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Expression, regulation, and function of inhibitor of apoptosis family genes in rat mesangial cells

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Background. The inhibitor of apoptosis (IAP) family of proteins regulates programmed cell death triggered by various stimuli. The purpose of this investigation was to examine the expression, regulation, and function of IAP genes in cultured rat mesangial cells.

Methods. Basal and inducible expression of c-IAP1, c-IAP2, XIAP, and TIAP mRNAs was examined in mesangial cells, isolated glomeruli, and other cell lines under unstimulated and tumor necrosis factor-α (TNF-α)-stimulated conditions. To examine a role of nuclear factor-κB (NF-κB) in the regulation of IAPs, expression of IAPs in NF-κB-inactive mesangial cells was compared with that in wild-type cells. To investigate roles of IAPs in mesangial cell apoptosis, NF-κB–inactive cells were stably supertransfected with c-IAP1 or c-IAP2, and the susceptibility of these cells to TNF-α–induced apoptosis was evaluated quantitatively.

Results. Substantial, constitutive expression of c-IAP2, XIAP, and TIAP was observed in serum-deprived rat mesangial cells and c-IAP2 and XIAP in isolated normal rat glomeruli. In response to TNF-α, expression of c-IAP1 and c-IAP2 was induced in HeLa cells and ECV304 endothelial cells, but not in mesangial cells. In contrast to previous reports on other cell types, the expression of IAPs in rat mesangial cells was independent of NF-κB; that is, expression levels of IAPs in NF-κB–inactive cells were same as those in NF-κB–active cells under both unstimulated and TNF-α–stimulated conditions. Even without the induction of IAPs, NF-κB–active mesangial cells were more resistant to TNF-α–induced apoptosis than NF-κB–inactive cells. Interestingly, overexpression of either c-IAP1 or c-IAP2 completely compensated for the lack of resistance to apoptosis in NF-κB–inactive cells.

Conclusions. IAPs are constitutively expressed in cultured rat mesangial cells and isolated normal rat glomeruli. IAPs can contribute to the survival of rat mesangial cells, but unexpectedly, these molecules are not involved in the TNF-α–induced, NF-κB–dependent cytoprotection in this cell type.

Key words: tumor necrosis factor-α, programmed cell death, nuclear factor-κB, cytoprotection, cell suicide, glomerular injury.

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Apoptosis is an evolutionarily conserved biological program for cell suicide by which unnecessary or damaged cells are eliminated from organisms. Dysregulation of apoptosis is implicated in the pathogenesis of a wide range of diseases, including inflammation, ischemic injury, infection, and malignant disorders. In the glomerulus, apoptosis of mesangial cells is observed in various glomerular diseases [1] and supposedly contributes to progression or resolution of glomerular injury [2–4].

The proinflammatory cytokine tumor necrosis factor-α (TNF-α) is an important mediator that regulates apoptosis of resident and infiltrating cells. Previous reports disclosed that TNF-α simultaneously induces intracellular signals with opposite directions, that is, the death signal and the survival signal [5]. The death signal triggers activation of caspases via the TNF receptor 1 (TNFR1)–TNFR-associated death domain (TRADD)–Fas-associated death domain (FADD) pathway. TNF-α also incites the survival signal that is mediated by nuclear factor-κB (NF-κB). Disruption of the NF-κB pathway results in apoptosis of various cells originally resistant to TNF-α [6, 7].

The molecular mechanism involved in the anti-apoptotic action of NF-κB is not well understood, but inhibitor of apoptosis (IAP) proteins are supposed to play important roles in the NF-κB–dependent cytoprotection. IAP is the family of ubiquitous intracellular proteins that protect cells from apoptosis triggered by various stimuli [8, 9]. The common structure of the IAP family includes motifs termed the baculovirus IAP repeat (BIR) and the C-terminal RING finger domain [8, 9]. It is known that the BIR domain is able to bind and inhibit caspases [10, 11]. To date, six IAPs have been identified in mammals: c-IAP1 (HIAP2/hMIHB) [12], c-IAP2 (HIAP1/hMIHC) [12], XIAP (hILP/MIHA) [12], TIAP/Survivin

[13, 14], NAIP [15], and BRUCE/Apollon [16, 17]. Many of these molecules inhibit apoptosis via direct interaction with caspases including caspases 3, 7, and 9 [13, 18–20].

Previous reports showed that overexpression of c-IAP1, c-IAP2, XIAP, NIAP, or TIAP/Survivin suppressed apoptosis induced by a wide range of stimuli, including TNF-α, Fas ligand, oxidative stress, anticancer drug, and growth factor deprivation [13–15, 21–23]. The regulation of IAP genes has not been fully elucidated, but some IAPs are known to be regulated by NF-κB and are involved in the anti-apoptotic effect of NF-κB in certain cell types [22–25]. For example, in human umbilical vein endothelial cells and human skin microvascular endothelial cells, the expression of c-IAP1, c-IAP2, and XIAP genes is up-regulated by TNF-α. Inactivation of NF-κB attenuates the induction of IAPs and sensitizes the cells to TNF-α–induced apoptosis [22].

