Transforming Growth Factor $\beta_1$ Rescues Serum Deprivation-induced Apoptosis via the Mitogen-activated Protein Kinase (MAPK) Pathway in Macrophages*

(Received for publication, October 5, 1998, and in revised form, January 8, 1999)

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Cell death and cell survival are central components of normal development and pathologic states. Transforming growth factor $\beta_1$ (TGF-$\beta_1$) is a pleiotropic cytokine that regulates both cell growth and cell death. To better understand the molecular mechanisms that control cell death or survival, we investigated the role of TGF-$\beta_1$ in the apoptotic process by dominant-negative inhibition of both TGF-$\beta_1$, and mitogen-activated protein kinase (MAPK) signaling pathways. Murine macrophages (RAW 264.7) undergo apoptosis following serum deprivation, as determined by DNA laddering assay. However, apoptosis is prevented in serum-deprived macrophages by the presence of exogenous TGF-$\beta_1$. Using stably transfected RAW 264.7 cells with the kinase-deleted dominant-negative mutant of TGF-$\beta_1$, we demonstrate that this protective effect by TGF-$\beta_1$ is completely abrogated. To determine the downstream signaling pathways, we examined TGF-$\beta_1$ effects on the MAPK pathway. We show that TGF-$\beta_1$ induces the extracellular signal-regulated kinase (ERK) activity in a time-dependent manner up to 4 h after stimulation. Furthermore, TGF-$\beta_1$ does not rescue serum deprivation-induced apoptosis in RAW 264.7 cells transfected with a dominant-negative mutant MAPK (ERK2) cDNA or in wild type RAW 264.7 cells in the presence of the MAPK kinase (MEK1) inhibitor. Taken together, our data demonstrate for the first time that TGF-$\beta_1$ is an inhibitor of apoptosis in cultured macrophages and may serve as a cell survival factor via TGF-$\beta_1$-mediated signaling and downstream intracellular MAPK signaling pathway.

Apoptosis, the process of programmed cell death, is an integral part of normal embryonic development, inflammatory response, and tumorigenesis (1). It is a highly regulated series of well coordinated events characterized by distinctive morphologic and biochemical changes involving nuclear and chromatin condensation, cell membrane blebbing, and loss of cellular integrity forming distinct apoptotic bodies, as well as endonuclease activity resulting in DNA fragmentation and ultimately cell death (2). Regulatory mechanisms controlling cell death is as fundamental as those regulating cell growth in achieving the homeostatic balance between cell survival and cell death and involve a complex interplay of specific regulatory genes in signaling cells to either live or die.

Transforming growth factor $\beta_1$ (TGF-$\beta_1$) is a 25-kDa polypeptide, belonging to a superfamily of multifunctional cytokines, that regulates cellular growth and differentiation and extracellular matrix production (3). Moreover, TGF-$\beta_1$ has been shown to be a potent modulator of apoptosis in a variety of cell types, including epithelial cells, hepatocytes, hematopoietic cells, and lymphocytes, which undergo programmed cell death in response to TGF-$\beta_1$ (4–7). We have previously reported the induction of apoptosis by TGF-$\beta_1$ in endothelial cells (8). However, more recent studies suggest that TGF-$\beta_1$ also possesses the ability to inhibit apoptosis, further affirming the multifunctional nature of this cytokine (9).

TGF-$\beta_1$ elicits multiple biological responses by interaction with two transmembrane receptor serine/threonine kinases known as TGF-$\beta$ type I receptor (TGF-$\beta$-RI) and TGF-$\beta$ type II receptor (TGF-$\beta$-RII) (3). TGF-$\beta$-RI is a constitutively active kinase, which binds TGF-$\beta_1$ directly and recruits TGF-$\beta$-RI to form a “heteromeric” complex, and the signaling cascade is initiated upon transphosphorylation of the GS domain of TGF-$\beta$-RI by TGF-$\beta$-RII (10). TGF-$\beta$-RI alone does not exhibit significant binding of TGF-$\beta_1$ ligand when assessed by cross-linking analysis, and TGF-$\beta$-II is unable to signal without TGF-$\beta$-RI (10). Thus, TGF-$\beta$-RI is required for initial ligand binding and phosphorylation of TGF-$\beta$-RI to initiate the signaling cascade. We have previously reported the critical role of TGF-$\beta_1$ signaling pathway to induce apoptosis in endothelial cells (8). Interference with TGF-$\beta$-II-mediated signal transduction by a dominant-negative mutant of TGF-$\beta$-RII blocked TGF-$\beta_1$-induced endothelial cell apoptosis and associated capillary morphogenesis in vitro (8).

