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p38β MAP Kinase Protects Rat Mesangial Cells From TNF-α-Induced Apoptosis

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Abstract p38 MAP kinases (p38) and c-Jun N-terminal protein kinases (JNK) have been associated with TNF-α-induced apoptosis. However, recent studies indicate that an early but brief activation of JNK and/or p38 may actually protect some cells from TNF-α-induced apoptosis. Whether the activation of JNK and p38 provides a pro- or anti-apoptotic signal for TNF-α has been controversial. In this study, we investigated the role of p38 in the regulation of TNF-α cytotoxicity in rat mesangial cells. Treatment of the cells with TNF-α alone had little effect on their viability, but they became very sensitive to apoptosis when treated with TNF-α in the presence of the p38 inhibitor SB 203580. These results suggested that the p38 pathway is critical for mesangial cells to survive the toxic effect of TNF-α. Using adenovirus-mediated gene transfer technique, we further demonstrated that p38β, but not p38α, is essential to protect the cells from TNF-α toxicity. It has been speculated that there is a synergetic interaction between the p38 and the nuclear factor-κB (NF-κB) pathways in protecting certain cells from apoptosis. However, expression of neither p38β nor its dominant negative mutant in mesangial cells interfered with TNF-α-induced translocation of NF-κB, the initial step of NF-κB activation. While it is unclear whether p38β regulates NF-κB transcription activity at other steps, it is apparent that p38β does not affect TNF-α-induced NF-κB activation at the stage of nuclear translocation. J. Cell. Biochem. 82: 556–565, 2001.

Key words: protein kinase; ERK; JNK; NF-κB; apoptosis

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine produced by many types of cells and originally identified on the basis of its cytotoxic effect on certain cells. TNF-α can elicit a wide range of physiological responses including inflammatory reactions, cell proliferation, and differentiation in addition to its ability to induce cell death [Beyaert and Fiers, 1994]. The elicited response appears to depend on the cell type and its state of differentiation. This pleiotropic effect of TNF-α is a consequence of the fact that it activates multiple signal transduction pathways that differentially regulate various cellular processes [Liu et al., 1996; Nagata, 1997].

Many tumor cells are sensitive to TNF-α-induced apoptosis (or programmed cell death), but normal cells are usually resistant. Some cells undergo apoptosis only when they are treated with TNF-α in the presence of other agents such as protein synthesis inhibitors [Sugarman et al., 1985; Nagata, 1997; Guo et al., 1998a]. An emerging hypothesis suggests that the reason why TNF-α selectively kills some cells without harming others is that TNF-α may activate two opposing signaling pathways: an apoptotic pathway and a protective pathway. Interactions between the two pathways, especially the balance of their activities in response to TNF-α will thus determine whether the cells survive or die [Wallach, 1997]. Although details of the signaling steps remain to be delineated, significant progress has been made in recent years in clarifying

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the apoptotic mechanism. The current model proposes that, upon the binding of TNF-α to its receptor, the cytosolic part of the receptor recruits the "death domain containing proteins" TRADD (TNF-α Receptor-Associated Death Domain proteins), which in turn activates a cascade of proteolytic enzymes known as caspases. Caspases execute the apoptotic process by hydrolyzing the key enzymes and proteins that are essential for cells to survive [Nagata, 1997]. On the other hand, the identities of the protective pathways against TNF-α toxicity and the mechanisms involved remain largely unknown. Among the TNF-α activated signaling pathways, mitogen-activated protein kinases (MAP kinases) have been thought to play important roles in the regulation of physiological effects of TNF-α. Three distinct subtypes of MAP kinases have been identified: (1) extracellular signal-regulated kinases (ERK), (2) c-jun N-terminal protein kinases (JNK), and (3) p38 kinases (p38). JNK and p38 MAP kinases are usually activated by extra- or intracellular stress and inflammatory cytokines, such as TNF-α [Kyriakis and Avruch, 1996; Zanker et al., 1996; Ono and Han, 2000]. Activation of JNK and p38 generally promotes cell death or inhibition of cell growth, while ERK, strongly activated by growth factors and hormones that stimulate cell growth, is involved in the regulation of cell proliferation [Hunter and Karin, 1992; Xia et al., 1995]. Activation of the p38 and JNK pathways has been associated with cell death caused by several stress stimuli [Brenner et al., 1997; Kummer et al., 1997; Frasch et al., 1998]. However, recent evidence indicates that their implication in the regulation of cell viability is much more complex than that originally thought. In certain cells, activation of JNK and p38 by stress signals seems to induce resistance against further damage by these stimuli [Roulston et al., 1998; Zhao et al., 1998; Assefa et al., 1999]. Therefore, the involvement of the p38 and JNK pathways in apoptosis has been highly controversial.

