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Dexamethasone inhibits TNF-α-induced apoptosis and IAP protein downregulation in MCF-7 cells

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1 Exposure of human mammary carcinoma cell line MCF-7 to TNF-α leads to apoptotic cell death within 24 h. In search for apoptosis-preventing signals, we identified glucocorticoids as potent death-preventing compounds. Ten nM dexamethasone provided a significant protective effect whereas 100 nM dexamethasone roughly blocked 80–90% of TNF-α-induced apoptosis.

2 Surprisingly, dexamethasone exerted a protective effect even when supplied several hours after TNF-α. This points to a powerful inhibition of even advanced apoptotic processes by dexamethasone.

3 To further pinpoint the anti-apoptotic glucocorticoid action, we investigated the expression levels of several members of the inhibitors of apoptosis (IAPs) family of proteins in response to TNF-α and dexamethasone. IAP proteins directly block caspase protease activities including caspase-3, caspase-7, and caspase-9. Exposure of MCF-7 cells to TNF caused an extensive downregulation of cIAP1, cIAP2, and XIAP protein levels. The decline of the IAP protein levels temporally paralleled the appearance of apoptotic DNA fragments which started 12–14 h following TNF-α addition and maximal effects were seen within 24 h.

4 Coincubation of cells with TNF-α and dexamethasone potently blocked cIAP1, cIAP2, and XIAP downregulation.

5 TNF-α-mediated IAP protein downregulation was not affected by proteasome inhibitors like lactacystin, ALLN or ALLM, whereas it was blocked by the broad-spectrum caspase inhibitor Z-VAD-fmk which also prevented TNF-α-induced apoptotic cell death. These data suggest that inhibition of IAP downregulation mediated by a caspase proteolytic activity constitutes the anti-apoptotic action of glucocorticoids in MCF-7 carcinoma cells.

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Abbreviations: BIR, baculovirus inhibitor repeat; DISC, death-inducing signalling complex; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FADD, Fas-associated death domain; IAP, inhibitor of apoptosis; LPS, lipopolysaccharide; NAIP, neuronal apoptosis inhibitory protein; NF-κB, nuclear factor κB; PARP, poly(ADP-ribose) polymerase; RIP, receptor interacting protein; TE, Tris-EDTA; TNF-α, tumour necrosis factor α; TNF-RI, tumour necrosis factor receptor I; TRADD, TNF receptor-associated death domain protein; XIAP, X-chromosome-linked inhibitor of apoptosis; Z-Asp-CH_2-DCB, Z-aspartyl-2,6-dichlorobenzoyloxymethylketone; Z-VAD-fmk, Z-valinyl-alanyl-DL-aspartyl-fluoromethylketone

Introduction

The highly versatile cytokine tumour necrosis factor α (TNF-α) is involved in various physiological and pathophysiological processes (Eigler *et al*., 1997). Stimulation of cells with TNF-α generates two seemingly conflicting signals: one that triggers apoptotic cell death and the other that antagonizes apoptotic signalling in part by activating the transcription factor NF-κB (Beg & Baltimore, 1996; van Antwerp *et al*., 1996; Wang *et al*., 1996). The cellular responses initiated by TNF-α occur through the high affinity binding of trimeric TNF-α to two distinct cellular receptors denoted as TNFR1 (p55) and TNFR2 (p75) (Baker & Reddy, 1996). Following trimerization of TNFR1 by TNF-α, apoptosis is activated through the release of silencer death domains and recruitment of a series of death domain-containing proteins such as TRADD, RIP, and FADD, resulting in the formation of the death-inducing signalling complex (DISC) (Lin *et al*., 1999; Kischkel *et al*., 1995). Signalling following DISC formation is forwarded to upstream apoptosis-initiating cascades, resulting in the activation of the initiating death proteases caspase 8 and/or caspase 10, followed by the activation of downstream caspases and the triggering of enhancing pathways. In the latter case, caspase-8-mediated cleavage of Bid, the translocation of cleaved Bid to mitochondria and the subsequent release of cytochrome *c* (Bradham *et al*., 1998) into the cytosol promote proapoptotic events (Gross *et al*., 1999; Luo *et al*., 1998; Li *et al*., 1998). Cytochrome *c* binds to Apaf-1 which recruits caspase-9 and as an active complex initiates cleavage and activation of downstream effector caspases (Rodriguez & Lazebnik, 1999). Once activated, caspases can cleave their substrates and other procaspases to generate

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active subunits. Executioner caspases such as caspase-3 and caspase-7 subsequently cleave distinct cellular proteins such as PARP, lamin, fodrin, and also Bcl-2 (Cryns & Yuan, 1998). Cleavage of distinct cellular proteins then leads to the characteristic morphological changes.

Regulation of apoptotic signalling is achieved in general by distinct protein families such as the Bcl-2 family as well as the IAP protein family. The Bcl-2 family of proteins consists of two subfamilies; pro-apoptotic members such as Bax, Bad, Bik, Bim, or Bcl-x_S which initiate or promote apoptosis-inducing signalling, and anti-apoptotic members such as Bcl-2, Bcl-x_L, Mcl-1, or A1 which block the activation of effector caspases and, therefore, the induction of an apoptotic morphology (Zamzami *et al*., 1998).

Apart from the Bcl-2 family of proteins more recently it has been shown that members of the IAP family of proteins, structurally related to the baculovirus p35 protein, prevent apoptosis induction in part *via* a direct inhibition of caspase-3, -7, and -9 (Deveraux & Reed, 1999; Roy *et al*., 1997). Structurally, human IAPs, which comprise cIAP1, cIAP2, XIAP(=hILP), NAIP, survivin, and Apollon, are characterized by one to three copies of the BIR domain. Human XIAP, cIAP1, and cIAP2 are able to block caspase-3 and -7 activity, however, the exact mechanisms by which IAPs interfere with apoptosis remain unclear. Upregulation of IAP gene expression in mammalian systems is achieved by the activation of NF-κB transcription factor activity in response to TNF-α and is suggested to participate in the anti-apopotic pathways elicited by TNF-α (Stehlik *et al*., 1998; Wang *et al*., 1998). However, regulation of IAP protein and mRNA expression levels are only poorly investigated.

Distinct pharmacological modulation of apoptosis is achieved by glucocorticoids such as dexamethasone. Depending on the cell type, dexamethasone either elicits apoptosis, e.g. in thymocytes or exerts potent anti-apoptotic effects as recently described for different cell types (Kull, 1988; Weller *et al*., 1997; Meßmer *et al*., 1999a, b; 2000). Although dexamethasone was shown to suppress upregulation of pro-apoptotic members of the Bcl-2 family such as Bcl-x_S and Bak (Sakamoto *et al*., 1995; Meßmer *et al*., 2000), and the downregulation of Bcl-x_L, as well as the activation of caspase activities in response to TNF-α (Meßmer *et al*., 2000), the anti-apoptotic mechanism still remains to be fully elucidated.