Although molecular cloning of the TGF-$\beta_1$ receptors have furthered our understanding of the mechanism of TGF-$\beta_1$ signaling, the downstream signaling pathways activated after the initial receptor interaction with ligand to mediate multiple TGF-$\beta_1$ responses remain poorly understood. Recent studies

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* This work was supported in part by Physicisn Scientist Award 5-K12-DK0129809 from the NIDDK, National Institutes of Health, Grant-in-aid 96015510 from the American Heart Association, and a Veterans Affairs Career Development Award (to M. E. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

** Supported by a Laboratory Scholarship from Toxicological Sciences and Environmental Health Sciences, and a Veterans Affairs Career Development Award (to M. E. C.).

† Supported by National Institutes of Health Grant R29 HL-55330, American Heart Association EIA, and National Institutes of Health Grant R01 AI-42365.

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1 The abbreviations used are: TGF-$\beta_1$, transforming growth factor $\beta_1$; TGF-$\beta$, transforming growth factor $\beta$; TGF-$\beta$-R, TGF-$\beta$ receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase.
support the involvement of the mitogen-activated protein kinase (MAPK) pathways in TGF-β signaling (11–14). Moreover, activation of the MAPK-dependent pathways has been implicated in the process of apoptosis (15, 16). Members of the MAPK family, like the TGF-β receptors, are structurally related serine/threonine kinases that are actively involved in cellular events such as growth, differentiation, and cellular responses to environmental stress (17, 18). There are three groups of the MAPK family members identified to date: the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), also known as p44 and p42 MAPKs, respectively; the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK); and the p38 (18, 19). The signal transduction cascades involved in the activation of MAPKs require a well coordinated series of three protein kinase reactions, propagating the phosphorylation and the activation of the next kinase in their respective pathways. The MAPKs require dual phosphorylation at the threonine and tyrosine sites by MAPK kinases, the MEKs and MKKs that are specific for ERK, JNK, and p38, which are in turn activated by MAPK kinase kinases (MKKKs) via serine/threonine phosphorylation (19, 20). The MAPK cascades display evolutionary conservatism and are implicated to play essential roles in the regulation of cell growth, differentiation, and apoptosis.

To better understand the molecular mechanism controlling cell death or survival, we investigated the role of TGF-β1 in the apoptotic process by dominant-negative inhibition of both TGF-β1 and MAPK signaling pathways. In this study, we utilized serum withdrawal or deprivation to induce apoptosis by decreased availability of cell survival factors. We show that serum deprivation induces apoptosis in murine macrophages (RAW 264.7) and that TGF-β1 is able to prevent serum-deprived macrophages from undergoing apoptosis. This “rescue” is inhibited in cells transfected with a dominant-negative mutant of TβRII (TβRIIΔ), suggesting the critical role of TβRII in TGF-β1 signaling to prevent serum deprivation-induced apoptosis. Furthermore, we demonstrate that TGF-β1 rapidly induces ERK1/ERK2 MAPK activity. TGF-β1 fails to rescue RAW 264.7 cells from serum deprivation-induced apoptosis upon stable transfection with a dominant-negative mutant MAPK (ERK2) cDNA or in the presence of the MEK1 inhibitor. Together, our data suggest that TGF-β1 rescues macrophages from serum deprivation-induced apoptosis via TβRII-mediated signaling and downstream intracellular MAPK signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Recombinant human TGF-β1 and TGF-α were obtained from Life Technologies, Inc. The p44/42 MAPK, phosophespecific p44/42 MAPK (Tyr-204), and phosophespecific phos (Ser-383) rabbit polyclonal antibodies, purified Elk-1 fusion protein, ERK2, and phosphospecific SAPK/JNK (Thr-183/Tyr-185) and phosphospecific p38 (Thr-180/Tyr-182) rabbit polyclonal antibodies were purchased from New England Biolabs, Inc. (Beverly, MA). The MEK1 inhibitor, PD098059, was also obtained from New England Biolabs, Inc.