We previously reported that cultured rat mesangial cells are resistant to TNF-α–induced apoptosis and that those cells become susceptible to the apoptosis when NF-κB is inactivated [26, 27]. Currently, the mechanisms involved in this phenomenon, especially molecules downstream of NF-κB, have not been identified. To investigate the cytoprotective action of NF-κB in TNF-α–stimulated mesangial cells further, the present study examined the roles of the IAP family. This investigation examined which IAPs are expressed in glomerular cells, whether or not NF-κB is required for the basal and inducible expression, and how the expression is involved in the NF-κB–mediated cytoprotection.

METHODS

Cells and isolated glomeruli

Clonal rat mesangial cells (SM41, SM43, and FM14) were established from isolated glomeruli of a male Sprague-Dawley rat (SM) or a male inbred F344 rat (FM) and were identified as being of the mesangial cell phenotype, as described in previous studies [28, 29]. SM43 cells generally were used for the experiments. HeLa cells and the human endothelial cell line ECV304 were purchased from European Collection of Animal Cell Cultures (Salisbury, UK). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 100 U/mL of penicillin G, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, and 10% fetal calf serum (FCS). Medium containing 1% FCS was generally used for experiments.

Normal glomeruli were isolated on ice from adult male Sprague-Dawley rats (250 to 300 g body weight) by the conventional sieving method and used for Northern blot analysis, as described previously [30].

Establishment of stable transfectants

Nuclear factor-κB–inactive mesangial cells, SM/IκBαM, were created by overexpression of a super-repressor mutant of IκBα, IκBαM, as described before [31]. IκBαM possesses mutations at the N- and C-terminal phosphorylation sites and is resistant to both constitutive and inducible degradation. When overexpressed, IκBαM effectively blocks NF-κB activation via inhibiting nuclear translocation of NF-κB complexes [7]. SM/IκBαM cells express IκBαM mRNA and IκBαM protein abundantly and exhibit blunted activation of NF-κB in response to IL-1β and TNF-α [26, 31].

SM/IκB·IAP1 and SM/IκB·IAP2 clones were created as follows. Using a modified calcium phosphate coprecipitation method, SM/IκBαM cells were stably supertransfected with an expression plasmid pEF-c-IAP1 or pEF-c-IAP2 (gifts of Dr. David L. Vaux, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) [12] that introduces the full-length human c-IAP1 or c-IAP2 cDNA together with the puromycin resistance gene. Stable transfectants were selected in the presence of puromycin (1.5 μg/mL), and stable clones SM/IκB·IAP1-1, SM/IκB·IAP1-4, SM/IκB·IAP2-2, and SM/IκB·IAP2-4 were established. As a control clone, SM/IκB·control cells were created by transfection of SM/IκBαM cells with pBabe-puro (a gift of Dr. H. Land, Imperial Cancer Research Fund, London, UK) [32] encoding the puromycin resistance gene alone.

Induction and assessment of apoptosis

Confluent mesangial cells were preincubated in 1% FCS for 24 hours and exposed to human recombinant TNF-α (250 U/mL; a gift of Dr. K. Noguchi, Teikyo University, Tokyo, Japan) for up to 40 hours. Morphologic examination was performed using phase-contrast microscopy. For the quantitative assessment of apoptosis, cells were fixed in 4% formaldehyde for 10 minutes, stained by Hoechst33258 (10 μg/mL; Sigma-Aldrich, Dorset, UK) for one hour, and subjected to fluorescence microscopy. Both attached and detached cells were used for the analysis.

Northern blot analysis

Confluent cells were cultured in 1% FCS for 24 hours and treated with or without TNF-α for up to 48 hours. Total RNA was extracted by a single-step method [33], and RNA samples were electrophoresed on 1.2% agarose gels and transferred onto nitrocellulose membranes. For hybridization, human c-IAP1 [12], human c-IAP2 [12], murine XIAP [12], and murine TIAP [13] cDNAs were labeled with ^{32}p-dCTP using the random priming method. As a loading control, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. The membranes were hybridized with probes at 65°C

overnight in a solution containing 4 × standard sodium citrate (SSC; 600 mmol/L sodium chloride, 60 mmol/L sodium citrate), 5 × Denhardt's solution, 10% dextran sulfate, 50 μg/mL herring sperm DNA, and 50 μg/mL poly(A), washed at 50°C and exposed to Kodak XAR films at −80°C. Intensity of IAP mRNAs was evaluated by densitometric analysis. Each value was normalized by the level of GAPDH mRNA and expressed as fold increase.

