

Correlation between Sustained c-Jun N-terminal Protein Kinase Activation and Apoptosis Induced by Tumor Necrosis Factor- α in Rat Mesangial Cells*

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Rat mesangial cells are normally resistant to tumor necrosis factor- α (TNF- α)-induced apoptosis. In this report we show that the cells can be made susceptible to the apoptotic effect of TNF- α when pretreated with actinomycin D, cycloheximide, or vanadate. c-Jun N-terminal protein kinase (JNK) has been thought to mediate apoptotic processes elicited by some stimuli, but its involvement in TNF- α -induced apoptosis has been controversial. JNK activation was investigated under conditions where the mesangial cells were either resistant or susceptible to TNF- α -induced apoptosis. TNF- α alone stimulated a single transient JNK activity peak. However, when the cells were pretreated with actinomycin D or cycloheximide, TNF- α stimulated a second sustained JNK activity peak. When the cells were pretreated with the phosphatase inhibitor vanadate, TNF- α -induced JNK activation was greatly prolonged. In all three cases, a sustained JNK activation was associated with the initiation of apoptosis. Our data suggest that a sustained activation of JNK induced by these reagents may be associated with blocking the expression of a phosphatase that inactivates JNK. Further studies reveal that the expression of mitogen-activated protein kinase phosphatase-1 (MKP-1) was induced by TNF- α , indicating that MKP-1 may be involved in protecting the cells from apoptosis by preventing a prolonged activation of JNK under normal conditions. Additional studies showed that extracellular signal-regulated protein kinase activation stimulated by TNF- α was unlikely to contribute to the resistance of mesangial cells to TNF- α cytotoxicity.

It elicits a wide range of biological responses including cell proliferation, differentiation, and apoptosis, depending on the cell type and its state of differentiation (1). Most cells express specific receptors for TNF- α . Two receptors have been characterized, designated as TNF- α receptor 1 (TNFR1) and TNF- α receptor 2 (TNFR2). After binding to these receptors, TNF- α elicits multiple signal transduction pathways that regulate different cellular processes (2).

Many tumor cells are sensitive to TNF- α -induced apoptosis, but normal cells are usually resistant. Some cells undergo apoptosis only when they are treated with TNF- α in the presence of other agents or when the cells are damaged (3–5). The well characterized morphological changes in the cells that undergo apoptosis include cell shrinkage, cytoplasmic blebbing, and DNA digestion. Recently, activation of interleukin 1-converting enzyme (ICE)-related proteases have been implicated as the “executors of the cell death” at the onset stage of the apoptotic process (6). However, the initial events in the signal transduction pathway responsible for the later phases of cell death are poorly understood. Among these signaling pathways, activation of transcription factors c-Jun and c-Fos (components of the AP-1 transcription complex) and NF- κ B are among the early cellular responses. After phosphorylation, c-Fos and c-Jun form a heterodimer to produce an active AP-1 transcription complex. Mitogen-activated protein kinases (MAP kinase) are among the protein kinases that are responsible for the phosphorylation of c-Fos and c-Jun (7). In mammalian cells, three distinct subtypes have been identified in the MAP kinase family: extracellular signal-regulated kinases (ERK), c-Jun N-terminal protein kinases (JNK), and p38 kinases. JNK and p38 kinases are strongly activated by extra- or intracellular stress and inflammatory cytokines including TNF- α (8, 9). It is thought that activation of JNK and p38 kinases generally promotes an inhibition of cell growth or promotion of cell death, whereas ERK is usually strongly activated by growth factors and hormones that stimulate cell growth and is, therefore, involved in the regulation of cell proliferation (9, 10). Strong and prolonged activation of JNK has been reported in response to a variety of stresses including UV light, ionizing irradiation, and hydrogen peroxide, any of which can trigger apoptosis (11–13). Because TNF- α strongly activates JNK but only weakly stimulates ERK in many cells, it is postulated that JNK activation is involved in TNF- α -induced apoptosis (14–17). However, several reports show that activation of JNK can be mediated through a noncytotoxic TRAF2 (TNF receptor-associated factor 2) pathway initiated by TNF- α , which is not linked to apoptosis (2, 18, 19). The role of the JNK pathway in TNF- α -induced signaling, therefore requires further elucidation.

NF- κ B is a transcription factor present as a heterodimer complexed with I- κ B in the cytoplasm of unstimulated cells.

Tumor necrosis factor- α (TNF- α)¹ is a multifunctional cyto-

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¹ The abbreviations used are: TNF- α , tumor necrosis factor- α ; ICE, interleukin 1-converting enzyme; NF- κ B, nuclear factor- κ B; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal protein kinase; I- κ B, inhibitor of nuclear factor- κ B; MBP, myelin basic protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; MKP, mitogen-activated protein kinase phosphatase.

Upon cell stimulation, I- κ B is phosphorylated and degraded, resulting in the release of NF- κ B, which is translocated to the nucleus where it initiates transcription activity (20). TNF- α is known to induce NF- κ B activation. Initially, it was thought that NF- κ B activation was involved in apoptosis induced by TNF- α until more recent findings suggested that activation of NF- κ B by TNF- α stimulates the synthesis of a survival factor, which protects the cell from apoptosis (21–23). This hypothesis provides a plausible explanation for the reason why some cells, notably primary cells, are resistant to TNF- α -induced apoptosis. Nevertheless, the putative protective factor has not been identified, and the role of NF- κ B activation in the overall progress of apoptosis has not been resolved.