**Constructs—**A truncated TβRII construct (TβRIIΔ), lacking the transmembrane spanning and extracellular domains, was generated by polymerase chain reaction (PCR) using a rat TβRII cDNA as the template, as described previously (8). Primer sequences were as follows: sense primer 5′-GTTTAAAGTCTAGCAGGGTGGTGCACTG-3′; antisense primer 5′-GGCCGGTCCAGTACAGCGTGTCAGTAAGG-3′. These contained the sequences for the restriction enzymes NheI and SstI, respectively, for directional cloning, and a stop codon in the antisense primer. The PCR-amplified product was cloned into the pMAmneo (CLONTECH), a glucocorticoid-inducible mammalian expression vector, containing a neomycin-resistant gene. Correct directionality and in-frame sequences of the PCR product ligated in pMAMneo were verified by restriction mapping with EcoRI, BamHI, and HindIII and by sequencing the diodeoxy chain termination technique using Sequenase 2.0 (United States Biochemical Corp.). The MAPK-WT (wild type ERK2) and the MAPK-TA (dominant-negative mutant of ERK2) constructs used in this study were provided by Dr. Andrew Larner (21).

**Culture and Transfection—**The murine peritoneal macrophage cell line, RAW 264.7, was obtained from ATCC (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) supplemented with 10% FBS (HyClone) and gentamicin (50 μg/ml) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. To generate clones that stably expressed TβRIIΔ, RAW 264.7 cells were transfected using Lipofectin (Life Technologies, Inc.) as follows. Cells, that were grown to approximately 50% confluency on 100-mm dishes (Falcon) were incubated with 10 μg of DNA (TβRIIΔ) and 50 μl of Lipofectin suspension in DMEM at 37 °C in 5% CO2. After a 5-h incubation, medium containing 20% FBS in DMEM was added to make a final concentration of 10% FBS and incubated further for 48 h. Then the DNA/Lipofectin-containing medium was changed to 10% FBS in DMEM (no antibiotics) and incubated for another 24 h. To select for transfectants, cells were treated up to 800 μg/ml G418 (Life Technologies, Inc.) in DMEM containing 10% FBS, and the medium was changed every 2–3 days. G418-resistant colonies emerged at approximately 10 days after transfection and were subcloned using ring cylinders, expanded, and maintained in DMEM containing 10% FBS, Geneticin (200 μg/ml), and gentamicin (50 μg/ml). Two independent, stably transfected clones expressing the TβRIIΔ, named 10-2 and 10-3, were expanded. Confirmation was made by mRNA extraction and reverse transcription-polymerase chain reaction using primer pairs that contain species-specific sequences that recognize only the transfected TβRIIΔ construct and not the endogenous wild-type TβRII.

To generate clones that stably expressed the MAPK-WT or the MAPK-TA, the corresponding constructs were co-transfected with pcDNAs (Invitrogen), a mammalian expression vector containing a neomycin-resistant gene, using Lipofectin, as described above. The stable transfectants were also selected in medium containing 800 μg/ml G418 and then subcloned and maintained in 200 μg/ml G418. Confirmation of mRNA expression was obtained by reverse transcription-PCR.

**Induction of Apoptosis/Genomic DNA Isolation and Analysis—**To induce apoptosis, cells grown on 100-mm dishes (Falcon) were incubated in DMEM containing 10% FBS and 95% O2 at 37 °C. Control cells were maintained in 5% CO2 and 95% O2 at 37 °C. Each of the experiments was repeated at least three times.

