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Tumor necrosis factor-α and lipopolysaccharide induce apoptotic cell death in bovine glomerular endothelial cells

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Background. The glomerular endothelial cell is a specialized microvascular cell type involved in the regulation of glomerular ultrafiltration. During gram-negative sepsis, glomerulonephritis, and acute renal failure, bacterial lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) may cause severe cell damage. Our aim was to study and compare the direct effects of TNF-α and LPS on the induction of apoptosis in bovine glomerular endothelial cells.

Methods. Primary bovine glomerular endothelial cells were stimulated with TNF-α or LPS, and apoptotic cell death was investigated by DNA fragmentation analysis, morphological studies, measurement of cytochrome c efflux and mitochondrial permeability transition, Bak, Bad, Bax, Bcl-2, Bcl-x_L protein expression, and caspase-3–like protease activity.

Results. TNF-α, as well as LPS, elicited apoptotic cell death both time and concentration dependently. Along with DNA ladder formation, we detected the formation of 50 kbp high molecular weight DNA fragments, nuclear condensation, and mitochondrial permeability transition. Concerning all parameters, LPS signaling proved to be more rapid than TNF-α. Mechanistically, TNF-α–induced cell death was preceded by an efflux of mitochondrial cytochrome c into the cytosol and, subsequently, by a marked increase in the proapoptotic protein Bak and a decrease in the anti-apoptotic Bcl-x_L protein content. Comparable but more pronounced effects were seen with LPS. Later, caspase-3–like protease activity was first detectable after 10 hours and was continuously increased up to 24 hours in both TNF-α– and LPS-stimulated cells. Correspondingly, we detected an extended cleavage of the nuclear enzyme poly(ADP-ribose) polymerase. Caspase inhibitors Z-Asp-CH_2-DCB and Z-VAD-fmk blocked both TNF-α– and LPS-induced apoptosis in a comparable manner. Only Z-Asp-CH_2-DCB was able to block apoptotic cell death completely.

Conclusion. Both bacterial LPS and TNF-α potently induced apoptotic cell death in glomerular endothelial cells. Direct endotoxin-induced apoptosis may therefore be relevant in the progression of acute renal failure, which is a frequent complication of gram-negative sepsis.

Key words: cytochrome c, Bak up-regulation, caspase-3, Bcl-x_L, glomerular ultrafiltration.

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Glomerular inflammatory diseases are a leading cause of end-stage renal failure. Most forms of human glomerulonephritis lack specific therapy, and the mechanisms involved in the initial injury to the kidney and the progression to renal scarring (glomerulosclerosis and interstitial fibrosis) are still not completely understood [1, 2]. Some forms of human glomerulonephritis may recover, allowing glomerular structure and function to return to normal. In progressive forms of glomerulonephritis, glomerular inflammation is sustained and eventually leads to end-stage renal failure. The glomerulus is a well-developed capillary network, and endothelial cell damage of glomeruli and kidney arterioles appears to play a pivotal role in glomerulonephritis and several other pathological situations [3].

It is generally accepted that both peripheral blood cells infiltrating the glomerulus and intrinsic glomerular cells (mesangial, endothelial, and epithelial cells) can synthesize and release cytokines and chemotactic factors [4, 5]. For example, in mesangial cells, tumor necrosis factor-α (TNF-α) induces the expression of interleukin (IL)-8 and IP-10 (a lipopolysaccharide- and interferon-γ-inducible protein) [6], two members of the chemotactic protein family, and platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) [7], two potent lipid factors. Several cytokines have been demonstrated to affect the behavior of infiltrating and intrinsic glomerular cells and may participate in renal damage.

Besides glomerulonephritis, acute renal failure is also a frequent complication of gram-negative sepsis. Septic shock or endotoxic shock is a consequence of a systemic infection by gram-negative bacteria, and in an experimental model, it can be elicited by intravenous infusion of bacterial lipopolysaccharide (LPS) [8]. The septic shock syndrome is characterized by systemic inflammation, multiple organ failure, circulatory collapse, and death. LPS causes systemic release of TNF-α, and both may mediate their deleterious effects in part by direct endothelial cell damage [9].

Tumor necrosis factor-α is an important autocrine and

paracrine factor in glomerular inflammatory reactions, produced by infiltrating monocytes/macrophages and by intrinsic glomerular mesangial cells. Continuous intravenous infusion of TNF-α into rabbits produced endothelial cell damage and accumulation of neutrophils in the glomerular capillary lumen, suggesting a chemotactic and/or toxic role for TNF-α *in vivo* [10]. However, the interaction between TNF-α and glomerular endothelial cells remains essentially unstudied.

Tumor necrosis factor-α as a pleiotropic cytokine produced by many cell types elicits a wide spectrum of cellular responses. One intensively investigated response is the induction of apoptotic cell death in many different cellular systems, including macrovascular endothelial cells [11, 12]. Apoptosis, or programmed cell death, is the natural form of cell death normally occurring in the absence of an inflammatory response. Apoptosis is mainly characterized by several morphological criteria, that is, compaction and segregation of nuclear chromatin, cytoplasmic condensation, and the formation of apoptotic bodies. Biochemical characteristics are oligonucleosomal DNA fragmentation, which appears on an agarose gel as a DNA ladder and cleavage of distinct proteins by the activation of several proteases.

The associated signaling pathways leading to apoptosis are the most intensively studied areas of TNF research [13]. TNF-α effects are transmitted via cross-linking of the membrane-bound receptor molecules TNF receptor I (TNFRI, p55 TNFR) and TNFRII (p75 TNFR). Both receptors lack intrinsic catalytic activity and are members of a family of cell surface glycoproteins, characterized by the presence of conserved cysteine residues in the extracellular ligand domain (TNFR superfamily). These include CD27, CD40, CD30, p75 NGFR, OX-40, and Fas (APO-1, CD95) [13]. The initiation of downstream signaling events in response to TNFR or Fas activation requires a coordinated network of intermediate signaling proteins interacting with the cytoplasmic domain of the receptors via the conserved "death domain" [14]. These include TNFR-associated factor 1 (TRAF1) and TRAF2, TNFR1-associated death domain protein (TRADD), receptor interacting protein (RIP), and Fas-associated death domain protein (FADD) [15]. Receptor ligation, the interaction with "death domain" proteins, and the activation of several second messenger systems such as ceramide, stress-activated protein kinase (SAPK), and nuclear factor κB (NF-κB) are events of the initiation phase of TNF-mediated apoptotic cell death. Cell-type differences determine this initiation phase similar to the subsequent effector phase, which defines the "decision to die." The Bcl-2 family of proteins has a major decisive regulatory function at this step. Bcl-2 is the prototypical member of this protein family that promotes cell survival along with Bcl-x_L, Bcl-w, Mcl-1, and A1 [16, 17]. On the other hand, the best characterized Bcl-2–related protein known to induce apoptotic cell death is the bax gene product, which shares its proapoptotic activity with Bad, Bak, Bcl-x_S, Bik, Bim, Hrk, and Bok. How Bcl-2–related molecules exert their apoptosis-regulatory function is currently intensively investigated and may involve protein dimerization, regulation of intracellular calcium fluxes [18], ion channel formation [19], and regulation of mitochondrial alterations [20]. The dissipation of the inner-mitochondrial transmembrane proton gradient ($\Delta\Psi_m$) and/or the release of cytochrome c into the cytosol mark the "point of no return" and lead of the degradation phase of apoptosis [17]. The latter represents the destruction pathways that require the activation of certain caspases and other proteases. Caspases are cysteine proteases related to the IL-1β–converting enzyme and share sequence homology with ced-3 [21]. Caspase-3 has been shown to play an important role as a downstream member of the protease cascade, and several proteins were characterized as caspase-3 substrates, such as poly(ADP-ribose) polymerase (PARP) [22].

The interaction of TNF-α and LPS with glomerular endothelial cells and the triggering of apoptosis in this cell type have not yet been studied. Our aim was to characterize the cytotoxic effects of TNF-α and LPS on bovine glomerular endothelial cells.

METHODS

Materials

Hoechst dye 33258, diphenylamine, LPS (*E. coli* serotype 0127:B8), haparin sodium, and proteinase K were purchased from Sigma (Deisenhofen, Germany). N-acetyl-aspartyl-glutamyl-valinyl-aspartyl-7-amino-4-coumarin (DEVD-AMC), 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). Enhanced chemiluminescence (ECL) detection reagents and 3,3′-dihexyloxacarbocyanide iodide [$DiOC_6(3)$] were ordered from Amersham (Braunschweig, Germany) and Molecular Probes (Leiden, The Netherlands), respectively. Bovine acidic fibroblast growth factor (aFGF) was purchased from R&D Systems (Wiesbaden, Germany), and recombinant human TNF-α (specific activity, 6.6×10^6 units/mg) was a generous gift from Knoll AG (Ludwigshafen, Germany). RPMI 1640, cell culture supplements, and fetal calf serum (FCS) were from GIBCO (Eggenstein, Germany). All other chemicals were of the highest purity grade commercially available.