Mesangial cells are a prominent cell type in the glomerulus, and they take part in the regulation of glomerular hemodynamics. They produce inflammatory cytokines such as TNF- α and are involved in the uptake and clearance of immune complexes from the glomeruli by phagocytosis (24). Cell death from apoptosis is prominent during the course of various renal diseases as well as in the early stages of kidney development. In the present study, the cytotoxic effect of TNF- α on rat mesangial cells has been investigated. Our results show that although TNF- α activated JNK, ERK, and NF- κ B, it was unable, by itself, to induce apoptosis. However, under conditions when JNK was activated in a sustained fashion, the cells underwent apoptosis. Of particular interest, our data suggest that a sustained activation of JNK may be associated with a diminished amount or activity of a phosphatase responsible for the dephosphorylation and inactivation of JNK, thereby triggering apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Recombinant TNF- α was obtained from Chemicon International Inc. (Temecula, CA). Anti-ERK2, anti-I- κ B- α , and anti-NF- κ B antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-c-Jun (Ser-63), anti-phospho-JNK antibodies were from New England Biolabs (Beverly, MA). Anti-c-Jun antibodies were from Oncogene (Uniondale, NY), and anti-phospho-ERK was from Promega (Madison, WI). Myelin basic protein (MBP) and PD098059 were purchased from Sigma. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay kit was from Boehringer Mannheim.

Cell Culture and Cell Viability Assay—Rat mesangial cells were isolated from male Sprague-Dawley rats under sterile conditions using the sieving technique as described (25). The cells were maintained in RPMI 1640 medium containing 20% fetal calf serum and 0.6 unit/ml insulin at 37 °C in a humidified incubator (5% CO₂, 95% air). Cells from 5–20 passages were used. After the cells were grown to 80–90% confluence, they were made quiescent by incubation for 16–18 h in insulin-free RPMI 1640 medium containing 2% fetal calf serum.

For cell viability assays, mesangial cells were grown in 12-well plates. The quiescent cells were treated with reagents for the indicated times. Uptake of neutral red dye was used as a measurement of cell viability (26). At the end of the incubations, the medium was removed, and the cells were incubated in Dulbecco's modified Eagle's medium with 2% fetal calf serum containing 0.001% neutral red for 90 min at 37 °C. The uptake of the dye by viable cells was terminated by removal of the media, washing the cells briefly with 1 ml 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, and solubilizing the internalized dye with 1 ml of a solution containing 50% ethanol and 1% glacial acetic acid. The absorbances, which correlate with the amount of live cells, were determined at 540 nm. During the cell incubations cell morphology was also examined under a light microscope.

Immunocytochemical Detection of Apoptosis and c-Jun Phosphorylation—Cells grown on 25-mm glass coverslips in six-well plates were fixed with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, after treatment with various reagents as indicated. DNA strand breaks were identified using a modified TUNEL assay kit. Briefly, the fixed cells were treated with terminal deoxynucleotidyl transferase, which incorporates fluorescein-tagged nucleotides onto 3'-OH termini of fragmented DNA. This was then visualized by adding anti-fluorescein antibody conjugated with horseradish peroxidase followed by diaminobenzidine. The positively stained dark colored nuclei were ana-

lyzed under a light microscope. Phosphorylation of c-Jun was detected with anti-phospho-c-Jun antibodies essentially following the immunocytochemistry protocol provided by the manufacturer (New England Biolabs).

Cell Lysate Preparation—The quiescent cells were treated with reagents for the indicated times, washed twice with ice-cold phosphate-buffered saline, and scraped into cell lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM Na₃VO₄, 50 mM pyrophosphate, 100 mM NaF, 1 mM EGTA, 1.5 mM MgCl₂, 1% Triton X-100, 10% glycerol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cells were incubated in lysis buffer for 30 min on ice with periodic vortexing and centrifuged at 15,000 \times *g* for 15 min. The supernatant was designated as cell lysate. Protein concentration was determined by the method of Bradford using BSA as standard (27). The gelatinous pellets that contained nuclei and cytoskeleton components were extracted with SDS sample buffer and heated at 100 °C for 5 min. The extract was designated as crude nuclear fraction.

Protein Kinase Assays—JNK activity was measured using a solid phase kinase assay method. GST-c-Jun(1–79) (GST-Jun) fusion protein was isolated from bacterial cells expressing pGEX-c-Jun plasmid (kindly provided by Dr. J. K. Westwick). GST-Jun bound to glutathione-agarose beads was used to affinity purify JNK. JNK activity was determined using GST-Jun as substrate (28). Briefly, 100 μ g of cell lysate was incubated with 2 μ g of GST-Jun-agarose beads at 4 °C for 2 h with rotation and centrifuged at 10,000 \times *g* for 1 min. The beads were washed three times with washing buffer (25 mM Hepes, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 0.05% (v/v) Triton X-100, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM β -glycerophosphate, and 10 mM NaF). The beads were then resuspended in 10 μ l of kinase buffer containing as final concentration 20 mM Hepes, pH 7.5, 10 mM MgCl₂, 1 mM Na₃VO₄, 20 mM β -glycerophosphate, 5 mM NaF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 40 μ M ATP, and 1 μ Ci of [γ -³²P]ATP. After incubation at room temperature for 20 min, the reaction was terminated by adding SDS sample buffer followed by heating at 100 °C for 3 min. The proteins were separated on 12% SDS-PAGE, and the phosphorylated proteins were detected by autoradiography. The kinase activity was quantified by scintillation counting of the radioactivity in the phospho-GST-Jun bands excised from the gels.

In-gel JNK assays were performed as described (29) with some modifications. The enzyme samples were added to SDS sample buffer and electrophoresed on SDS-PAGE as described by Laemmli (30) except that GST-Jun (20 μ g/ml) was added to the running gel solution before polymerization. SDS was removed by washing the gel three times with 25 mM Hepes, pH 7.5, containing 150 mM NaCl for 30 min at room temperature immediately after electrophoresis. The enzyme was allowed to renature in the washing buffer containing 0.5 mM dithiothreitol at 4 °C overnight. The gel was then incubated in the kinase reaction buffer as described above for 3–5 h with gentle shaking. The reaction was stopped by adding a solution containing 5% trichloroacetic acid (w/v) and 1% (w/v) sodium pyrophosphate. The gel was washed extensively with the same solution, dried, and autoradiographed.