Western blot analysis

Confluent cells cultured in 1% FCS were treated with or without TNF-α (250 U/mL) for up to 24 hours. After the treatment, Western blot analysis of c-IAP1 was performed using an anti–c-IAP1 antibody (R&D Systems, Minneapolis, MN, USA) following the instructions provided by the manufacturer. The antibody used can detect both human and rat c-IAP1.

Statistical analysis

Data were expressed as means ± SE. Statistical analysis was performed using the one-way analysis of variance (ANOVA) with Fisher's PLSD test to compare data in different groups. A P value of <0.01 was used to indicate a statistically significant difference.

RESULTS

Basal expression of IAPs in rat mesangial cells and isolated normal rat glomeruli

Expression of IAPs in rat mesangial cells was examined under the serum-deprived, basal culture condition. Rat mesangial cells (SM43) incubated in 1% FCS for 24 hours were subjected to analysis of c-IAP1, c-IAP2, XIAP, and TIAP expression. Northern blot analysis showed that mesangial cells constitutively expressed c-IAP2 (5.8 kb), XIAP (9.0 kb), and TIAP (1.4 kb) mRNAs (Fig. 1A). The size of mRNAs observed in rat mesangial cells was identical to that in human tissues (c-IAP2 and XIAP) and in mouse tissues (TIAP) [13, 15]. In contrast to these IAPs, the expression level of c-IAP1 (4.0 kb) was very low, and only a faint expression could be occasionally detected in rat mesangial cells.

Expression of IAPs was also examined in isolated normal rat glomeruli. Like cultured mesangial cells, normal glomeruli constitutively expressed c-IAP2 and XIAP (Fig. 1B). Expression of c-IAP1 and TIAP was not detectable in isolated normal glomeruli.

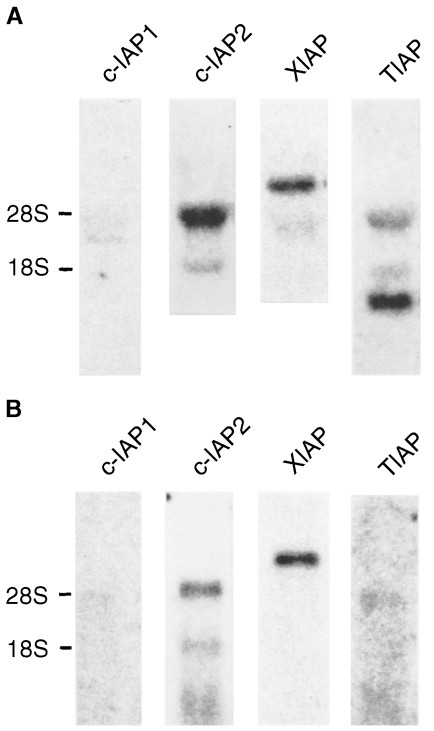

Fig. 1. Basal expression of inhibitor of apoptosis (IAP) genes in unstimulated glomerular cells. (*A*) Expression in cultured rat mesangial cells. Confluent SM43 mesangial cells were incubated in the presence of 1% FCS for 24 hours and subjected to Northern blot analysis of c-IAP1, c-IAP2, XIAP, and TIAP. The position of 28S and 18S ribosomal RNAs is indicated on the left. (*B*) Expression in isolated normal rat glomeruli. Normal glomeruli were isolated from adult rat kidneys and subjected to Northern blot analysis.

Lack of IAP induction in TNF-α–stimulated mesangial cells

Previous reports suggested that certain IAPs, including c-IAP1, c-IAP2, and XIAP, are induced by TNF-α in some cell types [22–25]. We next examined the effect of TNF-α on the expression of IAPs in mesangial cells and other cell lines. Rat mesangial cells, HeLa cells, and endothelial ECV304 cells were preincubated in 1% FCS for 24 hours and stimulated by TNF-α for four hours. Northern blot analysis showed that constitutive expression of c-IAP1, c-IAP2, XIAP, and TIAP was observed in HeLa cells (Fig. 2A) and ECV304 cells (Fig. 2B). In these cells, expression of c-IAP1 and c-IAP2 was increased 3- to 15-fold in response to TNF-α. In contrast, the induction of c-IAP1 and c-IAP2 by TNF-α was not observed in rat mesangial cells (Fig. 2C). Densitometric analysis of mRNA levels is summarized in Figure 2D. The lack of IAP induction in mesangial cells was not due to an abnormality in the TNF-α signaling because, like other cell types, rat mesangial cells (SM43) show activation of NF-κB in response to TNF-α [26]. In all cell types, expression of XIAP and TIAP was unaffected by the treatment with TNF-α.

