Basic Fibroblast Growth Factor Selectively Enhances TNF-α–Induced Apoptotic Cell Death in Glomerular Endothelial Cells: Effects on Apoptotic Signaling Pathways

UDO K. MESSMER,* VERENA A. BRINER, † and JOSEF PFEILSCHIFTER*
*Pharmacenter Frankfurt, Klinikum der Johann Wolfgang Goethe-University Hospital, Frankfurt am Main, Germany; and † Department of Medicine, Kantonsspital Luzern, Luzern, Switzerland.

Abstract. Endothelial cell damage of glomeruli and kidney arterioles seems to play a pivotal role in several pathologic situations, such as Gram-negative sepsis, glomerulonephritis, and acute renal failure. Bacterial lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) have been identified as potent inducers of apoptotic cell death in bovine glomerular endothelial cells. Both agents elicited apoptotic DNA laddering within 12 to 24 h. Basic fibroblast growth factor (bFGF) was generally described as a protective factor for endothelial cells against radiation-, TNF-α–, and UV-light–induced programmed cell death. Therefore, whether bFGF also affects apoptosis of microvascular endothelial cells was questioned. Surprising was that simultaneous treatment of glomerular endothelial cells with bFGF and either LPS or TNF-α left LPS-induced death unaffected, whereas TNF-α–induced death induction was potentiated, amounting to 48.9 ± 6.3% versus 22.4 ± 4.3% DNA degradation with TNF-α alone. Comparably, acidic FGF also selectively potentiated TNF-α–induced apoptosis. In mechanistic terms, bFGF synergistically increased TNF-α–induced mitochondrial permeability transition, the release of cytochrome c from mitochondria to the cytosol, and upregulation of the proapoptotic protein Bak and significantly enhanced activation of caspase-8 protease activity. In contrast, stress-activated protein kinase and nuclear factor κB activation, which represent primary signals of TNF/TNF receptor interaction, downregulation of the antiapoptotic protein Bcl-xL, and caspase-3–like protease activation, were unaffected. As bFGF did not affect LPS-induced apoptotic cell death, bFGF also left LPS-induced Bak upregulation and Bcl-xL downregulation unaffected. The results point to a selective bFGF-mediated enhancement of distinct proapoptotic pathways induced by TNF-α in glomerular endothelial cells.

Proliferation, differentiation, and cell death are dynamic processes that regulate cell homeostasis throughout life. Cell proliferation represents an essential mechanism during development and renewal of an organism and is mainly elicited and regulated by growth factors. For example, angiogenesis, the formation of new capillaries from preexisting blood vessels, is an important event in embryonic development and wound repair (1). Angiogenesis involves several steps, including invasion of endothelial cells into a stroma space and cell proliferation, which is regulated by angiogenic growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (1,2). Moreover, neuronal growth is regulated by neurotrophins, such as nerve growth factor, and any other cell by diverse factors; among them are insulin-like growth factor (IGF), epithelial growth factor, and transforming growth factor-β (3).

In contrast, cell death is the opposite process that eliminates unwanted or unnecessary cells. Cell death is thought to occur by two mechanisms, necrosis and apoptosis. Whereas necrosis occurs in response to physical injury, apoptosis is a genetically encoded and tightly regulated suicidal process in which the cell participates in its own destruction. Apoptosis is specifically induced by certain death factors; among them are Fas, tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), and different anticancer agents (4–6). Much attention has been spent in the past few years on the question of how mammalian cells exactly regulate pro- and antiapoptotic signaling (7). One prominent factor that has been investigated intensively is the cytokine TNF-α (8). Apoptosis signaling by TNF-α can be traced through protein–protein interactions involving proteins that contain specific protein interaction domains, such as the death effector domain. Among them are caspases 8 and 10 and receptor interacting protein-associated ICH-1/CED-3-homologous protein with a death domain, which associate with the TNF receptor-1 (TNF-RI) and potently link the receptor directly to the caspase cascade. Caspases 8 and 10 bind to Fas-associated death domain (FADD), which binds to TNF receptor-associated death domain protein, whereas receptor interacting protein-associated ICH-1/CED-3-homologous protein with a death domain binds to receptor interacting protein and can recruit caspase 2 (9). Apart from turning on the caspase cascade, caspase 8 cleaves Bid, which translocates to mitochondria and induces cytochrome c release (10,11).
Apoptosis signaling can be divided into induction, execution, and degradation phases, which are regulated by positive and negative control factors (7). Within the signaling phase of apoptosis, proapoptotic stimuli may be counteracted by antiapoptotic signals that mediate cell protection and survival. Antiapoptotic signaling is achieved by kinase-dependent signal transduction via the PKB/Akt pathway (12) or the Ras/Raf-Erk pathway (13), the activation of the nuclear factor κB (NF-κB) pathway (14,15), regulation of the Bcl-2 family of proteins (16), or expression/activation of the inhibitor of apoptosis protein family members, which act as direct caspase inhibitors (17). A central regulatory role is played by the Bcl-2 family of proteins whereby the Bcl-2/Bcl-xL subfamily members exert a strong antiapoptotic activity, whereas some others counteract cell survival. Proapoptotic Bcl-2 family members can be subdivided into (1) the Bax subfamily, characterized by three conserved domains (BH1, BH2, BH3) that compose Bax, Bak, and Bok, and (2) the BH3-only domain subgroup, which includes Bad, Bid, Bim, and others (18). How Bcl-2 family members exert their pro- or antiapoptotic activity is not exactly known. One important property is the formation of homo- and heterodimers within the Bcl-2 family besides the other suggested activities such as channel formation (19) and regulation of intracellular Ca²⁺ distribution (20).