**Genomic DNA isolation was performed using the Puregene kit (Gen-**

**Cell Survival Assay—**Determination of cell viability was done by trypan blue exclusion assay. Cells grown on 12-well plates to 90% confluency were induced to undergo apoptosis as described above, and at the indicated time periods, cells in each of the wells were collected, centrifuged, and resuspended in 0.5 ml of DMEM. Then aliquots of 0.1 ml were incubated with trypan blue dye (Life Technologies, Inc.) for 5 min followed by cell counting by hemocytometer. Both live (unstained) and dead (blue) cells were counted from the same randomly selected fields. The results were expressed as a percentage that did not take up the trypan blue dye in the total cell population. The experiments were performed in triplicate and repeated two times.

**Western Blot Analysis—**Total cellular extracts were obtained for the Western analyses by lysis of cells in buffer containing 1% Nonidet P-40, 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml aprotinin. Protein concentra-
increased cell survival with TGF-β serum deprivation for 24 h (6).

Molecular weight markers: 100 bp, lane 1; 1-kb ladder, lane 2.

FIG. 1. Serum deprivation-induced apoptosis in RAW 264.7 cells. Genomic DNA was isolated from RAW 264.7 cells incubated in the presence of 10% FBS (lane 3) or in 0.5% FBS (lane 4) for 24 h and fractionated on 1.5% agarose gel electrophoresis as described under “Experimental Procedures.” Molecular weight markers: 100 bp, lane 1; 1-kb ladder, lane 2.

RESULTS

Serum Deprivation Induces Apoptosis in RAW 264.7 Cells—We first determined whether RAW 264.7 cells underwent apoptosis following withdrawal of serum. Genomic DNA isolated from RAW 264.7 cells were assessed for the presence of DNA fragmentation by a “ladder” pattern on agarose gel electrophoresis, indicative of internucleosomal cleavage, a hallmark of apoptosis. The induction of genomic DNA fragmentation was observed in RAW 264.7 cells after 24 h of serum deprivation (Fig. 1).

TGF-β1 Rescues Serum-deprived RAW 264.7 Cells from Apoptosis—Given that previous studies have implicated the role of TGF-β1 as a modulator of apoptosis, we examined the effects of TGF-β1 on serum deprivation-induced apoptosis in RAW 264.7 cells. As shown in Fig. 2A, genomic DNA fragmentation was not observed in RAW 264.7 cells upon serum deprivation in the presence of exogenous TGF-β1 (lanes 3–5, 1, 10, and 100 ng/ml, respectively). This inhibition of DNA fragmentation by TGF-β1 was associated with increased cell survival, as shown in Fig. 3.

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TGF-β1, an analog of epidermal growth factor, chemically distinct from TGF-β1 and acting through a tyrosine kinase receptor system, failed to prevent DNA fragmentation in serum-deprived RAW 264.7 cells (data not shown). Furthermore, TGF-β1 did not rescue the apoptotic process elicited by other stimuli such as oxidative stress (Fig. 2B), indicating specificity of TGF-β1-mediated rescue from serum deprivation-induced apoptosis in RAW 264.7 cells.

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FIG. 2. Effect of TGF-β1 on serum deprivation-induced apoptosis in RAW 264.7 cells. A, genomic DNA was isolated from RAW 264.7 cells incubated for 24 h in the presence of 10% FBS (lane 1) or in 0.5% FBS (lane 1) or in 0.5% FBS with increasing doses of exogenous TGF-β1 (0, 1, 10, and 100 ng/ml, lanes 2–5) and fractionated on 1.5% agarose gel electrophoresis as described under “Experimental Procedures.” Molecular weight markers (MW), 1 kb; 100-bp ladder. B, genomic DNA was isolated from RAW 264.7 cells exposed to normoxia (lane 1) or to hyperoxia (95% oxygen) for 24 h in the presence of increasing doses of TGF-β1 (0, 1, 10, and 100 ng/ml, lanes 2–5) and fractionated on 1.5% agarose gel electrophoresis as described under “Experimental Procedures.” Molecular weight marker (MW), 1-kb ladder.
TGF-β Inhibits Apoptosis via ERK Pathway