The present work examined the response of MCF-7 cells to TNF-α with a special focus on the Bcl-2 and the IAP family of proteins. We show that XIAP, cIAP1, and cIAP2 were degraded in response to TNF-α. Moreover, we demonstrate that dexamethasone blocked IAP degradation concomitantly with inhibition of apoptosis induction.

Methods

Materials

Diphenylamine, heparin sodium, lactacystine, ALLN, ALLM, and dexamethasone were purchased from Sigma (Deisenhofen, Germany). Z-valinyl-alanyl-DL-aspartyl-fluoromethylketone (Z-VAD-fmk), and Z-aspartyl-2,6-dichlorobenzoyloxymethylketone (Z-Asp-CH_2-DCB) were delivered by Bachem (Heidelberg, Germany). ECL detection reagents were ordered from Amersham (Braunschweig, Germany). Recombinant human tumour necrosis factor-α (specific activity: 6.6×10^6 units mg^{-1}) was a generous gift from Knoll AG, Germany. RPMI 1640, cell culture supplements and foetal calf serum were from Gibco (Eggenstein, Germany). All other chemicals were of the highest grade of purity commercially available.

Cell culture and cell treatment

MCF-7 cells were cultured in DMEM medium containing 25 mM HEPES, 1000 mg l^{-1} glucose, pyridoxine, 5% foetal calf serum, penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), 2 mM N-acetyl-L-alanyl-L-glutamine and non-essential amino acids (delivered by Biochrom, Berlin, Germany). Subconfluent cells were incubated as indicated in the same medium.

Bovine glomerular endothelial cells were cultivated as described previously (Briner & Kern, 1994). In brief, approximately 10 g of renal cortex tissue were minced, passed through a sterile 240 μm stainless steel sieve, and suspended in HBSS. This suspension was then poured through a 180 μm stainless sieve followed by a 100 μm mesh. The glomeruli retained by the 100 μm sieve were washed three times in HBSS and were then incubated for 10 to 15 min at 37°C in HBSS containing 1 mg ml^{-1} collagenase (type V, Sigma, Deisenhofen, Germany). After digestion, glomerular remnants were sedimented at $500\times g$ for 5 min. The supernatant was centrifuged at $1000\times g$ for 5 min, and the pellet was suspended in RPMI 1640 medium containing 20% FCS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} heparin sodium, and 5 ng ml^{-1} of acidic fibroblast growth factor. Cells were plated on 0.2% gelatin-coated tissue culture plates. Primary cultures of endothelial cell clones were isolated with cloning cylinders, detached with trypsin-EDTA, and passaged at cloning density onto gelatin-coated 35-mm diameter plates. Individual clones of endothelial cells were characterized by positive staining for Factor VIII-related antigen and uniform uptake of fluorescent acetylated low-density lipoproteins (Ballermann, 1989). Negative staining for smooth muscle actin and cytokeratin excluded mesangial cell and epithelial cell contaminations, respectively. For the experiments, passages 9 to 19 of endothelial cells were used.

For experiments, endothelial cells were grown to confluency in 60-mm or 100-mm petri-dishes with RPMI 1640 medium containing 15% FCS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} heparin sodium, and 5 ng ml^{-1} of acidic fibroblast growth factor and incubated in RPMI 1640 containing 2% FCS, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

Morphological investigations

MCF-7 cells were grown in 60-mm culture plates to nearly confluency. Cells were stimulated, followed by fixation with 3% paraformaldehyde for 5 min onto glass slides. Samples were washed with phosphate-buffered saline, stained with Hoechst dye H33258 (8 μg ml^{-1}) for 5 min, washed with distilled water, and mounted in KAISER'S glycerol gelatin. Nuclei were visualized using a Zeiss Axiovert fluorescence microscope. For each preparation around 500 cells were counted by two different investigators which were blinded

toward the treatment. Each evaluation was repeated three times by three independent experiments.

Quantitation of DNA fragmentation

DNA fragmentation was essentially assayed as reported previously (Meßmer *et al*., 1998). Briefly, after incubation, cells were scraped off the culture plates, resuspended in 250 μl of 10 mM Tris containing 1 mM EDTA, pH 8.0 (TE-buffer), and incubated with an additional volume lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 4°C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at $13{,}000 \times g$. Pellets were resuspended in 500 μl TE-buffer and samples were precipitated by adding 500 μl 10% trichloroacetic acid at 4°C. Samples were pelleted at 4000 r.p.m. for 10 min and the supernatant was removed. After addition of 300 μl 5% trichloroacetic acid, samples were boiled for 15 min. DNA contents were quantitated using the diphenylamine reagent (Burton, 1956). The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

Western blot analysis

Cells were cultured and incubated as described. In general, cell lysis was achieved with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0) and sonication (Branson sonifier; 10 s, duty cycle 100%, output control 10%), followed by centrifugation ($4000 \times g$, 5 min), and Bradford protein determination (Bradford, 1976). For the analysis of mitochondrial cytochrome *c* efflux MCF-7 cells were harvested by trypsinization, pelleted by centrifugation, resuspended in 300 μl of homogenization buffer (mM): HEPES 20, pH 7.5, KCl 10, $MgCl_2$ 1.5, EDTA 1, EGTA 1, DTT 1, Pefabloc 4, 5 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, sucrose 250 and incubated for 10 min on ice. Cells were broken by 2×15 passages through a syringe fitted with a 25-gauge needle. The lysate was centrifuged at $750 \times g$ for 10 min at 4°C to pellet nuclei. The remaining supernatant was centrifuged for 15 min at $10{,}000 \times g$, the pellet was used as mitochondrial fraction and the supernatant as cytosolic fraction.