Although TNF-α elicits apoptotic cell death via the described signaling pathways, it also triggers other signals, such as NF-κB and stress-activated protein kinase (SAPK) activation, which at least in part counteract proapoptotic signaling (15,21). Moreover, whether TNF-α–induced apoptosis requires new protein expression in some cell systems also remains to be elucidated.

Apoptosis-counteracting signals provided by physiologic relevant receptor-dependent actions were described for different growth factors in diverse cell systems. Prominent factors are neurotrophic factors such as nerve growth factor and related factors that promote the survival of neurons (22) IGF-1 and bFGF, which can protect against nitric oxide–induced neuronal cell death (23). Especially bFGF was shown to promote survival of human umbilical vein endothelial cells against TNF-α–, radiation–, and serum deprivation–induced apoptosis (24). Protective signals provided by bFGF require Ras and the subsequent activation of the mitogen-activated protein kinase cascade (25), but the details of the activation process remain unresolved.

Previously, we characterized apoptotic signaling of bovine glomerular endothelial cells in response to TNF-α and bacterial LPS (26). Glomerular inflammatory diseases, glomerulosclerosis, and interstitial fibrosis are leading problems in critical health care. Moreover, glomerular endothelial cell apoptosis was described during severe forms of glomerulonephritis. TNF-α– and LPS–mediated apoptotic signaling involves mitochondrial cytochrome c release, Bak protein upregulation, Bcl-xL protein downregulation, and caspase-3 activation. Pharmacologic modulation of TNF-α– and LPS–mediated cell death was achieved by the administration of glucocorticoids that potently blocked critical signaling pathways, including the final death decision (27).

In our present work, we questioned whether bFGF and acidic FGF (aFGF), which work as growth factor supplements for glomerular endothelial cells, were able to modulate TNF-α– or LPS–induced apoptotic cell death. Surprising was that whereas LPS–induced death was not affected, TNF-α–induced death potentiation was potentiated by bFGF. This potentiating effect can be extended to an enhancement of cytochrome c release and Bak protein upregulation and accelerated caspase-8 activation. We therefore conclude that there is a highly specific regulation/modulation of certain signaling pathways and a different assignment of these pathways to specific agonists.

Materials and Methods

**Materials**

Diphenylamine, LPS (Escherichia coli serumotype 0127:B8), and heparin sodium were purchased from Sigma (Deisenhofen, Germany). N-acetyl-aspartyl-glutamyl-valinyl-aspartyl-7-aminoo-4-coumarin (DEVD-AMC), Z-valinyl-alanly-DL-aspartyl-fluoromethylketone (Z-VAD-fmk), and Z-aspartyl-2,6-dichlorobenzoylmethylketone (Z-Asp-CH₂-DCB) were delivered by Bachem (Heidelberg, Germany), and Z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH₂F (Z-IETD-FMK) was from Calbiochem (Bad Soden, Germany). Bovine aFGF and human bFGF were purchased from R&D Systems (Wiesbaden, Germany), and recombinant human TNF-α (specific activity: 6.6 × 10⁶ units/mg) was a generous gift from Knoll AG (Ludwigshafen, Germany). The SAPK/JNK (JNK) and Caspase-8 assay kit was from Upstate Biotechnology (Hamburg, Germany) and the caspase-8 kit was from AMS Biotechnology (Wiesbaden, Germany). [γ-³²P]ATP was from Amersham (Braunschweig, Germany); RPMI 1640, cell culture supplements and fetal calf serum were from Life Technologies (Eggenstein, Germany). All other chemicals were of the highest grade of purity commercially available.