FIG. 3. Time course of TGF-β<sub>1</sub> effect on cell survival in serum-deprived RAW 264.7 cells. Cells incubated in the presence of 10% FBS or in 0.5% FBS with and without exogenous TGF-β<sub>1</sub> (10 ng/ml) for the various time periods, as indicated, were assessed for cell viability by trypan blue exclusion assay as described under “Experimental Procedures.” The results were expressed as percentages of surviving cells that did not take up the trypan blue dye in the total cell population. Each data point is the mean of triplicate determinations ± S.E. *, there was a significant difference in survival of cells treated with TGF-β<sub>1</sub> compared with the respective control in 0.5% FBS without TGF-β<sub>1</sub> for each time period (p < 0.01, Student’s t test, n = 3). **, cell survival was significantly decreased upon serum deprivation in the absence of exogenous TGF-β<sub>1</sub> treatment (p < 0.05, analysis of variance).

FIG. 4. Inhibition of TGF-β<sub>1</sub> effect on serum deprivation-induced apoptosis by dominant-negative mutant TGF-β type II receptor (TβRII<sub>M</sub>). Genomic DNA was isolated from RAW 264.7 cells stably transfected with dominant-negative TβRII<sub>M</sub> and fractionated on 1.5% agarose gel electrophoresis as described under “Experimental Procedures.” Mutant 10-2 and 10-3 represent two independent clones of cells stably transfected with TβRII<sub>M</sub>. Lane 1, 10% FBS; lane 2, 10% FBS and 1 μM dexamethasone; lane 3, 0.5% FBS; lane 4, 0.5% FBS and 10 ng/ml TGF-β<sub>1</sub>; lane 5, 0.5% FBS, 10 ng/ml TGF-β<sub>1</sub> and 1 μM dexamethasone.

FIG. 5. Effect of TGF-β<sub>1</sub> on MAPK (ERK1/ERK2) activity in RAW 264.7 cells. A, lysates from RAW 264.7 cells incubated in the absence (Ctl) or in the presence of exogenous TGF-β<sub>1</sub> (10 ng/ml) for the indicated times were subjected to Western analysis using phosphospecific p44/42 MAPK (top panel) or p44/42 MAPK (bottom panel) antibodies as described under “Experimental Procedures.” Two bands are detected corresponding to phosphorylated forms of ERK1/ERK2 proteins (top panel) and two bands corresponding to total ERK1/ERK2 proteins (bottom panel); B, lysates from RAW 264.7 cells incubated in the absence (Ctl) or in the presence of exogenous TGF-β<sub>1</sub> (10 ng/ml) for the indicated times were subjected to Western analysis using phosphospecific SAPK/JNK (top panel) or phosphospecific p38 (bottom panel) antibodies as described under “Experimental Procedures.” C, immunocomplex kinase assay. Lysates from RAW 264.7 cells incubated in the absence (Ctl) or in the presence of exogenous TGF-β<sub>1</sub> (10 ng/ml) for the indicated times were analyzed for MAPK activity as described under “Experimental Procedures.” ERK activity was assayed by immunoprecipitation with phosphospecific antibody to MAPK (Tyr-204) followed by detection of phosphorylation of Elk-1 fusion protein at Ser-383 by Western blotting using a phosphospecific Elk-1 (Ser-383) antibody. For a positive control (+Ctl), 20 ng of active MAPK (ERK2) was incubated with a control extract.

stably transfected cells overexpressing a dominant-negative mutant of TβR-II (TβR-II<sub>M</sub>). Given that TGF-β<sub>1</sub> signal transduction requires heterodimerization of TβR-II and TβR-I and transphosphorylation of TβR-I by TβR-II, the truncated receptor TβR-II<sub>M</sub>, which is membrane-anchored but lacks the cytoplasmic serine/threonine kinase domain, competes for binding to TβR-I, hence acting in a dominant-negative fashion to inhibit TGF-β<sub>1</sub> signaling (8, 29). As predicted, complete inhibition of TGF-β<sub>1</sub> rescue from serum deprivation-induced apoptosis was observed in cells from two independent clones (10-2 and 10-3) expressing the truncated receptors, TβR-II<sub>M</sub> (Fig. 4). This occurred both with and without dexamethasone pretreatment. Although the TβR-II<sub>M</sub> construct was under a glucocorticoid-regulated promoter, “leakage” of promoter activity occurs during uninduced conditions and has been previously observed by us and other investigators (8, 24).

