

Interleukin-6 Inhibits Transforming Growth Factor- β -induced Apoptosis through the Phosphatidylinositol 3-Kinase/Akt and Signal Transducers and Activators of Transcription 3 Pathways*

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The multifunctional cytokine interleukin-6 (IL-6) regulates growth and differentiation of many cell types and induces production of acute-phase proteins in hepatocytes. Here we report that IL-6 protects hepatoma cells from apoptosis induced by transforming growth factor- β (TGF- β), a well known apoptotic inducer in liver cells. Addition of IL-6 blocked TGF- β -induced activation of caspase-3 while showing no effect on the induction of plasminogen activator inhibitor-1 and p15^{INK4B} genes, indicating that IL-6 interferes with only a subset of TGF- β activities. To further elucidate the mechanism of this anti-apoptotic effect of IL-6, we investigated which signaling pathway transduced by IL-6 is responsible for this effect. IL-6 stimulation of hepatoma cells induced a rapid tyrosine phosphorylation of the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) and its kinase activity followed by the activation of Akt. Inhibition of PI 3-kinase by wortmannin or LY294002 abolished the protection of IL-6 against TGF- β -induced apoptosis. A dominant-negative Akt also abrogated this anti-apoptotic effect. Dominant-negative inhibition of STAT3, however, only weakly attenuated the IL-6-induced protection. Finally, inhibition of both STAT3 and PI 3-kinase by treating cells overexpressing the dominant-negative STAT3 with LY294002 completely blocked IL-6-induced survival signal. Thus, concomitant activation of the PI 3-kinase/Akt and the STAT3 pathways mediates the anti-apoptotic effect of IL-6 against TGF- β , with the former likely playing a major role in this anti-apoptosis.

A balance between cell proliferation and apoptosis is critical for tissue homeostasis. Maintenance of the size of liver is a notable example of homeostasis which is heavily regulated by many growth factors and cytokines (1). Among these factors, transforming growth factor- β (TGF- β)¹ is a potent inducer of apoptosis in hepatocytes and several hepatoma cell lines, as

well as in regressing liver *in vivo* (2–6). TGF- β also inhibits liver cell proliferation *in vitro* (7) and plays a crucial role in terminating liver regeneration after partial hepatectomy (1). TGF- β exerts its biological effects through the action of two types of transmembrane serine/threonine kinase receptors. These receptors subsequently propagate the signal by phosphorylating the intracellular targets, Smads. Phosphorylated Smad2 or Smad3 can form a stable complex with Smad4, which then translocates to the nucleus to regulate transcriptional responses to TGF- β (8–10). Although the signal transduction pathway of TGF- β has been well studied, mechanism of its apoptotic effect is still not fully characterized. Nevertheless, induction of oxidative stress (6), activation of caspase-3 (3, 11), and inhibition of pRb expression (2) have been implicated in mediating TGF- β -induced apoptosis.

TGF- β -induced apoptosis in liver cells is blocked by growth factors such as insulin and insulin-like growth factor-1 as well as by elevated expression of insulin receptor substrate-1 (12). Our recent studies have revealed that the phosphatidylinositol 3-kinase (PI 3-kinase) and its downstream target, Akt, are responsible for the anti-apoptotic activity of insulin against TGF- β (13). PI 3-kinase was reported to suppress apoptotic cell death induced by a variety of stimuli (14–19). PI 3-kinase elicits this anti-apoptotic activity through the action of the serine/threonine kinase, Akt. Recent studies have demonstrated that activated Akt can phosphorylate the proapoptotic protein BAD (20, 21). This phosphorylation allows for BAD association with 14-3-3 and dissociation from BCL-X_L, which is then free to resume its function as a suppressor of apoptosis (22).

Interleukin-6 (IL-6) is a multifunctional cytokine acting in the immune system, hepatocytes, and neuronal cells (23, 24). In the liver, IL-6 induces synthesis of acute-phase proteins and plays central roles in preventing acute hepatitis and initiating liver regeneration (25, 26). Mice with targeted disruption of the IL-6 gene have impaired liver regeneration characterized by liver necrosis and failure (26). The signaling mechanism of IL-6 in hepatocytes is, however, not fully understood. In hematopoietic cells, binding IL-6 to the α subunit of its receptor triggers the recruitment of gp130, subsequently leading to the activation of the gp130-associated Janus kinases (JAKs) (27–29). JAKs phosphorylate gp130 on several tyrosine residues and these phosphotyrosines recruit various SH2 domain-containing proteins, such as STAT3 and SHP-2 (30–32). SHP-2 links cytokine receptor to the Ras/MAP kinase pathway and is essential for mitogenic activity, whereas STAT3 can induce BCL-2 and is involved in anti-apoptosis (33). In addition to JAK/STAT and Ras/MAP kinase pathways, IL-6 was recently shown to

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¹ The abbreviations used are: TGF- β , transforming growth factor- β ; PI 3-kinase, phosphatidylinositol 3-kinase; IL-6, interleukin-6; JAK, Janus kinase; STAT, signal transducer and activator of transcription; PAI-1, plasminogen activator inhibitor-1; SH2, Src homology domain 2;

MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

activate PI 3-kinase in prostate cancer cells (34). Whether IL-6 in liver cells activates these pathways remains to be investigated.