**Cell Culture and Cell Treatment**

Bovine glomerular endothelial cells were cultivated as described previously (28). 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-m 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 collagenase (type V, Sigma). After digestion, glomerular remnants were sedimented at 500 g for 5 min. The supernatant was centrifuged at 1000 g for 5 min, and the pellet was suspended in RPMI 1640 medium containing 20% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml heparin sodium, and 5 ng/ml aFGF. Cells were plated at 0.2% gelatin-coated tissue culture plates. Primary cultures of endothelial cell clones were isolated with cloning cylinders, detached with trypsin-ethylenediaminetraacetate (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 fluorescence acetylated low-density lipoproteins (29). 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 confluence in 60-mm 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 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 (30). Briefly, after incubation, cells were scraped off the culture plates, resuspended in 250 μl 10 mM Tris, 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 × g. Pellets were resuspended in 500 μl TE-buffer, and samples were precipitated by adding 500 μl 10% TCA at 4°C. Samples were pelleted at 4000 rpm for 10 min, and the supernatant was removed. After addition of 300 μl 5% TCA, samples were boiled for 15 min. DNA contents were quantitated using the diphenylamine reagent (31). The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

**Morphologic Investigations**

Glomerular endothelial cells were grown in 60-mm culture plates to near confluence. Cells were stimulated, followed by fixation with 3% paraformaldehyde for 5 min onto glass slides. Samples were washed with phosphate-buffered saline (PBS), 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 (Oberkochen, Germany). For each preparation, approximately 500 cells were counted by two investigators, who were blinded to the treatment. Each evaluation was repeated three times by three independent experiments.

**SAPK Activity Assay**

Glomerular endothelial cells were cultured in 100-mm diameter dishes and stimulated as indicated. Cells were scraped off the culture plates, and lysis was achieved in SAPK lysis buffer (20 mM Tris [pH 7.4], 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerocephosphate, 10% glycerol, 4 mM Pefabloc, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na3VO4, and 2 mM sodium pyrophosphate) and sonication (Branson sonifier, 10 s, duty cycle 100%, output control 10%) followed by centrifugation (2000 × g, 5 min). Protein concentration was determined by the Bradford assay (32). The solid-phase c-Jun kinase assay was performed as described (33) using a glutathione S-transferase (GST)-c-Jun (5-89) fusion protein coupled to glutathione-Sepharose beads as substrate. In brief, 4 μg GST-c-Jun (5-89) was coupled to glutathione-Sepharose in 0.5 ml SAPK lysis buffer for 30 min at 4°C. The beads were then centrifuged for 2 min at 13000 × g, washed twice with triton X-100 lysis buffer, and incubated for 2 h at 4°C with cell extracts containing 200 μg of protein. Thereafter, the complexes were washed twice with SAPK lysis buffer and once with 20 mM HEPES (pH 7.4), 20 mM MgCl2, and 20 mM β-glycerocephosphate before the kinase reaction was started by addition of 30 μl of kinase buffer (20 mM HEPES [pH 7.4], 20 mM MgCl2, 2 mM dithiothreitol [DTT], 20 mM β-glycerocephosphate, 0.1 mM Na3VO4, 20 mM p-nitrophenylphosphate, 10 μM ATP, and 2 μCi [γ-32P]ATP) to the complexes and incubated for 30 min at 30°C. To stop the reaction, 10 μl of 4x Laemmli sample buffer was added and the samples were heated for 5 min at 95°C. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and phosphorylated GST-c-Jun was detected and quantitated by Phosphor image analysis (Fuji) (Raytest, Straubenhardt, Germany).

**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 phenylmethylsulfonl fluoride [PMSF; pH 8.0]) and sonication (Branson sonifier; 10 s, duty cycle 100%, output control 10%), followed by centrifugation (4000 × g, 5 min) and Bradford protein determination (32). Proteins were normalized to 100 μg/lane (poly[ADP-ribose] polymerase [PARP]) or to 40 μg/lane (Bcl-2 proteins and TNF-RI), resolved on 7.5% (PARP and TNF-RI) or 12.5% polyacrylamide gels (Bcl-2 proteins) and blotted onto polyvinylidifluoride sheets. Sheets were washed twice with tris-buffered saline (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 with the mouse anti-PARP antibody (Biomol, Hamburg, Germany, clone C-II-10, 1 μg/ml in TBS + 0.5% skim milk), goat anti-human TNF-RI antibody (R&D Systems, 0.2 μg/ml, in TBS + 0.5% skim milk), mouse anti-Bcl-2 antibody (Immunotech, Marseilles, France, clone 83-8B, 1 μg/ml), rabbit anti-Bcl-x antibody (Transduction Laboratories, Affinity Research Products, Namhead, Exeter, UK, 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 MVGDSQEPGRGGPTSEQMK coupled to keyhole limpet hemocyanine by the m-mallimidobenzyl-N-hydroxysuccinimide method (1:2000 in TBS + 0.5% Skim milk), or rabbit anti-Bak antibody raised against a peptide WIARGGWAALNLG coupled to keyhole limpet hemocyanine by the m-mallimidobenzyl-N-hydroxysuccinimide 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 anti-mouse monoclonal antibodies (1:5000) or goat anti-rabbit monoclonal antibodies (1:5000) for 1.5 h at room temperature using the enhanced chemiluminescence method (Amersham). The primary bax 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.