TGF-β<sub>1</sub> Activates MAPK (ERK1 and ERK2) in RAW 264.7 Cells—Previous studies have suggested that TGF-β<sub>1</sub> exerts its biological effects via the MAPK signaling pathway in several cell culture systems (11–14). We first determined the levels of ERK1 and ERK2 protein expression in RAW 264.7 cells treated with exogenous TGF-β<sub>1</sub> (10 ng/ml) by Western analyses, using phosphospecific p44/42 MAPK and p44/42 MAPK antibodies. The phosphospecific p44/42 MAPK antibodies detect specifically the phosphorylated forms of ERK1/ERK2, whereas the p44/42 MAPK antibodies detect total (phosphorylation-state independent) ERK1/ERK2 proteins. As shown in Fig. 5A, increases in phosphorylation of ERK1 and ERK2 proteins were observed in cells, as early as 15 min after stimulation with exogenous TGF-β<sub>1</sub>. There were no appreciable increases in the activation of JNK or p38 within the same time periods of TGF-β<sub>1</sub> treatment (Fig. 5B).

We next examined whether this induction of ERK1 and ERK2 by TGF-β<sub>1</sub> was associated with an increase in MAPK activity using an immunocomplex kinase assay. Lysates from RAW 264.7 cells incubated in the presence or absence or exogenous TGF-β<sub>1</sub> (10 ng/ml) were subjected to immunoprecipitation using phosphospecific p44/42 MAPK antibodies. The resulting active ERK1/ERK2 immunoprecipitate was then allowed to phosphorylate Elk-1 fusion protein, and ERK activity was assayed by the detection of phosphorylated Elk-1 by
Western blot analysis. Exogenous TGF-β1 (10 ng/ml) induced the increase of the phosphorylated form of Elk-1 (Fig. 5C). Although there was some endogenous activity in the control untreated cells, TGF-β1 induced ERK activity within 15 min of TGF-β1 treatment, with marked ERK activity up to 4 h of TGF-β1 treatment.

Inhibition of Serum Deprivation-induced Apoptosis by TGF-β1—Involves the MAPK Pathway—Given that TGF-β1 activates the MAPK (ERK) pathway in RAW 264.7 cells, we next examined whether the MAPK signaling pathway mediates the TGF-β1 rescue of RAW 264.7 cells from serum deprivation-induced apoptosis. Our first strategy was to inhibit the MAPK pathway by genetic blockade utilizing a dominant-negative mutant MAPK (MAPK-TA). Fig. 6A demonstrates that TGF-β1 rescues serum deprivation-induced apoptosis in cells transfected with MAPK-WT, as was previously observed in wild-type RAW 264.7 cells expressing the TβR-II (TβR-II) containing fibroblasts and endothelial cells, as a result of decreased availability of cell survival factors (8, 25). We observed that macrophages also undergo apoptosis upon serum withdrawal or deprivation, as determined by detection of the characteristic genomic DNA laddering (Fig. 1). Remarkably, the presence of exogenous TGF-β1 prevented the macrophages from undergoing apoptosis upon serum withdrawal (Fig. 2A). This inhibition of DNA laddering by TGF-β1 was also associated with increased cell survival (Fig. 3). The anti-apoptotic activity of TGF-β1 was further confirmed by its inability to rescue cells from serum deprivation-induced apoptosis when its signaling receptors are blocked by a kinase-deleted dominant-negative mutant of TβR-II (TβR-IIm). Since signal transduction requires heterodimerization of TβR-II and TβR-I, the mutant receptor competes for binding to wild-type TβR-I, hence acting in a dominant-negative fashion (3, 8, 10). In stably transfected RAW 264.7 cells expressing the TβR-I, apoptosis occurred with serum deprivation, both in the presence or absence of exogenous TGF-β1 (Fig. 4), indicating that the anti-apoptotic effect by TGF-β1 is mediated by TβR-II kinase.