Cell death from apoptosis is recognized during the course of various renal diseases as well as in the early stages of kidney development. Mesangial cells are a prominent cell type of kidney glomeruli and regulate glomerular hemodynamics. We have used rat mesangial cells as a model to characterize apoptotic and necrotic cell death [Guo et al., 1998a,b; Guo et al., 1999a,b]. Our previous work revealed a close correlation between a sustained JNK activation and onset of apoptosis in mesangial cells treated with TNF-α in the presence of protein synthesis inhibitor or phosphatase inhibitors [Guo et al., 1998a,b], supporting the notion that the JNK pathway may be involved in induction of apoptosis only when the duration of its activation is prolonged. Roulston et al. [1998] recently reported that an early but brief activation of JNK and/or p38 may actually protect NIH-3T3 cells from apoptosis induced by TNF-α. Taken together, these results imply that the timing and extent of JNK/p38 activation may have different implications in mediating the physiological effect of TNF-α. In this study, the involvement of p38 MAP kinases in TNF-α-induced apoptosis has been investigated in mesangial cells. Our results showed that although TNF-α was unable, by itself, to cause cell death, it induced a rapid cell death by apoptosis when the p38 pathway was blocked. These results suggest that the p38 pathway is essential for protecting these cells from TNF-α cytotoxicity. Of particular interest, by using adenovirus-mediated gene transfer technique, we demonstrate that p38β isoform, but not p38α, is responsible for the protective effect displayed in mesangial cells. Our data provide an example that different members of the p38 family have distinct physiological functions.

MATERIALS AND METHODS

Materials

Recombinant TNF-α was obtained from Chemicon International Inc. (Temecula, CA). Anti-NF-κB (RelA subunit) antibodies and anti-p38 antibodies (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p38 and anti-phospho-JNK antibodies were from New England Biolabs (Beverly, MA). Anti-phospho-ERK antibodies were from Promega (Madison, WI). Anti-Flag antibodies (M2) were from Sigma (St Louis, MO). SB203580 was purchased from Calbiochem (San Diego, CA).

Cell Culture

Rat mesangial cells were isolated from male Sprague-Dawley rats under sterile conditions using the sieving technique as described [Kreisberg et al., 1978]. The cells were maintained in RPMI 1640 medium containing 20% fetal calf serum (FCS) and 0.6 U/ml of insulin at

37°C in a humidified incubator (5% CO_2, 95% air). Cells from 5 to 20 passages were used.

Adenovirus Infection of Mesangial Cells

Recombinant adenoviruses expressing wild type p38α, p38β and their dominant negative mutants p38α[AF] and p38β[AF] were constructed as described previously [Huang et al., 1997]. They were propagated in 293 cells. The titers of viral stocks were determined by plaque assay [Becker et al., 1994]. For viral infection, 60–70% confluent cells were incubated in RPMI 1640 medium containing 0.25% FCS for 3 h prior to addition of viral stocks. The cells were infected with recombinant viruses (~1,000 plaque-forming U/cell) for 3 h and then incubated in fresh RPMI 1640 medium containing 0.25% FCS for 24–30 h as indicated prior to experiments. A recombinant virus encoding green florescent protein (GFP) was used as the control.

Cell Viability Assay and Apoptosis Analysis

For the cell viability assay, mesangial cells were grown in 12-well plates. The cells were treated with reagents for the indicated times. Uptake of neutral red dye was used as a measurement of cell viability [Guo et al., 1998a]. At the end of the incubations, the medium was removed and the cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 2% FCS and 0.01% neutral red for 60 min at 37°C. The uptake of the dye by viable cells was terminated by removal of the media. The cells were washed briefly with 1 ml 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). The internalized neutral red was extracted with 1 ml of a solution containing 50% ethanol and 1% glacial acetic acid. The absorbencies, which correlate with the amount of live cells, were determined at 540 nm.

For analysis of apoptosis, the morphological changes of the cells were examined under a microscope periodically during the cell incubations. Apoptosis was further confirmed by DNA fragmentation analysis according to the procedures described by Sanchez-Alcazar et al. [1997] with some modification. Briefly after treatments, cells were collected and lysed in a hypotonic buffer containing 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.2% Triton X-100. DNA was extracted from cell lysate with phenol/chloroform method. Isolated DNA was analyzed by 1% agarose gel electrophoresis followed by staining of the gel with ethidium bromide.

