibitory factor in DU145 extracts (Fig. 5). Thus, the inactivity of DU145 extracts in the cell-free system has to be attributed to a deficiency of an activity. Because DU145 cells do undergo DNA fragmentation when cultured in the presence of anti-Fas mAb and CHX, the inactivity observed in the cell-free system might be an artifact. Alternatively, the nuclear apoptotic activity in DU145 depends on a factor(s) that is in place after induction of apoptosis in living cells but not after induction of apoptosis in cytoplasmic extracts. A recent study suggests that indeed there might be differences between the activation processes occurring in treated intact cells and in cell-free systems (49).

In conclusion, our data suggest that in prostatic carcinoma cell lines, Fas ligation triggers a cascade that leads from activated caspase-8 over the processing of Bid to the release of cyto c into the cytosol and activation of caspase-9. The release of cyto c is an early event in Fas-mediated apoptosis in prostatic carcinoma cell lines because it can be observed as early as 2 h after Fas ligation. The results from the cell-free system suggest that the apoptotic executioner signaling pathway(s) induced by cyto c is usually in place and comprises the activation of caspase-9, caspase-7, and caspase-3. In the cell-free system, caspase-3-like activity is essential for the nuclear apoptotic activity of extracts exerted on isolated nuclei, but additional factors other than just the level of active executioner caspases appear to influence the capability to induce DNA fragmentation.

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Andreas Gewies, Oskar W. Rokhlin and Michael B. Cohen

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