Cell culture and cell treatment

Bovine glomerular endothelial cells were cultivated as described previously [23]. In brief, approximately 10 g of renal cortex tissue were minced, passed through a sterile 240 μm stainless steel sieve, and suspended in

Hanks' balanced salt solution (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 minutes at 37°C in HBSS containing 1 mg/ml collagenase (type V; Sigma). After digestion, glomerular remnants were sedimented at 500 g for five minutes. The supernatant was centrifuged at 1000 *g* for five minutes, and the pellet was suspended in RPMI 1640 medium containing 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml heparin sodium, and 5 ng/ml of aFGF. 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-ethylenediaminetetraacetic acid (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 [24]. 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 or 100 mm Petri dishes with RPMI 1640 medium containing 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml heparin sodium, and 5 ng/ml of aFGF and incubated in RPMI 1640 containing 2% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Quantitation of DNA fragmentation

DNA fragmentation was essentially assayed as reported previously [25]. Briefly, after incubation, cells were scraped off the culture plates, resuspended in 250 μl 10 mM Tris and 1 mM EDTA, pH 8.0 (TE buffer), and were incubated with an additional volume lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 minutes at 4°C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 minutes at 13,000 *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 minutes, and the supernatant was removed. After the addition of 300 μl 5% trichloroacetic acid, samples were boiled for 15 minutes. DNA contents were quantitated using the diphenylamine reagent [26]. The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

DNA agarose gel electrophoresis

For the preparation of DNA for agarose gel electrophoresis, cells were cultured, harvested, lyzed, and centrifuged as described earlier here to separate DNA fragments from intact chromatin. Supernatants were precipitated overnight with two volumes of ice-cold ethanol and 50 μl 5 M NaCl at –20°C, centrifuged at 13,000 *g* for 15 minutes, followed by an incubation of the pellet in 500 μl TE buffer supplemented with 100 μg/ml RNase A at 37°C for 30 minutes. Samples were extracted with phenol: chloroform:isoamylalcohol (25:24:1) and once again with chloroform:isoamylalcohol (24:1). DNA was precipitated, and pellets were recovered by centrifugation (13,000 *g*, 15 min), air dried, resuspended in 10 μl TE buffer, supplemented with 2 μl sample buffer (0.25% bromophenol blue, 30% glyceric acid), and electrophoretically separated on a 1% agarose gel containing 1 μg/ml ethidium bromide for 2.5 hours at 100 V. Pictures were taken by ultraviolet transillumination.

Pulsed field gel electrophoresis

DNA degradation into oligonucleosomal-sized fragments was analyzed as described [27]. Glomerular endothelial cells were exposed to the apoptotic stimuli, scraped off the culture dishes, mixed with 1% low-melting agarose, and molded into plugs of 100 μl volume. Cellular protein was digested by incubation of the plugs with 1 mg/ml proteinase K in lysis buffer (10 mM Tris, 10 mM NaCl, 100 mM EDTA, 1% sodium lauryl sarcosine, pH 8.0) for 24 to 36 hours at 50°C. Pulsed field gel electrophoresis (PFGE) was carried out in a BioRad CHEF III system in 0.5 × TBE buffer at 11°C for 14 hours at a voltage gradient of 6 V/cm with a switch time ramped linearly from 35 to 80 seconds. After electrophoresis, the separated DNA fragments were visualized by ethidium bromide staining and ultraviolet transillumination.

Cytofluorometric determination of mitochondrial membrane potential

For the determination of the $\Delta\Psi_m$, $DiOC_6(3)$ (final concentration 10 nM) was used [28]. For these experiments, glomerular endothelial cells were cultured in 60 mm culture dishes and incubated with the different apoptotic stimuli, and for the last 15 minutes, 10 nM $DiOC_6(3)$ was added. Afterward, cells were harvested by trypsinization and were resuspended in 500 μl phosphate-buffered saline containing 10 μg/ml propidium iodide. Within 30 minutes, cells were analyzed using a Fluorescence-activated cell sorting (FACS) calibur (Becton Dickinson, Heidelberg, Germany). Cells exhibiting a normal forward/side scatter ratio were selected followed by the determination of the $DiOC_6(3)$/propidium iodide staining properties.

Analysis of mitochondrial cytochrome c efflux

Glomerular endothelial cells, incubated as described, were harvested by trypsinization, pelleted by centrifugation, resuspended in 300 μl of homogenization buffer

[20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM ethyleneglycol-bis(β-aminoethylether) N, N′-tetraacetate (EGTA), 1 mM dithiothreitol (DTT), 4 mM Pefabloc, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 250 mM sucrose], and incubated for 10 minutes on ice. Cells were broken by 2 × 15 passages through a syringe fitted with a 25 gauge needle. The lysate was centrifuged at 750 *g* for 10 minutes at 4°C to pellet nuclei. The remaining supernatant was centrifuged for 15 minutes at 10,000 *g*. The pellet was used as mitochondrial fraction, and the supernatant as cytosolic fraction. Protein was determined with the Bradford method [29], and 50 μg were used for Western blot analysis. Proteins were resolved on 14% polyacrylamide gels and blotted onto polyvinylidene difluoride (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/1% FCS. Filters were incubated with the mouse anticytochrome c antibody (clone 7H8.2C12, PharMingen, 1 μg/ml in TBS/2% skim milk/0.7% FCS) overnight at 4°C. Sheets were washed five times, and unspecific binding was blocked as described. Detection was by horseradish peroxidase-conjugated goat antimouse monoclonal antibody (1:5000) for 1.5 hours at room temperature using the ECL method (Amersham).

Morphological investigations

Glomerular endothelial cells were grown in 60 mm culture plates to nearly confluency. Cells were stimulated, followed by fixation with 3% paraformaldehyde for five minutes onto glass slides. Samples were washed with phosphate-buffered saline, stained with Hoechst dye H33258 (8 μg/ml) for five minutes, washed with distilled water, and mounted in Kaiser's glycerol gelatin. Nuclei were visualized using a Zeiss Axiovert fluorescence microscope.

Caspase-3 enzyme activity

For detection of caspase-3 activity, glomerular endothelial cells were incubated as indicated and lyzed in lysis buffer (10 mM Tris/HCl, 0.32 M sucrose, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM DTT, pH 8.0) for 30 minutes. Following sonication (10 sec, output control 1), lysates were centrifuged (10,000 *g* for five minutes at 4°C) and stored at –80°C. Protein determinations were performed with the Bradford method [29]. Caspase-3 activity was detected by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC. Cell lysates (50 μg protein) were incubated in 100 mM HEPES, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-propansulfonate (CHAPS), pH 7.5, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM DTT at 37°C with 12 μM DEVD-AMC in a total volume of 700 μl. Substrate cleavage and AMC accumulation was followed fluorometrically with excitation at 380 nm and emission at 460 nm.

Western blot analysis

Cells were cultured and incubated as described. Cell lysis was achieved with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM phenylmethylsulfonyl fluoride, pH 8.0) and sonication (Branson sonifier; 10 seconds, duty cycle 100%, output control 10%), followed by centrifugation (4000 × *g* for 5 min), and Bradford protein determination [29]. Proteins were normalized to 100 μg/lane (PARP) or to 40 μg/lane (Bcl-2 proteins), resolved on 7.5% (PARP) or 12.5% polyacrylamide gels (Bcl-2 proteins), 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-PARP antibody (clone C-II-10, 1 μg/ml, in TBS + 0.5% skim milk; Biomol, Plymouth, Meeting, PA, USA), mouse anti-Bcl-2 antibody (clone 83-8B, 1 μg/ml; Immunotech, Narseilla, France), rabbit anti–Bcl-x antibody (1:1000 in TBS + 0.5% skim milk; Transduction Laboratories, Lexington, KY, USA), mouse anti-Bad antibody (1:500 in TBS + 0.5% skim milk; Transduction Laboratories), rabbit anti-Bax antibody raised against a peptide MDGSGEQPRGGGPTSSEQIMK coupled to keyhole limpet hemocyanin (KLH) by the m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) method (1:2000 in TBS + 0.5% skim milk), and rabbit anti-Bak antibody raised against a peptide WIARGGWVAA LNLG coupled to KLH by the MBS method (1:1500 in TBS + 0.5% skim milk) overnight at 4°C. Sheets were washed five times, and unspecific binding was blocked as described. Detection was by horseradish peroxidase-conjugated goat antimouse monoclonal antibodies (1:5000) or goat antirabbit monoclonal antibodies (1:5000) for 1.5 hours 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 antibax; Calbiochem Ab-2 antibak) using mouse and human cell preparations (RAW 264.7 and U937). The antibodies exhibited no cross-reactivity with other Bcl-2 family members.

Statistical analyses

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

RESULTS

Induction of glomerular endothelial cell apoptosis by tumor necrosis factor-α and lipopolysaccharide

To investigate the influence of TNF-α and LPS on glomerular endothelial cell viability, bovine glomerular endothelial cells were isolated, cultured, grown to confluency, and left either untreated (control) or treated with increasing concentrations of human recombinant TNF-α or bacterial LPS. Internucleosomal DNA fragmentation was selected as a specific apoptotic marker. Figure 1A shows that TNF-α as well as LPS induced DNA degradation concentration dependently within 24 hours. As little as 1 ng/ml TNF-α was effective to initiate DNA fragmentation, and roughly 25 ng/ml TNF-α caused a maximal response amounting to 33.3 ± 9.3% (mean ± SD, $N = 4$) DNA cleavage. Similarly, LPS at a very low concentration of 1 ng/ml was able to decrease glomerular endothelial cell viability, whereas 10 to 30 ng/ml caused significant DNA fragmentation of roughly 30 to 40%. In addition, as shown in Figure 1C, the pattern of DNA fragmentation elicited by TNF-α and LPS generated the characteristic apoptotic DNA ladder, confirming the results obtained with the diphenylamine reaction. Moreover, additional results excluding necrotic cell death and arguing for programmed cell death are depicted in Figure 1B and Figure 2. Chromatin condensation, as well as high molecular weight fragmentation of genomic DNA, was also chosen as a specific marker for apoptotic cell death. Investigation of cell morphology using the Hoechst dye 33258 proved chromatin condensation in TNF-α- and LPS-stimulated versus control glomerular endothelial cells (Fig. 2 B and C vs. Fig. 2A). Similarly, stimulation of glomerular endothelial cells with 10 ng/ml TNF-α or 10 ng/ml LPS resulted in the formation of 50 kbp high molecular weight DNA fragments (Fig. 1B).