**Assay for NF-κB Binding Activity**

For electrophoretic mobility shift assays, glomerular endothelial cells were incubated for the times indicated. Cells were dissolved by trypsinization, and nuclear extracts were prepared as described (34). In brief, cells were washed two times with TBS (140 mM NaCl, 50 mM Tris [pH 7.2]) and resuspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N'-tetraacetic acid [EGTA], 1 mM DTT, 0.5 mM PMSF) followed by the addition of 1/16 volume of 10% NP-40 for 10 s. After 30 s, centrifugation pellets were resuspended in buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and lysed for 15 min. After 5 min of centrifugation, the supernatant was collected and protein concentrations were determined with the Bradford assay. Binding reactions were performed for 30 min at 30°C in 1 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.05% low-fat milk powder, 0.1 μg/μl poly dIdC × poly dIdC by using 5 μg of nuclear extract and 20,000 cpm of [32P]labeled oligonucleotide (5'-AAT-TCACAAAAGGAGCTTCCCTACATCCATTG-3'). DNA-protein complexes were separated from unbound DNA probe on native 4%
polyacrylamide gels, vacuum dried, and exposed to Phosphor image screens.

**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 MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 4 mM Pefabloc, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 250 mM sucrose), 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 g for 10 min at 4°C to pellet nuclei. The remaining supernatant was centrifuged for 15 min at 10,000 × g, the pellet was used as mitochondrial fraction, and the supernatant was used as cytosolic fraction. Protein was determined with the Bradford method (32), and 50 μg were used for Western blot analysis. Proteins...
were resolved on 14% polyacrylamide gels and blotted onto polyvinylidifluoride 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 anti-cytochrome c antibody (clone 7H8.2C12, PharMingen; Becton Dickinson, San Diego, CA); 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 anti-mouse monoclonal antibody (1:5000) for 1.5 h at room temperature using the enhanced chemiluminescence method.

**FACS Analysis**

For the determination of the ΔΩ, 3,3'-dihexyloxacarbocyanide iodide (DiOC₆(3)); final concentration 10 nM) was used (35). For these experiments, glomerular endothelial cells were cultured in 60-mm culture dishes, incubated with the different apoptotic stimuli, and for the last 15 min 10 nM DiOC₆(3) was added. Afterward, cells were harvested by trypan blue and resuspended in 500 µl of PBS containing 10 µg/ml propidium iodide. Within 30 min cells were analyzed using a FACS calibur (Becton Dickinson, Heidelberg, Germany). Cells that exhibited a normal forward/side scatter ratio were selected followed by the determination of the DiOC₆(3)/propidium iodide staining properties.

For cell cycle analysis, cells were cultured in 60-mm culture dishes, incubated, harvested by trypsinization, resuspended in 5% of PBS, supplemented with 2.1 ml of ethanol, and fixed for at least 2 h at −20°C. Afterward, pelleted cells were resuspended in 500 µl of PBS containing RNase and 10 µg/ml propidium iodide and incubated for 3 min at room temperature. Cells that exhibited a normal forward/side scatter ratio were selected followed by the determination of the propidium iodide staining properties.

**Caspase-3 and Caspase-8 Enzyme Activity**

For detection of caspase-3 activity, glomerular endothelial cells were incubated as indicated and lysed in lysis buffer (10 mM Tris/HCl, 0.32 M sucrose, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM DTT [pH 8.0]) for 30 min. After sonication (10 s, output control 1), lysates were centrifuged (10,000 × g, 5 min, 4°C) and stored at −80°C. Protein determinations were performed with the Bradford method (32). Caspase-3 activity was detected by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC. Cell lysates (50 µg of protein) were incubated in 100 mM HEPES, 10% sucrose, 0.1% 3-[3-chloroindomopropyl]-dimethylammonio] propa sulfonate (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM DTT at 37°C with 12 nM DEVD-AMC in a total volume of 700 µl. Substrate cleavage and AMC accumulation were followed fluorometrically with excitation at 380 nm and emission at 460 nm.