Immunocytochemical Analysis

For expression analysis of p38α, p38β and their dominant negative mutants, cells grown on 25 mm glass coverslips in 6-well plates were fixed with 4% paraformaldehyde in PBS after viral infection for the times stated in different experiments. The cells were stained with anti-Flag antibodies that recognize the Flag-tags in the expressed proteins and visualized with Texas Red-conjugated secondary antibodies under a florescence microscope. For the control virus, expressed GFP was examined without further treatment after fixation. For NF-κB localization, the cells were stained with anti-65 kD subunit (RelA) of NF-κB antibodies followed by florescein-conjugated secondary antibodies and examined under a florescence microscope. The images were captured with a CCD digital camera and analyzed with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) as described previously [Guo et al., 1999b].

Cell Lysate Preparation

After various treatments as described in different experiments, mesangial cells were washed twice with ice-cold PBS and scraped into cell-lysis buffer containing 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM Na_3VO_4, 1% SDS. The cells were heated for 3 min at 90°C then lysed by sonication (8 pulses, output control 3) using a Branson sonicator. The solution was centrifuged at 15,000*g* for 15 min. The supernatant was designated as whole cell lysate and used for Western-blot analysis. Protein concentration was determined using a protein assay kit purchased from Pierce.

Western-Blot Analysis

Protein samples were subjected to SDS–PAGE and the separated proteins were then transferred onto nitrocellulose membranes. Equal loading of proteins was checked by staining the membranes with Indian ink. The membranes were blocked with 5% nonfat dry milk in Tris-buffered-saline containing 0.05% Tween 20 and incubated with primary antibodies followed by HRP-conjugated secondary antibodies according to the manufacturer's instructions. The immunoblots were visualized

by an enhanced chemiluminescence (ECL) kit obtained from Amersham Pharmacia Biotech (Arlington Heights, IL).

RESULTS

Activation of MAP Kinases by TNF-α in Mesangial Cells

The early activation of MAP kinases is considered to be important in the regulation of activities of many key enzymes and transcription factors which ultimately regulate TNF-α-induced physiological responses [Beyaert and Fiers, 1994; Robinson and Cobb, 1997]. In response to TNF-α stimulation, all three subtypes of MAP kinases in mesangial cells were activated as determined by Western-blot analysis using the antibodies that only recognize phosphorylated forms of MAP kinases (Fig. 1). Activation of MAP kinases requires phosphorylation of a threonine and a tyrosine residue in their active sites. Therefore, phosphorylation of MAP kinases on these two residues has often been used to indicate their activation [Brenner et al., 1997; Guan et al., 1998; Guo et al., 1998a]. Figure 1 shows the time courses of activation of the three subtypes of MAP kinases. p38 activation was biphasic with the first activity peak detected as early as 5 min and reached a maximal activation at 15 min followed by a transient decline. A weaker but more sustained second activity peak appeared at 60 min after addition of TNF-α, which lasted for at least 1 h. (Fig. 1A). The activation of JNK and ERK was very transient with activation maximal at 15 min; thereafter, their activities declined to the level below detection (Fig. 1B and C). Figure 1D illustrates protein staining of the samples as used in panel A, B, and C to show equal amount of proteins loaded in each lane.

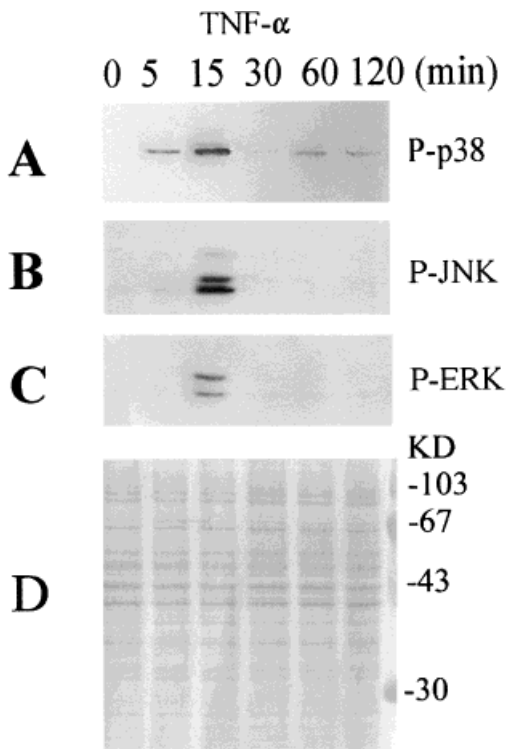

Fig. 1. Activation of p38, JNK, and ERK by TNF-α. Mesangial cells were stimulated with TNF-α (10 ng/ml) for different times as indicated. Activation of p38, JNK, and ERK was determined by Western-blot analysis using the antibodies that recognized only their phosphorylated forms (panel **A**, **B**, and **C** respectively). Panel **D** represents protein staining of a membrane as used in panel A, B, and C to show equal loading of proteins in each lane.