The lack of induction of c-IAP1, c-IAP2, and XIAP in mesangial cells was further confirmed using various time points. Mesangial cells preincubated in 1% FCS were stimulated by TNF-α for 0 to 48 hours and subjected to Northern blot analysis. Consistent with the result shown in Figure 2C, induction of IAPs was not observed at any time point (Fig. 2E).

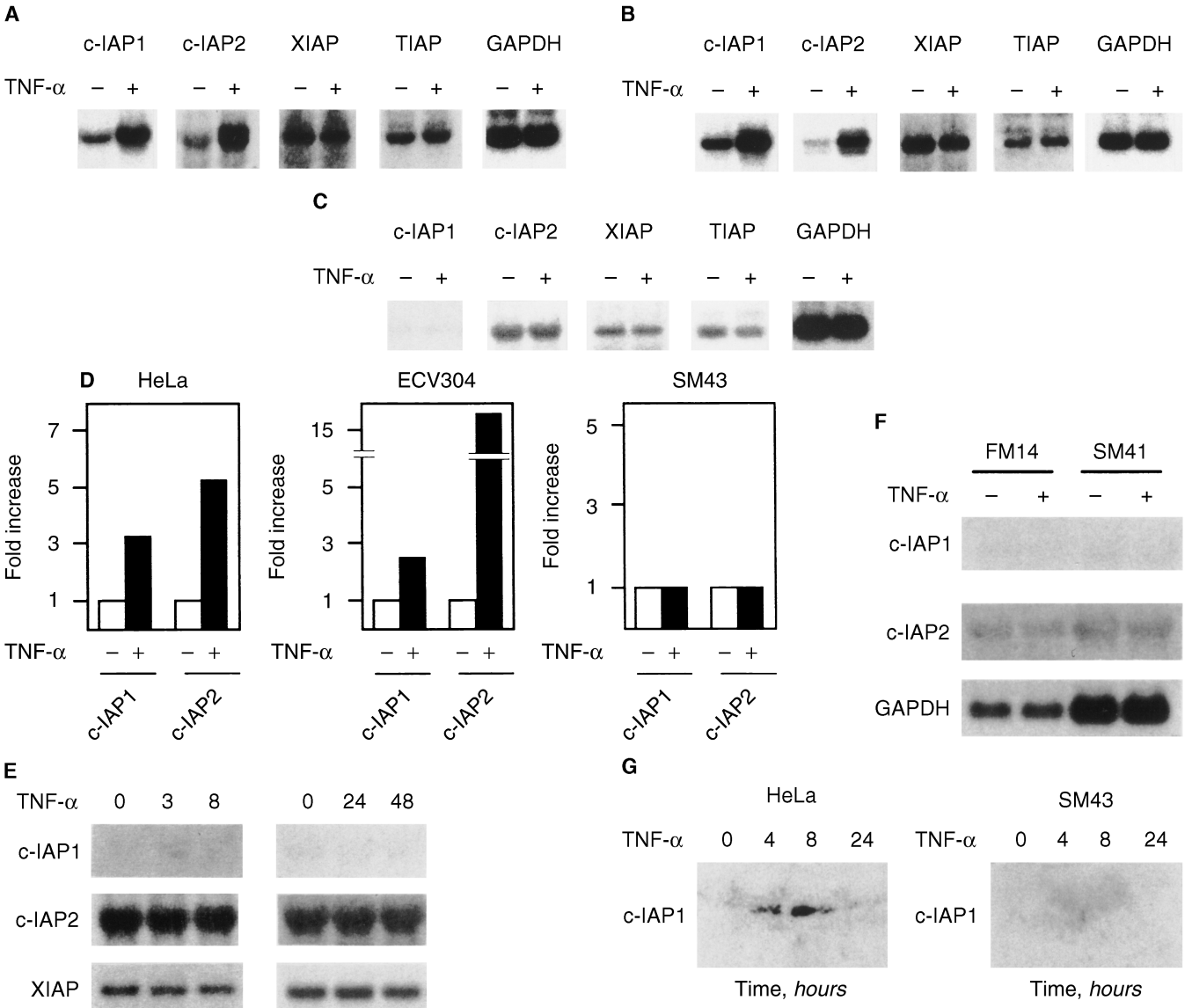

Fig. 2. Effect of tumor necrosis factor-α (TNF-α) on the expression of IAPs in HeLa cells, ECV304 cells and rat mesangial cells. (*A–F*) Northern blot analysis. HeLa cells (A), ECV304 cells (B), and rat mesangial cells (C and E, SM43; F, FM14 and SM41) were incubated in the absence (−) or presence (+) of TNF-α (250 U/mL) for 4 hours (A–D, F) or 3 to 48 hours (E), and expression of c-IAP1, c-IAP2, XIAP, and TIAP was examined by Northern blot analysis. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control. Intensity of c-IAP1 and c-IAP2 mRNAs was evaluated by densitometric analysis. Each value was normalized by the value of GAPDH mRNA and expressed as fold increase (*D*). (*G*) Western blot analysis. HeLa cells and mesangial cells (SM43) were stimulated with TNF-α for up to 24 hours, and Western blot analysis of c-IAP1 was performed.

