Hepatocyte Growth Factor Inhibits Apoptosis by the Profibrotic Factor Angiotensin II via Extracellular Signal-regulated Kinase 1/2 in Endothelial Cells and Tissue Explants

Young H. Lee, Ana P. Marquez, Ognnoon Mungunaksukh, and Regina M. Day

Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814

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Hepatocyte growth factor (HGF), an endogenous tissue repair factor, attenuates apoptosis in many primary cell types, but the mechanism is not completely understood. Our laboratory demonstrated that angiotensin (Ang) II activates the intrinsic apoptotic pathway in primary endothelial cells (ECs) via reduction of the antiapoptotic protein Bcl-xL. Ang II decreased Bcl-xL mRNA half-life by reducing its binding to nucleolin, a protein that normally binds a 3′ AU-rich region and stabilizes Bcl-xL mRNA. We hypothesized HGF may block apoptosis induced by Ang II. We used primary EC and ex vivo cultures of rat lung tissue to investigate HGF inhibition of Ang II-induced apoptosis. Our data indicated HGF abrogated Ang II-induced apoptosis by inhibiting cytochrome c release, caspase-3 activation, and DNA fragmentation. RNA-immunoprecipitation experiments demonstrated that HGF stabilized Bcl-xL mRNA by increasing nucleolin binding to the 3′-untranslated region that was associated with cytoplasmic localization of nucleolin. Cytoplasmic localization of nucleolin and Bcl-xL mRNA stabilization required HGF activation of extracellular signal-regulated kinase (ERK)1/2, but not phosphatidylinositol 3-kinase 3-kinase. HGF also blocked Ang II-induced caspase-3 activation and lactate dehydrogenase release in tissue explants in an ERK-dependent manner.

INTRODUCTION

Hepatocyte growth factor (HGF) was originally identified as a potent mitogen for mature hepatocytes but has since been demonstrated to exert potent motogenic, mitogenic, and morphogenic activities in cells of epithelial and endothelial origin (Rubin et al., 1993). HGF activity plays a critical role in embryogenesis and organogenesis, but in the adult HGF functions in a variety of organs as a tissue repair factor (Matsumoto and Nakamura, 1993; Rubin et al., 1993). HGF promotes normal repair and prevents aberrant wound healing in several organ systems, including the lung, heart, kidney, and liver (Matsumoto and Nakamura, 1993; Kawaida et al., 1994; Kosiak et al., 1999; Dohi et al., 2000; Kitta et al., 2001). Animal studies show that administration of HGF induces proliferation of epithelial and endothelial cells and is believed to contribute to normal tissue regeneration.

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Address correspondence to: Regina M. Day (rday@usuhs.mil).

Abbreviations: Ang, angiotensin; BSA, bovine serum albumin; EC, endothelial cell; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HGF, hepatocyte growth factor; PI3K, phosphatidylinositol 3-kinase; UTR, untranslated region.

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The simultaneous or delayed administration of HGF protein to mice with bleomycin-induced lung injury was demonstrated to block fibrotic remodeling, including the prevention of epithelial cell apoptosis, the inhibition of fibroblast foci, and the inhibition of collagen accumulation (Yaekashiwa et al., 1997; Dohi et al., 2000). Ectopic expression of HGF, using either adenoviral expression or in vivo plasmid transient transfection, also was shown to inhibit bleomycin-induced lung remodeling (Ebina et al., 2002; Watanabe et al., 2005).

Met, the HGF receptor, is a tyrosine kinase receptor with a single transmembrane domain. HGF binding to Met induces phosphorylation in the tyrosine kinase domain as well as in a carboxy-terminal multifunctional docking domain, the site of activation of downstream signaling pathways (Ponzetto et al., 1994). Among the pathways activated by HGF are the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways, both associated with cellular survival and growth (Brunet et al., 1999; Day et al., 1999; Lee et al., 2008). The activation of cell survival and proliferation is thought to be critical for the antifibrotic functions of HGF (Kitta et al., 2001; Matsumoto and Nakamura, 2001).