Inhibition of the p38 Pathway Causes Mesangial Cells Susceptible to the Apoptotic Effect of TNF-α

To evaluate the role of the p38 pathway in the regulation of TNF-α toxicity, SB203580 was used to block TNF-α-induced p38 activation. SB203580 is a pyridinyl imidazole derivative that is considered to be a selective inhibitor of p38. It has been shown that SB203580 and its related compounds selectively inhibited p38 without significant effect on JNK or ERK at concentrations up to 100 μM. [Gum et al., 1998]. This observation has been confirmed in rat mesangial cells by Guan et al. [1998]. As shown in Figure 2, treatment of cells with either TNF-α or SB293580 (30 μM) alone had no significant effect on cell viability (up to 12 h incubation

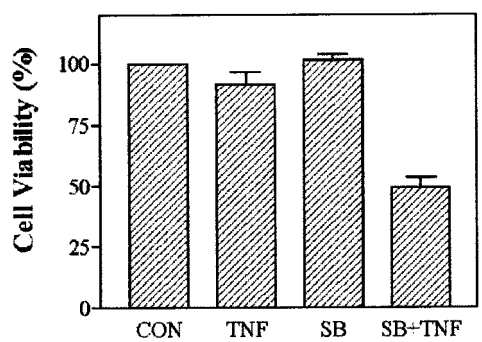

Fig. 2. Effect of TNF-α and SB203580 on cell viability. Mesangial cells were treated with 10 ng/ml of TNF-α (TNF), or 30 μM of SB203580 (SB), or pretreated with 30 μM SB203580 for 30 min followed by treatment with 10 ng/ml TNF-α for 3.5 h (SB + TNF). Cell viability was determined by the neutral red assay method. Results are means±SEM of three experiments performed in triplicate.

period, determined in a separate experiment). However, treatment of the cells with a combination of TNF-α and SB203580 caused a rapid cell death. Within 3–4 h, about 50% of the cells were dead of apoptosis as judged by morphological and biochemical standards, i.e., membrane blebbing, nuclear condensation, and DNA fragmentation (data not shown). These results indicate that the p38 pathway is critical for the prevention of TNF-α-induced apoptosis in mesangial cells.

Expression of p38α, p38β and Their Dominant Negative Mutants Using Adenovirus-Mediated Gene Transfer in Mesangial Cells

Although SB203580 has been shown to be highly selective for inhibition of p38 in an in vitro assay, it is possible that responses in whole cells may reflect some nonspecific effects. More specific approaches are necessary to confirm the results obtained from experiments using this compound. The involvement of the p38 pathway in the regulation of TNF-α toxicity was further investigated using adenovirus-mediated gene transfer technique. This approach has been proven to be a very effective technique for expressing cloned genes in many cells, especially in primary cells like mesangial cells where transfection with plasmid vectors is associated with technical difficulties. Figure 3 (photo) illustrates mesangial cells that were infected with the recombinant adenoviruses encoding green florescent protein (GFP) as the control (a), wild type p38β (b), and the dominant negative mutant p38β[AF] (c). The infection efficiency could reach as high as ~80% as determined by the number of the cells expressing the introduced genes. Panels A, B, and C represent the cells without viral infection as controls for panels a, b, and c respectively (the florescent particles seen in A, B, and C were caused by cell debris which emit "auto-florescence" under a florescent microscope). Similar efficiency was obtained in the cells that were infected with p38α and its dominant negative mutant p38α[AF] (data not shown). Expression of GFP in the control cells did not show an apparent toxic effect under the conditions used, thus ensuring the suitability of this technique for the present study.

The expression levels of the recombinant p38 were further analyzed by Western-blot using anti-Flag antibodies that recognize the Flag-tags in their sequences. A similar expression level was observed for p38α, p38α[AF], p38β, and p38β[AF] in the cell lysates prepared from the infected cells (Fig. 3, blot, upper panel). To assess the relative levels of Flag-tagged recombinant kinases and naturally expressed p38, the same protein samples were probed with an antibody that was raised against p38 (Santa, Cruz, C-20). As shown in Figure 3 (blot, lower panel), this antibody recognized a protein band