ERK activity was measured by the immunocomplex assay method. 100 μ g of whole cell lysate was incubated with 1.5 μ l of polyclonal anti-ERK2 serum for 1.5 h at 4 °C with rotation followed by addition of 5 μ l of protein A-agarose beads (50%). The suspension was further incubated for 1 h. Immunocomplexes were washed three times with washing buffer as for JNK purification. The ERK activity was determined under the same condition as for JNK assays except that GST-Jun was replaced with 2 μ g of MBP as substrate. ERK activation was also determined by a gel shift assay (31). The appearance of a slower migrating band on the gel caused by phosphorylation of threonine and tyrosine residues of ERK2 was used as an indication of its activation.

Western Blot Analysis—The protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions. The immunoblots were visualized by an enhanced chemiluminescence (ECL) kit obtained from Amersham.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from mesangial cells using TRI-reagent (Molecular Research Center, Inc.) as recommended by the manufacturer. Northern blot analysis was performed as described previously (32). *Hind*III/*Bam*HI fragment of pCEP4-MKP-1 (MAP kinase phosphatase-1) plasmid (kindly provided by Drs. N. Tonks and H. Sun) was used as a probe.

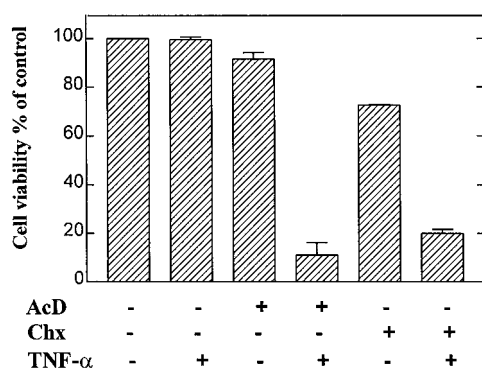


FIG. 1. Effects of TNF- α on the viability of mesangial cells in the presence and absence of actinomycin D or cycloheximide. The cells were stimulated with TNF- α (10 ng/ml) for 4 h, or they were pretreated with actinomycin D (AcD, 0.4 μ g/ml) or cycloheximide (Chx, 5 μ g/ml) for 1 h and then stimulated with TNF- α (10 ng/ml) for 4 h. The control experiments include cells without treatment or treated with actinomycin D or cycloheximide alone for 5 h. Cell viability was determined by the neutral red assay method. Results are the mean \pm S.E. of three experiments.

RESULTS

Effect of TNF- α on the Viability of Mesangial Cells in the Presence or Absence of Actinomycin D and Cycloheximide—Mesangial cells are essentially insensitive to TNF- α cytotoxicity at a concentration of 10 ng/ml (Fig. 1), which is sufficient to induce apoptosis in susceptible cells (4, 17). Prolonged incubation of mesangial cells with 10 ng/ml TNF- α for 48 h showed little deterioration of cell viability. However, when the cells were pretreated with the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide, cell viability was decreased dramatically (Fig. 1). The cells did not show demonstrable signs of apoptosis after they were treated with TNF- α , actinomycin D, or cycloheximide alone. Cell death resulting from preincubation with either actinomycin D or cycloheximide followed by TNF- α stimulation showed typical characteristics of apoptosis as examined under a microscope and as determined by the TUNEL analysis. At the end of the 4-h incubation, more than 50% of the cells attached to the coverslips were TUNEL staining-positive when the cells were pretreated with either actinomycin D or cycloheximide followed by stimulation with TNF- α , whereas under the same conditions only 5% of the cells were TUNEL staining-positive when the cells were treated with TNF- α only. This is the same as for the control experiments where the cells were not treated with any reagent. These results indicate that mesangial cells are normally resistant to TNF- α -induced apoptosis with this resistance depending on *de novo* protein synthesis, a property shown by other nontransformed cell types (3, 4).

Activation of JNK by TNF- α and the Effects of Actinomycin D and Cycloheximide—The JNK pathway has been implicated in the regulation of apoptosis induced by various stimuli (12, 13, 33). Two protein kinases with molecular masses of 54 and 46 kDa were activated by TNF- α as detected by an in-gel assay when GST-Jun was used as substrate in mesangial cells. They were identified as JNK2 and JNK1, respectively, based on their molecular weights. Their identities were further confirmed by Western blot analysis using anti-phospho-JNK antibodies that only recognized the activated JNK1 and JNK2 (data not shown). To investigate if there was a correlation between JNK activity and cell viability, the time course of JNK activation was determined in cells after treatment with TNF- α . As shown in Fig. 2A, the activation of JNK by TNF- α was very transient. Maximal activation was reached within 15 min, giving a 5–10 fold-stimulation, which diminished to the basal level by 30 min. However, when the cells were pretreated with actinomycin D or