Proteins were normalized to 40 μg lane^{-1} (Bcl-2 proteins) or to 50 μg lane^{-1} (IAP proteins), resolved on 7.5% (cIAP1, cIAP2), 10% (XIAP), 12.5% (Bcl-2 proteins) or 14% (cytochrome *c*) polyacrylamide gels and blotted onto PVDF sheets. Sheets were washed twice with TBS (140 mM NaCl, 50 mM Tris, pH 7.2) containing 0.1% Tween-20 before blocking unspecific binding with TBS/5% skim milk. Filters were incubated either with the mouse anti-cytochrome *c* antibody (clone 7H8.2C12, PharMingen, 1 μg ml^{-1} in TBS/2% skim milk/0.7% FCS), rabbit anti-hILP (=XIAP) antibody (Transduction Laboratories, clone 48, 1 μg ml^{-1} in TBS/0.5% skim milk), rabbit anti-cIAP1 antibody (Santa Cruz, clone H-83, 1 μg ml^{-1}, in TBS + 0.5% skim milk), rabbit anti-cIAP2 antibody (R&D, 1 μg ml^{-1}, in TBS + 0.5% skim milk), mouse anti-Bcl-2 antibody (Immunotech, clone 83-8B, 1 μg ml^{-1}), rabbit anti-Bcl-x antibody (Transduction Laboratories, 1 : 1000 in TBS + 0.5% skim milk), mouse anti-Bad antibody (Transduction Laboratories, 1 : 500 in TBS + 0.5% skim milk), rabbit anti-Bax antibody raised against a peptide MDGSGEQPRGGGPTSSEQIMK coupled to KLH by the MBS method (1 : 2000 in TBS + 0.5% skim milk), rabbit anti-Bak antibody raised against a peptide WIARGGWVAALNLG coupled to KLH by the MBS method (1 : 1500 in TBS + 0.5% skim milk), and rabbit antiserum raised against human erythrocyte GAPDH (Dimmeler *et al*., 1993) overnight at 4°C. Sheets were washed five times and unspecific binding was blocked as described. Detection was by horseradish peroxidase-conjugated goat anti-mouse monoclonal antibodies (1 : 5000) or goat anti-rabbit monoclonal antibodies (1 : 5000) for 1.5 h at room temperature using the ECL method (Amersham). The primary bak and bax antibodies were tested by comparing with antibodies commercially available (Santa Cruz clone P-19 anti-bax; Calbiochem Ab-2 anti-bak) using mouse and human cell preparations (RAW 264.7 and U937). The antibodies exhibited no cross-reactivity with other Bcl-2 family members.

Statistical analysis

Each experiment was performed at least three times and statistical analysis were performed using the two tailed Student's *t*-test or ANOVA and for multiple comparison the data were corrected by Dunn's Method.

Results

Dexamethasone blocks TNF-α-induced apoptosis

The human breast cancer cell line MCF-7 has been used as a model system in many different studies concerning apoptosis signal transduction. These cells die by apoptotic cell death in response to TNF-α (Mueller *et al*., 1996). To investigate the effects of glucocorticoids on TNF-α-induced MCF-7 cell apoptosis we first focused on the formation of apoptotic DNA fragmentation by using a colorimetric assay and the quantification of morphological alterations by microscopy. As shown in Figure 1A, 1 ng ml^{-1} TNF-α was required to initiate apoptosis induction within 24 h and 50 ng ml^{-1} of TNF-α induced a maximal response resulting in DNA cleavage in approximately 30% of the cells. Comparing these results with morphological investigations revealed that around 60% of all MCF-7 cells exhibited an apoptotic morphology within 24 h when exposed to 25 or 50 ng ml^{-1} TNF-α (Figure 1B) Dexamethasone concentration-dependently blocked TNF-α-induced apoptosis (Figure 1A,B). A significant inhibition was achieved with 10 nM dexamethasone, whereas 100 nM dexamethasone exerted approximately a 60% inhibitory effect on DNA cleavage as well as on the appearance of apoptotic cells.

Time kinetic studies clearly showed significant biochemical and morphological changes indicative of apoptosis such as low molecular weight DNA fragmentation and nuclear condensation, that were detectable as early as 14–15 h following TNF-α addition. Cotreatment of MCF-7 cells with TNF-α and 100 nM dexamethasone revealed that dexamethasone exerted its inhibitory effects throughout the time course investigated, showing that glucocorticoids act continuously on TNF-α-mediated apoptotic signal transduction.