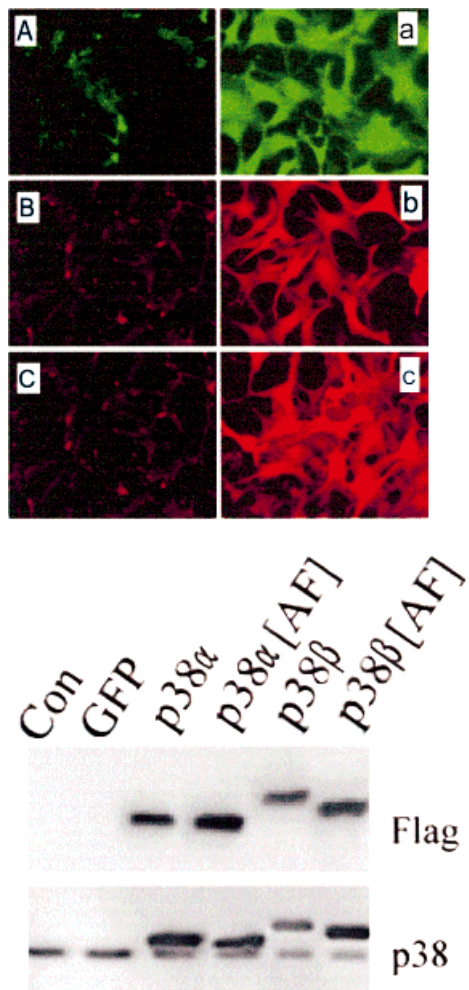

Fig. 3. Infection of mesangial cells with recombinant adenoviruses. Cells were infected with the viruses for 24 h, and then they were fixed with 4% paraformaldehyde. The cells infected with the control virus expressing GFP were visualized without further treatment after fixation (**a**). The cells expressing p38β (**b**) and p38β[AF] (**c**) were identified using anti-Flag antibodies and visualized with Texas Red-conjugated secondary antibodies under a florescence microscope. **A**, **B**, and **C** represent uninfected cells as controls. For Western-blot analysis, cell lysates prepared from the cells infected with recombinant viruses as indicated were probed with anti-Flag antibodies (blot, upper panel) or with anti-p38 antibodies (blot, lower panel). Immunoreactivity was detected with ECL. CON represents the cell lysate prepared from uninfected cells.

with a molecular weight corresponding to p38 MAP kinases, Flag-p38α, as well as Flag-p38α[AF]. It also cross-reacted with Flag-p38β and Flag-p38β[AF] (Fig. 3, blot, lower panel). It is apparent that this antibody could not distinguish between p38α and p38β. Nevertheless, this result shows that the amount of overexpressed kinases is about 3–4 fold of that of the naturally expressed p38 band (Fig. 3, blot, lower panel).

The Effects of Overexpression of p38α, p38β and Their Dominant Negative Mutants on Cell Viability

Four isoforms of the p38 MAP kinase subfamily have been identified and named p38α, p38β, p38γ, and p38δ respectively [Ono and Han, 2000]. p38α and p38β, but not p38γ and p38δ, are inhibited by the SB203580 [Jiang et al., 1997; Gum et al., 1998]. Therefore, it is likely that either p38α or p38β, or both are responsible for the protective effect against TNF-α toxicity displayed in normal mesangial cells. To identify which isoform is involved, the cells were infected with the recombinant viruses encoding wild types of p38α, p38β and their dominant negative mutants p38α[AF] and p38β[AF] respectively. p38α[AF] and p38β[AF] were generated by mutating the phosphorylation sites in the kinase domains. Phosphorylation of a threonine (T) and a tyrosine (Y) residue in p38 kinase domains is required for enzyme activity [Hunter and Karin, 1992; Zanker et al., 1996]. Mutation of these two sites (from T to A and from Y to F) created no-phosphorylatable kinases thus abolishing their activation. It has been shown that the mutated kinases can neither undergo autophosphorylation nor can they phosphorylate substrates in vitro kinase assays [Jiang et al., 1996, 1997]. As shown in Figure 4, infection of cells with p38α, p38α[AF] or p38β did not affect on cell viability significantly in response to TNF-α stimulation compared with controls. However, the cells expressing p38β[AF] were much more sensitive to TNF-α toxic effect under the same conditions (Fig. 4). There was no significant difference in cell viability among the infected cells in the absence of TNF-α (data not shown). The viability and morphology of the infected cells were further examined under a microscope. The cells expressing p38β[AF] displayed the typical features of apoptosis such as cell shrinkage and membrane blebbing (Fig. 5A) while the cells

