Interleukin-11 Up-Regulates Survivin Expression in Endothelial Cells through a Signal Transducer and Activator of Transcription-3 Pathway

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SUMMARY: Interleukin-11 (IL-11) reduces injury both in vivo and in vitro, but the mechanisms are unknown. Stimulation of serum- and growth factor-deprived HUVEC with IL-11 increased survivin mRNA and protein expression levels in a dose-dependent manner, with maximal induction at 50 to 100 ng/ml of IL-11. Survivin mRNA expression peaked after 3 to 6 hours of IL-11 treatment and decreased by 24 hours. Survivin protein expression was maximal at 6 hours of treatment and remained elevated through 24 hours. Survivin induction may be mediated by activation of protein kinase B/Akt, but IL-11 failed to activate this pathway in HUVEC. IL-11 did activate signal transducer and activator of transcription (STAT)-3 and IL-11 failed to induce survivin expression in HUVEC transduced with a dominant-negative STAT3 mutant, whereas control-transduced HUVEC responded normally. An IL-11 transgene caused increased survivin mRNA expression in mice compared with control littermates. Intradermal injection of IL-11 (500 ng) into human skin xenografts on immunodeficient mice up-regulated survivin protein in microvascular endothelium and epithelial keratinocytes. We conclude that IL-11 induces expression of survivin, an antiapoptotic protein, in vitro and in vivo, and identify STAT3 as a critical mediator of this response. (*Lab Invest 2001, 81:327–334*).

*I*nterleukin-11 (IL-11) is an interleukin-6 (IL-6)–type cytokine that interacts with a variety of hematopoietic and nonhematopoietic cell types. IL-11 has been shown to have protective effects in various in vivo models of injury, both in the gastrointestinal tract (Du et al, 1997; Keith et al, 1994; Orazi et al, 1996) and in the lung (Waxman et al, 1998). Human IL-11, expressed as a transgene in bronchial mucosa, markedly diminish hyperoxic lung injury in mice (Waxman et al, 1998). This effect was associated with a reduction in multiple parameters of lung injury, including alveolar-capillary protein leak, endothelial cell and epithelial cell membrane injury, lipid peroxidation, pulmonary neutrophil recruitment, inflammatory cytokine production, and DNA fragmentation (Waxman et al, 1998). The protective effects of IL-11 are not restricted to in vivo models of injury. Recently, we reported that pretreatment of cultured HUVEC with IL-11 can protect these cells from immune-mediated injury due to cytotoxic T lymphocyte or antibody (Ab) plus complement (Mahboubi et al, 2000). In vitro cytoprotection by

IL-11 is dependent on new protein synthesis (Mahboubi et al, 2000). Although it is likely that induction of specific gene products is required for cytoprotective effects of IL-11 in vitro, the exact mechanisms of cytoprotection or the identities of such putative anti-apoptotic proteins are not known.

IL-11 activates cells through a multimeric receptor containing a signal-transducing subunit, gp130, and a unique ligand binding subunit, IL-11-receptor-α (IL-11Rα) chain (Heinrich et al, 1998). Binding of IL-11 to IL-11Rα induces clustering of gp130, leading to phosphorylation of signal transducer and activator of transcription (STAT)-3 as well as p42 and p44 mitogen-activated protein kinases (MAPK) at concentrations of IL-11 as low as 1 ng/ml (Mahboubi et al, 2000). STAT1 is also activated by IL-11, but only at concentrations of IL-11 above 10 ng/ml (Mahboubi et al, 2000). Recently, phosphotidylinositol-3 kinase (PI-3 kinase) has been described to be activated by IL-6 and certain other cytokines that use gp130 (Chung et al, 2000; Oh et al, 1998; Qiu et al, 1998). Activation of the PI-3 kinase pathway results in the phosphorylation and subsequent activation of an antiapoptotic serine/threonine kinase Akt (also known as protein kinase B) (Hemmings, 1997).

Survivin is a member of the inhibitor of apoptosis protein family that is highly expressed in many human cancers, but is undetectable in normal adult tissues in vivo (Ambrosini et al, 1997; Reed and Reed, 1999). Survivin expression can be up-regulated in cultured
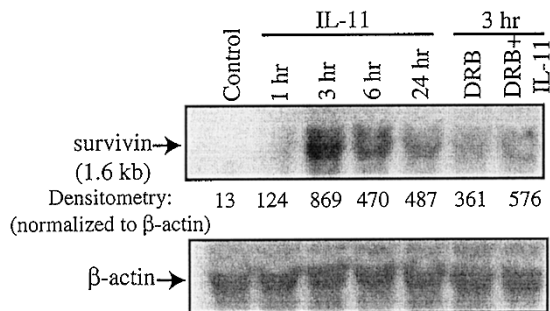
endothelial cells (EC) either by growth factors, such as vascular endothelial cell growth factor (VEGF), in a cell cycle–dependent manner (O'Connor et al, 2000; Tran et al, 1999) or by the vascular remodeling factor angiopoietin-1 in a cell cycle–independent manner (Papapetropoulos et al, 2000). In vivo, survivin expression is observed in untransformed EC that are involved in an angiogenic response (O'Connor et al, 2000), ie, in a setting where EC are exposed to VEGF and angiopoietin-1. The angiopoietin effect is mediated via the PI-3 kinase/Akt pathway (Papapetropoulos et al, 2000). Overexpression of survivin can reduce apoptosis induced either by growth factor withdrawal or by TNF/cycloheximide in cultured EC (O'Connor et al, 2000). These observations have led to the hypothesis that a physiologic role of survivin is to protect angiogenic endothelium from apoptosis.