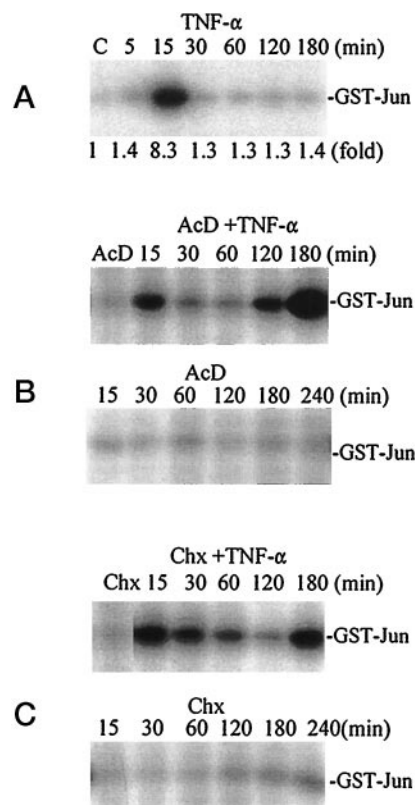


FIG. 2. Time course of JNK activation by TNF- α ; effects of actinomycin D and cycloheximide. Panel A, time course of JNK activation by TNF- α (10 ng/ml). Lane C represents cells that were not treated with TNF- α . The fold activation above the control (C) was determined by scintillation counting of the radioactivity in the phospho-GST-Jun bands excised from the gel. Panels B and C, effects of actinomycin D (AcD, 0.4 μ g/ml) and cycloheximide (Chx, 5 μ g/ml) on JNK activity, respectively. The cells were pretreated with actinomycin D or cycloheximide for 1 h and stimulated with TNF- α (10 ng/ml) for the times indicated. The lower panels of B and C represent experiments in which the cells were treated with actinomycin D or cycloheximide only. 100 μ g of cell lysate was used for affinity purification of JNK. The kinase activity was determined by solid phase kinase assay.

cycloheximide, a second JNK activation peak was detected. In the case of actinomycin D, the second activity peak started at 2 h and lasted at least for another hour with increased activity (Fig. 2B). In the case of cycloheximide, the second activity peak started at 3 h after the addition of TNF- α with a lower level of activation compared with the effect of actinomycin D (Fig. 2C). In addition to inducing the second peak of JNK activation, cycloheximide also potentiated the duration of the first TNF- α -induced activity peak of JNK compared with TNF- α alone as a control (Fig. 2A). The second activity peak of JNK was not caused directly by actinomycin D or cycloheximide because neither treatment had a significant effect on JNK activity (Figs. 2, B and C, lower panels). Although it is reported that cycloheximide by itself activates JNK in some cells (15), its effect on mesangial cells was rather weak. A similar result was also reported by Liu *et al.* in rat mesangial cells (34).

To test the effects of actinomycin D and cycloheximide on steps downstream of JNK, the phosphorylation state of c-Jun was examined by using antibodies that were able to recognize only phospho-c-Jun. As shown in Fig. 3, at the end of 3 h of incubation, about 90% of the cells were heavily stained with anti-phospho-c-Jun when the cells were pretreated with either actinomycin D or cycloheximide followed by stimulation with TNF- α , whereas under the same conditions, treatment of the cells with TNF- α , actinomycin D, or cycloheximide alone only showed a slight effect on c-Jun phosphorylation. The increased

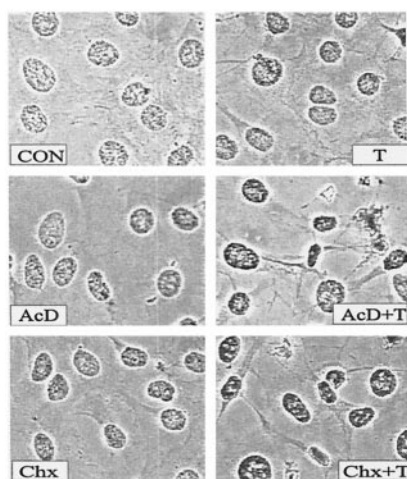


FIG. 3. Immunostaining of phospho-c-Jun stimulated by TNF- α ; effects of actinomycin D and cycloheximide. The concentrations of TNF- α , actinomycin D (AcD), and cycloheximide (Chx) were the same as described for Fig. 1. The cells were stimulated with TNF- α alone for 4 h (T), or they were pretreated with actinomycin D for 1 h and then stimulated with TNF- α for 3 h (AcD+T), or they were pretreated with cycloheximide for 1 h and then stimulated with TNF- α for 3 h (Chx+T). The controls include the cells without treatment (CON) or treated with actinomycin D or cycloheximide alone for 4 h. Phospho-c-Jun was detected by dark stained nuclei.

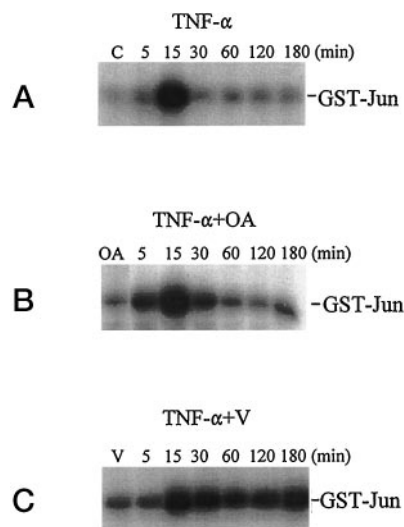


FIG. 4. Effect of phosphatase inhibitors on JNK activity stimulated by TNF- α . Panel A, activation of JNK by TNF- α (10 ng/ml) alone. Lane C represents cells in the absence of TNF- α . Panel B, effects of okadaic acid (OA, 250 nM) on JNK activity stimulated by TNF- α . Panel C, effect of vanadate (V, 500 μ M) on JNK activity stimulated by TNF- α . The cells were pretreated with okadaic acid or vanadate for 1 h and stimulated with TNF- α (10 ng/ml) for the times indicated. Lanes OA and V (in panels B and C) represent the cells treated with okadaic acid or vanadate alone, respectively. JNK activity was determined as described for Fig. 2.

activity of JNK and phosphorylation of c-Jun caused by TNF- α in the presence of actinomycin D or cycloheximide after a 3-h incubation corresponded to the onset of apoptosis (Fig. 1).