Although TGF-β1 has been shown in a number of systems to be a potent inducer of apoptosis, anti-apoptotic actions of TGF-β1 are less well known. Sachsenmeier et al. reported “protective” effects of TGF-β1 by inhibiting suspension-induced apoptosis in human keratinocytes following loss of adhesion (9). Treatment of keratinocytes with TGF-β1 attenuated suspension-induced DNA fragmentation. Moreover, inhibition of endogenous TGF-β1 by neutralizing antibody to TGF-β1 increased DNA fragmentation following suspension. Thus, TGF-β1 clearly possesses the ability to exert both pro-apoptotic and homeostasis of multicellular organisms and is carefully regulated by diverse signals that influence the decision of a cell between life and death. These signals may act to either promote or inhibit apoptosis, and the same signal may potentially have opposing effects on different cell types. One such signaling molecule that may possess both pro-apoptotic and anti-apoptotic activities is the multifunctional cytokine TGF-β1, both a potent stimulator and an inhibitor of cell proliferation. In the present study, we examined the role of TGF-β1 in modulating apoptosis in cultured macrophages (RAW 264.7 cells). In order to induce apoptosis, serum was withdrawn. Serum deprivation has been shown to provoke apoptosis in a variety of cells including fibroblasts and endothelial cells, as a result of decreased availability of cell survival factors (8, 25). We observed that macrophages also undergo apoptosis upon serum withdrawal or deprivation, as determined by detection of the characteristic genomic DNA laddering (Fig. 1). Remarkably, the presence of exogenous TGF-β1 prevented the macrophages from undergoing apoptosis upon serum withdrawal (Fig. 2A). This inhibition of DNA laddering by TGF-β1 was also associated with increased cell survival (Fig. 3). The anti-apoptotic activity of TGF-β1 was further confirmed by its inability to rescue cells from serum deprivation-induced apoptosis when its signaling receptors are blocked by a kinase-deleted dominant-negative mutant of TβR-II (TβR-IIm). Since signal transduction requires heterodimerization of TβR-II and TβR-I, the mutant receptor competes for binding to wild-type TβR-I, hence acting in a dominant-negative fashion (3, 8, 10). In stably transfected RAW 264.7 cells expressing the TβR-I, apoptosis occurred with serum deprivation, both in the presence or absence of exogenous TGF-β1 (Fig. 4), indicating that the anti-apoptotic effect by TGF-β1 is mediated by TβR-II kinase.

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anti-apoptotic effects in different cell systems, and the differential cellular responses likely are necessary for proper homeostasis of multicellular organisms. With the findings that TGF-β1 can promote cell survival in certain cell types, we were interested in exploring the potential downstream intracellular pathways responsible for these protective effects of TGF-β1 in macrophages.

Evidences that TGF-β1 is capable of activating MAPK-dependent pathways in mammalian cells have been reported. For instance, rapid activation of ERK1 by TGF-β1 has been demonstrated in intestinal epithelial cells and is associated with growth inhibitory effects of TGF-β1 (12). In other cell types, including HepG2, CHO, and MDCK cell lines, TGF-β1 has been shown to activate JNK/SAPK, and dominant-negative forms of various components of the JNK/SAPK pathway abolished TGF-β signaling (13). We examined whether TGF-β1 is capable of activating MAPK in macrophages and whether TGF-β1 signals rescue from serum deprivation-induced apoptosis via the MAPK-dependent pathway. ERK activity was assayed by two methods. First, increased phosphorylation of ERK1/ERK2 was determined by Western analyses using phosphospecific p44/42 MAPK antibodies that detect only the tyrosine 204-phosphorylated form of ERK1/ERK2. Next, ERK activity was determined by in vitro kinase assay. Following immunoprecipitation with p44/42 MAPK antibodies to select for the activated (phosphorylated) MAPK, detection of in vitro phosphorylation of a known substrate, Elk-1, was determined using phosphospecific antibodies that detect only the serine 383-phosphorylated Elk-1. Our results show that ERK1/ERK2 was activated within 15 min of stimulation with exogenous TGF-β1 in cultured RAW 264.7 cells, and this activation was sustained up to 4 h (Fig. 5C). Accordingly, the sharp increase in phosphorylation of Elk-1 is observed parallel with increased phosphorylated forms of ERK1/ERK2 (Fig. 5A). In contrast, we observed that TGF-β1 failed to activate JNK/SAPK or p38 within this same time period, indicating that TGF-β1 is capable of rapidly activating only the ERK pathway, but not the JNK/SAPK or p38 pathways, in RAW 264.7 macrophages (Fig. 5B).