A time kinetic study revealed that the 50 kbp high molecular weight DNA fragments were first visible seven hours after stimulation with 10 ng/ml LPS and 10 to 16 hours following the addition of TNF-α. From the experiments with high molecular weight DNA degradation, LPS clearly proved to be more rapid than TNF-α. These results were confirmed by a detailed time kinetic study comparing LPS- and TNF-α–induced DNA degradation by the diphenylamine reaction. As demonstrated in Figure 3, LPS-induced DNA cleavage first emerged after 8 hours and monotonically increased up to 24 hours, whereas the TNF-α–mediated signal was first evident after 12 to 14 hours. These data indicate that the LPS-mediated final death pathway was not triggered until an initiation time of seven to eight hours, and the TNF-α–mediated pathway needed more than 10 hours to enter the degradation phase. Extending these time kinetic studies to 48 hours revealed that cells exhibiting an apoptotic morphology increased up to 80% with LPS and 70% with TNF-α (data not shown), although DNA fragmentation measured by the diphenylamine assay no longer correlated with the extent of apoptosis (data not shown).

Next, we questioned whether glomerular endothelial cells had to be exposed to TNF-α or LPS for the whole time to induce an apoptotic response effectively or whether short time exposures were sufficient to elicit the final death program. Glomerular endothelial cells were exposed to TNF-α or LPS for 1 hour up to 24 hours, followed by removal of the cytokine or LPS after 1, 2, 4, 6, 8, 10, or 12 hours, respectively (Fig. 4). After changing the media, incubations were continued until DNA fragmentation was assayed after a total incubation time of 24 hours, when maximal alterations were measurable. In one set of experiments, glomerular endothelial cells were exposed to TNF-α/LPS for the entire period of 24 hours. TNF-α at a suboptimal concentration of 1 ng/ml or at a maximal effective concentration of 10 ng/ml produced substantial fragmentation when incubated for 24 hours. In contrast, incubations for one hour up to six hours, followed by apogen removal, only induced a weak response. Even if the cells were exposed for 8 hours up to 12 hours followed by cytokine washout, the apoptotic response was limited and did not fully reach maximal values. Similarly, exposure to LPS for 1 to 12 hours followed by changing the media and measurement of DNA fragmentation after 24 hours consecutively increased from low levels after a one hour exposure to close to optimal levels after 12 hours. Generally, to induce a maximal apoptotic response toward TNF-α and LPS, a continuous stimulation over several hours is needed.

Mitochondrial permeability transition and mitochondrial cytochrome c efflux in tumor necrosis factor-α– and lipopolysaccharide–induced apoptosis

Alterations in mitochondrial function in general, an efflux of cytochrome c into the cytosol, and induction of mitochondrial permeability transition in particular are proposed to play essential roles in apoptosis [30]. To characterize apoptotic signaling in glomerular endothelial cells and to compare the initiating apoptotic signals of TNF-α and LPS, we first measured the mitochondrial membrane potential by the uptake of the mitochondrial-specific dye $DiOC_6(3)$. Adherent cells were stimulated with TNF-α or LPS for different time periods followed by $DiOC_6(3)$ addition to the culture medium 15 minutes prior to harvesting the cells. After the appropriate incubation time, cells were trypsinized and characterized by FACS analysis. As shown in Figure 5A, control cells exhibited a low $DiOC_6(3)$ uptake capacity, reflecting a low mitochondrial membrane potential that varies, depending on the preparation, between 5 and 11%. TNF-α

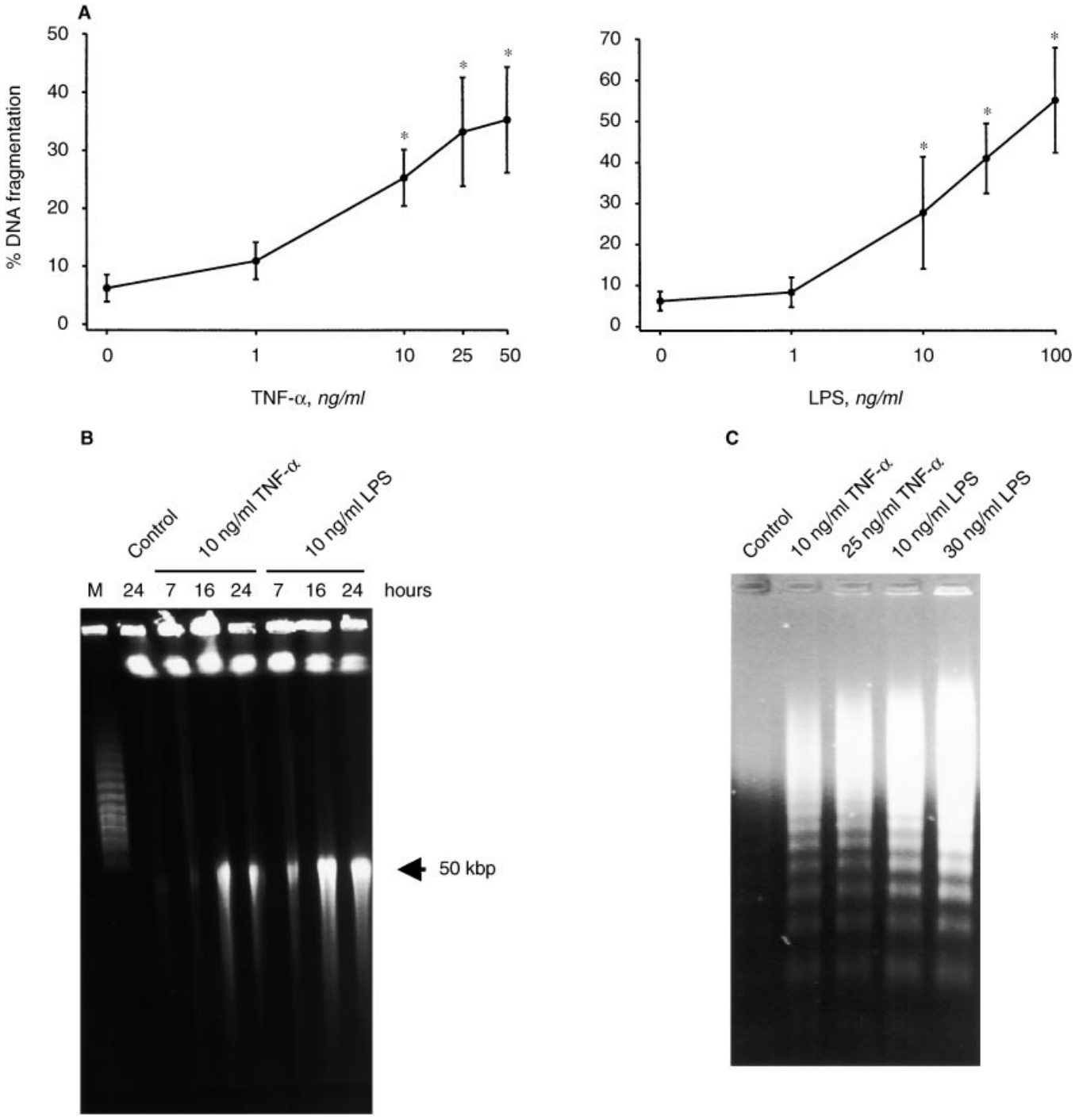

Fig. 1. Induction of apoptotic DNA fragmentation by tumor necrosis factor-α (TNF-α) and bacterial lipopolysaccharide (LPS). (*A*) Bovine glomerular endothelial cells were cultured as outlined in the **Methods** section and were incubated for 24 hours with increasing concentrations of TNF-α and LPS, respectively. DNA fragmentation was quantitated by the diphenylamine reaction. Values are means ± SD of four individual experiments. $*P < 0.05$ vs. control (analysis of variance and for multiple comparison the data were corrected by Dunn's method). The formation of large DNA fragments (*B*) and oligonucleosomal fragments (*C*) was assessed by field inversion and conventional gel electrophoresis, respectively. Cells were incubated with different TNF-α and LPS concentrations for 7 to 24 hours (B), as indicated or for 24 hours (C). The appearance of 50 kbp fragments and the formation of a DNA ladder point to apoptotic rather than necrotic cell death. The results are representative of three experiments.