Activation of JNK by TNF- α and the Effect of Protein Phosphatase Inhibitors—From the above data it appeared that a sustained JNK activation was necessary for the induction of apoptosis, whereas activation of JNK by TNF- α alone in mesangial cells was too transient to initiate the apoptotic process. To determine whether a sustained JNK activation is able to render the cells susceptible to TNF- α cytotoxicity using other approaches, mesangial cells were pretreated with two protein

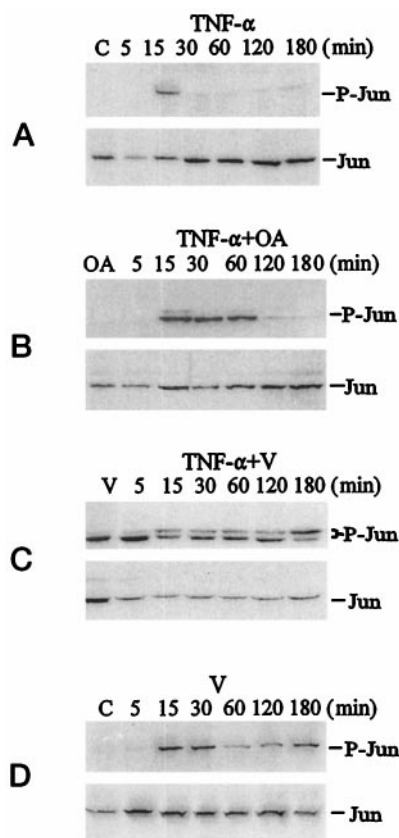


FIG. 5. Phosphorylation of c-Jun stimulated by TNF- α ; effects of protein phosphatase inhibitors. Panel A, mesangial cells were treated with TNF- α (10 ng/ml) alone. Panel B, the cells were pretreated with 250 nM okadaic acid (OA) and then stimulated with TNF- α . Panel C, the cells were pretreated with 500 μ M vanadate (V) and then stimulated with TNF- α . Panel D, the cells were treated with vanadate alone. The crude nuclear fractions were extracted with SDS sample buffer. The extracts were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Bands corresponding to c-Jun (Jun) and phospho-c-Jun (P-Jun) were identified by Western blot analysis using specific antibodies.

phosphatase inhibitors, vanadate and okadaic acid, which are known to inhibit the tyrosine phosphatases and type 1/type 2A serine/threonine phosphatases, respectively. These treatments presumably would inactivate protein phosphatases that dephosphorylate JNK, thereby prolonging the activation of JNK by TNF- α . As shown in Fig. 4, whereas pretreatment of the cells with okadaic acid (Fig. 4B) slightly potentiated the duration of JNK activation stimulated by TNF- α , pretreatment of the cells with vanadate (Fig. 4C) dramatically prolonged TNF- α -induced JNK activation. Control experiments showed that okadaic acid alone had little effect on JNK activity, but vanadate alone activated JNK about 2-fold. These results indicate that inactivation of JNK occurs mainly by a vanadate-sensitive tyrosine phosphatase.

The phosphorylation state of c-Jun was examined by Western blot analysis using antibodies that were able to recognize only c-Jun or phospho-c-Jun. As shown in Fig. 5, the phosphorylation of c-Jun correlated well with the duration of JNK activity observed in Fig. 4. Pretreatment of the cells with okadaic acid prolonged the phosphorylation state of c-Jun during stimulation by TNF- α for up to 1 h, whereas pretreatment of the cells with vanadate followed by stimulation with TNF- α caused the phosphorylation of c-Jun to last for at least 3 h. Interestingly, under this condition, phospho-c-Jun was recognized as a doublet that represents phosphorylation at multiple sites, causing a retardation of the protein band attributed to

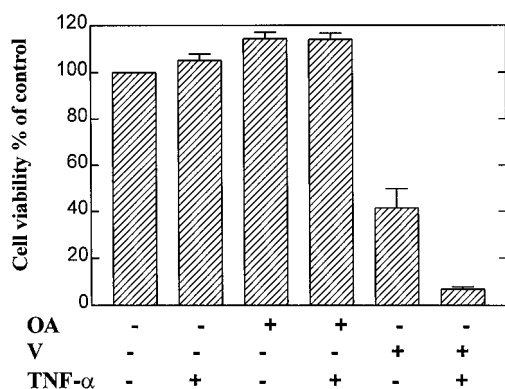


FIG. 6. Effect of TNF- α on cell viability in the presence and absence of okadaic acid and vanadate. Mesangial cells were stimulated with TNF- α (10 ng/ml) for 4 h, or they were pretreated with okadaic acid (OA, 250 nM) or vanadate (V, 500 μ M) for 1 h and then stimulated with TNF- α (10 ng/ml) for 4 h. Control experiments include cells without treatment or treated with okadaic acid or vanadate for alone for 5 h. Cell viability was determined by the neutral red assay method. Results are the mean \pm S.E. of three experiments.

hyperphosphorylation of c-Jun (35). Okadaic acid alone did not cause significant phosphorylation of c-Jun, whereas vanadate alone caused a sustained c-Jun phosphorylation, without the hyperphosphorylated band being detected (Fig. 5D).