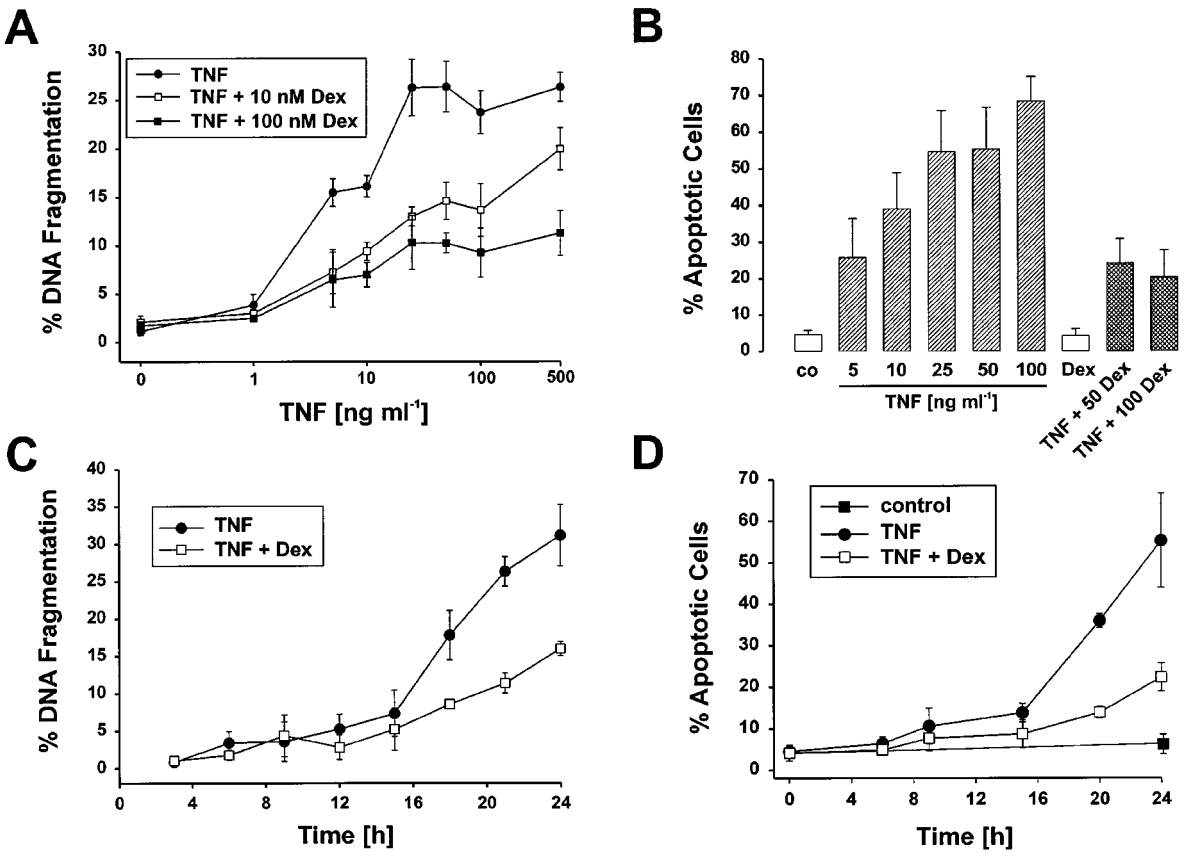

Figure 1 Dexamethasone blocks TNF-α-induced apoptotic DNA fragmentation and the appearance of cells exhibiting apoptotic morphology in MCF-7 cells. (A) MCF-7 cells were exposed for 24 h to (A) different concentrations of TNF-α in the absence or presence of either 10 nM or 100 nM dexamethasone, (B) various concentrations of TNF-α or 50 ng ml^{-1} TNF-α (TNF) plus 50 or 100 nM dexamethasone (50 Dex or 100 Dex, respectively) or 100 nM dexamethasone (Dex), (C,D) either 50 ng ml^{-1} TNF-α or 50 ng ml^{-1} TNF-α plus 100 nM dexamethasone. DNA fragmentation was determined by using the diphenylamine reaction (A,C) or apoptotic cells exhibiting characteristic chromatin condensation were counted by fluorescence microscopy (B,D). Data are means ± standard deviations of four individual experiments.

Next, we wanted to know whether dexamethasone had to be supplied at the same time as TNF-α or whether it would also be able to influence cell death signalling when added several hours after TNF-α. As shown in Figure 2, dexamethasone was still able to exert significant anti-apoptotic effects even when it was added 12 h following TNF-α and it still had a maximal inhibitory effect when added up to 6 h after TNF-α. To test whether dexamethasone pretreatment may enhance its anti-apoptotic potency, MCF-7 cells were pretreated for 2, 4 or 6 h with 100 nM dexamethasone followed by a 24 h treatment with 50 ng ml^{-1} TNF-α. As shown in Figure 2, dexamethasone pretreatment did not enhance its anti-apoptotic potency as compared to a cotreatment schedule.

Bid cleavage and mitochondrial cytochrome c*-release in TNF-α-induced apoptosis*

In order to define molecular targets for dexamethasone action, we analysed several apoptotic signalling pathways. First, an efflux of cytochrome *c* from the inner mitochondrial membrane space into the cytosol and alterations in mitochondrial function in general are proposed to be early apoptotic signals (Gross *et al*., 1999). We determined cytosolic cytochrome *c* protein levels by Western blotting. As shown in Figure 3A, in cytosolic preparations of control cells, only very low levels of cytochrome *c* were detectable. TNF-α induced mitochondrial cytochrome *c* efflux within 4–8 h which preceded apoptotic DNA fragmentation. Dexamethasone potently blocked TNF-α-induced cytochrome *c* efflux (Figure 3A). To exclude an interference of dexamethasone with cytochrome *c* protein expression, we performed cytochrome *c* Western blots in whole cell extracts. These experiments showed no significant changes in cytochrome *c* protein levels within the critical time periods (data not shown).

To investigate the impact of the very important Bcl-2 family of proteins, we first evaluated Bid cleavage. Bid normally gets cleaved by a caspase-8-dependent process and cleaved Bid elicits mitochondrial cytochrome *c* release. As shown in Figure 3A, TNF-α initiated Bid cleavage which started 9–14 h after TNF-α addition whereas dexamethasone blocked this process similar to cytochrome *c* efflux. However, contrary to many other experimental models, in MCF-7 cells cytochrome *c* release preceded Bid cleavage.

Next, we investigated whether gene products that may either promote cell survival, such as Bcl-2 and Bcl-x_L, or accelerate cell death, such as Bad, Bax, and Bak are involved in TNF-α-mediated apoptosis. To this end we determined protein levels

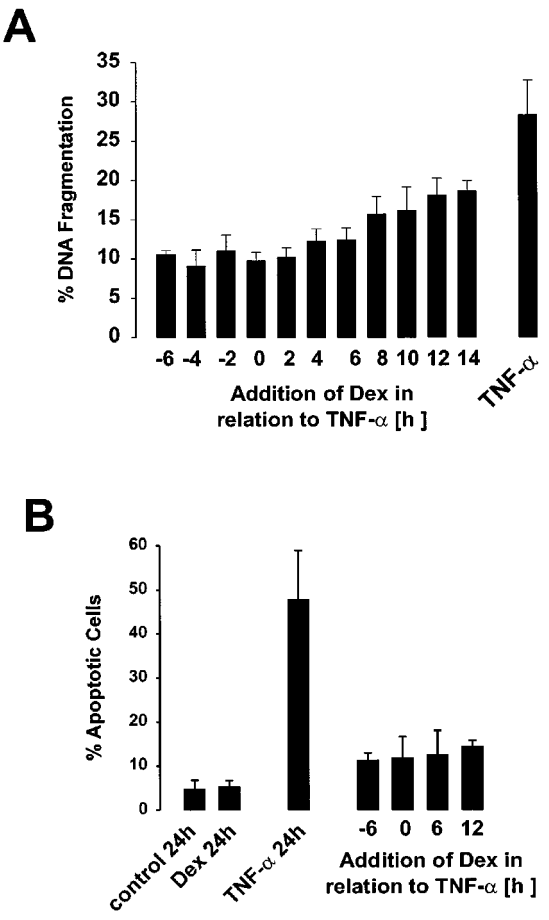

Figure 2 Time dependency of dexamethasone protection. MCF-7 cells were cultured as described in the experimental section and stimulated for 24 h with 50 ng ml^{-1} TNF-α. 100 nM dexamethasone were added at various times in relation to TNF-α application starting 6 h before the apoptotic inducer till 14 h after TNF-α application (TNF-α was added at time point 0) DNA fragmentation was determined by using the diphenylamine assay (A) while apoptotic cells exhibiting characteristic chromatin condensation were counted by fluorescence microscopy (B) Values are means±standard deviation out of three independent experiments.