In the liver, IL-6 acts as a hepatoprotecting and/or mitogenic factor, whereas TGF- β elicits an apoptotic and/or growth-arresting effect. In this study, we elucidate a cross-talk between signaling pathways induced by these two factors in liver cells. Our results indicate that IL-6 suppressed TGF- β -induced apoptotic death of hepatoma cells in a dose-dependent manner. IL-6 inhibited TGF- β -induced activation of caspase-3 but did not affect its induction of an extracellular matrix protein and a cell cycle inhibitor, suggesting that IL-6 signaling blocks only the apoptotic signaling of TGF- β . Furthermore, we demonstrate that the concomitant activation of the PI 3-kinase/Akt and the STAT3 signaling pathways mediates the anti-apoptotic effect of IL-6 against TGF- β .

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human hepatoma cell line Hep3B was cultured as described previously (11). Hep3B cells stably expressing the dominant-negative mutant of Akt were reported previously (13). Transfection was performed using LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. For transient transfection, cells were harvested at 48 h after transfection. For selecting of stable clones, G418 (700 μ g/ml) was added into culture medium at 48 h after transfection. The G418-resistant clones were individually picked, expanded, and assayed for expression of the transfected cDNAs by Western blotting.

Antibodies and Reagents—Mammalian expression plasmids for the two dominant-negative mutants of STAT3, STAT3D and STAT3F (35), were kindly provided by Dr. T. Hirano. Antibodies for p85 subunit of the PI 3-kinase, Akt, and phosphotyrosine were purchased from Santa Cruz Biotechnology. The anti-STAT3 antibody was from UBI. Wortmannin and LY294002, the two PI 3-kinase inhibitors, were from Sigma.

Apoptosis Assays—Apoptosis was quantitated with TUNEL and Cell-Death Detection ELISA assays. For TUNEL assay (36), cells seeded on chamber slides were serum starved for 24 h and then treated with or without various cytokines and/or agents for 18 h. Cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate. TUNEL assays were performed using the *In Situ* Death Detection Kit, Fluorescein (Roche Molecular Biochemicals) according to the manufacturer's instructions. Apoptotic cells were visualized with fluorescence microscopy. The Cell-Death Detection ELISA assay (Roche Molecular Biochemicals) measures the presence of soluble histone-DNA complex as a result of DNA fragmentation (37). For this assay, cells were seeded on 96-well plates at a density of 0.5×10^4 cells/well and were serum-starved and treated with various agents as for TUNEL assays. Cell-Death ELISA assays were performed according to the manufacturer's instructions.

Caspase-3 Activity Assays—Cells were seeded onto 6-well plates, serum starved for 24 h, and treated with or without TGF- β (5 ng/ml) and/or IL-6 (60 ng/ml) for various times. Cells were lysed in RIPA buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin and cell lysates were centrifuged at 14,000 rpm. Cell lysate containing 100 μ g of proteins in a 1-ml assay buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mM EGTA was incubated with a fluorogenic peptide substrate, Ac-DEVD-AMC (5.6 μ M) at 37 °C for 30 min. Fluorescence was quantified with a spectrofluorometer (excitation 380 nm, emission 460 nm).

Luciferase Assays—Reporter plasmids p15P113-luc (38) and p800luc (39), kindly provided by Drs. X.-F. Wang and R. Derynck, contain the luciferase expression unit driven by the TGF- β -responsive elements in p15^{INK4B} and plasminogen activator inhibitor-1 (PAI-1) promoters, respectively. These plasmids, together with a plasmid for constitutive expression of β -galactosidase, were transfected to Hep3B. One day after transfection, the cells were serum-starved for 10 h and then treated with TGF- β (5 ng/ml) and/or IL-6 (60 ng/ml) for 12 h. Luciferase and β -galactosidase activities were quantitated by the Luciferase Assay System and the β -galactosidase Enzyme Assay System (Promega), respectively.

PI 3-Kinase Assay—PI 3-kinase activities were assayed as described previously (40). Briefly, 10^7 cells were washed twice with ice-cold phosphate-buffered saline and lysed with 1 ml of lysis buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% Nonidet P-40, 10% glycerol,

1 mg/ml bovine serum albumin, 20 mM Tris, pH 8.0, 2 mM orthovanadate). Cell extracts were incubated with 1 μ g of anti-phosphotyrosine antibody overnight at 4 °C. The immunocomplex was precipitated with 50 μ l of protein A-Sepharose for 1 h at 4 °C, washed three times with lysis buffer, twice with LiCl buffer (0.5 M LiCl, 100 mM Tris, pH 7.6) and twice with TNE buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA). The immunocomplex was preincubated with 10 μ l of 20 mM Hepes (pH 7.4), containing 2 mg/ml PI (Sigma) on ice for 10 min. Kinase reaction was performed by the addition of 40 μ l of reaction buffer (10 μ Ci of [γ -³²P] ATP, 20 mM Hepes, pH 7.4, 20 μ M ATP, 5 mM MgCl₂) at room temperature for 15 min. The reaction was stopped by the addition of 100 μ l of 1 M HCl and extracted with 200 μ l of a 1:1 mixture of chloroform and methanol. The radiolabeled lipids were separated by thin-layer chromatography and visualized using a PhosphorImager.

In Vitro Akt Kinase Assay—Akt kinase assays were performed essentially as described (41). Briefly, cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM sodium fluoride, 1 mM NaVO₄, 1 mM sodium pyrophosphate, 2 μ M aprotinin, 2 μ M leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The HA-tagged Akt was precipitated from cell lysates with anti-HA antibody followed by protein A-Sepharose conjugated with rabbit anti-mouse antibody. The immunocomplexes were washed once with Akt kinase buffer containing 20 mM Hepes (pH 7.4), 1 mM dithiothreitol, 10 mM MnCl₂, 10 mM MgCl₂ and then incubated in 30 μ l of kinase buffer containing 5 μ M ATP, 100 μ g of histone 2B, and 10 μ Ci of [γ -³²P]ATP at 30 °C for 30 min. The kinase reaction was terminated by addition of an equal volume of 2 times SDS-PAGE sample buffer. The samples were boiled for 5 min prior to electrophoretic separation by SDS-PAGE.