To examine whether the lack of IAP induction is a general phenomenon in rat mesangial cells, a mesangial cell clone FM14 derived from a different rat strain (F344 rat) and another clone SM41 derived from the same rat strain (Sprague-Dawley rat) were tested. These cells were incubated in the presence or absence of TNF-α for four hours and subjected to Northern blot analysis. Consistent with the result of SM43 cells, stimulation of FM14 cells and SM41 cells with TNF-α did not induce expression of c-IAP1 and c-IAP2 (Fig. 2F).

The lack of IAP induction in TNF-α–stimulated mesangial cells was further confirmed using Western blot analysis. Mesangial cells and HeLa cells were stimulated by TNF-α for up to 24 hours and subjected to the analysis. Consistent with the Northern blot data, c-IAP1 protein was substantially induced in HeLa cells in response to

TNF-α. The level of c-IAP1 protein was increased four to eight hours after the stimulation and declined after 24 hours (Fig. 2G, left). In contrast, c-IAP1 protein was not induced by TNF-α in mesangial cells (Fig. 2G, right).

NF-κB–independent regulation of IAPs in mesangial cells

We previously showed that TNF-α induces activation of NF-κB in SM43 mesangial cells [26]. The lack of IAP induction by TNF-α raises a possibility that in rat mesangial cells, IAPs are regulated independently of NF-κB. To examine this possibility, expression of IAPs in wild-type mesangial cells (SM43) was compared with that in NF-κB–inactive mesangial cells (SM/IκBαM) under basal and TNF-α–stimulated conditions. SM43 cells and SM/IκBαM cells were preincubated in 1% FCS for 24 hours and stimulated with or without TNF-α for four hours. Northern blot analysis showed that basal levels of all IAPs were not different between SM43 cells and SM/IκBαM cells (Fig. 3A). Furthermore, SM/IκBαM cells expressed IAPs at the same levels as those of SM43 cells even under the TNF-α–stimulated condition (Fig. 3B). Of note, although SM/IκBαM cells are susceptible to TNF-α–induced apoptosis [25, 26], apoptosis was not induced by the short-term (4 hours) treatment with TNF-α.

Previous reports suggested that IAPs are involved in the inducible, cytoprotective machinery in TNF-α–exposed cells [22, 25]. To confirm that inducible expression of IAPs is not necessary for the NF-κB–dependent cytoprotection in mesangial cells, TNF-α–induced apoptosis was examined in SM43 cells and SM/IκBαM cells. As shown in Figure 3 C and D, SM43 cells that have intact NF-κB function were resistant to TNF-α–induced apoptosis. In contrast, NF-κB–inactive SM/IκBαM cells exposed to TNF-α exhibited shrinkage of the cytoplasm and condensation/fragmentation of the nuclei typical of apoptosis. Quantitative analysis using Hoechst staining showed that TNF-α did not significantly induce apoptosis of SM43 cells (% apoptosis: 2.0 ± 0.2% in untreated cells and 3.8 ± 1.1% in TNF-α–treated cells; means ± SE). In contrast, TNF-α significantly induced apoptosis of SM/IκBαM cells from 4.2 ± 0.8% (untreated) to 22.0 ± 0.5% (TNF-α–treated, $P < 0.01$; Fig. 3E).

Roles of IAPs in mesangial cell survival

To examine the function of IAPs in rat mesangial cells, SM/IκBαM cells were stably supertransfected with an expression plasmid encoding c-IAP1 or c-IAP2. Northern blot analysis showed that the established clones SM/IκB·IAP1-1, SM/IκB·IAP1-4, SM/IκB·IAP2-2, and SM/IκB·IAP2-4 expressed high levels of exogenous c-IAP1 and c-IAP2 transcripts (Fig. 4A). Using the established clones, susceptibility to TNF-α–induced apoptosis was examined. SM/IκB·IAP1, SM/IκB·IAP2, and SM/IκB· control clones were treated with TNF-α for 24 hours and subjected to microscopic analyses. Phase-contrast microscopy showed that overexpression of c-IAP1 or c-IAP2 protected SM/IκBαM cells from apoptotic changes induced by TNF-α (Fig. 4B). Quantitative analysis using Hoechst staining showed that the apoptosis induced by TNF-α (18.3 ± 1.1%) was markedly suppressed by overexpression of c-IAP1 (4.4 ± 1.4% in clone1-4, $P < 0.01$; Fig. 4C). Similarly, overexpression of c-IAP2 dramatically reduced the percentage of apoptotic cells from 30.0 ± 3.5% to 5.3 ± 0.2% (clone2-2, $P < 0.01$; Fig. 4D). Similar suppressive effects of c-IAP1 and c-IAP2 were also observed in another set of clones, SM/IκB·IAP1-1 and SM/IκB·IAP2-4 (data not shown).