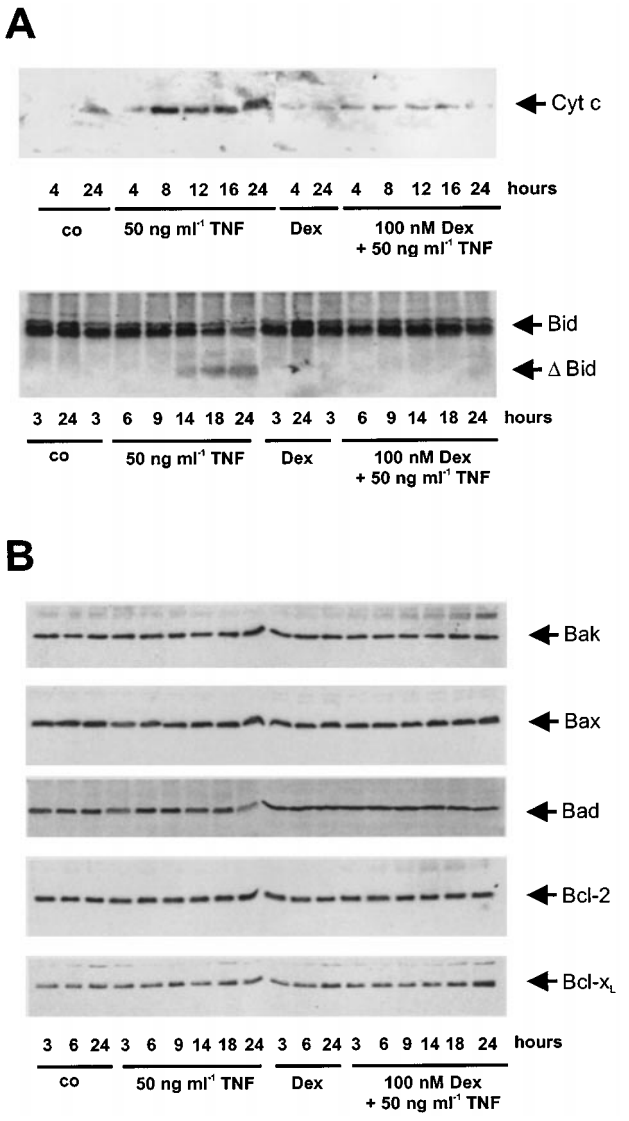

Figure 3 Effects of TNF-α and dexamethasone on Bcl-2-like protein expression and mitochondrial cytochrome *c* release. (A) MCF-7 cells were incubated with 50 ng ml^{-1} TNF-α, 100 nM dexamethasone plus 50 ng ml^{-1} TNF-α, 100 nM dexamethasone (Dex), or vehicle (control) for the times indicated. The determination of Bid cleavage was done by Western blot analysis whereas for the determination of cytochrome *c* release MCF-7 cells were harvested by trypsinization, and a cytosolic extract was prepared as described in the method section. The samples were then subjected to Western blot analysis for cytochrome *c*. (B) Bak, Bax, Bad, Bcl-2, and Bcl-x_L protein levels were determined by Western blot analysis. Values are representative of three individual experiments.

by exposing cells to TNF-α or TNF-α/dexamethasone for different time periods. Contrary to Bid, neither TNF-α nor dexamethasone significantly elicited changes in the protein levels of Bak, Bax, Bad, Bcl-2 or Bcl-x_L (Figure 3B).

IAP protein degradation during TNF-α-induced apoptosis is blocked by dexamethasone

Besides Bcl-2 and Bcl-2-related anti-apoptotic factors, the IAP family of proteins are important regulators of apoptotic signalling cascades by blocking caspase activities. Therefore, IAPs may potentially be targets for dexamethasone action. To determine the fate of cIAP1, cIAP2, and XIAP, MCF-7 cells were stimulated with TNF-α for different time periods. After stimulation, whole cell lysates were prepared and analysed by Western blotting using different antibodies against the individual IAP proteins. As expected, full-length cIAP1 was detected as a protein with approximately 70 kDa (Figure 4A). Within 9–14 h after TNF-α addition, the level of full-length cIAP1 started to decline. Apparently, a 53 kDa protein fragment appeared that was recognized by the anti-cIAP1 antibody. The appearance of the 53 kDa fragment started after 9 h. Simultaneous addition of dexamethasone potently blocked the disappearance of the full-length cIAP1 protein and concomitantly the appearance of the 53 kDa cleavage fragment (Figure 4A). Comparable results were obtained for cIAP2 which is cleaved in response to TNF-α in a similar fashion. An approximately 55 kDa cleavage fragment appeared concomitantly with the disappearance of the full-length cIAP2 protein. Dexamethasone also potently blocked cIAP2 cleavage and the appearance of the 55 kDa

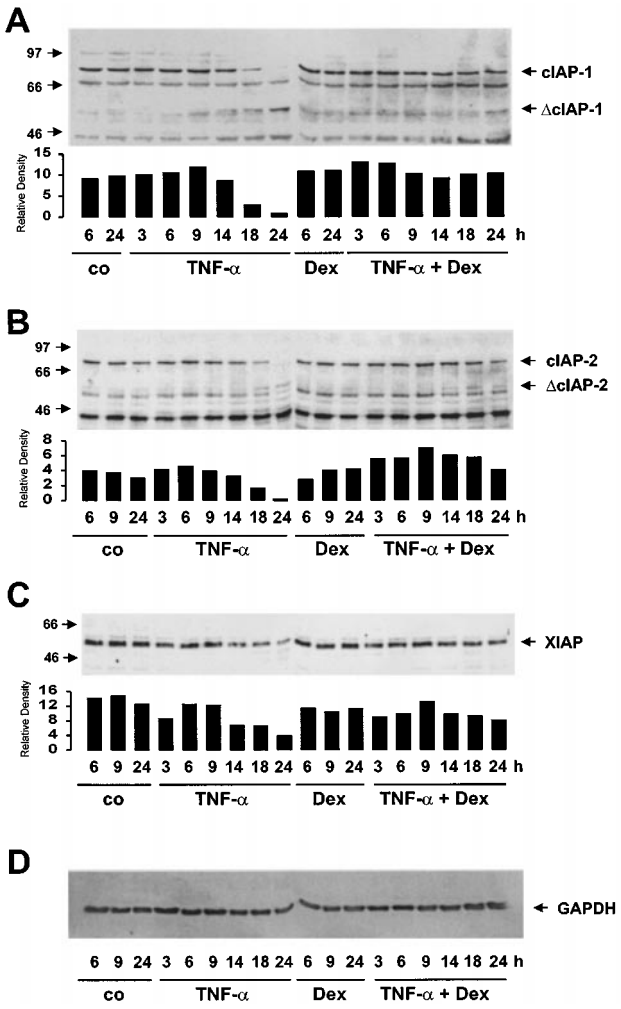

Figure 4 TNF-α mediates cIAP1, cIAP2, and XIAP protein cleavage concomitant with apoptotic cell death induction: inhibition by dexamethasone. Cells were treated for the times indicated with 50 ng ml^{-1} TNF-α (TNF-α), 100 nM dexamethasone (Dex), 50 ng ml^{-1} TNF-α plus 100 nM dexamethasone (TNF-α + Dex), or vehicle as a control (co). Proteins were normalized to 50 μg lane^{-1} and cell lysates were subjected to Western blot analysis for cIAP1 (A), cIAP2 (B), XIAP (C), and GAPDH (D) proteins followed by ECL detection. Each blot is representative of three similar experiments.

cleavage fragment. Regarding XIAP protein expression, we detected a reduction in the expression of full-length XIAP protein starting 9–14 h after TNF-α addition and an inhibition of this process by dexamethasone (Figure 4C). XIAP cleavage fragments were not detected by our antibody.