Angiotensin (Ang) II was first studied for its role in blood pressure homeostasis but has more recently been shown to play a key role in fibrosis of lung, liver, kidney, and heart (Konigshoff et al., 2007; Wynn, 2008). Ang II is believed to play a dual role in the progression of dysfunctional wound repair by promoting the apoptosis of tissue epithelial and endothelial cells as well as inducing the growth and transdifferentiation of myofibroblasts (Filippatos et al., 2001; Marshall et al., 2004). Local synthesis of Ang II has been observed
in fibrotic plaques, and human myofibroblasts from patients with idiopathic pulmonary fibrosis were found to generate Ang II (Wang et al., 1999a). Furthermore, the inhibition of Ang II signaling was associated with the reduction of epithelial and endothelial cell apoptosis in an animal model of pulmonary fibrosis (Wang et al., 2000). Studies showed that Ang II induced apoptosis via the intrinsic apoptotic pathway in lung epithelial cells (Wang et al., 1999b).

Our laboratory recently demonstrated that Ang II activates the intrinsic apoptotic pathway in primary endothelial cells (ECs), including the release of cytochrome c, the activation of caspase-3, and caused DNA laddering (Lee et al., 2010). Ang II treatment of ECs resulted in the loss of Bcl-xL protein through the destabilization of its mRNA. We found that Ang II induced Src homology 2 domain-containing protein tyrosine phosphatase- (SHP-2-) dependent nuclear translocation of nucleolin, thus preventing its binding to the Bcl-xL mRNA 3'-untranslated region (UTR), leading to Bcl-xL mRNA instability and degradation and allowing the activation of the intrinsic apoptotic pathway.

Here, we examined the effects of HGF on Ang II-induced apoptosis in primary cultures of ECs. HGF inhibited the release of cytochrome c and activation of caspase-3 in response to Ang II. HGF protected the Bcl-xL mRNA from Ang II-induced degradation in an ERK1/2-mediated nucleolin-dependent pathway. Together, these data suggest that HGF protects endothelial cells against Ang II-induced apoptosis via activated ERK1/2-mediated cytoplasmic localization of nucleolin that stabilizes Bcl-xL mRNA, thus inhibiting the intrinsic apoptotic pathway.

### MATERIALS AND METHODS

#### Reagents

Angiotensin II was purchased from Bachem (Torrance, CA). Antibodies against cytochrome c and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-cleaved caspase-3 was purchased from Cell Signaling Technology (Danvers, MA). The Reagents sections were the gift of Dr. Tim Bowden (University of Arizona, Tucson, AZ; Zhang et al., 2008a). Purified human HGF was the gift of Dr. G. F. Vande Woude (Van Andel Research Institute, Grand Rapids, MI). Dominant-negative Ras in an adenoviral expression vector was provided by Dr. M. L. Culler (Univer-Sified Services University of the Health Sciences, Bethesda, MD). Constitutively active and dominant-negative (DN) mitogen-activated protein kinase kinase (MEK) 1 in an adenoviral expression vector was the gift of Dr. Y. J. Suzuki (Georgetown University, Washington, DC).

#### Cell Culture

Bovine pulmonary artery ECs were purchased from American Type Culture Collection (Manassas, VA). Passages 2–8 cells were used for all experiments and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen). Cells were grown and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Gemini Bioproducts, Woodland, CA), 1% penicillin/streptomycin, and 0.5% Fungizone (Invitrogen).}

**DNA Laddering Assay**

Cells were harvested and pelleted at 1000 x g. Pellets were resuspended and incubated on ice in lysis buffer (1x Tris-EDTA and 0.2% Triton, pH 8.0) for 15 min. Resuspended pellet was centrifuged (14,000 x g for 10 min at 4°C), and supernatant containing the fragmented DNA was collected. RNase A (final concentration, 60 mg/ml) was added and incubated for 30 min at 37°C. SDS was added to a final concentration of 0.5% along with 150 μg/ml protease K and incubated 2 h at 30°C. Then, 0.1 volume of 5 M NaCl and 1 volume of ice cold isopropanol were added, and samples were incubated on ice for 10 min. The samples were centrifuged at 13,000 x g for 15 min at 4°C. The DNA pellet was briefly dried and dissolved in 20 μl of TE buffer, followed by electrophoresis (~2 h at 20 V) in 1.5% agarose.