DISCUSSION

The inhibitors of apoptosis (IAPs) are ubiquitous intracellular proteins that regulate apoptotic processes. Previous reports showed that IAPs are able to inhibit apoptosis induced by a wide range of stimuli, including TNF-α, anti-Fas antibodies, oxidative stress, pro-apoptotic members of the Bcl-2 family, cytochrome C, anticancer drugs, and serum withdrawal [15, 18, 22, 25, 34, 35]. Because of the ubiquitous distribution and the inducible property, the IAP family is supposed to serve as the important cytoprotective machinery under pathophysiologic situations. Currently, however, expression of IAPs in the kidney, especially in individual nephron segments, is unknown. This report examined the expression of IAPs in glomerular cells. The results showed that substantial expression of c-IAP2 and XIAP, but not c-IAP1, was observed in unstimulated rat mesangial cells and isolated normal rat glomeruli. This is consistent with previous reports showing that expression of c-IAP2 and XIAP, but not c-IAP1, was detectable in human adult kidneys [15, 21]. In addition to c-IAP2 and XIAP, expression of TIAP was also observed in cultured mesangial cells. The biological significance of the constitutively expressed IAPs in glomerular cells is currently unknown, but a recent report showed the crucial roles of constitutive IAPs in the survival of thymocytes [36]. The basal expression of IAPs may be important for the maintenance of glomerular cell survival under the physiological situation. Our current data using IAP-transfected mesangial cells may support this possibility.

Previous investigations suggested that certain IAPs, including c-IAP1, c-IAP2 and XIAP, are induced by TNF-α in some cell types [22–25]. In the present study, we examined whether IAPs are induced by TNF-α in rat mesangial cells. Consistent with the previous reports, c-IAP1 and c-IAP2 were markedly induced by TNF-α in HeLa cells and ECV304 cells. However, unexpectedly, the induction of c-IAP1 and c-IAP2 was not observed in TNF-α–stimulated mesangial cells. The lack of induction was not due to defects in the TNF-α signaling because