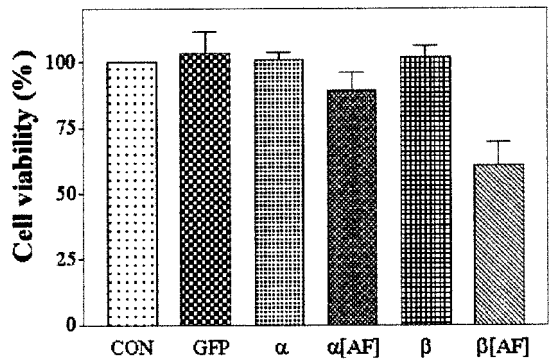

Fig. 4. Effects of overexpression of p38α, p38β and their dominant negative mutants on cell viability. Cells were infected with viruses expressing p38α (α), p38β (β) or p38α[AF] (α [AF]) and p38β[AF] (β [AF]) for 24 h, then they were incubated with 10 ng/ml TNF-α for 18 h. Cell viability was determined by neutral red assays. Uninfected (CON) or cells infected with adenovirus encoding GFP (GFP) were used as controls. Results are means±SEM of four experiments performed in triplicate.

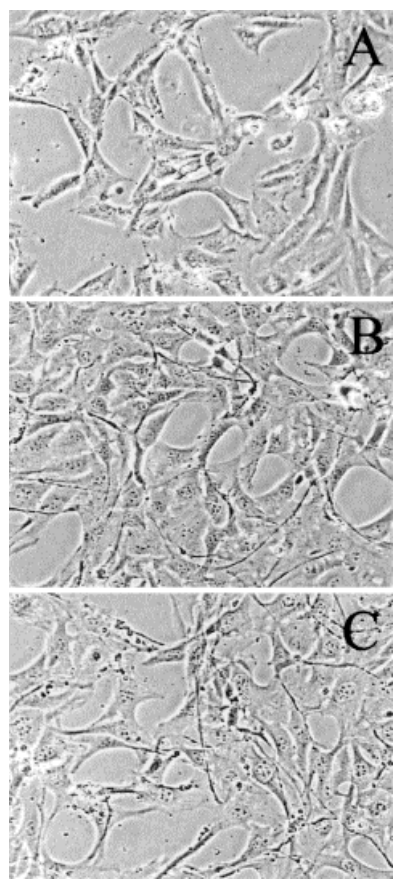

Fig. 5. Morphology of apoptotic cells caused by expressing p38β[AF] in response to TNF-α treatment. Cells were infected with recombinant viruses encoding p38β[AF] (**A**), p38β (**B**), or GFP (**C**) for 24 h followed by incubation with 10 ng/ml TNF-α for an additional 18 h. The morphology of the cells was examined under a phase-contrast microscope.

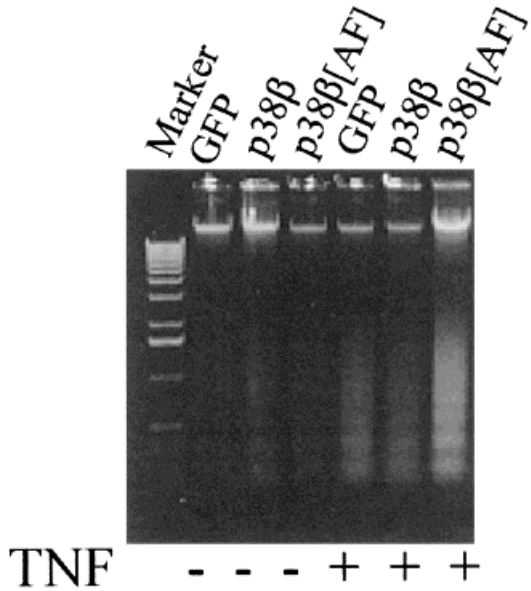

Fig. 6. DNA fragmentation analysis. Cells were infected with the recombinant viruses as indicated for 24 h, then they were incubated with or without 10 ng/ml TNF-α for an additional 18 h. DNA was isolated from the collected cells and analyzed by 1% agarose gel electrophoresis.