**RNA Isolation and Reverse Transcription (RT)**

Total RNA was obtained from bovine ECs by using the RNeasy kit (Qiagen, Valencia, CA). Genomic DNA was removed using the RnaFree DNAse set (Qiagen, Valencia, CA). RNA concentrations were measured spectrophotometrically at 260 nm (ND-1000 spectrophotometer; NanoDrop, Wilmington, DE, RNA (1.0 μg) was subjected to RT with GeneAmp RNA polymerase chain reaction (PCR) kit according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA).

**Semi-quantitative RT-PCR**

We used 1 μl of cDNA from the RT reaction for the PCR reaction containing 0.4 μM each primer, 200 μM each dNTP, 1 U of Taq DNA polymerase, and 1x PCR buffer (Bio-Rad Laboratories, Hercules, CA). The PCR primers for Bcl-xL mRNA were as follows: 5'-GGT ATT GGT GAG TCG CAT GAT CG and 5'-GCT GCA TTAG TTC CTC CAG TAG AG. PCR reactions were optimized for annealing temperatures using a temperature gradient in an iCycler (Bio-Rad Laboratories). Reactions were carried out for 25 cycles by using the following conditions: 95°C 1 min, 60°C 45 s, and 72°C 1.5 min. The last cycle extension was for 10 min at 65°C. PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: 5'-GAA GCT CGT -3'-CCA CTT GGT GAC TTT CAT CAA TGG AAA for cDNA amplification and 3'-CCA CTT GGT GAC TTT CAT CAA TGG AAA and 5'-CCA CTT GAT GGT GAC TTT CAT CAA TGG AAA for cDNA amplification. PCR reactions were analyzed on a 1.5% agarose gel in Tris EDTA buffer, and bands were visualized using ethidium bromide.

**Mitochondria and Mitochondria-free Cytosolic Protein Extraction**

Mitochondria extraction was performed using the mitochondria isolation kit for cultured cells according to the manufacturer’s protocol (Pierce Chemical).
Determination of mRNA Half-Life

Bovine ECs were treated as indicated above and then incubated with 5 μg/ml actinomycin D for time courses between 30 min and 8 h. Total RNA was isolated and the level of Bcl-xL mRNA was determined by quantitative PCR or by semi-quantitative RT-PCR (primers listed above). Levels of GAPDH mRNA were determined by gel electrophoresis and used for normalization of the semi-quantitative RT-PCR (primers are listed above). ImageJ software (http://www.uhnresearch.ca/facilities/wcif/index.htm) was used for quantification.

Plasmid Transfection

One day before transfection, cells were plated at 1.4 × 10^6 cells/well in a 12-well plate. DNA (1 μg/well) was transfected using the FuGENE 6 transfection reagent (Roche Applied Science, San Francisco, CA), according to the manufacturer’s instructions, in serum-free, antibiotic-free medium. For luciferase transfection, transfection mixtures contained a ratio of Runtell-Bcl-xL reporter construct to luciferase control vector (RSV-Luc) of 6:1 to normalize transfection efficiency.

Dual-Luciferase Assay

Transfected cells were washed twice with cold PBS, lysed with passive lysis buffer, and assayed for firefly and Renilla luciferase activities using the dual-luciferase assay (Promega, Madison, WI) according to the manufacturer’s instructions in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

RNA-Immunoprecipitation (IP)