For detection of caspase-8 activity, glomerular endothelial cells were exposed to TNF-α or TNF-α plus bFGF for the times indicated followed by cell lysis and caspase-8 activity determination according to the manufacturer’s protocol using isoeyucyl-glutamyl-threonyl-aspartyl-7-amido-4-trifluoromethyl-coumarin as substrate.

**Results**

**Effect of bFGF on TNF-α- and LPS-Induced Apoptotic DNA Fragmentation in Glomerular Endothelial Cells**

We previously demonstrated that TNF-α and LPS potently induce apoptotic cell death in isolated bovine microvascular glomerular endothelial cells (26). To explore effects of bFGF on apoptosis induction, we first coincubated glomerular endothelial cells with various concentrations of TNF-α and bFGF or LPS and bFGF and monitored DNA fragmentation. As shown in Figure 1A, TNF-α concentration-dependently induced apoptotic DNA cleavage within 24 h. Exposing the cells under comparable conditions to up to 10 ng/ml bFGF revealed no apoptotic response. Coexposure to various concentrations of TNF-α and 1 ng/ml bFGF or 10 ng/ml bFGF resulted in a strong enhancement of TNF-α-induced DNA cleavage by bFGF (Figure 1A), whereas 0.5 ng/ml bFGF exhibited a medium response and concentrations below 0.1 ng/ml were ineffective (Figure 1A). These results obtained by the quantitative diphenylamine reaction were supported by agarose gel electrophoresis, which clearly showed enhanced DNA ladder formation in incubations in which TNF-α was simultaneously applied with bFGF when compared with TNF-α alone. Similar to TNF-α, LPS also concentration-dependently elicited apoptotic DNA fragmentation, resulting in approximately 30 and 45% DNA degradation when using 10 ng/ml or 30 ng/ml LPS, respectively (Figure 1B). In contrast, bFGF showed no modulatory effects on LPS-induced apoptosis induction, verified quantitatively by the diphenylamine assay and qualitatively by agarose gel electrophoresis. Similar to biochemical parameters, morphologic studies using H33258 nuclear staining revealed enhancement of TNF-α–induced apoptosis by bFGF, whereas LPS-induced apoptosis was not affected (Figure 1, C through E, and data not shown). Concerning the effects of aFGF on...
TNF-α– and LPS-induced apoptosis, we observed comparable effects as with bFGF (data not shown).

Next, we wanted to know how bFGF is able to modulate TNF-α–mediated apoptotic signaling. Therefore, we first compared in a time kinetic study TNF-α– and TNF-α/bFGF-induced DNA fragmentation. As demonstrated in Figure 2, TNF-α– as well as TNF-α/bFGF-induced DNA cleavage first emerged after 8 to 10 h and increased up to 24 h, with TNF-α/bFGF resulting in a more pronounced DNA cleavage.

In the experiments described so far, bFGF and the apoptotic inducer TNF-α were administered at the same time and cells were continuously exposed to the agents during the entire incubation period. Next, bFGF was administered at various time intervals before and after the addition of TNF-α to define the time window required for an effective apoptosis-enhancing effect. As demonstrated in Figure 3, A and B, when a suboptimal concentration of TNF-α (1 ng/ml) was used, bFGF displayed a maximal enhancing effect when it was added 8 h before TNF-α, and this effect gradually declined when bFGF was added 8 h after TNF-α. Concerning the bFGF concentration dependence, there was no significant difference between a low and a high concentration of bFGF. In contrast, when TNF-α was applied at an effective concentration (10 ng/ml), bFGF significantly but in a less dramatic way enhanced apoptosis induction (Figure 3, C and D). By using this experimental setting, there was no difference between high or low concentrations of bFGF or the administration of bFGF 8 h before or 8 h after TNF-α.

Effects of TNF-α or TNF-α Plus bFGF on SAPK/JNK Activity, NF-κB Activation, and TNF-RI Expression

After binding to its specific receptors on the cell surface, TNF-α exerts its apoptotic action via several signaling cascades. To define the interaction target of bFGF, we selected distinct pathways for further experiments. Activation of protein kinases, such as SAPK cascade, and activation of the transcription factor NF-κB represent early intracellular signaling pathways after TNF-α receptor activation. First, within 10 min after