RESULTS

IL-6 Protects Hepatoma Cells from TGF- β -induced Apoptosis—Previous studies revealed that human hepatoma cell line Hep3B is highly sensitive to the apoptotic activity of TGF- β (4). To investigate whether IL-6 affects apoptosis induced by TGF- β , Hep3B cells were co-treated with 5 ng/ml TGF- β and IL-6 at various concentrations. Using an ELISA assay measuring the presence of soluble histone-DNA complex resulted from DNA fragmentation, we observed that IL-6 suppressed TGF- β -induced apoptosis in a dose-dependent manner (Fig. 1, A and B). The anti-apoptotic effect of IL-6 was readily detected at a dose as low as 0.1 ng/ml (Fig. 1A). At a concentration of 60 ng/ml, IL-6 elicited a maximal protection. Notably, in the absence of TGF- β , IL-6 did not promote nor suppress cell survival (Fig. 1B). These findings suggest the existence of a cross-talk between the signaling pathways transduced by TGF- β and IL-6.

IL-6 Does Not Affect the Ability of TGF- β to Induce PAI-1 and p15^{INK4B} Genes—To explore the mechanism by which IL-6 signaling prevents TGF- β -induced apoptosis, we investigated whether IL-6 could block all cellular responses to TGF- β or affect only a subset of the activities. The PAI-1 and p15^{INK4B} genes are highly induced by TGF- β and are frequently used as indicators for the effects of TGF- β on production of extracellular matrix and cell cycle arrest, respectively (42, 43). The reporter plasmid p800luc, containing the TGF- β responsive element in PAI-1 promoter (39), was used to evaluate the induction of PAI-1 gene. When Hep3B cells were transiently transfected with this reporter, TGF- β induced a \sim 3.5-fold increase in luciferase activity. This induction was not significantly affected by co-treatment with IL-6 at 60 ng/ml (Fig. 2A), i.e. a dosage that showed a maximal inhibition of TGF- β -induced apoptosis (Fig. 1B). Similar results were observed using the p15P113-luc reporter (38) containing the p15^{INK4B} promoter (Fig. 2B). Our data thus indicate that IL-6, although preventing apoptosis induced by TGF- β , did not affect the transcriptional up-regulation of PAI-1 and p15^{INK4B} genes in response to TGF- β . IL-6 signaling is likely to block a step specific to the apoptotic activity of TGF- β .

IL-6 Signaling Prevents TGF- β -induced Activation of Caspase-3—Our previous studies demonstrated that caspase-3

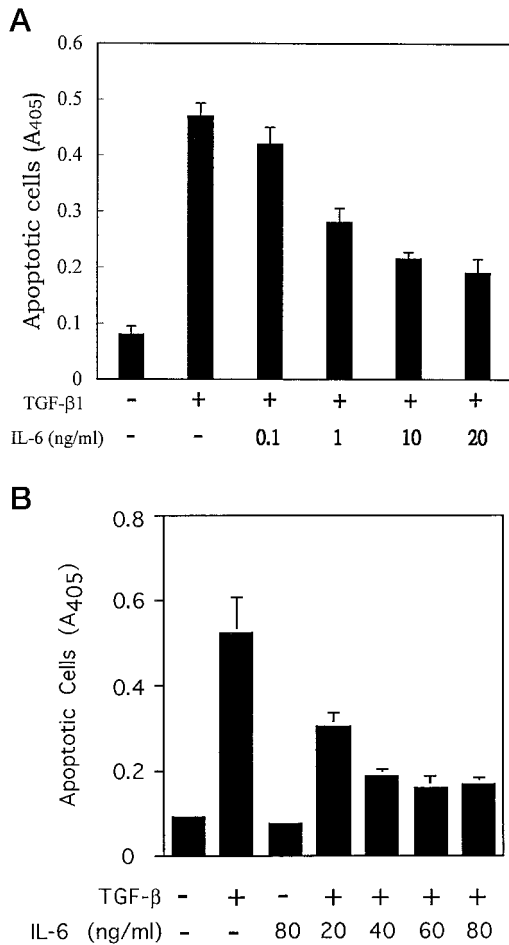


FIG. 1. IL-6 suppresses TGF- β -induced apoptosis in hepatoma cells. Hep3B cells were treated with TGF- β (5 ng/ml) and IL-6 at low (A) or high concentrations (B) as indicated. Apoptotic cells at 18 h after treatment were determined by the Cell-Death Detection ELISA. Data from three independent experiments are presented as mean \pm S.D.

is activated upon TGF- β treatment of Hep3B cells (13). To evaluate whether IL-6 could block this activity of TGF- β , cell extract derived from Hep3B treated with or without TGF- β and/or IL-6 was analyzed for caspase-3 activity by measuring cleavage of a fluorogenic substrate, Ac-DEVD-AMC. As shown in Fig. 3, TGF- β -induced caspase activity was abolished by co-treatment of IL-6. This finding suggests that IL-6 signaling prevents the apoptotic activity of TGF- β by inhibiting the activation of caspase-3.