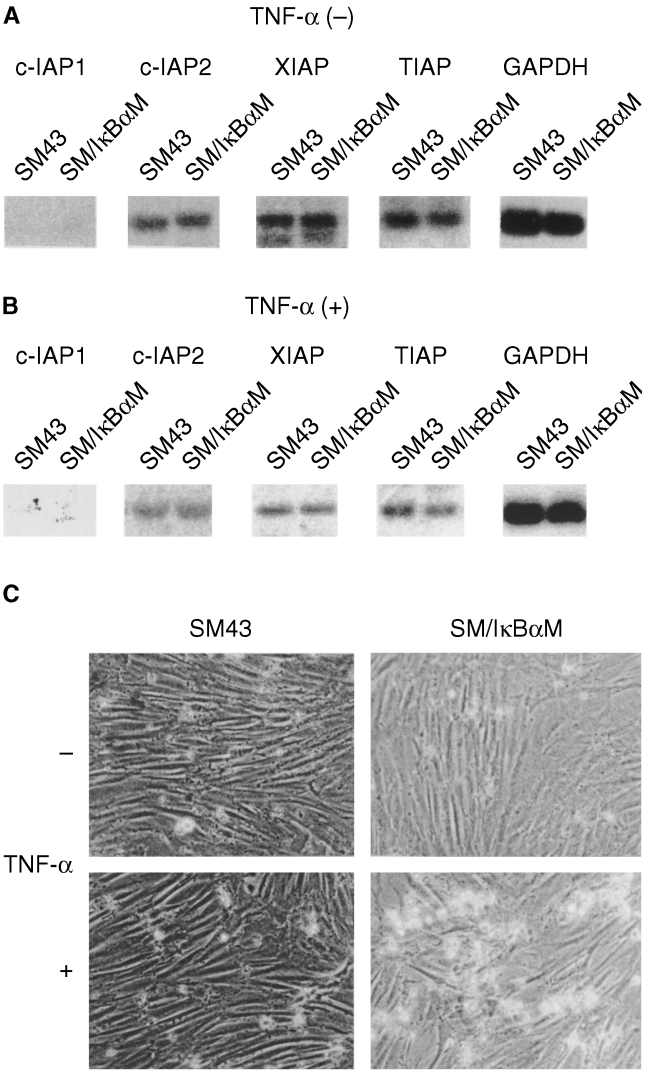

Fig. 3. Roles of nuclear factor-κB (NF-κB) in the IAP expression and apoptosis in rat mesangial cells. (*A* and *B*) Expression of IAPs in NF-κB–active and NF-κB–inactive mesangial cells. Wild-type mesangial cells (SM43) and NF-κB–inactive mesangial cells overexpressing a super-repressor mutant of IκBα (SM/IκBαM) were cultured in the absence (A) or presence (B) of TNF-α for four hours, and expression of IAP genes was examined by Northern blot analysis. (*C–E*) Susceptibility to TNF-α–induced apoptosis in NF-κB–active and NF-κB–inactive mesangial cells. Confluent cultures of SM43 cells and SM/IκBαM cells were incubated in the absence (−) or presence (+) of TNF-α for up to 40 hours. Apoptosis was evaluated by phase-contrast microscopy (C) and fluorescence microscopy (Hoechst 33258 staining; D and E). Both attached and detached cells were used for the quantitative assessment of apoptosis (E). Symbols are: (□) SM43; (▨) SM/IκBαM. Data are shown as means ± SE. An asterisk indicates a statistically significant difference ($P < 0.01$).

(*1*) substantial NF-κB activation occurred in response to TNF-α in SM43 mesangial cells [26] and (*2*) SM43 cells underwent apoptosis by the treatment with TNF-α, if NF-κB was inactive (Fig. 3 C–E). One possible explanation is that the regulation of IAPs in rats is different from that in other species, especially human. Indeed, in contrast to rat mesangial cells, c-IAP1 and c-IAP2 were substantially up-regulated by TNF-α in human mesangial cells (A. Furusu and M. Kitamura, unpublished observation). It is worthwhile to note that in the majority of previous reports, human cells were generally used to investigate transcriptional regulation of IAPs.

The expression of c-IAP1, c-IAP2, and XIAP is regulated by NF-κB in some cell types. For example, in human umbilical vein endothelial cells and fibrosarcoma cells, expression of c-IAP1, c-IAP2, and XIAP genes is NF-κB-dependent [22, 24]. However, the requirement of NF-κB for the regulation of IAPs is somewhat controversial. In Jurkat cells, Chu et al showed that c-IAP2 but not c-IAP1 is regulated by NF-κB [25]. Our present study found that expression of c-IAP1, c-IAP2, and XIAP in rat mesangial cells was independent of NF-κB. This is because (*1*) TNF-α triggered activation of NF-κB [26] but did not induce any IAPs (Fig. 2C), and (*2*) the expression levels of any IAPs in NF-κB–inactive cells were the same as those in wild-type cells under both unstimulated and TNF-α–stimulated conditions (Fig. 3 A, B). These results provide further evidence for the NF-κB–independent regulation of IAPs in certain cell types.

It has been proposed that IAPs are important molecules for the TNF-α–inducible, NF-κB–dependent cytoprotection [22, 25]. We found that NF-κB–inactive mesangial cells showed high susceptibility to the TNF-α–induced apoptosis, but they expressed all IAPs at the same levels