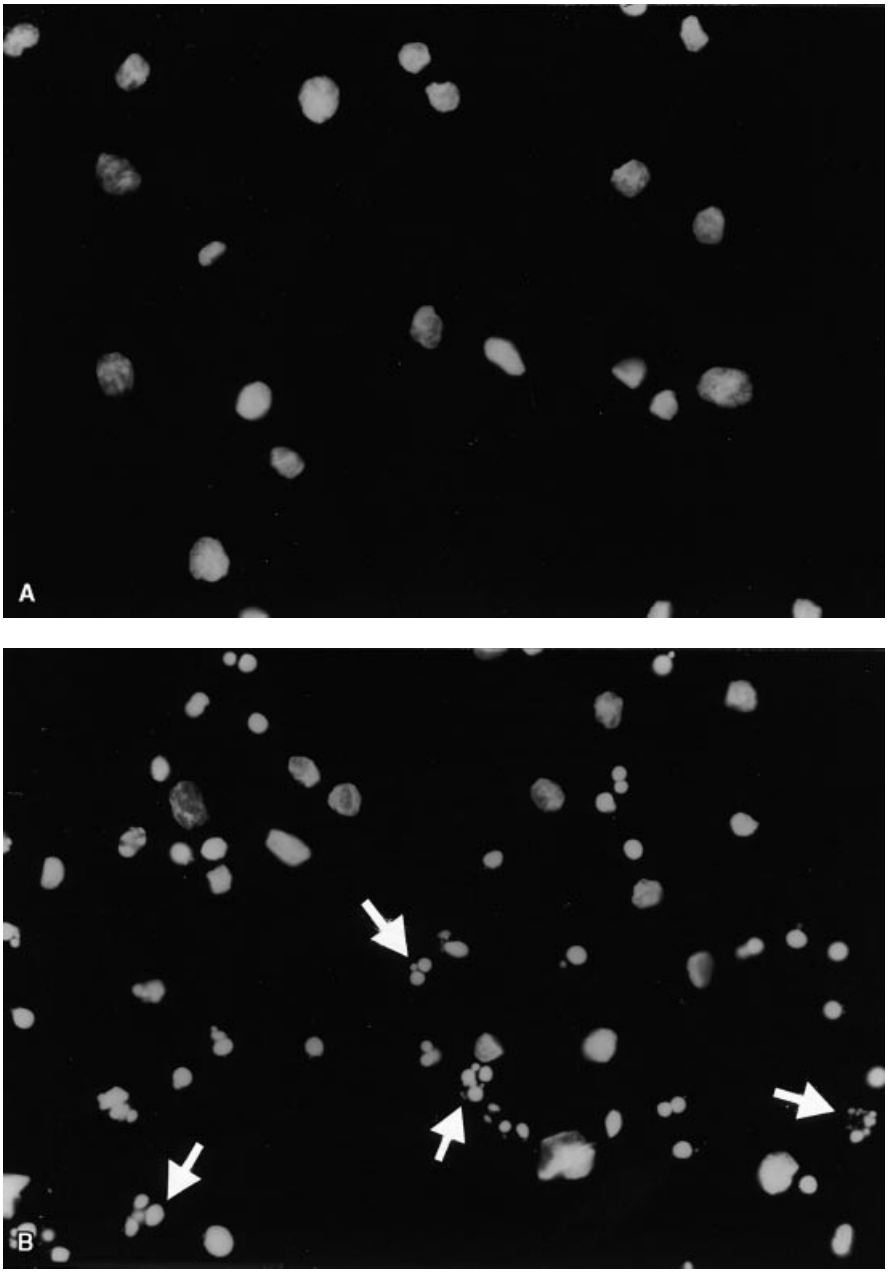

Fig. 2. Chromatin condensation and nuclear fragmentation in glomerular endothelial cells induced by TNF-α and LPS. Cells were cultured and incubated for 24 hours with vehicle (control, *A*), 10 ng/ml TNF-α (*B*), and 30 ng/ml LPS (*C*), fixed and stained using the DNA-specific fluorochrome H33258. Control cells exhibited a normal nuclear morphology characterized by diffuse chromatin structure and, therefore, only weak fluorochrome staining (A). (B and C) Approximately 25 to 50% of the stimulated cells exhibited an apoptotic morphology, characterized by chromatin condensation and fragmentation. Results are representative of three different experiments.

and LPS slightly increased the percentage of cells with a decreased mitochondrial membrane potential after six hours, an early time point at which no significant signs of cell degradation were detectable. After 18 and 24 hours, roughly 20 and 35%, respectively, of cells stimulated with TNF-α and approximately 30 and 46%, respectively, of LPS-stimulated glomerular endothelial cells exhibited a significant decrease in mitochondrial $\Delta\Psi$, which was equivalent to the amount of apoptotic DNA degradation and the appearance of morphologically altered cells.

Then we examined whether or not TNF-α and LPS induced the release of mitochondrial cytochrome c into the cytosol, and whether this process would be an early apoptotic signal. As shown in Figure 5B (left panel), TNF-α induced mitochondrial cytochrome c efflux within four to six hours, whereas in control incubations, cytosolic cytochrome c remained undetectable. Cytochrome c release was maximal within 15 hours and clearly preceded end-stage apoptotic changes. Further on, we compared cytochrome c release induced by TNF-α, TNF-α plus cycloheximide, and LPS. Similar to other reports, cycloheximide enhanced TNF-α-induced apoptotic DNA fragmentation [2.9 ± 2.5% vs. 24.1 ±

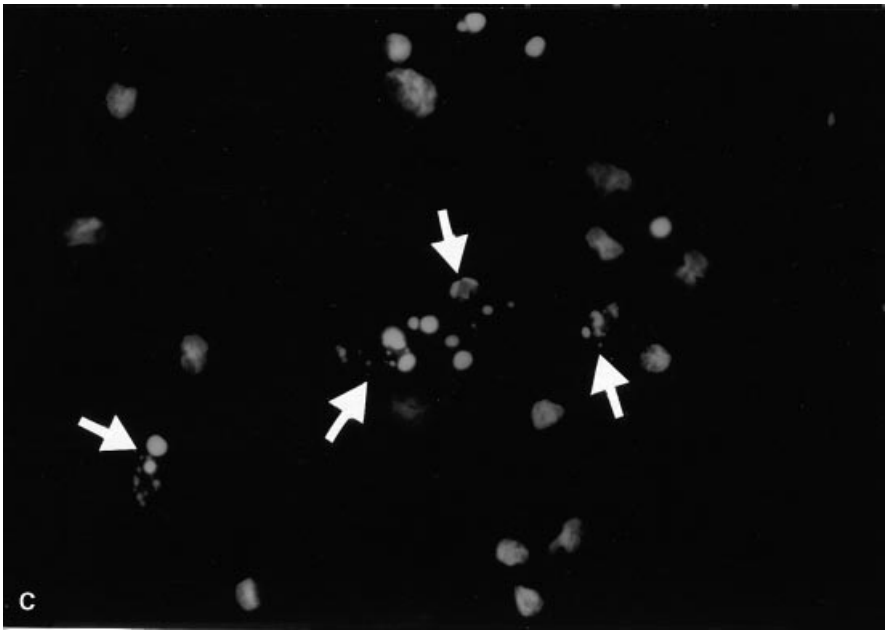

Fig. 2. (continued)

10.9% vs. 63.6 ± 8.05% (means ± SD, $N = 4$) DNA cleavage with 1 μM cycloheximide, 10 ng/ml TNF-α, and 1 μM cycloheximide plus 10 ng/ml TNF-α, respectively] and consequently, TNF-α-induced cytochrome c release (Fig. 5B right panel), whereas 1 μM cycloheximide alone left the cytochrome c release unaffected (data not shown). Also, LPS concentration dependently induced a rapid cytochrome c efflux within a few hours. These findings thus demonstrate that mitochondrial cytochrome c efflux is indeed an early signal in TNF-α- and LPS-induced apoptosis of glomerular endothelial cells occurring before the characteristic late phase processes.

Tumor necrosis factor-α and lipopolysaccharide induced an up-regulation of Bak and a down-regulation of Bcl-x_L

The family of Bcl-2–related proteins, biologically the most relevant class of apoptosis-regulatory gene products, is known to crucially affect apoptosis signaling pathways. Therefore, 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 Bax, Bak, and Bad, were involved in TNF-α- and LPS-induced glomerular endothelial cell apoptosis. For these experiments, we performed time kinetic studies exposing cells to 10 ng/ml TNF-α or 30 ng/ml LPS, and determined the protein expression of Bad, Bak, Bax, Bcl-x_L, and Bcl-2. The amount of the Bad protein doubled within eight hours after either TNF-α and LPS (3.1 ± 1.7-fold and 2.1 ± 0.8-fold vs. control, means ± SEM, $N = 3$), whereas the classic apoptosis modifier Bax slightly increased within 24 hours (1.4 ± 0.2-fold and 1.7 ± 0.2-fold vs. control, means ± SEM, $N = 3$), and unexpectedly, Bcl-2 also temporarily increased slightly within the 24-hour incubation period (1.35 ± 0.26-fold and 1.16 ± 0.13-fold vs. control after four hours of incubation with TNF-α or LPS, respectively, means ± SEM, $N = 3$; Fig. 6). Highly significant and dramatic changes were detected investigating Bak and Bcl-x_L protein expression. Bak protein TNF dependently increased up to sixfold within 10 to 24 hours, while LPS did so dependently, and we detected a 16-fold up-regulation within 8 to 24 hours following LPS addition. Concomitantly, the anti-apoptotic Bcl-x_L protein declined within 10 to 24 hours to approximately 60% of the control value with TNF-α (Fig. 6) and within 8 to 24 hours to approximately 30% with LPS (Fig. 7). These data suggest an involvement of Bak, which is closely related to Bax, and a concomitant decline of the Bak antagonist Bcl-x_L in the pro-apoptotic signaling of TNF-α and LPS.