To verify that cIAP1 cleavage is a general feature of proapoptotic pathways, we determined cIAP1 protein levels in bovine glomerular endothelial cells which undergo apoptotic cell death in response to TNF-α, as well as to bacterial LPS (Meßmer *et al*., 1999b). Glomerular endothelial cells exposed to 30 ng ml^{-1} LPS or 10 ng ml^{-1} TNF-α die by apoptosis within 15–24 h following apoptogen addition. With the same time course, cIAP1 full-length protein was downregulated (Figure 5). In contrast to human MCF-7 cells, in bovine glomerular endothelial cells the antibody only recognized bovine full-length protein but not a 53 kDa cleavage product.

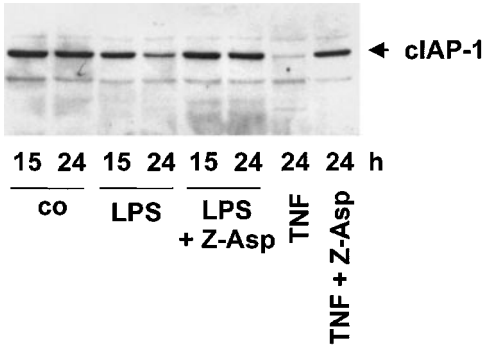

Figure 5 TNF-α and LPS mediate caspase-dependent cIAP1 protein cleavage concomitant with apoptotic cell death induction in glomerular endothelial cells. Bovine glomerular endothelial cells were treated for the times indicated with 30 ng ml^{-1} LPS, 30 ng ml^{-1} LPS plus 100 μM Z-Asp-CH_2-DCB (Z-Asp), 25 ng ml^{-1} TNF-α (TNF-α), or 25 ng ml^{-1} TNF-α plus 100 μM Z-Asp-CH_2-DCB (Z-Asp), or vehicle as a control (co). Proteins were normalized to 50 μg lane^{-1} and cell lysates were subjected to Western blot analysis for cIAP1 protein followed by ECL detection. The blot is representative of three similar experiments.

Effects of serine- and caspase-protease inhibitors on IAP protein degradation

A number of intracellular proteins were cleaved by caspase proteases during apoptosis, including PARP, Bid, and Bcl-2 (Cryns & Yuan, 1998). As IAPs directly bind and inhibit caspase-3, -7, and -9, we evaluated whether IAP cleavage was mediated by caspase proteases. Glomerular endothelial cells were treated with TNF-α or LPS in the presence of Z-Asp-CH_2-DCB, a broad-spectrum caspase-inhibitor which significantly blocked glomerular endothelial cell apoptosis (Meßmer *et al*., 1999a). As shown in Figure 5, Z-Asp-CH_2-DCB completely abrogated cIAP1 protein downregulation in response to both stimuli.

Similar experiments using two different broad-spectrum caspase-inhibitors (Z-Asp-CH_2-DCB and Z-VAD-fmk) were also performed with human MCF-7 cells. First, we determined the impact of the caspase inhibitors on apoptotic DNA fragmentation. As shown in Figure 6A, Z-VAD-fmk completely blocked TNF-α-induced DNA cleavage whereas Z-Asp-CH_2-DCB was ineffective. Consequently, only Z-VAD-fmk abrogated cIAP1 (Figure 6B,C), cIAP2 (Figure 6D), and XIAP cleavage (Figure 6E) whereas Z-Asp-CH_2-DCB was ineffective. This indicates that IAP cleavage was mediated by caspases in glomerular endothelial cells as well as MCF-7 cells.

Moreover, serine proteases and especially the ubiquitin-proteolytic pathway are also major systems for selective protein degradation in eucaryotic cells (Ciechanover *et al*., 2000). To investigate whether IAP degradation may also depend on the ubiquitin proteolytic pathway we used lactacystine, ALLN, ALLM, and MG132 as protease inhibitors. To test the impact of these inhibitors on TNF-α-mediated cell death induction, MCF-7 cells were exposed to TNF-α in the presence of different concentrations of lactacystine (Figure 7A), ALLN, ALLM, MG132 (data not shown) and determined the extent of DNA fragmentation. However, neither lactacystine (Figure 7A), nor ALLN,