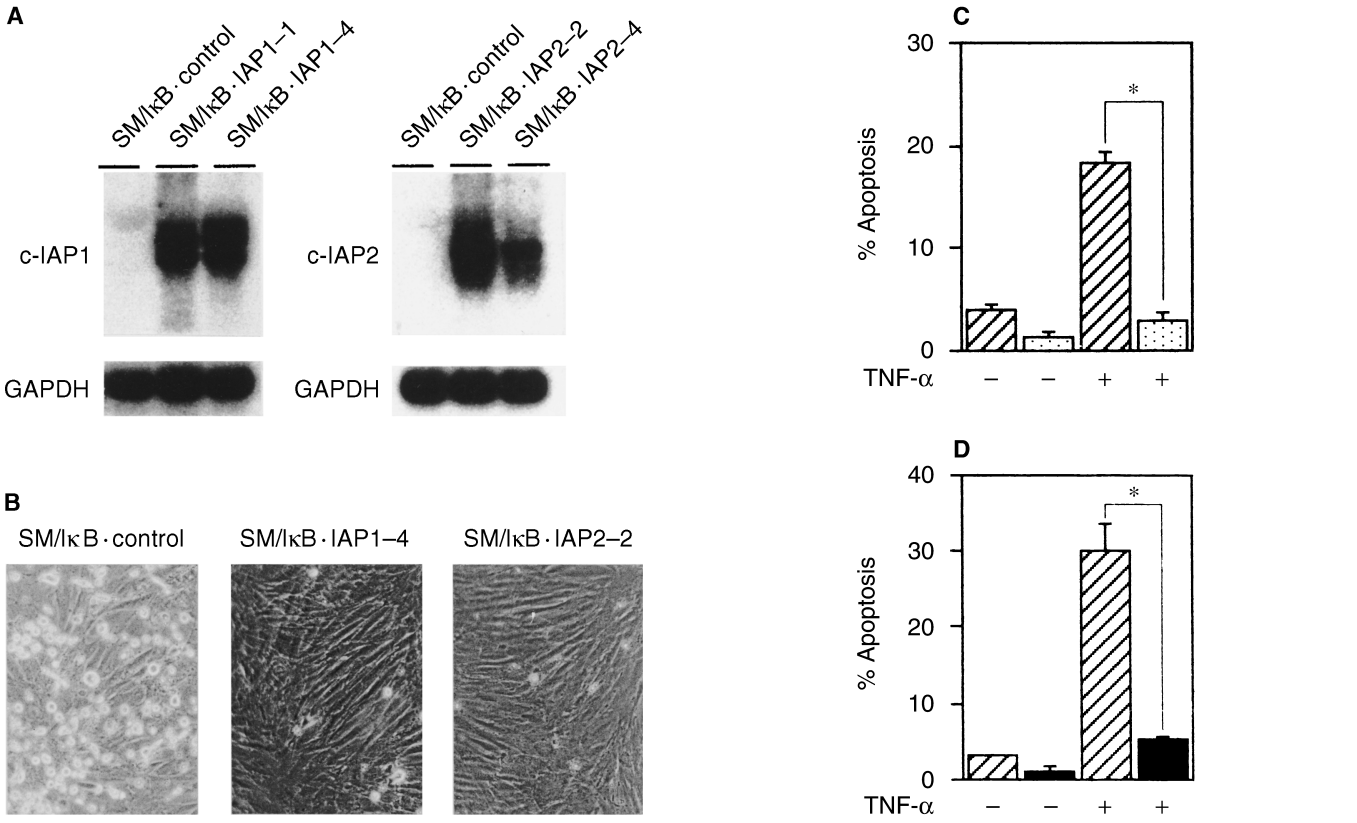

Fig. 4. Effects of IAP overexpression on TNF-α–induced apoptosis of rat mesangial cells. (*A*) Expression of exogenous c-IAP1 and c-IAP2 in the established transfectants. SM/IκBαM cells were stably super-transfected with c-IAP1 or IAP2 cDNA, and stable clones SM/IκB·IAP1-1, SM/IκB·IAP1-4, SM/IκB·IAP2-2, and SM/IκB·IAP2-4 were established. The expression of exogenous IAPs was examined by Northern blot analysis. Vector-transfected SM/IκB·control cells were used as a control. (*B–D*) Susceptibility to TNF-α–induced apoptosis. Confluent cultures of SM/Iκ·control, SM/Iκ·IAP1-4, and SM/Iκ·IAP2-2 cells were exposed to TNF-α for 24 hours, and microscopic analysis was performed. (B) Phase-contrast microscopy. (C and D) Quantitative analysis of apoptosis using Hoechst 33258 staining. Symbols are: (▨) SM/IκB·control; (▩) SM/IκB·IAP1-4; (■) SM/IκB·IAP2-2. Data are shown as means ± SE. Asterisks indicate statistically significant differences ($P < 0.01$).

as those in NF-κB–active mesangial cells. This result indicates that other NF-κB–inducible molecules are involved in the TNF-α–inducible cytoprotection in rat mesangial cells.

Currently, the types of downstream molecules that are involved in the NF-κB–mediated cytoprotection in mesangial cells are unknown; however, several candidates can be postulated. Those include *IEX*-1L, A20, Bfl-1/A1, and manganese superoxide dismutase (MnSOD). *IEX*-1L is an immediate-early response gene induced by TNF-α, and the induction is mediated by NF-κB. In Jurkat cells, it plays a key role in cellular resistance to TNF-α–induced apoptosis [37]. Similarly, anti-apoptotic proteins A20 and Bfl-1/A1 are induced by NF-κB and are able to inhibit TNF-α–induced apoptosis in MCF-7 cells and HeLa cells [38–40]. MnSOD, a mitochondrial enzyme involved in the scavenging of superoxide radicals, is induced by NF-κB [41]. Because superoxide anion, but not other reactive oxygen intermediates, is the crucial mediator for TNF-α–induced apoptosis of rat mesangial cells [27], it may contribute to the NF-κB–mediated cytoprotection. Indeed, previous reports showed that MnSOD is essential for cellular resistance to TNF-α in 293 cells [42]. Further investigation will be required to examine roles of these molecules in the TNF-α–inducible, NF-κB–dependent cytoprotection in rat mesangial cells.

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APPENDIX

Abbreviations used in this article are: BIR, baculovirus IAP repeat; FADD, Fas-associated death domain; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IAP, inhibitor of apoptosis; IκBαM, super-repressor mutant of IκBα; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; TNFR1, tumor necrosis factor receptor 1; TRADD, TNFR-associated death domain.

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