PI 3-Kinase Is Involved in the Antiapoptotic Signaling of IL-6—Our previous studies indicated not only that the PI 3-kinase/Akt pathway mediates the anti-apoptotic signal of insulin against TGF- β but also that this signal leads to a blockage of caspase-3 activation in response to TGF- β (13). Therefore, we sought to investigate whether IL-6 induces PI 3-kinase in Hep3B cells and whether the anti-apoptotic activity of IL-6 is mediated through the function of PI 3-kinase. We observed that IL-6 treatment of Hep3B cells stimulated tyrosine phosphorylation of the regulatory subunit of PI 3-kinase, p85, as determined by immunoprecipitations with an anti-phosphotyrosine antibody followed by Western blotting using an anti-p85 antibody (Fig. 4A). Furthermore, IL-6 induced a substantial increase of PI 3-kinase activity in immunoprecipitates with the anti-phosphotyrosine antibody (Fig. 4B). Both tyrosine phosphorylation of the p85 and PI 3-kinase activity were readily evident within 5 min and declined at 60 min after exposure to IL-6, indicating a rapid activation of PI 3-kinase by

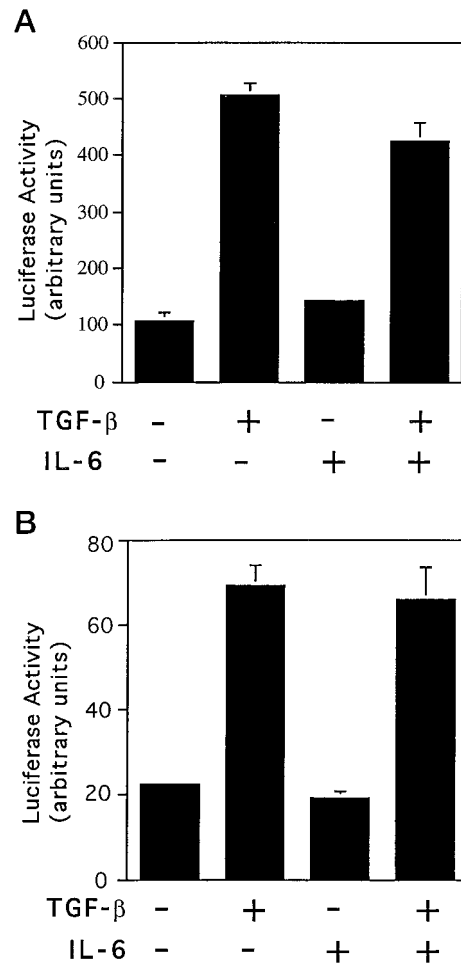


FIG. 2. IL-6 does not affect TGF- β -induced activation of PAI-1 and p15^{INK4B} promoters. Hep3B cells transiently transfected with the reporter plasmid were treated with or without TGF- β (5 ng/ml) and/or IL-6 (60 ng/ml) for 12 h. The reporter plasmid p800luc containing the PAI-1 promoter was used in A, whereas the p15P113-luc reporter was used in B. For each transfection, luciferase activity was normalized to transfection efficiency and cell survival by using β -galactosidase activity as an internal control. Values are mean \pm S.D. of three independent transfections.

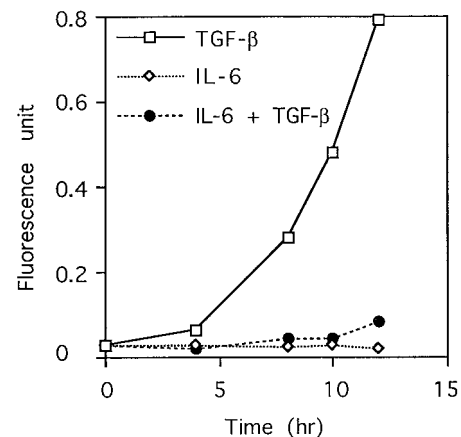


FIG. 3. IL-6 inhibits TGF- β -induced caspase-3-like activity. Caspase-3 activities in Hep3B cells treated with various agents were measured by the amount of fluorescence generated from the cleavage of Ac-DEVD-AMC.

IL-6. To determine the involvement of PI 3-kinase in the anti-apoptotic effect of IL-6, two specific inhibitors of PI 3-kinase, wortmannin and LY294002 (44, 45), were used. As shown in