Figure 3. Time dependency of bFGF on the TNF-α effect. Bovine glomerular endothelial cells were stimulated for 24 h with 1 ng/ml TNF-α (A and B) or 10 ng/ml TNF-α (C and D). As indicated, 1 ng/ml bFGF (A and C) or 10 n/ml bFGF (B and D) were added at various times in relation to TNF-α application starting 8 h before the apoptotic inducer until 8 h after TNF-α application. All experiments reached a total TNF-α exposure period of 24 h. The amount of DNA fragmentation was determined with the diphenylamine reaction as outlined in the Materials and Methods section. Values are means ± SEM of at least four individual experiments. *P < 0.05 compared with incubations without bFGF (ANOVA and for multiple comparison, the data were corrected by Dunn’s method).
TNF-α addition, SAPK is activated in glomerular endothelial cells, peaks 30 min after TNF-α, and returns to control values within 4 h (Figure 4A). A similar and comparable time-kinetic response was detected when bFGF was applied together with TNF-α, whereas bFGF alone remained inactive. Second, NF-κB transcription factor activity was triggered within 30 min after TNF-α addition and remained increased up to 16 h. A comparable time-kinetic activity was obtained with TNF-α/bFGF (Figure 4B). As bFGF has no effect on these distinct primary TNF-α signaling pathways and bFGF also exerts an enhancing effect when applied several hours after TNF-α (Figure 3), we conclude that bFGF may modulate pathways downstream or independent of SAPK or NF-κB activation. Therefore, we also questioned whether bFGF may increase TNF-RI expression in glomerular endothelial cells. As shown in Figure 4C, neither TNF-α nor TNF-α/bFGF exhibited significant modulatory effects of TNF-RI protein expression.

**Effect of bFGF on TNF-α–Induced Apoptotic Signaling**

Mitochondrial permeability transition and mitochondrial cytochrome c efflux, with the resulting activation of caspases 9, 3, and 7, seems to be a pivotal control point for apoptosis induction by several apoptogens in different cell types (36). To characterize further the apoptosis-accelerating effect of bFGF, we measured its effects on mitochondrial permeability transition induced by TNF-α. The mitochondrial membrane potential was measured by the uptake of the mitochondrial-specific dye DiOC₆(3). Adherent cells were stimulated with TNF-α in the presence or absence of bFGF for different time periods followed by DiOC₆(3) addition to the culture medium 15 min before harvesting the cells. Approximately 7 to 10% of the cells that grew for up to 24 h under control conditions exhibited a low DiOC₆(3) uptake capacity (Figure 5A). TNF-α increased the cell population with a reduced mitochondrial membrane potential within 10 to 24 h from 12% to approximately 38%. Coincubation of cells with bFGF and TNF-α resulted in an accelerated decline in the mitochondrial membrane potential affecting approximately 21 to 52% of all cells at 10 and 24 h, respectively. Comparably, mitochondrial cytochrome c efflux in response to TNF-α starts within 2 to 6 h and proceeds up to 24 h, and this process is markedly accelerated by simultaneously adding bFGF (Figure 5B).

Next, we focused on an important group of apoptosis regulatory factors, the Bcl-2 family of proteins. Regarding some proapoptotic members of this protein family, we show in Figure 6 that Bax and Bad protein levels were not altered by TNF-α or TNF-α/bFGF. In contrast, Bak protein showed a strong induction with TNF-α within 10 to 24 h, which is potentiated by coinoculation with bFGF (Figure 6). Focusing on the antiapoptotic subfamily of Bcl-2-related proteins, Bcl-2 protein levels were not altered by either TNF-α or TNF-α/bFGF, whereas Bcl-xL markedly declined within 10 to 24 h after TNF-α stimulation. Surprising is that although bFGF accelerated TNF-α–induced Bak protein upregulation, TNF-α–induced Bcl-xL protein downregulation remained unaffected by bFGF (Figure 6). These data point to a selective modulation of certain proapoptotic pathways by bFGF.

**Effect of bFGF on LPS-Induced Bak Protein Upregulation and Bcl-xL Protein Downregulation**

To verify whether bFGF specifically accelerates TNF-α–mediated proapoptotic pathways that lead to Bak upregulation and subsequently to enhanced apoptotic cell death, we ques-
tioned whether bFGF, which leaves LPS-induced apoptosis unaffected, shows any effect on LPS-mediated Bak upregulation or Bcl-xL downregulation. As demonstrated in Figure 7, 10 ng/ml LPS or 30 ng/ml LPS promoted approximately a fourfold Bak protein upregulation and a concomitant Bcl-xL protein downregulation after 18 h and 24 h. Simultaneous addition of bFGF and LPS to glomerular endothelial cells modulated neither LPS-induced Bak upregulation nor Bcl-xL downregulation (Figure 7). These results are in line with those obtained with the diphenylamine assay (Figure 1B) and support our suggestion that bFGF selectively affects TNF-α–induced apoptosis at least in part by affecting Bak protein expression.