Cells were harvested by centrifugation at 1000 × g for 3 min and resuspended in 10 ml of PBS. RNA and protein complexes were cross-linked by adding of 1% (vol/vol), final concentration, of formaldehyde and incubation at room temperature for 10 min with gentle mixing. Cross-linking was quenched by addition of glycine (pH 7.0, 0.125 mol/l, final concentration) at room temperature for 5 min. Cells were washed twice with 10 ml of PBS containing protease and RNase inhibitors. The pellet was resuspended in 0.2 ml of NP-40 buffer [5 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 8.0, 80 mM KCl, 0.5% NP-40, protease inhibitors, and RNase inhibitor] and incubated on ice for 10 min. Nuclei were pelleted by centrifugation at 1400 × g for 5 min at 4°C. Supernatant was sonicated three times for 20 s each at output level 6 of a sonic model 100 dismembrator (Thermo Fisher Scientific, Waltham, MA). The samples were cleared by centrifugation at 14,000 rpm for 10 min at 4°C and diluted 10-fold into IP buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 17 mM Tris, pH 8.1, 167 mM NaCl, protease inhibitors, and RNase inhibitor) to a final volume of 1 ml per immunoprecipitation reaction. A 1% aliquot was saved as an input sample. Nucleolin antibody (1:1000 dilution) was added to each tube, and immune complexes were allowed to form by gentle mixing on a rotating platform at 4°C overnight. To collect immune complexes, 50 μl of Sepharose beads was added and mixed gently for 2 h followed by centrifugation at 60 × g for 2 min at 4°C. The immune complexes were washed twice with 5 ml each with low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl), LiCl (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl). The tubes were washed twice with 1 ml of TE buffer, and 0.5% SDS was added and incubated for 1 h at 65°C. Immune complexes were eluted in 500 μl of elution buffer [1% SDS, 0.1 M NaHCO₃, and RNase inhibitor]. NaCl was added to a final concentration of 200 mM, and then samples were placed at 65°C for 2 h to reverse the cross-linking. Next, 20 μl of 1 M Tris-Cl, pH 6.5, 10 μl of 0.5 M EDTA, and 20 μg of proteinase K were added to each sample and incubated at 42°C for 45 min. RNA was extracted using phenol/chloroform with glycerol as a DNA-carrier. DNA was removed with DNase (QiAGEN). RT-PCR was performed using 1 μl of the cDNA reaction for 25 cycles: denaturing was performed at 95°C for 1 min, annealing for 45 s at 60°C, and polymerase reaction for 1.5 min at 72°C. Primers were as follows: Bcl-xL ARF-2, 5’-ACC TTC TTC ATG TGT CGT GGG-3’ and 5’-GCG GAA GAT GGT CAG AAA C-3’. Bcl-xL ARE-GGTT TGA GGG CTG CAC and 5’-ACT GCC TGC CAC AGA CCA GC.

Ex Vivo Lung Explants

All treatment of animals was according to National Institutes of Health, Department of Defense, and institutional guidelines. Sprague Dawley rats (Taconic Farms, Germantown, NY) were housed one female with litter per cage in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal rooms were maintained at 21 ± 2°C, 50 ± 10% humidity, and 12:12 light/dark cycle. Commercial rat chow (Harlan Teklad Rodent Diet 8604; Harlan, Indianapolis, IN) and water were freely available. Rat pups were obtained on postnatal day 10 or 11. Animals were anesthetized with pentobarbital and decapitated, or killed with Fatal Plus. The surface of the anterior chest wall and upper abdomen were excised, incised with a small nick was made to insert a 22-gauge needle with a short piece of polyethylene tube attached. Through a midline abdominal incision the chest cavity was exposed and the animal was exsanguinated by severing the abdominal aorta. The right ventricle was punctured and the lungs were perfused with sterile PBS to remove the blood. The trachea, lungs, and the heart were then aseptically dissected from the animal.

To obtain lung slices for the ex vivo culture, the lungs were inflated with 1% low melting point agarose dissolved in RPMI 1640 medium. The agarose was instilled via the trachea by using a syringe to fully inflate the lungs. The lung-heart blocks were placed in sterile cell culture plates at 37°C for at least 30 min to solidify the agarose. The heart was then excised from the lung, and each lobe of the lung was embedded on a cutting board with 1% agarose to prevent movement. The agarose-filled and embedded lungs were then cut into 500-μm-thick slices with a McIlwain tissue chopper (Geneq, Anjou, QC, Canada). Slices were incubated in RPMI 1640 medium containing 10% FBS, 1% streptomycin, and 1% penicillin/streptomycin for 1 h at 37°C in a humidified chamber with 5% CO₂. Lung slices were transferred to a 24-well cell culture plate and treated for 16–24 h in 37°C humidified chamber with 5% CO₂.

Statistical Analysis

Means ± SD were calculated, and statistically significant differences between two groups were determined using the Student’s t test. For three or more groups, statistical analysis was performed using one-way analysis of variance, followed by the Bonferroni’s post analysis, as appropriate; p < 0.05 was considered statistically significant. For mRNA half-life, linear regression was calculated and confidence intervals were determined. Statistical software for all analysis was SigmaStat 3.1 (Systat Software, Point Richmond, CA).