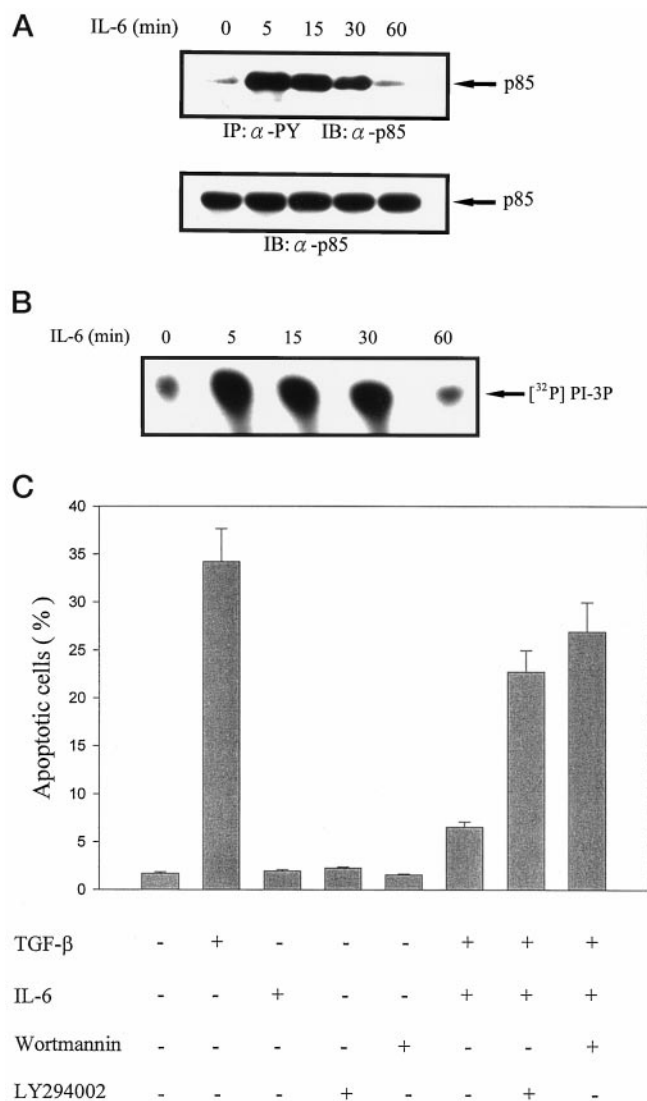


FIG. 4. PI 3-kinase is involved in the anti-apoptotic effect of IL-6 against TGF- β . *A*, induction of tyrosine phosphorylation of p85 subunit of the PI 3-kinase by IL-6. Hep3B cells were treated with IL-6 (60 ng/ml) for various time intervals as indicated. *Upper panel*, cell lysates with equal amount of proteins were immunoprecipitated with an anti-phosphotyrosine (PY) antibody. The immunoprecipitates were separated on SDS-PAGE and subjected to Western blot with antibody against p85. *Lower panel*, cell lysates with equal amounts of proteins were subjected to Western blot with the anti-p85 antibody to verify the same amount of p85 in all samples. *B*, activation of PI 3-kinase activity by IL-6. Cells were treated as indicated and lysates with equal amounts of protein were subjected to immunoprecipitations with anti-phosphotyrosine antibody. The immunocomplex was used for PI 3-kinase assays. *C*, wortmannin and LY294002, two specific inhibitors of PI 3-kinase, block the protection of IL-6 against TGF- β -induced apoptosis. Hep3B cells were treated with various agents as indicated. The concentrations of wortmannin and LY294002 used were 10 nM and 25 μ M, respectively. Apoptotic cells were stained with the TUNEL assay and identified under a fluorescent microscope. Data are presented as means of three experiments.

Fig. 4C, wortmannin markedly diminished the anti-apoptotic activity of IL-6 at a dose of 10 nM. A similar finding was observed with 25 μ M LY294002. However, these two inhibitors did not by themselves induce apoptosis in the absence of TGF- β . Taken together, our results demonstrated a critical role of PI 3-kinase in the anti-apoptotic signaling of IL-6 against TGF- β .

Akt Is Activated following IL-6 Stimulation and Is Involved in the Antiapoptotic Signaling of IL-6—Having demonstrated the involvement of PI 3-kinase in the anti-apoptotic signaling

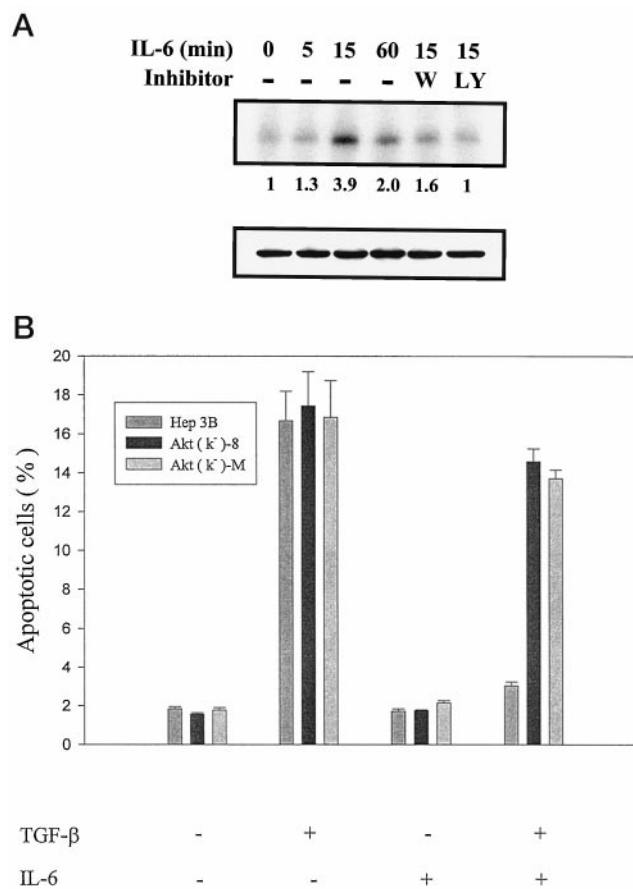


FIG. 5. Akt, a downstream effector of PI 3-kinase, mediates the survival signal transduced by IL-6. *A*, IL-6 stimulates Akt activity. *Upper panel*, Hep3B cells transiently transfected with an HA-tagged Akt were treated with 60 ng/ml IL-6 in the presence or absence of 10 nM wortmannin (W) or 25 μ M LY294002 (LY) for various time intervals as indicated. Cell lysates were subjected to immunoprecipitations with an anti-HA antibody followed by *in vitro* kinase assays using histone 2B as a substrate. *Lower panel*, the same immunoprecipitates were used for Western blotting with an anti-Akt antibody to verify equal amount of proteins were loaded. Akt kinase activity in each lane was quantitated and normalized to the equal amount of proteins and was shown under the *upper panel*. *B*, dominant-negative Akt inhibits the anti-apoptotic effect of IL-6. Parental Hep3B and stable transfectants expressing the dominant-negative Akt, Akt(K⁻), were treated with various agents as indicated and apoptosis was assayed as for Fig. 4C.