Caspase-3–Like and Caspase-8–Like Protease Activation in Response to TNF-α and bFGF

Mammalian caspases, which compose a group of at least 14 members that can promote apoptosis (37), are recognized to participate in initial and final death pathways. Especially the caspase-3–like protease subfamily (caspases 3, 7, and 9) exerts degradative functions in programmed cell death of many cells. Therefore, we measured caspase-3–like activity by using DEVD-AMC as a fluorogenic substrate. TNF-α time-dependently increased caspase-3–like protease activity starting 8 h...
after TNF-α addition and grew continuously during the 24-h incubation period (Figure 8A). Unexpected was that although bFGF accelerates TNF-α–induced apoptosis, caspase-3–like protease activation was altered neither in its time course nor in its intensity. Similarly, TNF-α–induced cleavage of the nuclear protein PARP, which is generally regarded as a caspase-3–like protease substrate detectable in parallel to caspase-3–like protease activation, was not significantly influenced by bFGF (Figure 8B). To prove whether the accelerating effect of bFGF in general is caspase independent or depends on certain non–caspase-3–like activities, we used broad-spectrum caspase inhibitors such as Z-Asp-CH₂-DHB and Z-VAD-fmk. As shown in Figure 8C, Z-Asp-CH₂-DHB completely blocked TNF-α/bFGF-induced programmed cell death, thus verifying that the accelerating effect of bFGF also depends on caspase activation, which, however, seems to be different from caspase-3–like proteases. Therefore, we also tested caspase-8 activation, which may represent either a proximal or a terminal activity in the caspase protease cascade. Caspase-8 activity was detectable as early as 6 h after TNF-α or TNF-α/bFGF addition and raised up to 24 h (Figure 9A). As also shown in Figure 9A, bFGF significantly potentiated TNF-α–induced caspase-8 activity, where the most significant effect was visible at the 14-h time point ($P < 0.02$). Furthermore, we wanted to know whether TNF-α– or TNF-α/bFGF-induced apoptosis depends on caspase-8 activation. Therefore, we coincubated glomerular endothelial cells with either TNF-α or TNF-α/bFGF and 50
mM of the specific caspase-8 inhibitor Z-IETD-fmk and measured cell death induction by monitoring DNA cleavage with the diphenylamine assay. Surprising was that Z-IETD-fmk blocked only 20 to 30% of the TNF-α or TNF-α/bFGF effects (Figure 9B), which indicates either that the inhibitor is not available in sufficient quantities in the cells to block caspase 8 activity fully or that additional Z-IETD-fmk–insensitive caspases are involved in TNF-α– or TNF-α/bFGF-induced apoptosis.

Discussion

The major finding of the present study is that bFGF, which normally induces cell proliferation and differentiation of large vessel-derived endothelial cells, paradoxically exerts a selective apoptosis-enhancing effect on TNF-α–mediated cell death induction in glomerular capillary endothelial cells. Glomerular endothelial cells play a pivotal role in the inflammatory processes within the glomerulus, e.g., by coordinating the recruitment of inflammatory cells to sites of tissue injury (38). LPS activates many of the proinflammatory and procoagulant responses of endothelial cells, and endothelial injury in general is thought to play a crucial role in the pathogenesis of septic shock as a result of Gram-negative bacteria (39,40). Although endothelial cells are a prime target of LPS and vascular complications of septic shock as a result of Gram-negative bacteria are related to endothelial injury, LPS also targets immune cells such as macrophages and elicits proinflammatory mediators such as TNF-α (41). We clearly presented in a previous report that both LPS and TNF-α directly induce apoptosis of cultured bovine glomerular endothelial cells (26). The relevance of glomerular endothelial cell apoptosis in vivo was also documented in progressive glomerulonephritis and in mesangioproliferative glomerulonephritis (42,43). Although we have shown that TNF-α– and LPS-induced cell death can be blocked by glucocorticoids, which endogenously exert an antiinflammatory role, interactions with growth factors and other cytokines have not been addressed. So far, only one report documents a role of bFGF in experimental models of glomerulonephritis. In the rat mesangioproliferative anti-Thy 1.1 glomerulonephritis, mesangial cell damage induces the release of constitutively expressed glomerular bFGF. With the use of a neutralizing anti-bFGF antibody or a functional peptide receptor antagonist, mesangial cell injury was prevented. In contrast, cell death was accelerated with bolus injections of bFGF in the disease model (44,45).

The opposite, antiapoptotic function of bFGF on endothelial cell death was described in several reports. Human umbilical vein endothelial cells die by apoptosis in response to TNF-α or by growth factor and serum deprivation, whereas aFGF and bFGF were able to prevent this process (24,46). bFGF was also shown to prevent serum deprivation-induced apoptosis in retinal pigmented epithelial cells (47) and in a human lens epithelial cell line (48). Moreover, osteoblast survival was promoted by different growth factors, such as IGF-I, IGF-II, insulin, and bFGF (3).