RESULTS

HGF Inhibits Ang II-induced Intrinsic Apoptosis

HGF attenuates cellular apoptosis associated with fibrotic remodeling in the lung (Yaekashiwa et al., 1997; Dohl et al., 2000), but the mechanism of this activity is not completely understood. Previously, our laboratory reported that the profibrotic agent Ang II induces expression of the proapoptotic pathway in primary ECs, with DNA fragmentation occurring within 24 h (Lee et al., 2010). We investigated the effect of HGF on Ang II-induced apoptosis in ECs by using the neutral comet assay, which detects chromosomal fragmentation as a function of apoptosis. Treatment with Ang II alone resulted in 65% apoptosis within 24 h. Pretreatment with HGF inhibited the Ang II-induced apoptosis, resulting in only 20% apoptosis (p < 0.05) (Figure 1A). To confirm that the cells are indeed undergoing apoptosis, we performed DNA laddering experiments. Data show that 25 ng/ml HGF pretreatment inhibited Ang II-induced DNA laddering (Figure 1B). HGF also prevented Ang II-induced caspase-3 activation and mitochondrial release of cytochrome c, as determined by western blot (Figure 1C).

HGF Inhibits Ang II Reduction of Bcl-xL mRNA Half-Life

The down-regulation of anti-apoptotic members of the Bcl-2 family, relative to pro-apoptotic family members is sufficient to induce apoptosis. Our laboratory demonstrated that Ang II treatment of EC decreased levels of the anti-apoptotic protein Bcl-xL, and thereby reduced interaction of Bcl-xL with the pro-apoptotic Bax protein (Lee et al., 2010). Pretreatment of EC with HGF before exposure to Ang II restored Bcl-xL mRNA expression to basal levels (Figure 2A) and preserved its interaction with Bax (Figure 2B).

We demonstrated previously that Ang II decreases the half-life of Bcl-xL mRNA (Lee et al., 2010). We therefore hypothesized that HGF would prevent Ang II-induced degradation of Bcl-xL mRNA. The level of Bcl-xL mRNA in the cells pretreated with HGF was stabilized in the presence of Ang II treatment (Figure 2C). These data suggest that one mechanism by which HGF prevents Ang II-induced decrease of Bcl-xL protein levels is through stabilization of the Bcl-xL mRNA.

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HGF-activated ERK1/2 Is Required for Inhibition of Ang II-induced Apoptosis

Binding of the Met receptor by HGF leads to the activation of two major antiapoptotic pathways, the ERK1/2 MAPK pathway and the PI3K pathway. We demonstrated that these pathways are both activated by HGF in ECs (Lee et al., 2008). We investigated whether HGF uses the ERK1/2 or the PI3K pathway to inhibit Ang II-induced apoptosis. EC were

Figure 1. HGF inhibits Ang II-induced intrinsic apoptosis in ECs. ECs were grown to 80% confluence and starved in 0.01% FBS media overnight. Cells were treated with HGF (25 ng/ml) for 20 min before 10 μM Ang II for 24 h. (A) Neutral comet assay on HGF and Ang II-treated EC was done. The percentage of apoptotic cells are indicated. Data show means ± SD, n = 4. Asterisk (*) indicates statistical significance from control, p < 0.05. Dagger (†) indicates statistical significance from Ang II alone, p < 0.05. (B) Equal amounts of DNA were electrophoresed in agarose gels to visualize DNA laddering; representative data are shown. (C) Cells were lysed and mitochondria were removed from the cytosolic components. Cytosolic lysates were western blot for cytochrome c and cleaved caspase-3. Right panels indicate densitometry, normalized to β-actin. Data show mean band density normalized to actin ± SD, n = 4. Asterisk (*) indicates statistical significance from control, p < 0.05. All experiments were repeated at least four times.