We report here that IL-11 induces survivin expression in HUVEC in vitro and in vivo xenograft and transgenic models in vivo. This pathway appears independent of Akt signaling because IL-11 does not activate this enzyme in HUVEC. In contrast, we find that this response is dependent upon signaling through STAT3.

Results

IL-11 Increases Survivin mRNA and Protein Levels in Cultured HUVEC

We examined the effects of IL-11 on survivin expression in cultured HUVEC. Under standard culture conditions, ie, in the presence of 20% FCS and 50 μg/ml of fibroblast growth factor-1 (FGF-1), survivin mRNA and protein were basally expressed in HUVEC. The levels declined as the cells were made quiescent by serum and growth factor withdrawal for 36 to 48 hours (Fig. 1, control). Treatment of quiescent cells with IL-11 caused a time-dependent increase in survivin mRNA levels above untreated cells (Fig. 1), which peaked 1 to 3 hours after stimulation and remained sustained up to 24 hours (Fig. 1). Preincubation of

cells with the transcriptional inhibitor, 5, 6, dichlorobenzimidazole riboside (DRB) (20 μM) blocked upregulation of survivin induced by IL-11 (Fig. 1). These data suggest that IL-11 increases survivin mRNA by increasing transcription of the survivin gene.

Consistent with the expected molecular weight of survivin, an affinity-purified rabbit polyclonal Ab to survivin immunoblotted a single band of about 16.5 kDa from HUVEC (Fig. 2). Expression of endogenous survivin protein in quiescent HUVEC fell to levels that were minimally detectable by immunoblotting 36 to 48 hours after serum and growth factor were withdrawn (Fig. 2A, control). IL-11 induced the expression of survivin protein in quiescent HUVEC in a time-dependent manner, which peaked approximately 6 hours after stimulation and remained elevated for up to 24 hours (Fig. 2A). Survivin induction by IL-11 was concentration-dependent, showing a maximal effect at 50 to 100 ng/ml (Fig. 2B).

IL-11 Does Not Induce Serine Phosphorylation of Akt in HUVEC

Induction of survivin expression by angiopoietin-1 in EC is mediated by PI-3 kinase/Akt-dependent pathway (Papapetropoulos et al, 2000). To assess the activation of Akt in HUVEC we tested whether IL-11 treatment results in phosphorylation of Akt as detected by immunoblotting using a serine 437 phospho-Akt–specific Ab. Stimulation of HUVEC with either 100 or 500 ng/ml of IL-11 did not induce Akt phos-
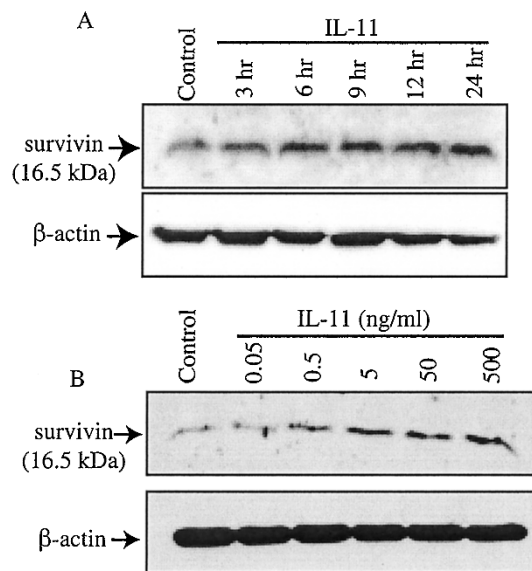

Figure 1.