of IL-6, we next investigated whether the serine/threonine kinase Akt, a downstream target of PI 3-kinase, is a critical component of this anti-apoptotic signaling pathway. Attempts to evaluate the activity of endogenous Akt were hindered by the lack of an antibody that could efficiently immunoprecipitate Akt from the lysates of Hep3B cells. Therefore, an HA-tagged Akt (19) was transiently transfected into Hep3B cells. The transfected cells were treated with IL-6 for various time intervals and cell lysates were used for immunoprecipitations with an anti-HA antibody followed by *in vitro* kinase assays. As shown in Fig. 5A, IL-6 substantially increased Akt activity, and the kinetics of Akt activation resembled that of the IL-6-induced tyrosine phosphorylation of p85 (Fig. 4A). Furthermore, both LY294002 and wortmannin efficiently suppressed the IL-6-induced Akt activity, indicating that the activation of Akt by IL-6 is mediated through PI 3-kinase. To assess the role of Akt in the anti-apoptotic activity of IL-6 against TGF- β , we evaluated whether a dominant-negative mutant of Akt can block IL-6-induced protection. Our previous study generated Hep3B cells stably expressing a kinase-defective mutant of Akt (Akt 179A) (13), which can inhibit wild type Akt in a dominant-

negative fashion (19). Akt(K⁻)-8, a stable transfectant of Hep3B expressing a high level of such mutated Akt, exhibited a markedly reduced protection of IL-6 against TGF- β (Fig. 5B). A similar but less prominent reduction of IL-6-induced anti-apoptosis was observed using a mixture of eight stable clones, Akt(K⁻)-M (13), expressing a lower level of the mutated Akt (Fig. 5B). Taken together, our findings indicate that the PI 3-kinase/Akt pathway is activated by IL-6 and mediates the anti-apoptotic effect of IL-6.

STAT3 Elicits a Weak Anti-apoptotic Effect against TGF- β —In hematopoietic cells, STAT3 mediates the survival signal of cytokines through its ability to induce BCL-2 (33). To investigate whether STAT3 is involved in suppressing TGF- β -induced apoptosis by IL-6, a STAT3 dominant-negative mutant (STAT3D) carrying EE to AA substitutions at positions 434 and 435 (35) was introduced to Hep3B cells. Seven stable transfectants were generated and Fig. 6A displayed the expression

levels of the endogenous, wild type STAT3 and the mutant proteins in these cells. A high expressor (STAT3D-1) and a mixture of all seven clones (STAT3D-M) were analyzed in terms of their responsiveness to IL-6-induced anti-apoptosis. As shown in Fig. 6B, IL-6 induced a slightly reduced protection against TGF- β -induced apoptosis in both STAT3D-1 and STAT3D-M cells, comparing to the parental Hep3B cells. This reduction was consistently observed in three independent experiments (Table I). In addition, this reduced protection was also observed from Hep3B cells stably expressing a second STAT3 dominant-negative mutant, STAT3F (35) (data not shown). Thus, our results suggest that STAT3 is involved in the anti-apoptotic signaling of IL-6.

PI 3-Kinase/Akt and STAT3 Pathways Act Cooperatively—As described earlier, both PI 3-kinase/Akt and STAT3 pathways were involved in the IL-6-induced anti-apoptosis. We therefore determined exactly how this anti-apoptosis could be affected by blocking both pathways. For this purpose, parental Hep3B and the STAT3D transfectants were treated with or without TGF- β , IL-6, and/or LY294002. As shown in Fig. 7, the anti-apoptotic activity of IL-6 against TGF- β was completely abolished in the STAT3D transfectants treated with LY294002. This complete inhibition of IL-6-induced anti-apoptosis was not observed in parental Hep3B cells treated with LY294002. In three independent experiments (Table II), we consistently observed a more complete abrogation of the anti-apoptotic activity of IL-6 in STAT3D transfectants than in parental Hep3B cells. These findings allow us to infer that PI 3-kinase/Akt and STAT3 pathways act cooperatively to mediate the anti-apoptotic effect of IL-6.

DISCUSSION

IL-6 is a pleiotropic cytokine capable not only of inducing growth and differentiation in many cell types but also of stimulating acute-phase protein synthesis in liver cells (23, 24). However, the anti-apoptotic effect of IL-6 has not been well documented. In this study, we demonstrated that IL-6 suppressed TGF- β -induced apoptotic death of hepatoma cells in a dose-dependent manner. The anti-apoptotic effect of IL-6 correlated with its inhibition of TGF- β -induced activation of caspase-3. IL-6 stimulation, however, did not alter the effect of TGF- β on the induction of PAI-1 and p15^{INK4B} genes. Our results suggest that IL-6 interferes with only a subset of TGF- β signaling without affecting general TGF- β signaling.