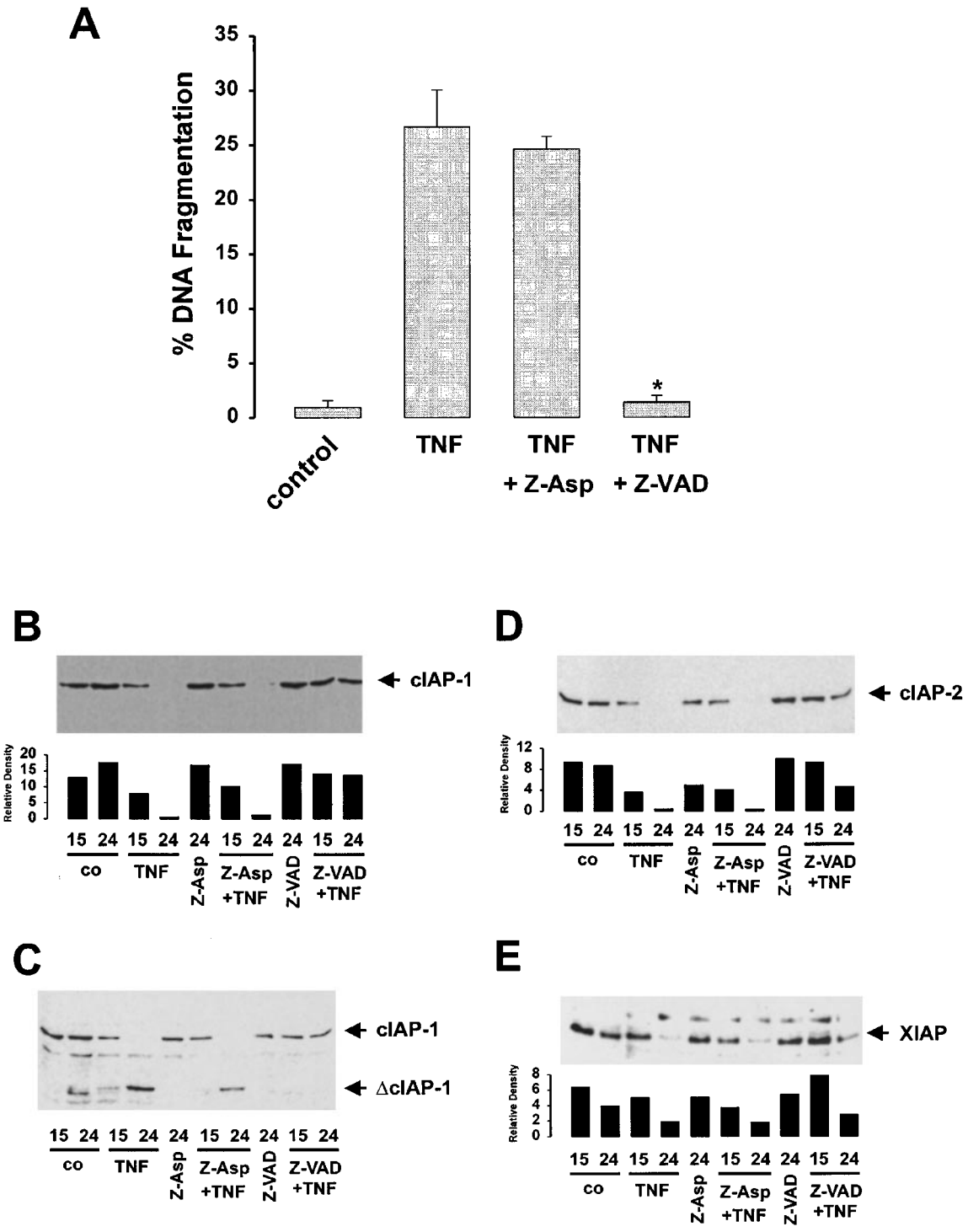

Figure 6 IAP protein cleavage is blocked by the caspase protease inhibitor Z-VAD-fmk. (A) MCF-7 cells were treated for 24 h with 50 ng ml^{-1} TNF-α, 50 ng ml^{-1} TNF-α plus 100 μM Z-Asp-CH_2-DCB or 100 μM Z-VAD-fmk or vehicle (control) as indicated. DNA fragmentation was quantified by using the diphenylamine assay. (B–E) Cells were exposed for various times to 50 ng ml^{-1} TNF-α or to 50 ng ml^{-1} TNF-α plus 100 μM Z-Asp-CH_2-DCB or 100 μM Z-VAD-fmk, or vehicle as a control (co) as indicated. Subsequently, proteins were normalized to 50 μg lane^{-1} and cell lysates were subjected to Western blot analysis for cIAP1 using the antibody from R&D Systems or from Santa Cruz (B and C, respectively), cIAP2 (D) or XIAP proteins (E) followed by ECL detection and video densitometry. Each blot is representative of three similar experiments.

ALLM, MG132 showed any effect on TNF-α-induced MCF-7 apoptosis. In line with these observations the protease inhibitors also failed to influence cIAP1, cIAP2 or XIAP protein degradation (Figure 7B).

Discussion

We and others have documented, that MCF-7 breast carcinoma cells die in response to TNF-α within 24 h by