Figure 2. HGF inhibits Ang II-induced reduction of Bcl-xL protein and mRNA. ECs were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. Cells were pretreated with HGF (25 ng/ml) for 20 min before treatment with 10 μM Ang II for 16 h. (A) Cell lysates were immunoblotted for Bcl-xL protein expression; blots were stripped and re-probed for β-actin. Bottom panel shows densitometry of Bcl-xL normalized to β-actin from three independent experiments. (B) Equal amounts of protein from cell lysates were immunoprecipitated for Bax and western blotted for Bcl-xL. Blots were stripped and blotted for Bax. Bottom panel shows densitometry of Bcl-xL normalized to Bax from three independent experiments. (C) Cells were pretreated with HGF (25 ng/ml) for 20 min before treatment with 10 μM Ang II for 8 h. After the treatment, 5 μg/ml actinomycin D was added. At various time points, total RNA was extracted, and the levels of Bcl-xL mRNA were determined by RT-PCR. GAPDH was determined by RT-PCR as a control. For all graphs, data show means, ± SD, n = 3. Asterisk (*) indicates statistical significance from control, p < 0.05. At least three independent experiments were performed.
treated with Ang II and HGF with either the MEK1/2 inhibitor U0126 that blocks activation of ERK1/2 or LY294002 that inhibits PI3K activity. Ang II treatment alone induced ~75% apoptosis, and HGF pretreatment inhibited the cell death to ~20%, slightly above control levels (Figure 3A). U0126 treatment with HGF before the addition of Ang II eliminated HGF protection, and apoptosis was not statistically different from the addition of Ang II alone. LY294002 treatment had no significant effect on HGF inhibition of Ang II-induced apoptosis. When treated with both U0126 and LY294002 treatment before HGF and Ang II, the level of apoptosis was not significantly different from the level of apoptosis when U0126 was used alone. Addition of HGF, U0126, or LY294002 in the absence of Ang II did not induce significant apoptosis. Similar results were seen when using cleaved caspase-3 as a marker of apoptosis (Figure 3B). Ang II induced an ~3.5-fold increase in the band density of cleaved caspase-3 compared with control. HGF treatment reduced caspase-3 activation to basal levels. The inhibitory activity of HGF was significantly blocked by pretreatment with either U0126 or LY294002, although U0126 treatment demonstrated a greater inhibition of HGF activity. Addition of U0126 with LY294002 completely blocked HGF inhibition of Ang II-induced caspase-3 activation. The addition of HGF, U0126, or LY294002 in the absence of Ang II did not increase caspase activation above basal levels.

RAS and MEK1/2 are upstream signaling molecules of ERK1/2 pathway in Met signaling (Zhang and Vande Woude, 2003). Therefore, we used DN RAS to determine whether cell survival by HGF is also RAS dependent. DN RAS suppressed HGF-induced cell survival (Figure 3C). We next investigated the effect of overexpression of constitutively active (CA) MEK1 in ECs on Ang II-induced apoptosis. CA MEK1 overexpression provided complete inhibition of Ang II-induced apoptosis and did not affect the antiapoptotic activity of HGF (Figure 3D). Together these results suggest that HGF activation of Ras is required for its antiapoptotic activity, and activation of MEK1 alone is sufficient to inhibit Ang II-induced apoptosis.

HGF Protects the Interaction between Cytoplasmic Nucleolin and the 3'-UTR of the Bcl-xL mRNA

Nucleolin protein binds to 3'-UTR of Bcl-xL mRNA to stabilize the mRNA and prevent its degradation (Zhang et al., 2008a). Our laboratory showed that Ang II causes displacement of nucleolin from the Bcl-xL mRNA in ECs, resulting in a decreased half-life of the mRNA (Lee et al., 2010). RNA-IP was performed to test whether HGF prevents Ang II-induced displacement of nucleolin from Bcl-xL mRNA. Ang II

Figure 3. HGF-activated ERK1/2, but not PI3K, is necessary to inhibit apoptosis. (A and B) ECs were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. Cells were then pretreated with U0126 (10 μM), LY294002 (10 μM), HGF (25 ng/ml), or a combination for 20 min before treatment with 10 μM Ang II for 24 h. (A) Neutral comet assay. (B) Immunoblotting, using equal amounts of protein from whole cell lysates, was performed to determine the levels of cleaved caspase-3. Blots were stripped and blotted for β-actin to control for protein loading. (C and D) ECs were grown to 80% confluence and infected with adenovirus DN RAS or CA MEK for 48 h. Cells were then pretreated with HGF (25 ng/ml) for 20 min before treatment with 10 μM Ang II for 24 h. Neutral comet assay was performed. Data show means ± SD, n = 4. Asterisk (*) indicates statistical significance from control, p < 0.05. Dagger (†) indicates statistical significance from Ang II alone, p < 0.05. Experiments were repeated at least four times.
Figure 4. HGF stabilizes Bcl-xL mRNA binding to nucleolin. ECs were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. Cells were then pretreated with HGF (25 ng/ml) for 20 min before treatment with 10 μM Ang II for 24 h. (A) Cells were fixed and RNA-IP was performed using the C23-nucleolin antibody. RNA–protein complex was reverse cross-linked for 2 h, and proteinase K and DNase were added. Phenolchloroform RNA extraction was performed for cDNA synthesis. PCR was performed for the second AU-rich region of the 3'-UTR of Bcl-xL. Bottom panel shows densitometry of Bcl-xL mRNA band. Data show means ± SD, n = 3. Asterisk (*) indicates statistical significance from control, p < 0.05. (B) Cells were transfected with Renilla-Bcl-xL 3'-UTR and cotransfected with a luciferase vector RSV-Luc reporter (data not shown). HGF (25 ng/ml) before treatment with 10 μM Ang II for 16 h. Luciferase and Renilla assays were performed on cell lysates. Data show means ± SD. Asterisk (*) indicates statistical significance from control, p < 0.05, n = 3. Experiments were repeated at least three times.