This study provides several insights into the anti-apoptotic mechanism of IL-6. The PI 3-kinase/Akt and the JAK/STAT3 pathways are both activated by IL-6 and function cooperatively to achieve the maximal anti-apoptotic effect of IL-6 against TGF- β . Previous investigations have indicated that the PI 3-kinase/Akt is involved in preventing apoptosis induced by various growth factors in many different cell types (14–21),

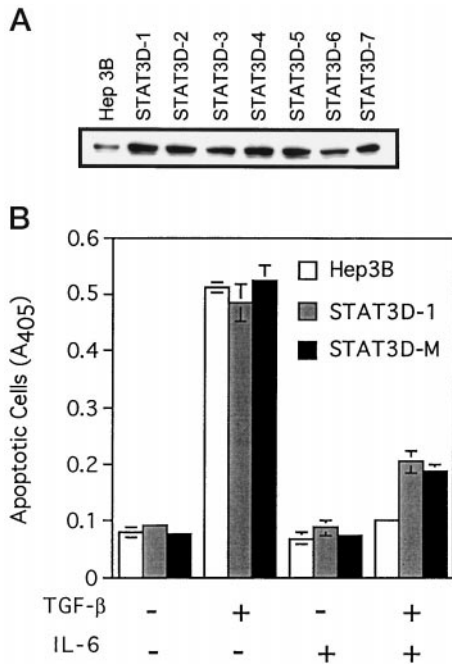


FIG. 6. The anti-apoptotic activity of IL-6 can be partially inhibited by a dominant-negative mutant of STAT3. A, expression of the dominant-negative STAT3 (STAT3D) in 7 stable transfectants assayed by Western blot using an anti-STAT3 antibody. An equal amount of proteins was loaded in each lane, and therefore the first lane represented the level of endogenous, wild type STAT3. B, expression of STAT3D attenuates the survival signal transduced by IL-6. STAT3D-M is a mixture of all stable transfectants shown in A. Cells were treated with various agents as indicated and apoptosis was assayed by the Cell-Death Detection ELISA.

TABLE I
Apoptotic death of Hep3B and STAT3D stable transfectants induced by TGF- β and/or IL-6

Cell	Treatment	Apoptotic cells ^a		
		Experiment 1	Experiment 2	Experiment 3
Hep3B	TGF- β	0.081 ± 0.009	0.063 ± 0.002	0.089 ± 0.007
	IL-6	0.511 ± 0.009	0.465 ± 0.041	0.679 ± 0.048
	IL-6 + TGF- β	0.069 ± 0.011	0.070 ± 0.009	0.076 ± 0.003
		0.102 ± 0.003	0.088 ± 0.005	0.142 ± 0.011
STAT3D-1	TGF- β	0.091 ± 0.007	0.076 ± 0.006	0.079 ± 0.005
	IL-6	0.486 ± 0.033	0.471 ± 0.022	0.703 ± 0.019
	IL-6 + TGF- β	0.088 ± 0.013	0.065 ± 0.003	0.073 ± 0.008
		0.205 ± 0.019	0.152 ± 0.009	0.298 ± 0.021
STAT3D-M	TGF- β	0.078 ± 0.005	0.069 ± 0.004	0.096 ± 0.009
	IL-6	0.523 ± 0.028	0.489 ± 0.039	0.638 ± 0.049
	IL-6 + TGF- β	0.075 ± 0.003	0.077 ± 0.010	0.086 ± 0.002
		0.189 ± 0.012	0.161 ± 0.013	0.212 ± 0.025

^a Apoptotic cells were quantitated by Cell-Death Detection ELISA kit. The number represents absorbance at A₄₀₅.

whereas STAT3 is a critical mediator of the anti-apoptotic effect of oncostatin M in osteosarcoma cells (46) and the survival signals transduced by gp130 in B cells (33). The anti-apoptotic mechanism of PI 3-kinase/Akt is at least partially attributed to phosphorylation of the BCL-2 family member BAD by Akt (20, 21). The phosphorylated BAD is then associated with 14-3-3, which sequesters BAD from BCL-X_L, thereby promoting cell survival (22). Regulating the BCL-2 family member is also considered as one of the anti-apoptotic mechanisms of STAT3, which was reported to be capable of inducing BCL-2 in pro-B cells (33). Thus, both anti-apoptotic signaling pathways transduced by IL-6 are likely to converge to BCL-2 family members, which could act upstream of caspase-3 (47, 48). This is consistent with our finding that IL-6 blocked the TGF- β -induced activation of caspase-3. In addition to induction of BCL-2, STAT3 can directly up-regulate the transcription of p21, which is implicated in the anti-apoptosis (46). In our system, an increased expression of p21 upon IL-6 stimulation was also observed (data not shown). Whether p21 and BCL-2 are induced independently by STAT3 and exactly how p21 promotes survival remain to be investigated.

IL-6 was reported to protect multiple myeloma plasma cells from anti-Fas- and dexamethasone-induced apoptosis (49). Inhibition of JNK/SAPK pathway is involved in IL-6-induced protection against anti-Fas but not dexamethasone. These results support a hypothesis that multiple mechanisms are involved in the IL-6-induced anti-apoptosis. However, blockage of JNK/SAPK pathway is unlikely to account for the mechanism by which IL-6 suppresses TGF- β -induced apoptosis, since TGF- β failed to induce JNK/SAPK activity in Hep3B cells (data not shown). Thus, distinct signaling pathways could mediate IL-6-induced protection from apoptosis induced by different stimuli.