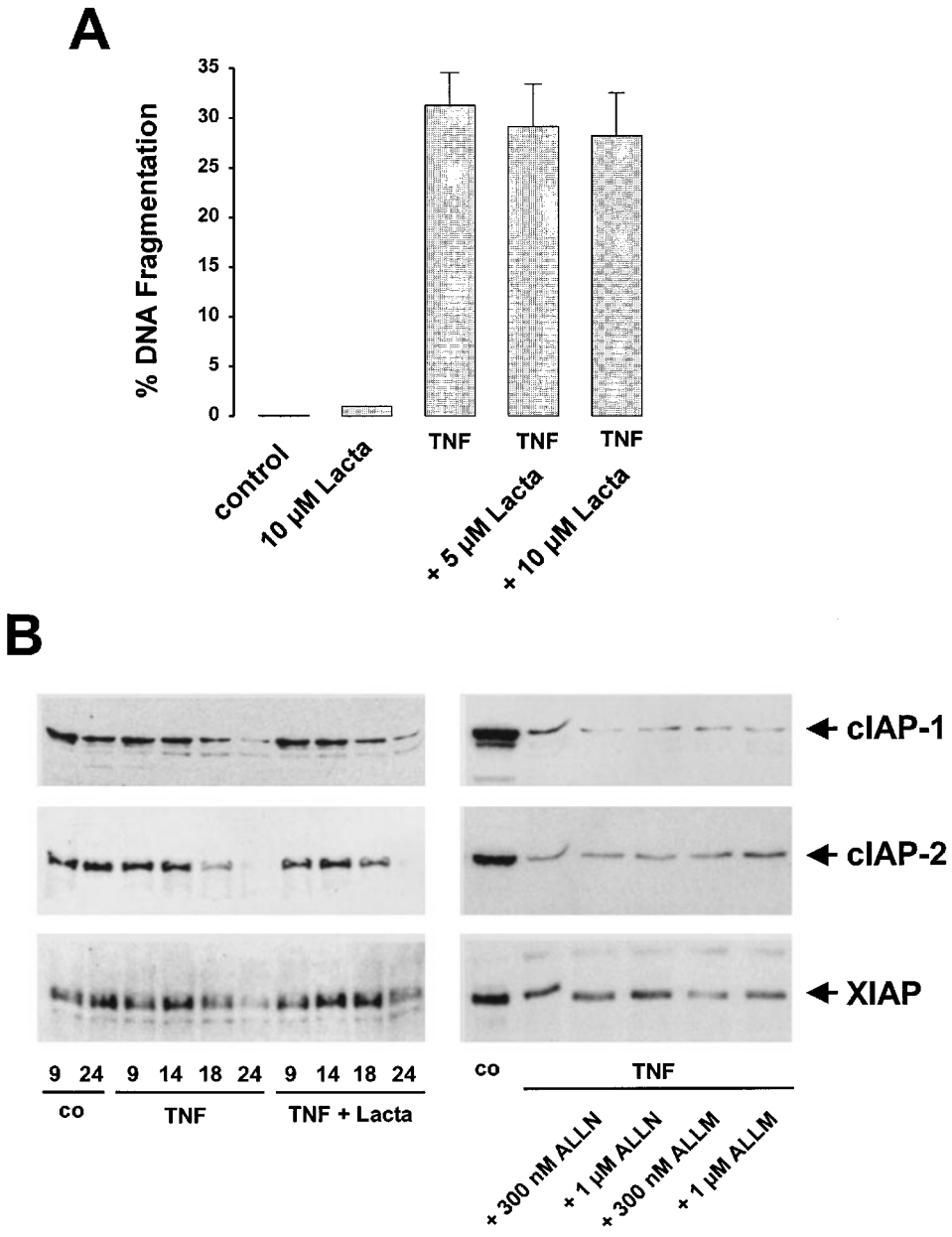

Figure 7 Effect of proteasome inhibitors on TNF-α-induced apoptosis and IAP protein cleavage in MCF-7 cells. (A) Cells were exposed for 24 h to 50 ng ml^{-1} TNF-α (TNF) plus 5 μM or 10 μM lactacystine, 10 μM lactacystine (Lacta) or vehicle (control) as indicated. DNA cleavage was determined by the diphenylamine assay. Values are means ± standard deviation out of four experiments. (B) MCF-7 cells were exposed for various times to 50 ng ml^{-1} TNF-α or to 50 ng ml^{-1} TNF-α plus 10 μM lactacystine or different concentrations of ALLN or ALLM, or vehicle as a control (co) as indicated. Subsequently, proteins were normalized to 50 μg $lane^{-1}$ and cell lysates were subjected to Western blot analysis for cIAP1, cIAP2 or XIAP proteins followed by ECL detection and video densitometry. Each blot is representative of three similar experiments.

apoptotic cell death. Unexpectedly, cytochrome *c* release preceded Bid cleavage in MCF-7 cells by about 6–8 h. One possible explanation would be that the detection of cytochrome *c* release was more sensitive than the detection of Bid cleavage. However, more likely is that the effector phase of apoptosis induction is regulated by amplification loops: Cytochrome *c* release can be divided into two phases, one initial phase which may be independent on Bid cleavage and a second phase which can be triggered by a caspase-dependent Bid cleavage process (Tang *et al*., 2000; Granville *et al*., 1999). Biphasic effects can be explained by the existence of feedback loops for amplification of apoptosis-associated mitochondrial cytochrome *c* release which was shown to depend on caspase-3-mediated Bid cleavage (Slee *et al*., 2000; Chen *et al*., 2000). However, as MCF-7 cells were devoid of an active or inactive caspase-3 protein (Kurokawa *et al*., 1999), one can imagine the participation of other caspase family members. Moreover, in contrast to glomerular endothelial cells (Meßmer *et al*., 1999a), the protein levels of other Bcl-2 protein family members (Bad, Bik, Bax, Bcl-2, Bcl-x_L) did not change during TNF-α-mediated apoptosis induction in MCF-7 cells.

A very important finding of the present work is that induction of apoptotic cell death was accompanied by the inactivation of anti-apoptotic factors, i.e. different members of the IAP family of proteins. cIAP1, cIAP2, and XIAP are direct inhibitors of distinct caspases and especially cIAP1 and cIAP2 are regulated on the level of gene expression by NF-κB (Stehlik *et al*., 1998). However, actually there are only a few reports on the regulation of IAP protein stability. Caspase-mediated cleavage of XIAP results in fragments with distinct specificities for caspases (Deveraux *et al*., 1999; Johnson *et al*., 2000). The two cleavage fragments of XIAP either comprise the BIR1 and BIR2 domains and the BIR3 and RING domains of XIAP. Further experiments revealed that BIR3/RING is a specific inhibitor of caspase-9 whereas BIR1-2 is specific for caspase-3 and -7 (Deveraux *et al*., 1999). However, cleavage products of XIAP have a reduced ability to inhibit caspases and caspase-mediated degradation of the BIR1-2 fragment may further reduce its anti-apoptotic potential (Deveraux *et al*., 1999). We also detected XIAP cleavage during TNF-α-mediated MCF-7 cell death, though our antibody was not able to detect the cleavage products. XIAP cleavage probably causes a decrease in the anti-apoptotic potential of MCF-7 cells. A further reduction may be achieved by cIAP1 and cIAP2 degradation. To the best of our knowledge, this is the first report describing the cleavage of cIAP1 and cIAP2 during TNF-α-induced apoptosis. In both cell types examined a cleavage product of about 55 kDa emerges correspondingly to IAP degradation. The existence of these cleavage products points to the involvement of caspases. Two experiments using Z-VAD-fmk as a broad-spectrum caspase inhibitor in MCF-7 cells and Z-Asp-CH_2-DCB as a broad-spectrum caspase inhibitor in glomerular endothelial cells points to a direct correlation between inhibiton of apoptosis, blockade of caspase activity and inhibition of cIAP degradation and to an involvement of caspases in this process. Since two independent reports documented that cIAP1 functions as a ubiquitin-protein ligase and promotes *in vitro* mono-ubiquitination of caspases-3 and -7 (Yang *et al*., 2000; Huang *et al*., 2000), we checked the involvement of ubiquitin protease family members by using lactacystin and other inhibitors of this protease. According to these data, we can exclude the involvement of ubiquitin protease family members in IAP degradation.

In the context of anti-cancer therapy, the inhibition of apoptotic signalling by steroids/glucocorticoids such as dexamethasone may be an important process (Perillo *et al*., 2000). Although dexamethasone was described as an inducer of apoptotic cell death in thymocytes, several authors also reported anti-apoptotic activities (Kull, 1988; Cox, 1995; Cox & Austin, 1997; Pagliacci *et al*., 1993; Chang *et al*., 1997; Naumann *et al*., 1998; Wen *et al*., 1997; Iida *et al*., 1998; Huang & Cidlowski, 1999). The anti-apoptotic effect of dexamethasone did not depend on a distinct cell type or a certain pro-apoptotic signal. However, the mechanisms of the survival effect remained to be clarified.

In MCF-7 cells the inhibitory action of glucocorticoids does not require the induction of a long lasting protective signal such as the synthesis of a long-lived anti-apoptotic protein. This suggestion results from our observation that a pretreatment of 2–6 h was not more inhibitory than simultaneous or an even 6 h posttreatment regimen. This finding is comparable to that found in glomerular endothelial cells (Meßmer *et al*., 2000) where we suggested a direct interaction with a signalling pathway downstream of primary receptor actions. Dexamethasone completely blocked Bid cleavage as well as the degradation of XIAP and cIAP1 or cIAP2 whereas mitochondrial cytochrome *c* release was significantly affected but not blocked completely. This finding supports the hypothesis that TNF-α-induced apoptotic signalling may be regulated by amplification loops and dexamethasone probably blocks a critical perpetuating signal within this amplification loop.

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