Time-dependent induction of survivin mRNA by IL-11 in HUVEC. Confluent HUVEC were made quiescent by culture in media containing 0.1% FCS for 36 hours, after which new media without (control) or with IL-11 (100 ng/ml) was added at the indicated time intervals. Survivin and β-actin RNA were examined by Northern hybridization. In the indicated samples, 5, 6, dichlorobenzimidazole riboside (DRB; 20 μM) was added 30 minutes before the addition of IL-11. Numbers below the survivin panel indicate relative levels based on densitometry. Data are from one of two experiments with similar outcome.
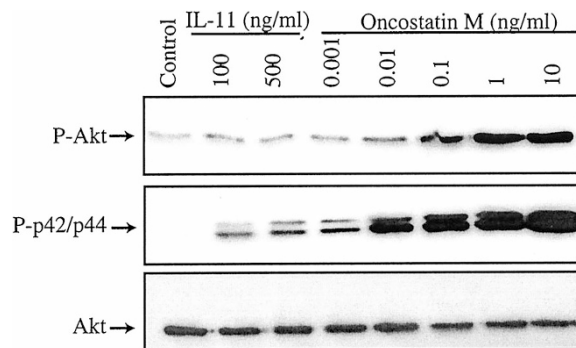

Figure 2.

IL-11 induces survivin protein expression in HUVEC. A, Time-dependent expression of survivin protein. The experimental conditions are the same as for Figure 1. At the indicated treatment intervals, quiescent HUVEC were harvested and analyzed for survivin and β-actin protein expression using immunoblotting as described in "Materials and Methods." B, IL-11 dose-dependent expression of survivin protein. HUVEC were made quiescent as described in Figure 1 and stimulated with various concentrations of IL-11. After 12 hours of treatment, cells were harvested and analyzed for survivin and β-actin using immunoblotting as described in "Materials and Methods." For both A and B, data are from one of three experiments with similar outcome.

phorylation (Fig. 3). However, IL-11 was active because it clearly increased phosphorylation of p42/p44 MAPK in these cells (Fig. 3). IL-11 at concentrations less than 100 ng/ml was also unable to cause phosphorylation of Akt (data not shown). Our previous studies had shown that oncostatin M is a much stronger activator of gp130 signaling responses in HUVEC than is IL-11 (Mahboubi et al, 2000). Stimulation of HUVEC with oncostatin M did increase Akt phosphorylation in a dose-dependent manner (Fig. 3). These data demonstrate that gp130 signals in HUVEC can result in Akt activation, but that this requires a stronger signal than that provided by IL-11.

### Down-Negative STAT3 Inhibits IL-11–Induced Survivin Expression in HUVEC

IL-11 activates STAT3 in HUVEC (Mahboubi et al, 2000). Therefore, we investigated the functional role of STAT3 in IL-11–induced survivin expression in these cells. For this purpose, we stably transduced HUVEC with retroviruses encoding either enhanced green fluorescent protein (EGFP), STAT3(Y705F)-FLAG, or STAT1(Y701F)-hemagglutinin (HA). The M2 monoclonal anti-FLAG Ab and anti-STAT3 Ab were used to confirm expression of STAT3(Y705F)-FLAG in transduced cells with this gene (Fig. 4A), whereas a monoclonal Ab to HA or STAT1 Ab was used to demonstrate the expression of STAT1(Y701F)-HA in transduced cells (Fig. 4B). STAT3-FLAG and STAT1-HA were only detected in cells transduced with STAT3(Y705F)-FLAG gene (Fig. 4A, upper panel) and STAT1(Y701F)-HA (Fig. 4B, upper panel), respectively. Single protein bands were detected in cells transduced with EGFP gene corresponding to endogenous levels of STAT3 and STAT1 protein by using antibodies to STAT3 and STAT1, respectively (Fig. 4, A and B, lower panels). This contrasts with the appearance of an additional protein band of slightly higher molecular weight detected in cells transduced with either STAT3(Y705F)-FLAG gene or STAT1(Y705F)-HA gene (Fig. 4B, lower panel).

We next investigated the tyrosine phosphorylation of endogenous STAT3 in EGFP, or STAT3(Y705F)-FLAG–transduced HUVEC. To do this, the cells were stimulated with various doses of IL-11 (0.5 to 500 ng/ml) for 10 minutes and the cell lysates were analyzed by immunoblot using specific P-STAT3 Ab. IL-11 dose-dependently induced tyrosine phosphorylation of STAT3 in EGFP-transduced HUVEC, in agreement with our previous observations using untransduced HUVEC (Fig. 5) (Mahboubi et al, 2000). The concentration of IL-11 required to cause tyrosine phosphorylation of endogenous STAT3 protein was increased by about 100-fold in STAT3(Y705F)-FLAG–transduced cells (Fig. 6), confirming that this mutation acts as a dominant negative (dn). Parallel experiments were conducted in STAT1(Y705F)-HA–transduced HUVEC. Despite the fact that STAT1(Y701F)-HA protein was expressed in HUVEC at roughly similar levels as STAT3(Y705F)-FLAG (Fig. 4B), neither IL-11–mediated tyrosine phosphorylation of endogenous STAT1 nor IL-11–mediated induction of survivin was abrogated by dn-STAT1 in these cells (data not shown).