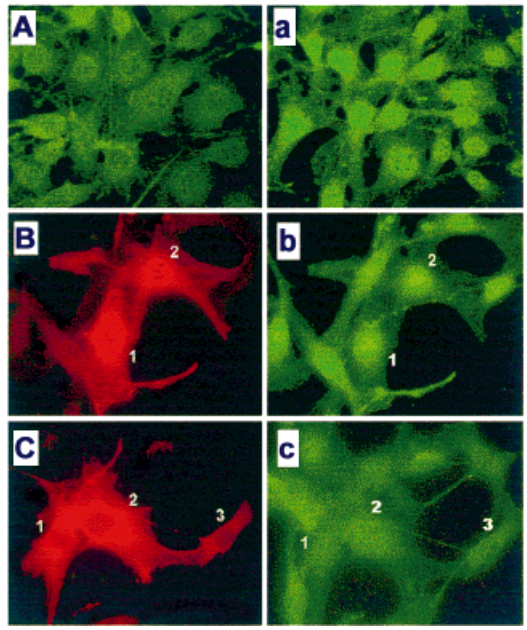

Fig. 7. TNF-α induced translocation of RelA in normal cells and in the cells expressing p38β[AF]. A/a, Cells were stained with anti-RelA (65 kDa subunit of NF-κB) antibodies after fixation and visualized with florescein-conjugated secondary antibodies. **A:** normal cells without treatment; (**a**), normal cells treated with 10 ng/ml TNF-α for 20 min. **B/b:** The cells were infected with p38β[AF] for 24 h followed by the treatment with TNF-α for 20 min and were double stained with anti-Flag antibodies and anti-NF-κB antibodies. **B:** Texas Red-conjugated secondary antibodies were used to detect p38β[AF]. b: Florescein-conjugated secondary antibodies were used to localize NF-κB (b). **C/c:** The cells were infected with p38β[AF] for 24 h but without TNF-α stimulation. Other treatments were the same as for B/b. The cells in B/b and C/c are photographed from same fields under a microscope. The same numbers in B/b or C/c represent the same cells.

expressing p38β or GFP showed normal morphology when treated with TNF-α (Fig. 5B and C, respectively). Apoptotic cell death was further confirmed by DNA fragmentation analysis. The amount of degraded DNA was significantly higher in the cells expressing p38β[AF] in response to TNF-α treatment (Fig. 6). These results are in agreement with those illustrated in Figures 4 and 5.

Expression of p38β or p38β[AF] Does not Interfere With TNF-α-Induced NF-κB Nuclear Translocation

NF-κB is a transcription factor that has been thought to be involved in the induction of a protective factor against TNF-α-induced apoptosis in several types of cells [Beg and Baltimore, 1996; Van Antwerp et al., 1996; Baichwal and Baeuerle, 1997]. It exists as a heterodimer composed of a RelA subunit and an IκB subunit in the cytoplasm of unstimulated cells. Upon cell stimulation, IκB is phosphorylated and degraded, resulting in the release of RelA from the complex, which migrates to the nucleus where it initiates transcription activity. There are indications that the p38 pathway and the NF-κB pathway may coordinately regulate some cellular processes [Read et al., 1997; Vanden Berghe et al., 1998]. If this is the case in TNF-α signaling in mesangial cells, one can speculate that expression of p38β[AF] might interrupt the normal interaction between the p38β and the NF-κB pathways, which may be necessary for the synthesis of protective factors. We examined the effect of overexpression of p38β[AF] on TNF-α-induced RelA nuclear translocation, the initial step for NF-κB transcription activity. As illustrated in Figure 7, RelA was evenly distributed in the resting cells (Fig. 7A). After treatment with TNF-α, RelA was highly concentrated in the nuclei (Fig. 7a), indicating that TNF-α induced a RelA translocation from cytosol to nuclei. Figure 7B shows two cells expressing p38β[AF] as detected with anti-Flag antibodies (labeled as 1 and 2) while Figure 7b shows the same cells (labeled as 1 and 2) stained with anti-RelA antibodies to localize

RelA after TNF-α treatment. The majority of RelA was detected in the nuclei (Fig. 7b). Therefore, expressing p38β[AF] did not interfere with the TNF-α-induced RelA nuclear translocation seen in normal cells (Fig. 7a). Expression of p38β[AF] also had no effect on the cellular localization of RelA in the resting cells shown in Figure 7C and c. In Figure 7C, cells 1, 2, and 3 are expressing p38β[AF]. Essentially there is no difference was detected in the localization of RelA among the three cells (Fig. 7c, 1, 2, and 3) and the cells not expressing p38β[AF] (Fig. 7c, the unnumbered cells). Similarly, overexpression of wild type of p38β affected neither TNF-α-induced RelA nuclear translocation nor its localization in unstimulated cells (data not shown).

DISCUSSION

We have previously reported that TNF-α stimulated a rapid but very transient JNK activation without causing apoptosis in mesangial cells, while a sustained JNK activation was associated with the onset of apoptosis under conditions in which cells were treated with TNF-α in the presence of protein synthesis inhibitor or phosphatase inhibitor [Guo et al., 1998a, b]. We hypothesized that the JNK pathway may mediate the TNF-α toxic effect, but the timing and duration of JNK activation seemed to be critical determinants as to whether or not it was involved in inducing apoptosis. Interestingly, it was recently reported that the early and brief activation of JNK and/or p38 stimulated by TNF-α may actually protect NIH-3T3 cells from TNF-α toxicity [Roulston et al., 1998]. This conclusion was based on the observation that overexpression of the dominant negative mutants of MKK4 or MKK6, the upstream kinases of JNK and p38 respectively, made these cells more sensitive to TNF-α-induced cell death. However, the relative contribution to the protective effect from JNK and p38 could not be evaluated from this study because of the cross activation of JNK by MKK6 and p38 by MKK4 [Roulston et al., 1998]. Clearly the roles of JNK and p38 in TNF-α-induced apoptosis require further investigation.