Effect of TNF- α on the Viability of Mesangial Cells in the Presence or Absence of Phosphatase Inhibitors—To investigate whether there was a correlation between the duration of JNK activation and cell viability, the mesangial cells were incubated with TNF- α in the presence of okadaic acid and vanadate. As shown in Fig. 6, preincubation of the cells with okadaic acid followed by stimulation with TNF- α had no effect on cell viability. In contrast, pretreatment of the cells with vanadate followed by TNF- α stimulation dramatically increased cell death. Cells that were treated with vanadate alone also resulted in significant cell death (Fig. 6). It should be noted that vanadate alone also caused JNK activation at a low level (\sim 3-fold) but in a sustained manner, in conjunction with a sustained phosphorylation of c-Jun (Fig. 5). Cells from parallel experiments were examined for apoptosis by the TUNEL analysis. Under the conditions tested, only the cells treated with vanadate or vanadate plus TNF- α showed the morphological characteristic of apoptosis. At the end of the 4-h incubation period, about 25% of the cells attached to the coverslips were TUNEL staining-positive when the cells were treated with vanadate alone, and 35% of the cells were TUNEL staining-positive when treated with vanadate and TNF- α . Only 5–7% of the cells were TUNEL staining-positive when the cells were treated with okadaic acid alone or in combination with TNF- α . These results indicate that cell death resulted from apoptosis and was paralleled by a sustained activation of JNK and c-Jun phosphorylation state.

Activation of ERK by TNF- α and the Effect of Protein Phosphatase Inhibitors—It is known that TNF- α activates both JNK and ERK, but generally JNK is activated more strongly than ERK, as reported in a number of cell types (14, 15, 36). TNF- α activated both ERK1 and ERK2 as determined by Western blot analysis using antibodies that only recognized the active ERK1 and ERK2. The effect of TNF- α on ERK2 activation in mesangial cells is shown in Fig. 7. The activation of ERK2 was transient with the activity peaking at 15 min followed by a decline to the basal level after 30–60 min. Similar kinetics were obtained when ERK2 activation was measured by the gel shift assay or when the activation of ERK2 was quantified using the kinase assay method, in which ERK2 was immunoprecipitated and its activity determined using MBP as

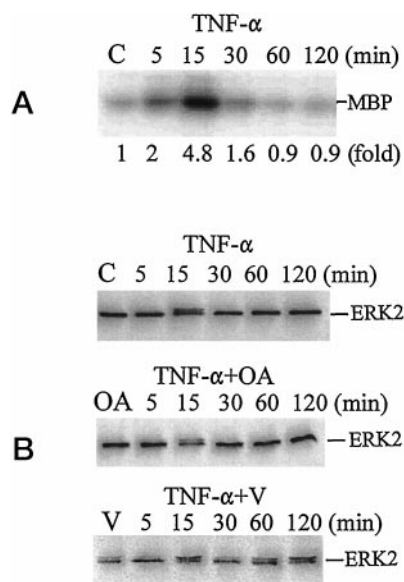


FIG. 7. Activation of ERK by TNF- α and effect of protein phosphatase inhibitors. Panel A, time course of ERK activation by TNF- α . The cells were treated with TNF- α (10 ng/ml) for the times as indicated. 100 μ g of cell lysate was used for purification of ERK2 by immunoprecipitation. The kinase activity was determined by an immunocomplex kinase assay. Fold activation above control was determined by liquid scintillation counting of radioactivity in the MBP bands excised from the gel. Panel B, effects of okadaic acid (OA) and vanadate (V) on ERK2 activation. The cells were stimulated with TNF- α (10 ng/ml) alone or pretreated with okadaic acid (250 nM) or vanadate (500 μ M) and then stimulated with TNF- α (10 ng/ml) for the times as indicated. 20 μ g of cell lysate from each sample was separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. ERK activation was indicated by a gel shift assay using anti-ERK2 antibodies.

substrate (Fig. 7). The maximum activation of ERK2 at 15 min was 3–5 fold above basal levels (Fig. 7A), whereas the maximum activation of JNK was 8–10-fold (Fig. 2A). The effects of protein phosphatase inhibitors on ERK2 activity showed a pattern similar to that for JNK with okadaic acid having little effect and vanadate profoundly prolonging ERK2 activation, as judged by the gel shift assay (Fig. 7B).

Effects of PD098059 on the Activation of JNK and ERK by TNF- α —Whereas the JNK pathway has been implicated in pro-apoptotic processes, the ERK pathway has been thought to be anti-apoptotic (10). Activation of the ERK pathway has been shown to suppress apoptosis induced by various agents including TNF- α , but selectively blocking ERK activation greatly potentiates the apoptotic process (10, 37). To test the possibility that ERK activation may contribute to the resistance of mesangial cells to TNF- α cytotoxicity, PD098059, a compound known to inhibit ERK kinase specifically, thereby blocking ERK activation (38), was used to block ERK activation by TNF- α . As shown in Fig. 8, pretreatment of mesangial cells with 30 μ M PD098059 effectively abolished the activation of ERK induced by TNF- α , but JNK activity was not affected. Control experiments showed that PD098059 alone had no effect on ERK or JNK activity. These results are in agreement with its reported effect on ERK and JNK activities in other cells (39). Additionally, neither cell viability nor morphology of the mesangial cells was affected by pretreatment with PD098059 compared with cells that were treated with TNF- α alone. The above results indicate that ERK activation stimulated by TNF- α is unlikely to contribute to the resistance of the mesangial cells to TNF- α cytotoxicity.