Finally, we examined the effect of expression of dn-STAT3 protein on the IL-11–mediated induction of survivin protein. IL-11 significantly increased survivin expression in EGFP-transduced HUVEC (Fig. 6). In contrast, IL-11 stimulation of STAT3(Y705F)-FLAG–transduced cells did not result in up-regulation of
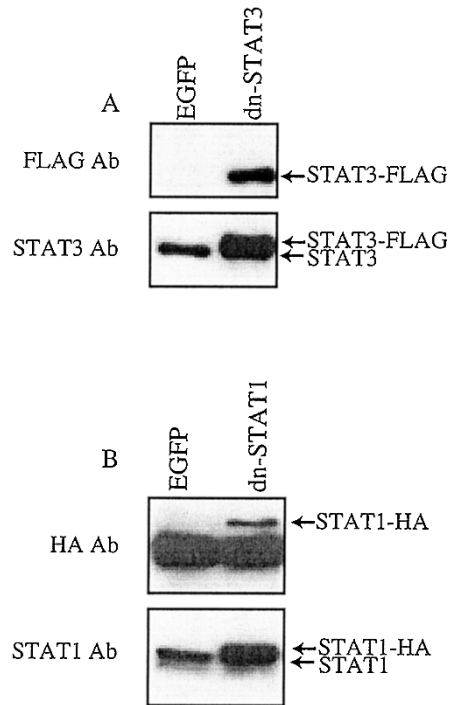
Figure 4.
Overexpression of dn-STAT3 and dn-STAT1 in HUVEC. HUVEC were transduced by retroviruses encoding either EGFP, dn-STAT3 (STAT3[Y705F]-FLAG), or dn-STAT1 (STAT1[Y701F]-HA) as described in "Materials and Methods." Cell lysates were analyzed for either STAT3-FLAG (Fig. 6A, upper panel), total STAT3 (Fig. 6B, lower panel), STAT1-HA (Fig. 6B, upper panel), or total STAT1 (Fig. 6B, lower panel) by immunoblotting as described in "Materials and Methods." Data are from one of two independent sets of transductants with similar outcome.
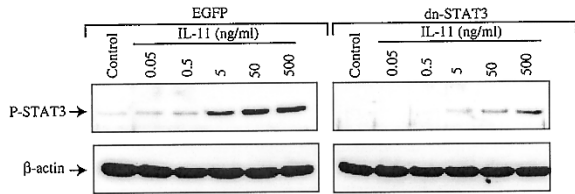
Figure 3.
IL-11 does not stimulate Akt phosphorylation in HUVEC. Quiescent HUVEC were incubated with various doses of IL-11 and oncostatin M (positive control) for 15 minutes and analyzed for either Akt phosphorylation (P-Akt, ser^{473}, upper panel), p42/p44 MAPK phosphorylation (middle panel, P-p42/p44, Thr^{202}/Tyr^{204}), or total Akt (lower panel) by immunoblotting as described in the "Materials and Methods." Data are from one of two experiments with similar outcome.
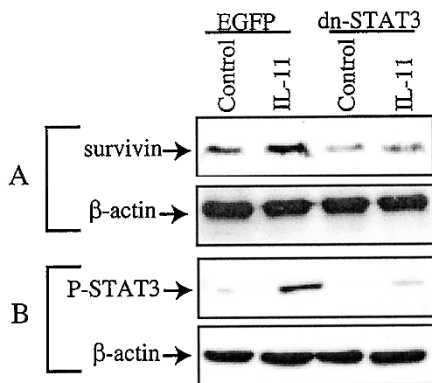
Figure 5.
Effect of dn-STAT3 on phosphorylation of tyrosine 705 of endogenous STAT3 by IL-11. HUVEC were transduced by retroviruses encoding either EGFP or dn-STAT3 (STAT3[Y705F]-FLAG) and stimulated with various concentrations of IL-11 for 10 minutes. Cell lysates were analyzed for either STAT3 phosphorylation (P-STAT3, Tyr^{705}, upper panel) or β-actin (lower panel) by immunoblotting as described in "Materials and Methods." Data are from one of two independent sets of transductants with similar outcome.

survivin (Fig. 6). These data indicate that IL-11 mediates up-regulation of survivin through a STAT3-dependent pathway.

IL-11 Up-Regulates Survivin mRNA and Protein In Vivo

Mice transgenic for human IL-11 regulated by the CC10 promoter express this protein at high levels in the lung (Ray et al, 1997). We performed Northern hybridization to compare the levels of survivin mRNA in the lungs of IL-11 transgene (+) and IL-11 transgene (−) control littermates. In Northern blots, a survivin-specific single-strand probe detected a predominant 1.9-kb transcript (Fig. 7, upper panel). Higher levels of survivin transcript were detected in IL-11 transgene (+) animals than in littermate controls (Fig. 7, upper panel). The amount of 28S and 18S ribosomal RNA confirmed comparable loading of mRNA per sample (Fig. 7, lower panel). These data indicate that survivin gene expression is induced by IL-11 in vivo.