Figure 5. HGF blocks nucleolin translocation to the nucleus. ECs were grown to 80% confluence and were placed in low serum (0.01% FBS) overnight. Cells were then pre-treated with HGF (25 ng/ml) for 20 min before treatment with 10 μM Ang II for 24 h. After Ang II ≥ HGF treatment, cytosolic and nuclear fractions were used for nucleolin immunoblotting. Blots were stripped and blotted for β-actin to control for protein loading. Data show means ± SD, n = 3. Asterisk (*) indicates statistical significance from control, p < 0.05. Experiments were repeated at least three times.

HGF Stabilization of the Binding of Nucleolin to the Bcl-xL 3'-UTR Requires ERK1/2 Pathway

Prior studies of HGF-induced cell survival suggested the involvement of two major pathways for this activity: the ERK1/2 and PI3K/Akt pathways (Day et al., 1999; Fan et al., 2000). To determine which pathway HGF uses for stabilization of the nucleolin–Bcl-xL mRNA complex, cells were treated with either the MEK1/2 inhibitor U0126 or PI3K inhibitor LY294002 before the addition of HGF and Ang II. Pretreatment with U0126 blocked the effect of HGF on Ang II-induced localization of nucleolin to the nucleus (Figure 6A and B). However, LY294002 treatment did not affect HGF inhibition of Ang II-induced nucleolin translocation. We next examined the effect of inhibiting the ERK1/2 pathway on the Bcl-xL mRNA levels. In agreement with our previous findings, quantitative RT-PCR showed that the MEK1/2 inhibitor U0126 blocked HGF inhibition of Ang II-induced down-regulation Bcl-xL mRNA (Figure 6C). Again, LY294002 treatment did not alter the level of the Bcl-xL mRNA induced by HGF treatment (Figure 6C). Using the Renilla expression construct containing the Bcl-xL 3'-UTR, we observed that HGF inhibited Ang II-induced decrease of Renilla expression (Figure 6D). Interestingly, levels of Renilla-Bcl-xL 3'-UTR levels were increased by HGF treatment alone to ~20% over control levels. ERK1/2 inhibition prevented HGF inhibition of Ang II destabilization of the Renilla reporter (Figure 6D). We found that LY294002 does not prevent HGF inhibition of Ang II destabilization of the Renilla reporter (data not shown).
To confirm the role of the Ras/MEK1/2/ERK1/2 pathway, EC were infected with adenovirus DN Ras or CA MEK1. When the cells were infected with DN Ras, the level of Bcl-xL decreased significantly even with the HGF treatment (Figure 6E). In addition, dominant-negative RAS inhibited HGF-induced ERK activation, showing that ERK activation by HGF is downstream of RAS activation (Figure 6E). In contrast, constitutively active MEK1 ex-
HGF Inhibits Ang II-induced Apoptosis in Ex Vivo Rat Lung Tissue

Ex vivo lung slice cultures have been used as an alternative to in vivo experiments for measuring cellular signaling mechanisms. In the ex vivo method, an isolated organ sustains the architecture and functionality of the tissue, thus representing a closer model to in vivo than the in vitro monoculture models from a single cell type. Studies have shown that alveolar cells divide and differentiate in ex vivo lung slice cultures (Kinnard et al., 1994), and this technique has been used for apoptosis and cell survival studies (Li et al., 2003; Lang et al., 2007; Hackett et al., 2008). The ex vivo lung model has been demonstrated to act as a reliable model for in vivo studies of the role of Ang II signaling inducing lung cell apoptosis downstream of profibrotic stimuli (Li et al., 2003; Mancini and Khalil, 2005). We therefore used lung explants with Ang II treatment with or without HGF to verify our EC monoculture findings. A lactate dehydrogenase (LDH)-cytotoxicity assay was performed to evaluate cell death. Ang II treatment induced an ~2.5-fold increase in LDH levels compared with the untreated control (Figure 7A). Cotreatment of Ang II with HGF reduced LDH to basal levels.