The mechanisms by which bFGF exerts its antiapoptotic functions remains unsettled. There is only scant information on this topic; for example, in bovine aortic endothelial cells, protein kinase C seems to be involved in bFGF-mediated protection against radiation-induced apoptosis (49). In retinal epithelial cells, bFGF protection depends on ERK-2 activation (47), and in brain capillary endothelial cells signaling via FGFR 1 depends on extracellular matrix (50). The unexpected
finding of the present study that bFGF selectively triggered a superinduction of TNF-α–elicited apoptotic cell death in glomerular endothelial cells suggests that cell type-specific signaling capacities determine the final action of bFGF as an pro-
or antiapoptotic modulator.

Until now, apoptosis signal transduction in bovine glomerular endothelial cells elicited by TNF-α and LPS seemed to be very similar (26). Both agents require an exposure period of approximately 10 h to exert irreversibly their proapoptotic action. Signaling pathways include release of mitochondrial cytochrome c into the cytosol, mitochondrial permeability transition, Bak protein upregulation, Bcl-xL protein downregulation, and caspase-3–like protease activation (26). In general, TNF-α–mediated apoptotic signal transduction is thought to require a coordinated interaction between the TNF receptor and certain receptor-associated proteins that contain a death domain. Some prominent and important proteins are FADD and caspase 8. Caspase 8 functions as a proximal caspase, which elicits the executioner caspase cascade and finally leads to cell death. In contrast, although LPS was characterized to induce apoptosis in endothelial cells, only fragmentary information is available concerning its signaling pathways. One interesting finding was that LPS requires CD14 binding and subsequently FADD activation, suggesting a similar mechanism as TNF-α although TNF receptor would not be involved in LPS-mediated death (51). However, as both TNF-α and LPS require a long incubation period (approximately 10 h) to elicit apoptotic cell death effectively and irreversibly, signaling pathways such as enhancing or regulatory loops that control the progression of proapoptotic signaling should be expected (Figure 10). Furthermore, as bFGF does not influence LPS signaling whereas TNF-α–mediated apoptotic cell death was synergistically enhanced, we propose at least in part that there are different apoptosis signaling pathways for TNF-α and LPS in bovine glomerular endothelial cells (Figure 10).

Apart from our current report using glomerular endothelial cells, a proapoptotic activity of bFGF was described for the human breast cancer cell line MCF-7 where bFGF itself promoted apoptosis and increased the rate of drug-induced cell death (52), in nontransformed but not in transformed cells where it results in the production and release of apoptosis inducing factor (53), and in glomerular mesangial cells (45). Recombinant bFGF downregulated Bcl-2 mRNA and protein levels in MCF-7 cells and caused an increase in Bak protein levels (52). In glomerular endothelial cells, only some distinct pathways, such as caspase-8 activation, Bak protein upregulation, and in part mitochondrial cytochrome c release or mitochondrial permeability transition, were enhanced by bFGF, whereas Bcl-xL downregulation, caspase-3 activation, and PARP cleavage were unaffected. Although bFGF targets pathways that result in a potentiation of TNF-α–induced caspase-8 activation, our studies with an irreversible, cell-permeable caspase-8 inhibitor revealed that enhancement of caspase-8 activation represents only one facet of bFGF action. Moreover, bFGF neither affected TNF-α–induced NF-κB activation nor modulated TNF-RI expression. Therefore, as TNF-α activates at least two apoptotic signaling cascades (54), we suggest that bFGF also targets at least one initiating/regulatory (loop) pathway that is required to trigger cell death irreversibly (Figure 9). This regulatory pathway affects in part mitochondrial cytochrome c release, Bak protein levels, and (caspase) protease pathways distinct from caspase 3. The involvement of certain caspase protease activities distinct from caspase 3 seems reasonable because DEVD-CHO blocked caspase 3 activity in glomerular endothelial cells but did not block apoptotic cell death, whereas broad-spectrum caspase inhibitors also blocked apoptosis induction (26). That bFGF may target a regulatory loop rather than an initiating signal fits with the results documenting that bFGF also modulates TNF-α–mediated apoptosis when it was added several hours after TNF-α. Further studies that will identify certain proximal pathways or distinct individual caspases will improve our understanding of the paradoxical apoptosis enhancing activity of bFGF.

Acknowledgments

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 553), the Paul and Ursula Klein Stiftung, and the Dr. med. h.c. Erwin Braun Stiftung. The authors thank Ulrike Müller for expert technical assistance.

References


40. Kitamura H, Shimizu A, Masuda Y, Ishizaki M, Sugisaki Y, Yamanaka N: Apoptosis in glomerular endothelial cells during...