Involvement of the caspase protease family in tumor necrosis factor-α- and lipopolysaccharide-induced apoptosis

Because in almost all apoptotic signaling cascades the caspase protease family was identified as an essential part of the executioner pathways, we finally focused on TNF-α- and LPS-mediated caspase activation in glomerular endothelial cells. First, as demonstrated in Figure 8A, cells were incubated with TNF-α or LPS in combination with two different broad spectrum caspase inhibitors (Z-Asp-CH_2-DCB and Z-VAD-fmk). Again, oligonucleosomal DNA fragmentation was selected as a reliable end-point marker of apoptosis. Z-Asp-CH_2-DCB

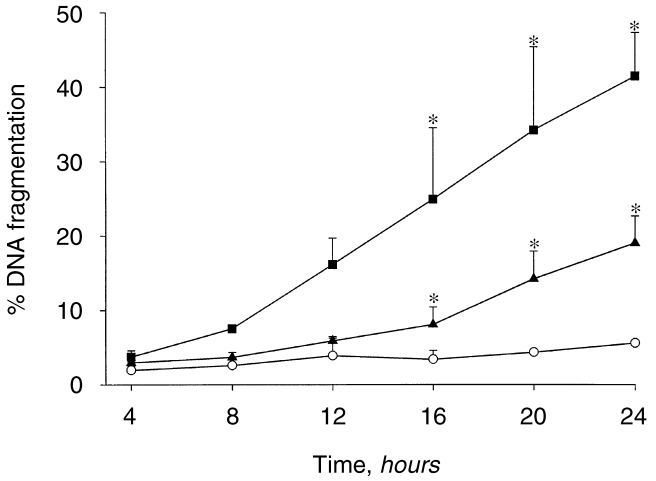

Fig. 3. Time-dependent DNA fragmentation in glomerular endothelial cells, induced by TNF-α and LPS. Cells were incubated with 10 ng/ml TNF-α (▲), 30 ng/ml LPS (■), or vehicle (control; ○), as indicated. DNA fragmentation was determined using the diphenylamine reaction. Values are means ± SD of at least four individual experiments. *$P <$ 0.05 vs. corresponding control (analysis of variance and for multiple comparison the data were corrected by Dunn's method).

as well as Z-VAD-fmk potently suppressed TNF-α– and LPS–induced apoptosis in a comparable manner (IC_{50} approximately 10 μM for Z-Asp-CH_2-DCB and IC_{50} approximately 40 μM for Z-VAD-fmk). However, only Z-Asp-CH_2-DCB was able to block apoptotic DNA fragmentation totally. This indicates an essential participation of caspase proteases in both signaling cascades.

Next, we investigated the effect of TNF-α and LPS on caspase-3–like protease activity. In control cells that were incubated with vehicle, there was no detectable caspase-3–like protease activity during the whole incubation period (Fig. 8B). Performing a time kinetic study, caspase-3–like activity was first detectable 8 hours following LPS addition and 10 to 12 hours past TNF-α addition (Fig. 8B). In both cases, caspase-3–like protease activity consecutively increased up to 24 hours. To evaluate whether caspase-3 would be an essential part of the apoptotic signaling cascade, glomerular endothelial cells were coincubated with 10 ng/ml TNF-α and 50 μM Ac-DEVD-fmk, an irreversible caspase-3–like protease inhibitor. DNA degradation amounted to 32.46 ± 13.07% in the absence and 25.63 ± 4.82% (mean ± SD, $N = 5$) in the presence of Ac-DEVD-fmk. To evaluate whether the poor inhibitory effect of DEVD-fmk was due to an inefficient inhibitor uptake or to a dispensability of caspase-3, we exposed cells for 24 hours to 10 ng/ml TNF-α and 50 μM Ac-DEVD-fmk. Afterwards, we monitored caspase-3–like protease activity in cell lysates by fluorometrically measuring the cleavage of Ac-DEVD-AMC. Caspase-3–like protease activity amounted to 1118 ± 245 nmol AMC/mg protein × min with 10 ng/ml TNF-α versus 2.2 ± 2.1 nmol AMC/mg protein × min with 10 ng/ml TNF-α plus 50 μM Ac-DEVD-fmk following a 24-hour incubation period. These results indicate that the caspase-3–like protease inhibitor indeed penetrated the cell and almost completely blocked caspase-3.

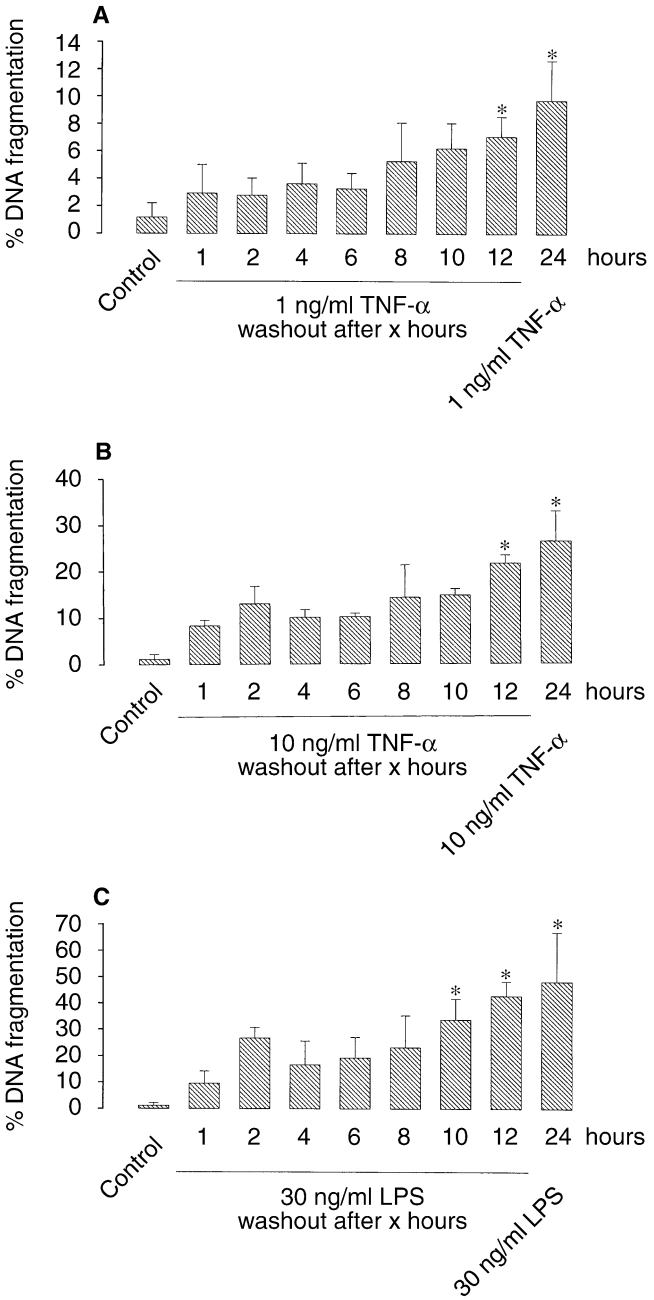

Fig. 4. DNA fragmentation in relation to TNF-α and LPS exposure times. Bovine glomerular endothelial cells were exposed to vehicle (control), 1 ng/ml TNF-α (*A*), 10 ng/ml TNF-α (*B*), and 30 ng/ml LPS (*C*) for the times indicated. Afterward, TNF-α and LPS were washed out, and in all cases, incubations were continued up to a total incubation time of 24 hours. DNA fragmentation was determined with the diphenylamine reaction as outlined in the **Methods** section. Values are means ± SD of four individual experiments. *$P < 0.05$ vs. corresponding control (analysis of variance and for multiple comparison the data were corrected by Dunn's method).