Activation of NF- κ B by TNF- α in the Presence and Absence of Protein Phosphatase Inhibitors—Recent evidence favors the hypothesis that activation of NF- κ B initiates transcription of a

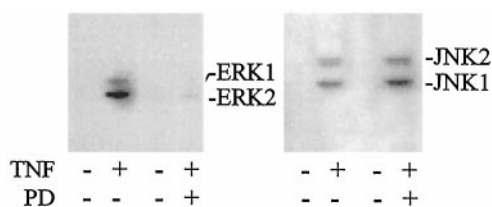


FIG. 8. Effect of PD098059 on ERK and JNK activation stimulated by TNF- α . Mesangial cells were stimulated with TNF- α (10 ng/ml) for 15 min after incubation with or without PD098059 for 1 h. 20 μ g of cell lysate from each sample was separated on 12% SDS-PAGE and transferred to nitrocellulose membranes. ERK activation was determined by Western blot analysis using anti-phospho-ERK antibodies that recognized both active ERK1 and ERK2. JNK activation was determined by Western blot analysis using anti-phospho-JNK antibodies that recognized both active JNK1 and JNK2.

gene encoding a factor that protects the cells from apoptosis (21–23). Although our primary hypothesis is that a sustained activation of JNK caused by inhibition of a JNK phosphatase with vanadate is the primary factor involved in TNF- α -induced apoptosis, it is possible that treatment of the cells with vanadate may also interfere with the activation of NF- κ B by TNF- α . This may have the effect of blocking the synthesis of the protective factor, thereby rendering the cells susceptible to TNF- α cytotoxicity. To investigate this possibility, the effect of vanadate on the activation of NF- κ B by TNF- α was determined by Western blot analysis using anti-I- κ B α antibodies. The degradation of I- κ B α was used as an indirect indication of NF- κ B activation (40). As illustrated in Fig. 9A, TNF- α treatment induced an initial decrease of I- κ B α , which was observed after 15 min but then returned to starting levels after 2 h. This biphasic response pattern has also been seen in other cells (40). Pretreatment of the cells with either okadaic acid or vanadate failed to block the degradation of I- κ B α induced by TNF- α , with okadaic acid pretreatment actually showing some potentiation of the TNF- α effect (Fig. 9B). The activation of NF- κ B was further confirmed by immunocytochemistry using antibodies against the 65-kDa subunit of NF- κ B. In all cases, the 65-kDa subunit translocated from the cytosol to the nucleus,² indicating that vanadate and okadaic acid did not block the activation of NF- κ B induced by TNF- α . Based on these observations, it is likely that vanadate exerts its effect through the inhibition of phosphatases rather than interfering with the NF- κ B activation induced by TNF- α .

Induction of MKP-1 mRNA by TNF- α —Our results thus far suggest that a sustained activation of JNK induced by TNF- α in the presence of actinomycin D and cycloheximide may be associated with blocking the expression of a phosphatase that inactivates JNK. To investigate if TNF- α could elicit the synthesis of such a phosphatase, we investigated the gene expression of MKP-1 in rat mesangial cells. MKP-1 is the most prominent and best characterized member of MKP family which has been shown to inactivate JNK *in vitro* and *in vivo* (41–43). As shown in Fig. 10, the mRNA of MKP-1 was strongly induced by TNF- α at the same time, which coincided with the inactivation of JNK (Fig. 2). This result strongly supports our hypothesis that a TNF- α -inducible phosphatase may be responsible for protecting mesangial cells from apoptosis by suppressing a prolonged activation of JNK. MKP-1 could be the phosphatase or one of such phosphatases.

DISCUSSION

Mesangial cells are insensitive to TNF- α -induced apoptosis under conditions that will induce apoptosis in TNF- α susceptible cells. Several hypotheses have been proposed to explain

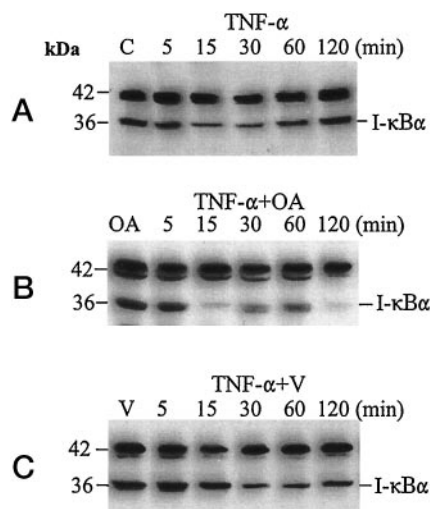


FIG. 9. Degradation of I- κ B induced by TNF- α and effects of protein phosphatase inhibitors. Panel A, mesangial cells were treated with TNF- α (10 ng/ml) alone. Lane C represents cells in the absence of TNF- α . Panel B, cells were pretreated with 250 nM okadaic acid (OA) and stimulated with TNF- α . Panel C, the cells were pretreated with 500 μ M vanadate (V) and stimulated with TNF- α . I- κ B α was analyzed by Western blot using anti-I- κ B α antibodies. Degradation of I- κ B α was indicated by decrease in intensity of the 36-kDa I- κ B α bands. Lanes OA and V (in panels B and C) represent the cells treated with okadaic acid or vanadate alone, respectively.

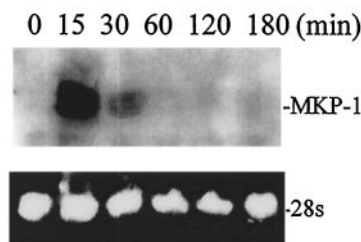


FIG. 10. Induction of MKP-1 mRNA by TNF- α . Mesangial cells were stimulated with 10 ng/ml TNF- α for the times indicated. The total mRNA (20 μ g) was analyzed by Northern blot using MKP-1 cDNA as a probe. The equal loading of mRNA was judged by 28 S rRNA.

how normal cells achieve this cellular resistance to TNF- α . First, TNF- α may activate an anti-apoptotic signaling pathway that counteracts the cytotoxicity of the apoptotic pathway (37, 44). Second, TNF- α may elicit the synthesis of a protective factor, causing the cells to become insensitive to TNF- α cytotoxicity (21, 23). Third, the apoptotic pathway inducible by TNF- α may not be sufficiently activated to initiate the apoptotic process. However, to date none of these hypotheses has been fully established. The present investigation was initiated to gain some insight into how mesangial cells achieve their resistance to TNF- α cytotoxicity.