Figure 6.
Effect of overexpression of dn-STAT3 protein on induction of survivin by IL-11. HUVEC were transduced by retroviruses encoding either EGFP or dn-STAT3 (STAT3[Y705F]-FLAG). A, Transduced cells were made quiescent as described in Figure 2 and stimulated with IL-11 (5 ng/ml) for 12 hours and analyzed for either survivin (upper panel) or β-actin (lower panel) protein by immunoblotting as described in "Materials and Methods." B, Transduced HUVEC were stimulated with IL-11 (5 ng/ml) for 10 minutes and analyzed for either STAT3 phosphorylation (P-STAT3, Tyr^{705}, upper panel) or β-actin (lower panel) by immunoblotting as described in "Materials and Methods." Data are from one of three experiments involving two independent sets of transductants with similar outcome.
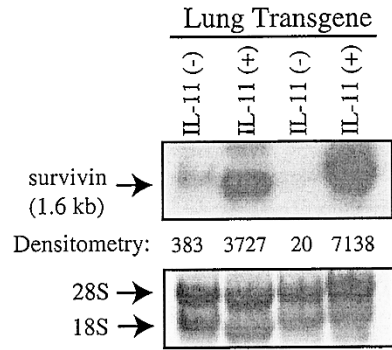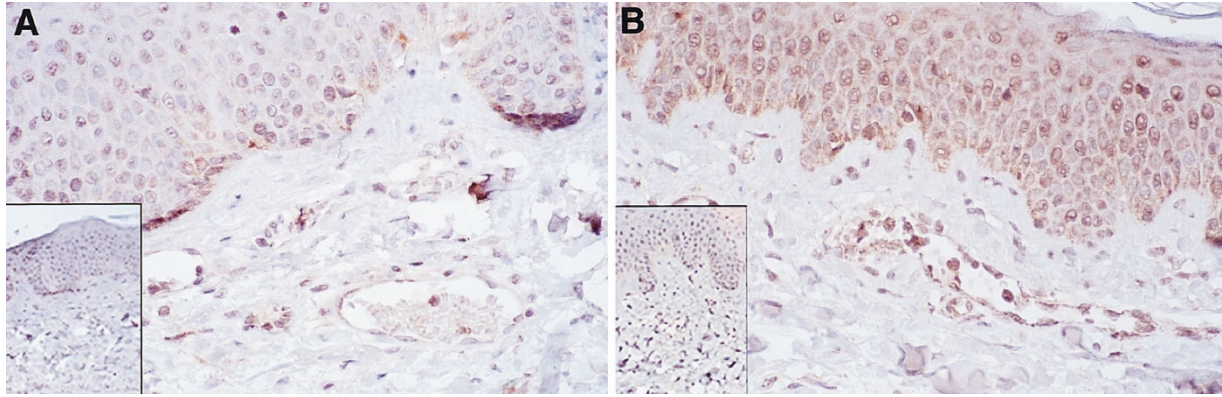

Figure 7.
Survivin mRNA in lungs of CC-10-IL-11 transgene (+) and transgene (−) mice. Total lung mRNA was isolated and analyzed by Northern blotting as described in "Materials and Methods" (upper panel). The amount of 28S and 18S RNA on the Nylon filters was detected by methylene blue staining and was used for comparison of sample loading (lower panel). Numbers below the survivin panel indicate relative levels based on densitometry. Northern blots of two pairs of animals out of four pairs examined, all with similar results, are shown.

To further establish whether IL-11 could increase survivin protein in vivo, we used a human skin xenograft model developed in severe combined immunodeficient (SCID)/beige mice (Murray et al, 1998). Immunostaining of human skin grafts with survivin Ab demonstrated that intradermal injection of IL-11 into human skin grafts increased survivin protein expression in these grafts (Fig. 8, panel A vs panel B). Up-regulation of survivin was detected both in the epidermal keratinocytes and in the dermal microvascular endothelium. These data establish that IL-11 can increase survivin protein expression in vivo.

Discussion

In this report we have shown that IL-11 up-regulates survivin expression in vitro and in vivo. The induction of survivin mRNA by IL-11 in endothelial cells is mediated, at least in part, by increasing transcription of the survivin gene. IL-11 stimulates survivin expression in EC via a STAT3-dependent pathway.