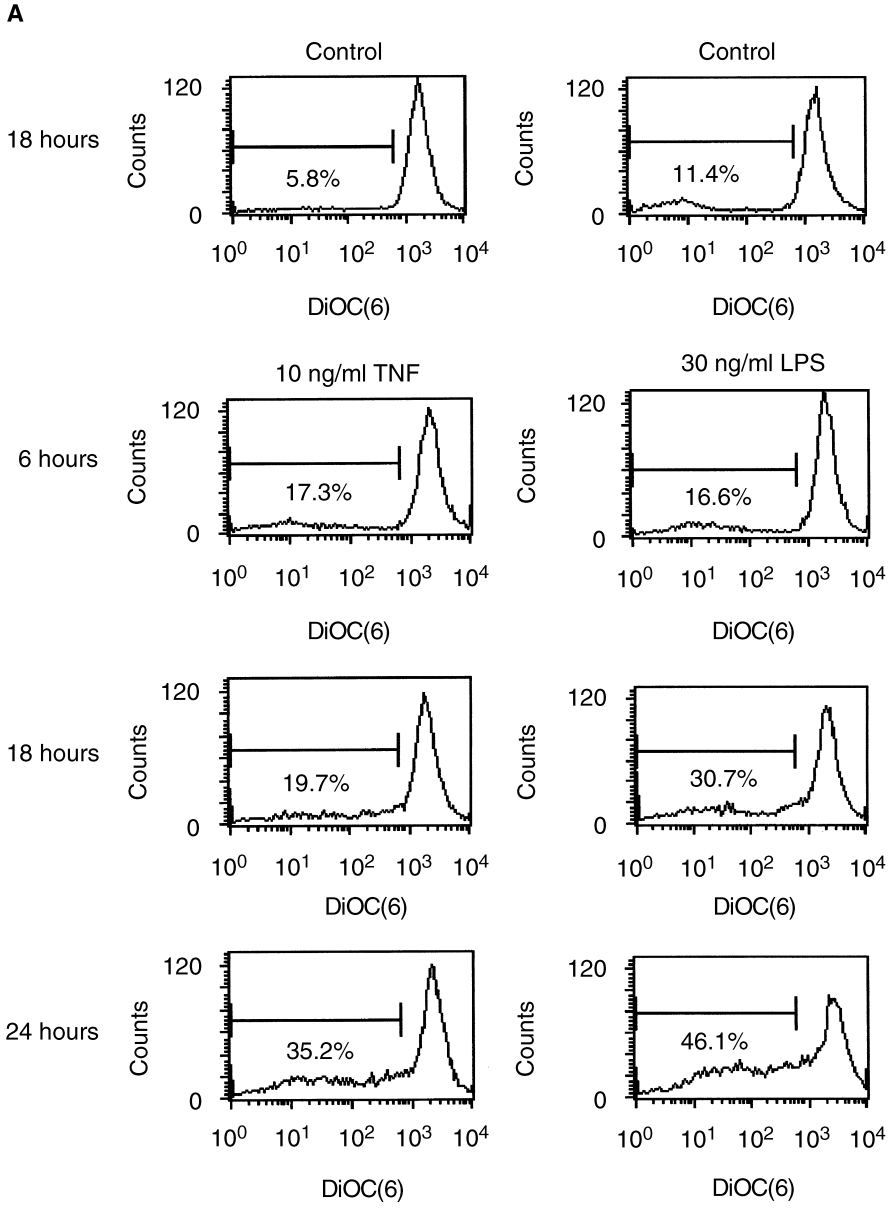

Fig. 5. Kinetics of TNF-α– and LPS–induced mitochondrial permeability transition and cytochrome c release into the cytosol. Glomerular endothelial cells were cultured as described in the **Methods** section. (*A*) During the last 15 minutes of the incubation, 10 nM $DiOC_6(3)$ was added to the culture medium, followed by cell trypsinization and counter staining with 10 μg/ml propidium iodide. Cells were analyzed with a FACS calibur (Becton Dickinson), and all cells exhibiting an intact forward/side scatter ratio and propidium iodide exclusion were selected. Data are representative of three independent experiments giving similar results. (*B*) Glomerular endothelial cells were incubated with 10 ng/ml TNF-α for the times indicated (left panel), 10 ng/ml TNF-α, 10 ng/ml TNF-α plus 1 μg/ml cycloheximide, 100 ng/ml LPS, 30 ng/ml LPS, or vehicle (control) for seven hours (right panel), harvested by trypsinization, and a cytosolic extract was prepared as described in the **Methods** section. The samples were then subjected to Western blot analysis for cytochrome c.

Together with the only moderate inhibition of TNF-α-induced DNA fragmentation, these data suggest that caspase-3 is dispensable for TNF-α-induced glomerular endothelial cell death. Similar results were also obtained with LPS (data not shown).

One substrate for many caspases is the nuclear enzyme PARP. In agreement with the activation of caspase-3–like proteases, the 116 kDa PARP holoenzyme was cleaved into the 85 kDa subunit in response to TNF-α and LPS (Fig. 8C).

DISCUSSION

This study demonstrates that both TNF-α and LPS individually are able to induce glomerular endothelial cell death. Cell death was characterized as apoptotic cell death by the following features: oligonucleosomal DNA degradation, DNA ladder formation, the appearance of 50 kbp high molecular weight DNA fragments, as well as nuclear compaction and segregation. Conclusively, glomerular endothelial cell apoptosis may contribute or be associated with glomerular inflammatory diseases caused by increased levels of TNF-α or LPS.

Endothelial cell apoptosis induced by TNF-α was recently reported for other microvascular structures, as well as for macrovascular endothelial cells such as human umbilical vein endothelial cells (HUVECs). However, HUVECs only hardly undergo programmed cell death in response to TNF-α alone [31, 32], but extensively die in response to TNF-α in combination with cycloheximide

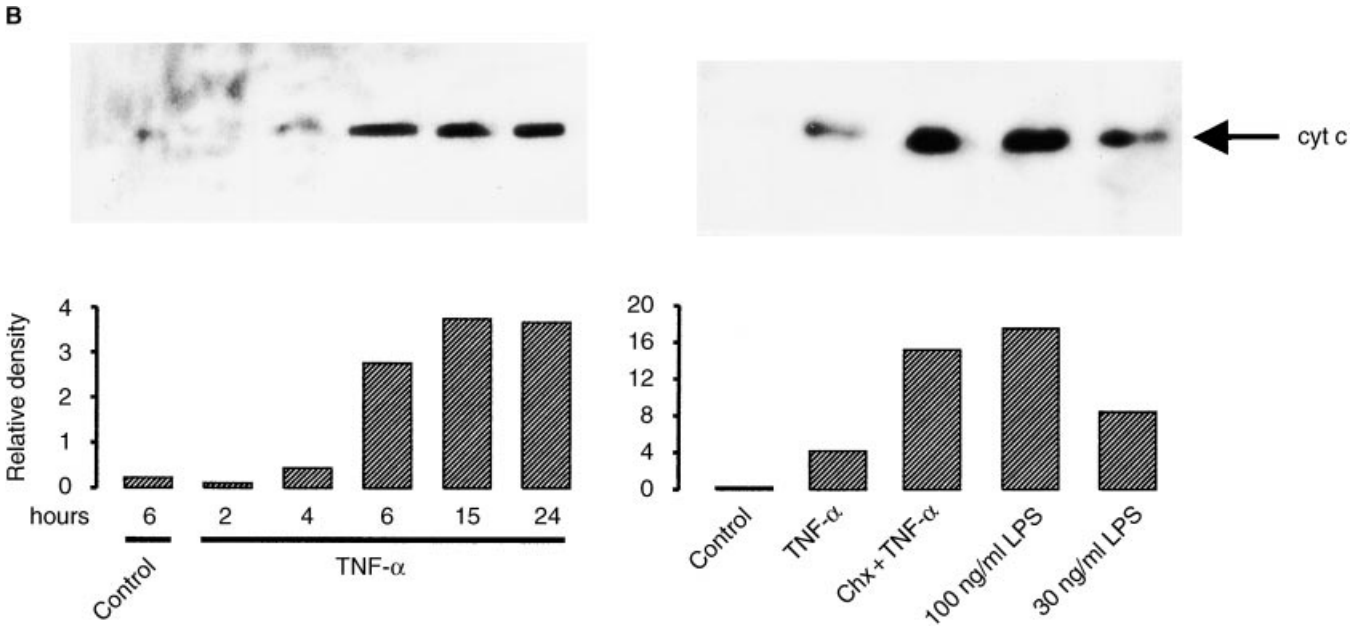

Fig. 5. (continued)

or actinomycin D or in the absence of growth factors [11]. The commitment of individual cells to die is known to depend on age [33, 34] or on the expression of anti-apoptotic genes such as members of the inhibitor of apoptosis (IAP) protein family [31], the A1 protein [35], or the A20 protein [33, 36].

Lipopolysaccharide is well known as a proinflammatory mediator, but as a direct cell death inducer, it was only poorly described. For example, porcine endothelial cells did not respond to LPS alone but underwent apoptotic cell death in response to LPS in the presence of sodium arsenite or heat shock [37]. Consistently, the induction of a heat shock response in porcine aortic endothelial cells leads to apoptosis only in those cells previously exposed to endotoxin [38]. In contrast, our experiments point to a potent induction of apoptotic cell death in glomerular endothelial cells in response to small quantities of LPS.

Tumor necrosis factor-α- or LPS-induced apoptotic endothelial cell death *in vivo* may either depend on the presence of protective growth factors such as vascular endothelial growth factor (VEGF) [39] or basic fibroblast growth factor (bFGF) [40] or, according to Figure 4, on the exposure time to the individual apogens. Although TNF-α rapidly binds to its receptor and activates a first line of signal transduction pathways such as SAPK [Meßmer et al, unpublished observations] or NF-κB [31] within one hour, the initial receptor binding and exposure of the cells to TNF-α or LPS for up to six hours was not sufficient to trigger apoptosis efficiently within 24 hours. Repetitive binding and enhancing loops may lead to the point of no return. Differences in primary pathways either between TNF-α and LPS or between glomerular endothelial cells and tumor cell lines like U937, Jurkat, MCF-7, which rapidly die in response to TNF-α within five to eight hours [41], may correlate with diverse biological functions.