Based on our current understanding of the roles of the different subtypes of MAP kinases in the regulation of growth and development, it has been proposed that the balance and/or threshold level of activation of one pathway *versus* the other, *e.g.* ERK *versus* JNK/p38 kinase, may determine whether a cell is going to proliferate or undergo apoptosis (45). For example, in PC12 cells, concurrent activation of JNK/p38 kinase pathways and inhibition of the ERK pathway induces apoptosis, whereas direct and selective activation of the ERK pathway prevents apoptosis (10). In L929 cells, fibroblast growth factor-2 suppresses TNF- α -induced apoptosis by causing an activation of ERK, and this effect could be reversed by inhibiting the ERK pathway (37). The present study shows that in mesangial cells, ERK was activated in a manner similar to the activation of JNK by TNF- α , although the magnitude of its

² Y.-L. Guo, K. Baysal, and J. R. Williamson, unpublished results.

activation was lower than that of JNK (3-fold versus 8-fold). However, when ERK activation by TNF- α was selectively inhibited by pretreatment of mesangial cells with PD098059, cell viability was not affected. This result argues against the possibility that activation of ERK by TNF- α contributed to the resistance of mesangial cells to TNF- α cytotoxicity. Therefore, the anti-apoptotic effect attributable to activation of the ERK pathway seems to be dependent on cell type and stimulus.

An obligatory role of the JNK pathway in apoptosis has been best documented in various stress-induced apoptosis models (11, 13, 17, 46). The duration of JNK activation is thought to be a critical factor determining cell proliferation or apoptosis with transient activation leading to cell proliferation or differentiation and prolonged JNK activation causing apoptosis (11). In mesangial cells, the fact that activation of JNK by TNF- α lasted less than 15 min implies that it may not be activated long enough to initiate the apoptotic process. Interestingly, when the cells were treated with actinomycin D or cycloheximide after stimulation with TNF- α , a second strong activation of JNK was observed, which coincided with the onset of apoptosis. The above result indicates that the JNK pathway may play a role in TNF- α -mediated apoptosis only if the activating stimulus is sufficiently prolonged. This observation resembles a similar pattern observed between JNK activation and cell death in Fas-induced apoptosis in a neuroblastoma cell line. Goillot *et al.* (47) found that JNK activation induced by Fas was biphasic. The transient first activity peak was detected at 15 min, then the activity decreased to basal level followed by a second peak starting 2 h after stimulation. The second activation peak of JNK was critical for the initiation of the apoptotic process. In our experiments with mesangial cells, no second activity peak of JNK was detected within 3 h of treatment with TNF- α alone. The simplest and most likely interpretation of these results is that under normal conditions the extent of JNK activation is constrained in some manner, possibly by activation of a JNK phosphatase. It is hypothesized that actinomycin D or cycloheximide blocks the expression of a phosphatase that is responsible for limiting the activation of JNK. According to this conjecture, the second activity peak of JNK would only be detectable when the induction of the phosphatase was blocked. This speculation is further supported by results obtained from pretreatment of the cells with vanadate. Vanadate inhibits both protein tyrosine phosphatases and members of the MKP family, and its addition to mesangial cells resulted in a sustained activation of JNK by TNF- α . Thus in each situation so far investigated, when JNK activation was sustained, the cells underwent apoptosis. These results suggest that a meaningful correlation exists between the duration of JNK activation and cell death.

The best model described to date for TNF receptor family-induced apoptosis is that once the ligand binds to the receptor (*e.g.* TNF- α to the TNF receptor or Fas ligand to Fas), the cytosolic part of the receptor recruits the "death domain-containing proteins" TRADD (TNFR-associated death domain protein) or FADD (Fas-associated death domain protein), which in turn activates a cascade of ICE proteases which executes the apoptotic process (2, 5). However, this model does not include an involvement of JNK in the apoptotic pathway activated by TNF- α or the Fas ligand. Schievella *et al.* (48) found that ERK and JNK activation induced by TNF- α could operate through a pathway other than TRAF2. They identified a novel death domain protein, MADD, which interacts with the TNF- α receptor and activates both ERK and JNK. This finding provides evidence that the JNK pathway is potentially involved in apoptosis. Similarly, a Fas-binding protein, Daxx, has been characterized. It interacts with the Fas death domain and activates

JNK as well as the apoptotic process (49). These findings provide the missing link between JNK activation and apoptosis in the TNF- α receptor and Fas signaling pathways. It is apparent that more than one pathway initiated by the TNF- α receptor family can lead to cell death. It has been proposed, for instance, that JNK activation could lead to another ICE protease cascade in addition to the earlier characterized FADD-FLICE-ICE cascade (49).

Our findings suggest a novel explanation that may account for the fact that mesangial cells protect themselves from TNF- α cytotoxicity under normal conditions. This proposed mechanism entails the induction of phosphatase by TNF- α , which contributes toward protecting the cells from TNF- α -induced apoptosis by suppressing a sustained JNK activation in a way similar to the way MKPs cause inactivation of JNK stimulated by stresses. It has been shown that some MKPs are inducible by various stresses that activate JNK (41–43). If this mechanism is involved, the duration of JNK activation would be regulated by MKP through a feedback mechanism. Therefore, induction of MKP to inactivate JNK serves as a protective mechanism against stress damage caused by various agents (41, 42, 50). The TNF- α -inducible MKP may play a similar role in protecting the cell from TNF- α cytotoxicity. This hypothesis is strongly supported by the fact that the expression of MKP-1 was induced by TNF- α in mesangial cells at the same time as the inactivation of JNK. Therefore, we conclude that MKP-1 induced by TNF- α may at least partially be responsible for protecting mesangial cells from apoptosis by suppressing a prolonged activation of JNK.

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