This study was designed to investigate the involvement of the p38 pathway in the regulation of TNF-α cytotoxicity in rat mesangial cells. In response to TNF-α stimulation, p38 was activated as early as 5 min. The fact that blocking TNF-α-induced p38 activation with SB203580 significantly reduced cell viability indicates the p38 pathway is essential for protecting the cells from the TNF-α toxic effect. This result also implies that either p38α or p38β or both are involved since they are the subtypes of p38 that are inhibited by SB203580. Further results obtained from expression of the dominant negative mutants of p38α and p38β experiments provide substantial evidence to support the possibility that p38β, but not p38α, may be responsible for the protective effect. Dominant negative effects of p38α[AF] and p38β[AF] used in this study have been confirmed by their ability to selectively block some physiological responses that are regulated by the wild type kinases. For instance, in a recent study it has been shown that p38α[AF], but not p38β[AF] selectively inhibited the expression of urokinase plasminogen activator and urokinase receptor in breast cancer cells [Huang et al., 2000]. In cardiomyocytes, p38α[AF], but not p38β[AF], suppressed apoptosis caused by overexpression of wild type p38α. In contrast, p38β[AF] abolished the characteristic feature of hypertrophy and anti-apoptotic effect associated with the expression of wild type p38β [Wang et al., 1998]. In agreement with our results, a protective role of p38β against cell death caused by other stimuli has previously been observed. Overexpression of p38β in Jurkat and HeLa cells reduced cell death induced by Fas ligand and UV-light. On the contrary, overexpression of p38α augmented apoptosis [Nemoto et al., 1998]. We did not observe an apparent effect of expression of p38α or p38α[AF] on the viability of mesangial cells exposed to TNF-α treatment. Similarly, Huang et al. [1997] showed that infection of Jurkat T cells with p38α or p38α[AF] did not alter their sensitivity to Fas ligand induced apoptosis. Taken together, it is probable that p38β may function as a common apoptosis suppressor in different cells while the role of p38α in the regulation of cell viability may vary between different cell types as well as between different stimuli.

Specific targets of p38 isoforms, such as transcription factors, may be critical for transducing their specific signals and thus regulating different cellular responses. To our knowledge, no definitive isoform-specific substrates for p38 have been identified. Therefore, clarification of how different subtypes of p38 selectively affect

cellular events is a challenging task. The NF-κB pathway protects several cell types including mesangial cells from TNF-α-induced apoptosis [Sugiyama et al., 1999]. Recent evidence suggests that there is a synergetic interaction between the p38 pathway and the NF-κB pathway in regulating certain cellular activities. For example, the p38 inhibitor SB203580 blocked TNF-α-induced NF-κB transcription activity in L929 cells [Vanden Berghe et al., 1998]. In myocytes, expression of the p38 upstream kinase MKK6 activated p38 and protected the cells from apoptosis with a concurrent NF-κB activation. Treatment of these cells with SB203580 abolished NF-κB activation and caused cell death when they were stimulated with TNF-α [Zechner et al., 1998]. It is conceivable that overexpression of the dominant negative mutant of p38β may interfere with TNF-α-induced NF-κB activation, thus rendering mesangial cells sensitive to TNF-α toxic effect. However, our results showed that expression of neither p38β nor p38β[AF] affected the TNF-α-stimulated nuclear translocation of the RelA subunit of NF-κB. While it is unclear if p38β regulates NF-κB transcription activity at other steps, it is apparent that p38β does not affect TNF-α-induced NF-κB activation at the stage of RelA nuclear translocation. Whether there is a synergism between the p38β and the NF-κB pathways or the two pathways act independently in protecting mesangial cells from TNF-α cytotoxicity needs further investigation.

In summary, the data presented in this study is the first to show that p38β but not p38α can protect mesangial cells against the toxicity of TNF-α. Our results demonstrate an intriguing possibility that members of the p38 family have divergent physiological functions. They may also provide a plausible explanation of why both pro- and anti-apoptotic effect have been attributed to the p38 MAP kinases in the literature. The differential expression of p38α/p38β in different cell types, and/or their selective activation by different stimuli may explain the conflicting observations in previous studies.

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