Apoptotic signaling is mediated by a conserved cell death apparatus that requires several families of cell death regulators and executioners [17]. Individual signaling and death regulation differ between the different cell lines and species. Cytochrome c release from the innermitochondrial membrane space into the cytosol and mitochondrial permeability transition is a first-line apoptotic response shown in many cell death pathways [30]. As these processes do not occur under all circumstances and seem to be dispensable in some apoptotic signaling pathways [42], our findings importantly demonstrate mitochondrial cytochrome c release to be one early and strong signal in glomerular endothelial cell death. The amount of cytochrome c released within six to seven hours correlated with the extent of the subsequent appearance of apoptotic markers. Cycloheximide strongly accelerated TNF-α-induced apoptotic cell death and correspondingly induced a fourfold increase in cytoplasmic cytochrome c (Fig. 5B). Similarly, LPS proved to be a potent apoptotic stimulus and elicited cytochrome c release concentration dependently. Remarkably, cytochrome c efflux clearly preceded apoptotic changes such as DNA fragmentation and also caspase-3–like protease activation, and in fact, according to several publications, cytochrome c is required for these activities [43]. Re-

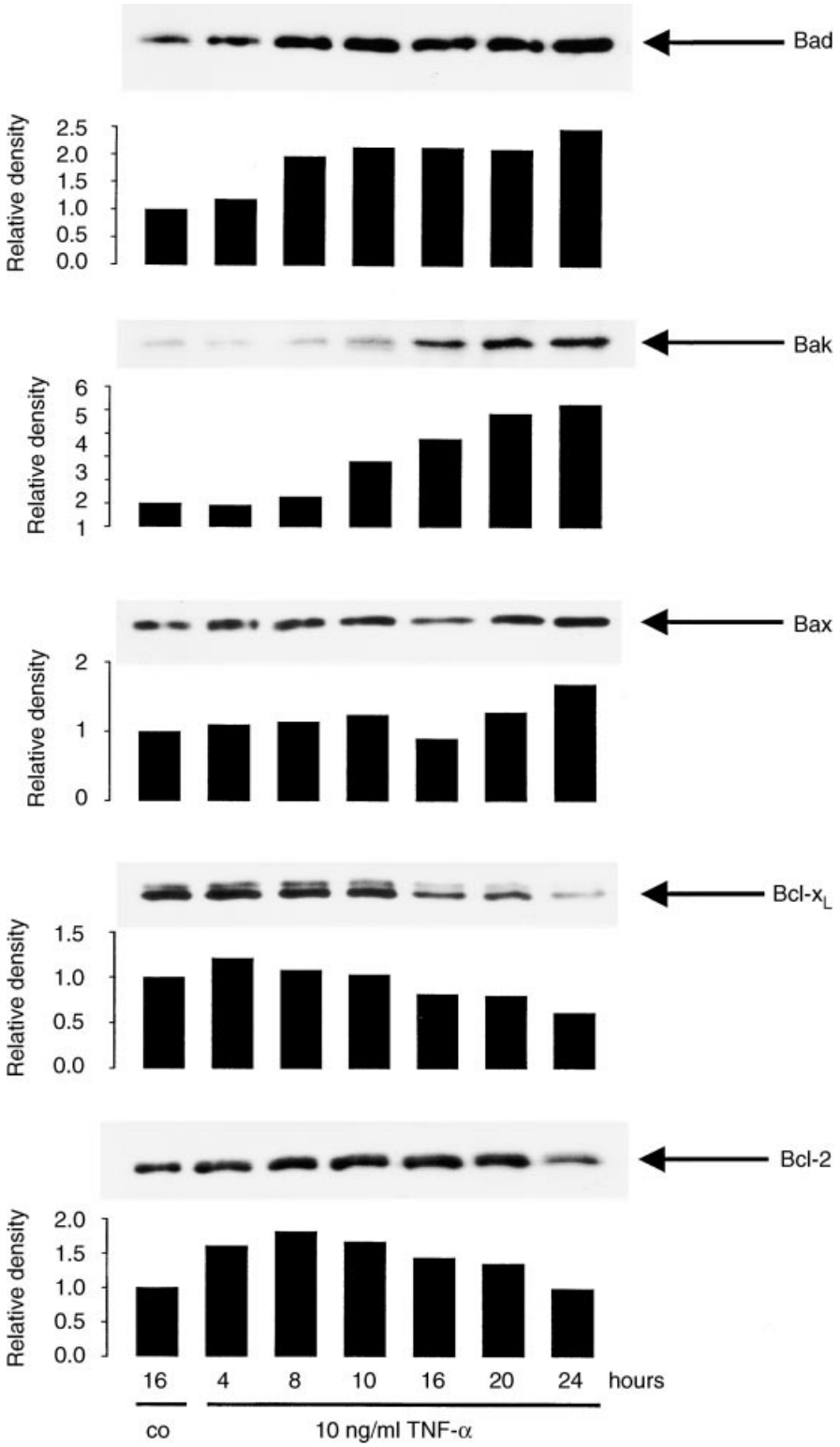

Fig. 6. Bak up-regulation and Bcl-x_L down-regulation in response to TNF-α treatment. Glomerular endothelial cells were exposed to 10 ng/ml TNF-α for the times indicated. Subsequently, cell lysates were subjected to Western blot analysis for Bad, Bak, Bax, Bcl-x_L, and Bcl-2 protein using several antibodies described in the **Methods** section followed by ECL detection. The blots are representative of at least three independent experiments.

cently, a human CED-4 homologue [apoptotic protease-activating factor 1 (Apaf-1)] has been identified to form a complex with cytochrome c, dATP, and caspase-9 and has been shown to be able to promote the activation of caspase-3 [44]. In line are our experiments that caspase-3 activation first emerged a few hours following cytochrome c release. In turn, the activation of caspases, that is, caspase-3 as a terminator caspase initiate their deadly

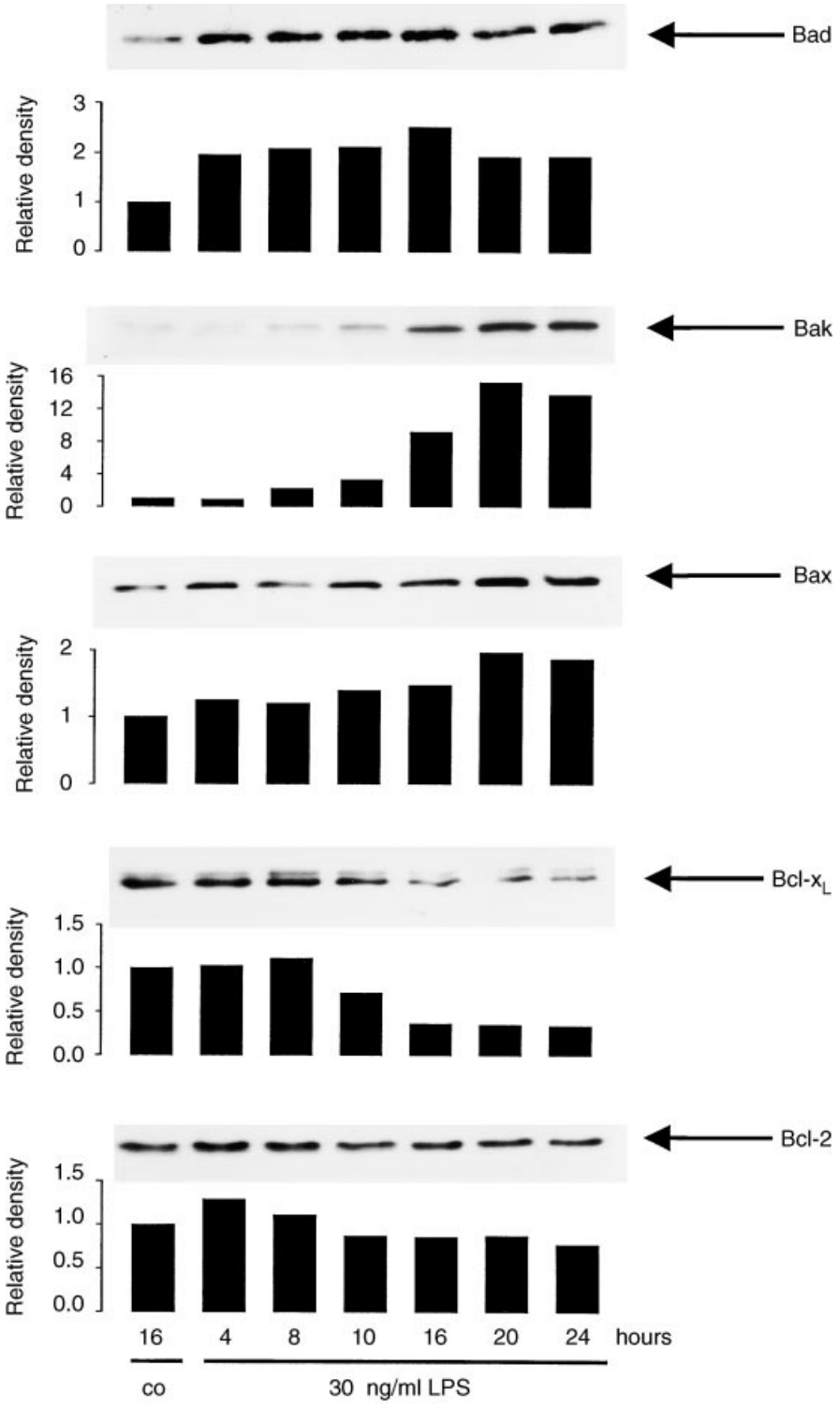

Fig. 7. Bak up-regulation and Bcl-x_L down-regulation in response to LPS treatment. Cells were exposed to 30 ng/ml LPS for the times indicated. Subsequently, cell lysates were subjected to Western blot analysis for Bad, Bak, Bax, Bcl-x_L, and Bcl-2 protein using several antibodies described in the **Methods** section followed by ECL detection. The blots are representative of at least three independent experiments.