Previous studies have demonstrated that survivin is up-regulated in endothelial cells by mediators of angiogenesis such as VEGF, FGF, and angiopoietin (O'Connor et al, 2000; Papapetropoulos et al, 2000; Tran et al, 1999). However, survivin is not regulated by proinflammatory cytokines such as interleukin-1 and TNF (O'Connor et al, 2000). IL-11 is not a mitogen for EC nor does it regulate the inflammatory functions of EC either positively or negatively. Although it shares with angiogenic factors the capacity to induce survivin, IL-11 seems to do so via a different signaling pathway because it does not activate Akt. Previous studies demonstrated that both overexpression of dn-STAT3 and dn-Akt inhibited the antiapoptotic effects of IL-6, another gp130-signaling cytokine, against transforming growth factor-β–induced apoptosis in human hepatoma cell line (Chen et al, 1999). Similarly, overexpression of dn-STAT3 completely abolishes gp130-mediated survival signals in a pro-B

Figure 8.
Representative photomicrographs (original magnification, ×40) of human skin grafts demonstrating the IL-11 up-regulated survivin protein expression in vivo. Human skin grafts on SCID/beige mice were harvested 24 hours after interdermal injection of either saline control (A) or 500 ng of IL-11 (B). Skin grafts were immunostained with either control Ab (A and B, insets) or antisurvivin Ab (A and B) as described in "Materials and Methods." Similar findings were observed in three additional pairs of animals providing two independent experiments.

cell line (Fukada et al, 1996). These results imply that both STAT3 and PI-3 kinase/Akt pathways are important for gp130-mediated antiapoptotic signals (Chen et al, 1999; Chung et al, 2000; Fukada et al, 1996).

The targets of STAT3 activation are not fully understood. STAT3 activation via gp130 increases expression of Bcl-2, an antiapoptotic protein, in pro-B cells (Fukada et al, 1996). Therefore, up-regulation of Bcl-2 is considered as one of the antiapoptotic mechanisms of STAT3. In addition, overexpression of STAT3 in osteoblastic cells potentiated both IL-6– and oncostatin M–mediated induction of p21 (Cip1, also known as WAF1, sd11) which also protects against apoptosis (Bellido et al, 1998). Although IL-11 activates STAT3 in HUVEC, IL-11 did not increase the expression of Bcl-2 or p21 (unpublished observations, KM) in these cells. These data are consistent with the idea that STAT3 may induce the expression of a different set of genes, depending on the cell type involved.

IL-11 significantly reduced epithelial and endothelial cell membrane injury associated with hyperoxia in the lung (Waxman et al, 1998). Cell death responses with features of necrosis and apoptosis play important roles in the pathogenesis of hyperoxic lung injury (Mantell et al, 1999). Interestingly, IL-11 diminished DNA fragmentation, a marker of apoptosis and necrosis, in the lung of mice exposed to 100% O_2 (Waxman et al, 1998). The exact mechanisms by which IL-11 decreases apoptosis in vivo are not clearly understood. An interesting finding of our study is the demonstration that IL-11 (+) transgenic mice express higher survivin mRNA levels and that administration of IL-11 induced de nova survivin expression in human skin xenografts. Thus, survivin protein expression may contribute to these effects.

A major question raised by the present study is to what extent survivin induction contributes to the cytoprotective actions of IL-11 we have previously reported in HUVEC. In in vitro experiments, IL-11 exerted a cytoprotective action on HUVEC cultured in complete media (ie, media containing serum and FGF-1) (Mahboubi et al, 2000), conditions under which

survivin is already expressed before the addition of IL-11. In this setting, it is therefore likely that IL-11 activates additional protective responses. We are unable to assess the protective effects of IL-11 in quiescent HUVEC because, although these cells are useful for evaluation of gene expression, they are extremely fragile and not suited for evaluation of cytoprotection. In vivo, survivin is not basally expressed in endothelium, and the induction of survivin in EC by IL-11 could contribute to cytoprotection. In this context it is intriguing that in addition to canonical pathways of cell cycle progression, survivin induction can be triggered by nonmitogenic stimuli, including angiopoietin (Papapetropoulos et al, 2000) or IL-11 (this study). Although survivin expression in proliferating cells has been linked to both the control of apoptosis and prevention of ploidy (Li et al, 1999), the presence of survivin in IL-11–stimulated, noncycling endothelium suggests a preferential role in cytoprotection, independently of cytokinesis. This question may be addressed by future studies using the mouse lung or human skin models described in this report.