Previous studies demonstrated that dominant-negative inhi-

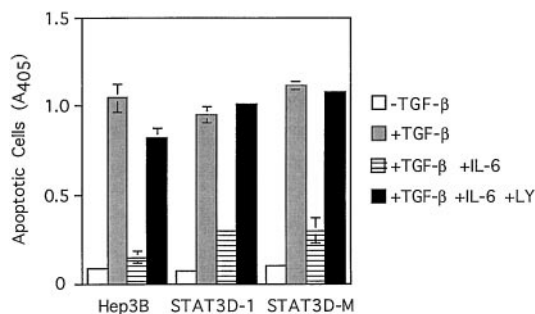


FIG. 7. The anti-apoptotic effect of IL-6 is completely abolished by blocking both PI 3-kinase and STAT3 signaling pathways. Hep3B, STAT3D-1, and STAT3D-M were treated with or without TGF- β (5 ng/ml), IL-6 (60 ng/ml), and LY294002 (25 μ M) and apoptosis was assayed as for Fig. 6B.

tion of STAT3 activity completely abolishes gp130-mediated survival signal in a pro-B cell line (33). In the case of Hep3B cells, however, the same dominant-negative mutants only partially blocked the anti-apoptotic effect of IL-6 against TGF- β . Interestingly, in mouse leukemia M1 cells, STAT3 is involved in the differentiation and growth arrest but not required for the anti-apoptotic signal (35, 50). These findings highlight the significance of cellular context in determining the biological functions of STAT3. STAT3 may induce the expression of a distinct set of genes depending on cell type involved. Another determinant of the biological consequences of STAT3 activation is likely to be the concomitant activation of other STAT family members and/or other signaling pathways, such as the PI 3-kinase/Akt pathway, upon ligand binding to the cytokine receptors.

A recent investigation indicated that IL-6 treatment of prostate cancer cell line LNCaP induces an increase in tyrosine phosphorylation of the p85 subunit of PI 3-kinase and its kinase activity (34). Accordingly, we observed a rapid induction of p85 tyrosine phosphorylation and PI 3-kinase activity by IL-6 in Hep3B cells. In addition, two specific inhibitors of PI 3-kinase, wortmannin and LY294002, blocked the anti-apoptotic effect of IL-6, implying the activation of PI 3-kinase by IL-6. Furthermore, we demonstrated for the first time that the serine/threonine kinase Akt is activated upon IL-6 treatment. A similar induction of PI 3-kinase and Akt activities was found in cardiac myocytes stimulated with leukemia inhibitory factor, a cytokine transducing signal via gp130 (51). Thus, in addition to JAK/STAT and Ras/MAP kinase pathways, the PI 3-kinase/Akt could be an important signaling pathway activated by various cytokines. The mechanism of IL-6-induced activation of PI 3-kinase remains unclear, although JAK can bind PI 3-kinase upon activation of gp130 (51).

In the liver, IL-6 plays a crucial role in anti-inflammatory responses to prevent liver injury (25, 52) and is a key growth factor to initiate liver regeneration (1, 26). This study demonstrates another important activity of IL-6 in the liver, namely, anti-apoptosis. We propose that the ability of IL-6 to suppress apoptosis induced by TGF- β could be physiologically important. TGF- β is thought to be a terminator of liver regeneration through its growth-inhibitory and apoptotic effects (1). However, the mRNA of TGF- β is induced at an initiation stage of liver regeneration (53), indicating that hepatocytes can proceed with regeneration despite the increase in the concentrations of TGF- β . Accordingly, hepatocytes isolated from actively regenerating liver are resistant to TGF- β (54). This raises the possibility that the effect of TGF- β is blocked by other growth factors and/or cytokines as part of their regeneration-promoting effects. IL-6 is a logical candidate of these factors, because the timing of its induction correlates with the resistance of

TABLE II
Apoptotic death of Hep3B and STAT3D stable transfectants induced by various agents

Cell	Treatment	Apoptotic cells ^a		
		Experiment 1	Experiment 2	Experiment 3
Hep3B	TGF- β	0.089 \pm 0.005	0.063 \pm 0.008	0.072 \pm 0.014
	IL-6 + TGF- β	1.046 \pm 0.079	0.782 \pm 0.061	0.682 \pm 0.023
	IL-6 + TGF- β + LY	0.148 \pm 0.035	0.097 \pm 0.011	0.103 \pm 0.034
	IL-6 + TGF- β + LY	0.821 \pm 0.056	0.582 \pm 0.006	0.454 \pm 0.008
STAT3D-1	TGF- β	0.069 \pm 0.012	0.081 \pm 0.006	0.083 \pm 0.013
	IL-6 + TGF- β	0.947 \pm 0.044	0.843 \pm 0.035	0.746 \pm 0.031
	IL-6 + TGF- β	0.295 \pm 0.017	0.267 \pm 0.012	0.192 \pm 0.005
	IL-6 + TGF- β + LY	1.012 \pm 0.021	0.796 \pm 0.048	0.712 \pm 0.027
STAT3D-M	TGF- β	0.105 \pm 0.017	0.077 \pm 0.002	0.069 \pm 0.015
	IL-6 + TGF- β	1.112 \pm 0.023	0.874 \pm 0.044	0.775 \pm 0.026
	IL-6 + TGF- β	0.300 \pm 0.070	0.356 \pm 0.041	0.254 \pm 0.014
	IL-6 + TGF- β + LY	1.078 \pm 0.014	0.942 \pm 0.025	0.689 \pm 0.036

^a Apoptotic cells were quantitated by Cell-Death Detection ELISA kit. The number represents absorbance at A₄₀₅.

hepatocytes to TGF- β (55). Additional studies would be required to further define the physiological roles of the anti-apoptotic activity of IL-6 against TGF- β in liver cells.

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