assault on the cell by selectively cleaving a number of "death substrates." One classic substrate with an uncertain functional significance in apoptosis is the nuclear enzyme PARP [45]. We demonstrated PARP cleavage that occurred in parallel to caspase-3–like protease activation (Fig. 8). However, whether caspase-3 represents an essential part in TNF-α- and LPS-mediated apoptotic signaling in glomerular endothelial cells remained questionable because the specific caspase-3–like inhibitor DEVD-fmk only partially affected TNF-α/LPS-induced

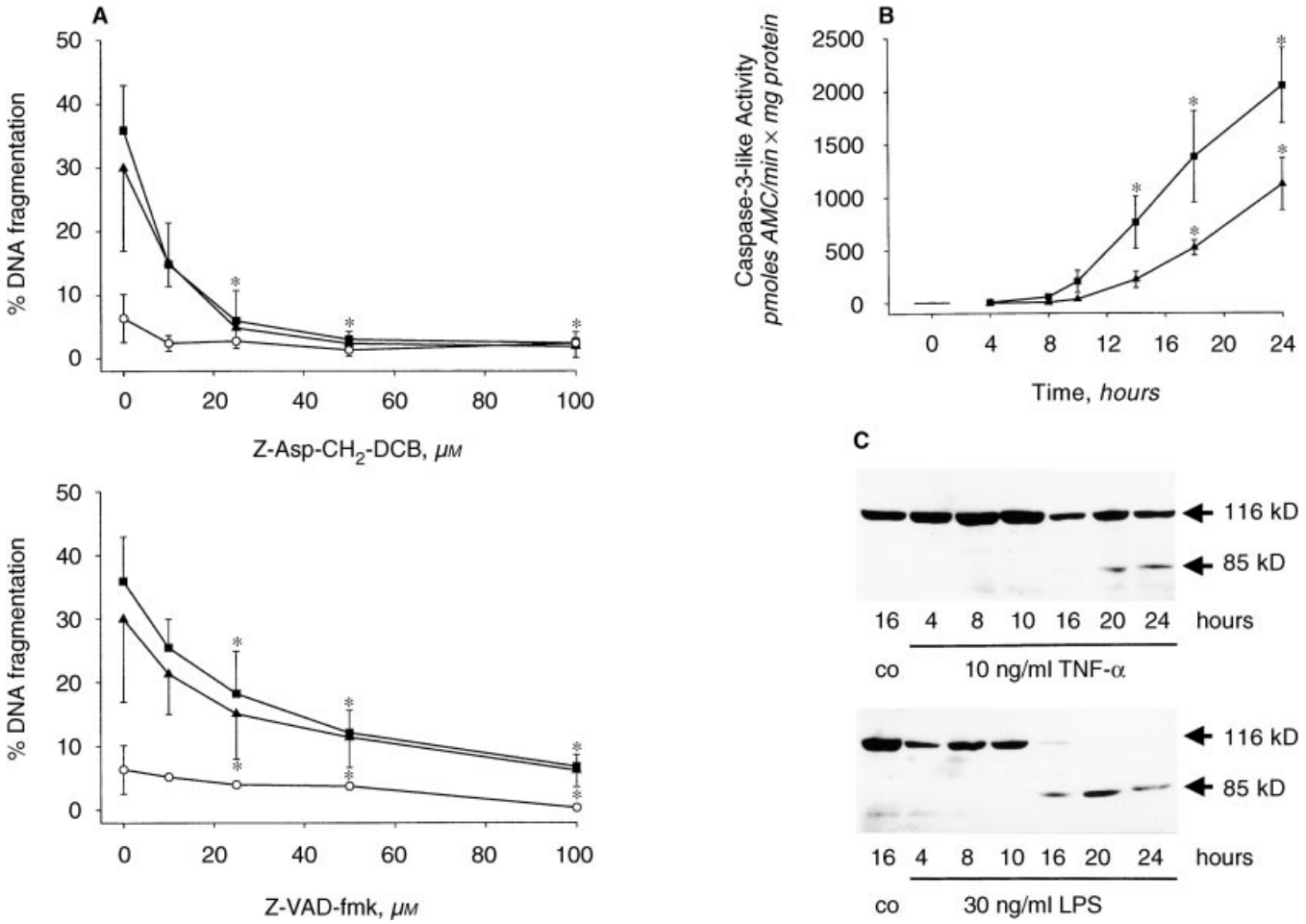

Fig. 8. Involvement of caspase-3–like protease activity in TNF-α- and LPS-induced glomerular endothelial cell apoptosis. (*A*) Glomerular endothelial cells were incubated for 24 hours with no (control, ○), 10 ng/ml TNF-α (▲) or 30 ng/ml LPS (■) in the presence of increasing concentrations of the caspase protease inhibitors Z-Asp-CH_2-DCB (upper panel) and Z-VAD-fmk (lower panel), which were added 30 minutes prior to the death agonists. DNA fragmentation was quantitated by the diphenylamine reaction. Values are means ± SD of four individual experiments. (*B and C*) Cells were incubated with 10 ng/ml TNF-α (▲) or 30 ng/ml LPS (■) for the times indicated. The line at zero signifies control. Caspase-3–like activity was determined using the fluorogenic substrate DEVD-AMC (B), and PARP cleavage (116 kDa holoenzyme and 85 kDa cleavage fragment) was monitored by Western blot analysis using the monoclonal anti-PARP antibody C-II-10 (C). Data are means ± SD or are representative of three independent experiments, respectively. $^*P < 0.05$ vs. corresponding control (analysis of variance and for multiple comparison the data were corrected by Dunn's method).

apoptosis. This may be due to a dispensability of caspase-3 in TNF-α/LPS signaling leading to cell death. Nevertheless, the efficient blockage of the final death pathways by broad spectrum caspase inhibitors (Fig. 8A) suggests a requirement of certain caspase family members.

Although cytochrome c induces death processes and caspase activation represents the terminal degradation signals, the Bcl-2 family proteins are highly organized cell death regulators. Each member of the Bcl-2 family is expressed in a cell type-, differentiation-, and activation stage-specific fashion, and a network of pairs of interacting proteins (Bcl-2/Bax, Bcl-x_L/Bak, etc.) influences cell fate [17]. According to a recent report, Bcl-x_L forms a ternary complex with caspase-9 and Apaf-1 and is able to block caspase-3 activation and apoptotic cell death [46]. Moreover, Bcl-2 and Bcl-x_L may block mitochondrial permeability transition and cytochrome c release [20, 47]. However, in our hands, Bcl-2 and Bcl-x_L protein levels were constant during the first hours at which cytochrome c was released into the cytosol. Therefore, in glomerular endothelial cells, cytochrome c efflux is an early signal that may not be blocked by Bcl-2/Bcl-x_L. Regarding the death execution pathway, Bcl-x_L protein content declined and Bak protein was up-regulated. Bak, a closely related Bax protein, can specifically suppress Bcl-x_L and Bcl-2 function and induces cell death by an unknown mechanism. Bcl-x_L as a Bak antagonist was down-regulated in parallel to caspase-3 activation, which may be due to the finding that Bcl-x_L can be degraded by a caspase-3–like activity [48]. Conclusively, in glomerular

endothelial cells, the ratio of the Bcl-x_L/Bak protein was dramatically shifted in favor of Bak, and the death execution and degradation process may depend on the expression of the proapoptotic Bak protein. Ongoing experiments will evaluate this hypothesis in TNF-α- and LPS-induced glomerular endothelial cell apoptosis.

In summary, glomerular endothelial cells undergo apoptotic cell death in response to long-term TNF-α/LPS exposure. Both signals seem to require similar apoptotic signaling pathways, such as cytochrome c release, Bcl-x_L down-regulation and Bak up-regulation, as well as caspase activation. Although both signals seem to be qualitatively comparable, LPS proved to be more potent than TNF-α. These results suggest a relevance of endotoxin- and TNF-α-induced death of glomerular endothelial cells in the progression of glomerular diseases.

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E-mail: Pfeilschifter@em.uni-frankfurt.de

APPENDIX

Abbreviations used in this article are: aFGF, acidic fibroblast growth factor; apaf-1, apoptotic protease activating factor 1; bFGF, basic fibroblast growth factor; $\Delta\Psi_m$, innermitochondrial transmembrane proton gradient; DEVD-AMC, N-acetyl-aspartyl-glutamyl-valinyl-aspartyl-7-amino-4-coumarin; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanide iodide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; EGTA, ethyleneglycol-bis(β-aminoethylether)-N,N′ tetraacetate; FADD, Fas associated death domain protein; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; HUVEC, human umbilical vein endothelial cells; IP, inducible protein; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; NF-κB, nuclear factor κB; PAF, platelet activating factor; PARP, poly(ADP-ribose) polymerase; PFGE, pulsed field gel electrophoresis; RIP, receptor interacting protein; SAPK, stress-activated protein kinase; TNF-α, tumor necrosis factor-α; TNFI, TNF receptor I; TNFR, TNF receptor; TRADD, TNF receptor associated death domain protein; TE buffer, Tris-EDTA buffer; Z-Asp-CH_2-DCB, Z-aspartyl-2,6-dichlorobenzoyloxymethylketone; Z-VAD-fmk, Z-valinyl-alanyl-DL-aspartyl-fluoromethylketone.

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