Finally we have established a linkage between STAT3 and survivin gene activation. This result implies that other growth factor receptors, or nonreceptor type tyrosine kinases, or oncogene products that are capable of activating STAT3 could also induce survivin expression. This may have more general implications for the overexpression of survivin in cancer, where it constitutes the top fourth "transcriptome" in the most common human neoplasia (Velculescu et al, 1999). Recent data demonstrated that a constitutively active form of STAT3 was sufficient to produce anchorage-independent cell proliferation and tumor formation in nude mice, thus emphasizing its oncogenetic potential (Bromberg et al, 1999). The dramatic up-regulation of survivin expression via STAT3 activation reported here suggests that this pathway may be also operative during oncogenic transformation, further contributing to dysregulation of the apoptosis in cancer. The generality of the survivin-STAT3 pathway and its conse-

quences for vascular responses and tumor growth warrant further exploration.

Materials and Methods

Reagents, Cells, and Animals

Recombinant human IL-11 was provided by Genetics Institute (Andover, Massachusetts). Recombinant human oncostatin M was purchased from R&D Systems (Minneapolis, Minnesota). FGF-1, commonly called endothelial cell growth supplement, was obtained from Collaborative Research/Becton Dickinson (Bedford, Massachusetts) and used in conjunction with porcine intestinal heparin (Sigma, St. Louis, Missouri). The transcriptional inhibitor, DRB, polybrene, and puromycin were obtained from Sigma. Mouse monoclonal Ab to β-actin and M2 monoclonal anti-FLAG Ab were obtained from Sigma. Rabbit polyclonal Ab reactive to STAT3, phosphotyrosine-STAT3, STAT1, phosphotyrosine STAT1, Akt, phosphoserine Akt, and phosphothreonine/phosphotyrosine-p42/p44 MAPK were purchased from New England Biolabs (Beverly, Massachusetts). Rabbit polyclonal Ab to survivin was made against recombinant survivin protein as described previously, and affinity purified (Grossman et al, 1999). Mouse monoclonal anti-HA Ab was purchased from Boehringer Mannheim (Indianapolis, Indiana).

Human EC were isolated from umbilical veins as previously described (Gimbrone, 1976; Thornton et al, 1983) and cultured on gelatin (J. T. Baker, Phillipsburg, New Jersey)–coated tissue culture plastic at 37° C in 5% CO_2-humidified air in Medium 199 containing 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Grand Island, New York), 50 μg/ml FGF-1, and 100 μg/ml porcine intestinal heparin (Sigma). The Phoenix-Ampho packaging cell line was obtained from Dr. G. Nolan (Stanford University, Stanford, California) and cultured in DMEM media containing 10% FCS 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

CC-10-IL-11 transgenic mice that overexpress IL-11 in a lung-specific fashion, were produced on CBA/C57BL6 background mice as described previously (Waxman et al, 1998). C.B-17 SCID/beige mice (Taconic Farms, Germantown, New York) were used at 5 to 8 weeks of age. The animals were housed in microisolator cages and were fed sterilized food and water.

RNA Extraction and Northern Blotting

To extract RNA from HUVEC, cells were lysed directly in a culture dish with TRI Reagent (0.2 ml/10^6 cells). For RNA extraction from mouse lung, the animals were anesthetized under a protocol approved by the Yale Animal Care and Use Committee. The lungs were perfused with PBS (pH 7.4) in situ, removed from the animal, and homogenized in TRI Reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's specifications. For Northern blot analysis, 10 to 20 μg of total RNA was separated on 1% agarose gels with 7% formaldehyde, transferred to Nylon filters (Hybond-N; Amersham Pharmacia Biotech, Piscataway, New Jersey), ultraviolet cross-linked (Stratagene, La Jolla, California) and hybridized with either ^{32}P-labeled survivin cDNA probe (Ambrosini et al, 1997) or ^{32}P-labeled β-actin cDNA probe (Clontech, Palo Alto, California) in ExpressHyb hybridization solution (Clontech). After washing, the filter was exposed for autoradiography. Autoradiograms were scanned by using a laser densitometer (Fast Scan, Series 300; Molecular Dynamics, Sunnyvale, California).

Immunoblotting

Cells were washed twice with ice-cold PBS containing 1 mM sodium orthovanadate and 1 mM sodium fluoride and lysed with ice-cold RIPA lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/ml leupeptin, 1 mM sodium orthovanadate). Cell lysates were clarified by centrifugation at 10,000 $\times g$ for 15 minutes, and protein concentrations of supernatant were determined by using a Bio-Rad assay kit (Bio-Rad, Hercules, California). Lysates were prepared for SDS-PAGE by adding an equal volume of 2X SDS-PAGE sample buffer (100 mM Tris-Cl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol) and heating the mixture in a boiling water bath for 3 minutes. Twenty micrograms of protein was separated on SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane by electrophoresis (Immobilon P; Millipore, Bedford, Massachusetts). After blocking with Tris-buffered saline Tween (10 mM Tris-HCl, pH 8.0, 0.150 mM NaCl, 0.05% Tween 20) containing 5% milk for 1 hour at room temperature, the membranes were incubated overnight at 4° C with blocking solution containing the indicated Ab. Membranes were washed and incubated with a suitable horseradish peroxidase (HRP)–conjugated detecting reagent (Jackson Immuno Research, West Grove, Pennsylvania) and HRP activity was detected using an enhanced chemiluminescence kit, according to the manufacturer's instructions (Pierce, Rockford, Illinois). Autoradiograms were scanned using a laser densitometer (Fast Scan, Series 300; Molecular Dynamics, Sunnyvale, California).