The ERK pathway is the prototypical MAPK pathway induced by epidermal growth factor stimulation and implicated in the regulation processes of cellular proliferation and differentiation (18, 26). Evidence for its potential importance in the modulation of apoptosis has been provided by studies in cardiac myocytes. Cardiostrophin 1 (CT-1), a member of the interleukin 6 family of cytokines, is a potent cardiac survival factor capable of inhibiting apoptosis in cardiac myocytes via the activation of an anti-apoptotic signaling pathway that requires MEKs (MAPK/ERK kinases) (27). We determined whether the ERK pathway was involved in the apoptotic rescue of macrophages by TGF-β1, using two independent approaches to block the ERK signaling pathway. Our transfection studies with the dominant-negative mutant MAPK (ERK2) in RAW 264.7 cells resulted in the blockade of TGF-β1 anti-apoptotic effects (Fig. 6B). To further support these findings, we utilized an MEK1-specific inhibitor, PD98059, which blocks MEK1 activation by Raf, thus preventing downstream activation of ERK1/ERK2, but does not inhibit JNK/SAPK or p38 protein kinase activation. In addition, the PD98059 has been shown to have little effect on other kinases, including cAMP-dependent kinase, protein kinase C, and other serine and threonine kinases (28–30).

In our studies, PD98059 effectively prevented the anti-apoptotic effects of TGF-β1 in serum-deprived macrophages and provides further evidence for the requirement of the ERK pathway in the survival function of TGF-β1 (Fig. 7B).

Interestingly, studies supporting our current findings that MAPK-dependent pathways are responsible for promoting the survival effects of TGF-β1 have been documented for other cytokines in neuronal cells and cardiac myocytes. Nerve growth factor promotes the survival of neuronal (PC-12) cells via activation of the ERK pathway to mediate and initiate rescue from apoptosis induced by serum deprivation, and JNK/SAPK activation along with inhibition of MAPK (ERK) are required for modulating and inducing apoptosis (31). The MAPK pathways have also been found to be necessary for CT-1 effects on promoting survival of serum-deprived cardiac myocytes and blocking MAPK activation by transfection of a dominant-negative mutant MEK or by treatment with PD98059 inhibited the survival effect of CT-1 (27). Furthermore, in the present report, we observed, even in the presence of serum, induction of apoptosis in RAW 264.7 cells upon blockade of the MAPK signaling pathways either by a dominant-negative mutant of MAPK (ERK) (Fig. 6B) or by MEK1 inhibitor, PD98059 (Fig. 7A). Thus, based on our studies and those previous studies, it is plausible that the MAPKs (ERK) represent a common pathway targeted by anti-apoptotic cytokines to promote cell survival.

It will be of great interest to determine whether similar cytokine-induced MAPK-dependent signaling pathways operate in vivo to promote cell survival, and the present findings may potentially have important clinical implications. Apoptosis in vivo is followed almost inevitably by rapid uptake into adjacent phagocytic cells and represents a critical process in tissue remodeling, regulation of immune response, or resolution of inflammatory reactions (32). The importance of TGF-β1 in the regulation of inflammation is well demonstrated by the observations that TGF-β knock-out mice have severe and generalized inflammatory disorders (33, 34). We have identified TGF-β1 as an inhibitor of apoptosis in cultured macrophages and may serve as a cell survival factor via the MAPK-dependent pathway. This would provide macrophages with a cellular defense mechanism to be selectively spared from toxicity and cell death, a process that would be critical in the resolution of inflammation. Survival of macrophages is required to perform their duties of phagocytosis and elimination of adjacent harmful or injured cells, including lymphocytes, that have undergone apoptosis, and disorders that compromise macrophage survival could contribute to chronic inflammatory diseases.

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**TGF-β, Inhibits Apoptosis via ERK Pathway**