Construction of Retroviral Vectors Expressing EGFP, dn-STAT3, or dn-STAT1 and Stable Transduction of dn-STAT3, dn-STAT1, or EGFP

dn-STAT3 and dn-STAT1 were made by replacing tyrosine 705 and 701, in the respective wild type sequences, with phenylalanine (Kaptein et al, 1996; Walter et al, 1997). A C-terminal FLAG–tagged version of human dn-STAT3 [STAT3(Y705F)-FLAG] cDNA in expression vector pSL1180 was obtained from Dr. X. Y. Fu (Department of Pathology, Yale University School of Medicine), and the cDNA insert (2400 bp) was isolated by PCR and subcloned into the

LZRSpBMN-Z retroviral vector (kindly provided by Dr. A. L. M. Bothwell, Section of Immunobiology, Yale University School of Medicine) by using 5' *Hin*dIII and 3' *Not*I cloning sites. An N-terminal HA-tagged version of human dn-STAT1 (STAT1[Y701F]-HA) cDNA in expression vector pSG91 was also obtained from Dr. X. Y. Fu and the cDNA insert (2400 bp) was isolated by PCR and subcloned into the LZRSpBMN-Z retroviral vector using 5' *Bam*HI and 3' *Not*I cloning sites. LZRSpBMN-Z retroviral vector DNA containing EGFP was kindly provided from Dr. A. L. M. Bothwell (Zheng et al, 2000). These retroviral vectors containing EGFP, (STAT3[Y705F]-FLAG), or (STAT1[Y701F]-HA) were directly transfected into the Phoenix-Amphoteric packaging cell line using lipofectamine plus reagent (Life Technologies). Two days after transfection, Phoenix cells were selected in the media containing puromycin (1 μg/ml). Puromycin-resistant cells were used to condition medium, providing a source of retroviral stock.

Infection of HUVEC was accomplished by serial infections over two weeks, without drug selection, as previously described by Zheng et al (2000). A round of viral infections in the presence of polybrene (8 μg/ml) was performed for 5 hours with HUVEC at passage one. The normal growth medium was replaced and cells were maintained overnight. The infection was repeated the next day. Cells were carried in culture for one week and then the process of double infection was repeated. Using this protocol the percentage of HUVEC-expressing transduced genes was more than 95%.

Skin Grafting

Discarded normal adult human abdominal skin was obtained through the Yale Medical School Department of Pathology under protocol approved by the Human Investigation Committee. Human skin was transplanted to SCID/beige mice as previously described under a protocol approved by Yale Animal Care and Use Committee (Murray et al, 1998). In brief, superficial portions of the skin were harvested in 0.5-mm-thick sheets using a Goulian dermatome knife, gauge size, 0.016 (Weck, Research Triangle Park, North Carolina), and cut into approximately 1 cm^2 pieces, which were kept in RPMI 1640 (Life Technologies) at 4° C until transplantation (not longer than 6 hours). SCID/beige mice were anesthetized by inhalation of methoxyfluride (Pitman-More, Mundelein, Illinois). The skin was shaved, two skin segments of equal size to human grafts were excised from the back of each mouse. The skin reproducibly engrafted with a more than 95% success rate and was allowed to heal for 5 weeks. IL-11 (500 ng/20 μl of saline) or vehicle control (20 μl of saline) was injected intradermally, Twenty-four hours later, the animals were killed under anesthesia and the skin grafts were removed and prepared for immunohistochemistry, as described below.

Immunohistochemistry

Each human skin graft was fixed in buffered formalin and used to prepare 3-μm paraffin-embedded sections. Sections were deparaffinized in xylene and rehydrated in graded alcohol with quenching of endogenous peroxidase activity by treatment with 2% H_2O_2 in methanol. For immunostaining, the slides were boiled for 5 minutes in a standard pressure cooker, blocked in 10% normal goat serum, and incubated with either affinity-purified rabbit antisurvivin Ab or rabbit IgG (0.5 μg/ml) for 24 hours at 4° C. After washes, the slides were incubated with biotin-conjugated goat antirabbit IgG (Vector Laboratory, Burlingame, California) for 30 minutes at 22° C, followed by streptavidin-conjugated peroxidase (Zymed Laboratory, San Francisco, California) and 3', 3'-diaminobenzidine (Zymed Laboratory). Sections were counterstained with hematoxylin (Sigma).

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