Ang II activated proapoptotic proteins in the lung slice explants, consistent with our findings in EC cultures (Figure 7B). The data demonstrate that HGF inhibited cleaved caspase-3 induced by Ang II treatment. Ang II treatment also lowered the level of Bcl-xL protein level, but with HGF, the level of Bcl-xL protein was similar to the control (Figure 7B). Interestingly, treatment of the lung explants with HGF alone reduced levels of caspase-3 and increased levels of Bcl-xL protein compared with basal levels. This suggests that HGF prevents cell death that might occur from the processing of the tissue for slice cultures.

HGF Activated ERK1/2 Inhibits Ang II-induced Apoptosis in Ex Vivo Rat Lung Slices

To confirm that HGF activation of ERK1/2 is necessary to inhibit Ang II-induced apoptosis in explant tissue, we first used chemical MEK1/2 inhibitor U0126. Ang II treatment of ex vivo rat lung explants increased levels of cleaved caspase-3, but pretreatment with HGF prevented cleavage of caspase-3 (Figure 8A, top). When the lung slices were pre-treated with U0126 before HGF and Ang II treatments, the level of active caspase-3 increased. To verify that U0126 can enter and inhibit MEK in the tissue, a control experiment was performed, showing that phosphorylated ERK1/2 is present in the tissue and can be inhibited by U0126 (Figure 8A, bottom). Next, we used overexpression of DN MEK1 to verify our findings using pharmacological inhibition of MEK to block ERK1/2 activation. Lung explant tissues were infected with either adenovirus-encoded green fluorescent protein (GFP), as a control, or adenovirus-encoded DN MEK1. Adenovirus-GFP was used to confirm viral incorporation and gene expression in the lung ex vivo explants (data not shown). After infection, lung explants were treated with HGF and Ang II. Overexpression of GFP did not affect HGF activation of ERK1/2, but overexpression of DN MEK1 blocked this activation (Figure 8B, left). Adenovirus-GFP tissues treated with Ang II contained increased active caspase-3, but HGF treatment significantly reduced cleaved caspase-3 levels (Figure 8B, right). Adenovirus-DN MEK1-infected tissue showed increased levels of caspase-3 activation in the presence of Ang II as well as HGF (Figure 8B, right). Together, the data suggest that HGF uses the ERK1/2 pathway to inhibit apoptosis in lung explant tissue.

DISCUSSION

HGF has been demonstrated to function as an antifibrotic factor, but the mechanism of this activity is not completely understood. Here, we demonstrate that HGF inhibits the apoptotic activity of Ang II, a key factor in the progression of fibrotic organ diseases (Filippatos et al., 2001; Marshall et al., 2004; Konigshoff et al., 2007; Wynn, 2008). We found previously that Ang II induces the intrinsic mechanism of apoptosis in EC through the reduction of Bcl-xL protein and mRNA levels (Lee et al., 2010). In our current work, we demonstrate using both in vitro and ex vivo models that HGF inhibits Ang II-induced apoptosis, blocking cytochrome c release from the mitochondria, caspase-3 activation, and DNA fragmentation. This blockade involves the stabilization of anti-apoptotic Bcl-xL mRNA via the binding of nucleolin. Our data indicate that HGF inhibition of Ang II-induced apoptosis, including stabilization of Bcl-xL mRNA, is dependent upon the activation of ERK1/2.

In cancer cells, the mechanism of HGF-induced cell growth and inhibition of apoptosis has been shown to involve two major pathways, ERK1/2 and PI3K (Day et al., 1999; Fan et al., 2000; Xiao et al., 2001). Others have shown that HGF can inhibit the intrinsic apoptotic pathway by dual mechanisms, involving phosphorylation and deactivation of proapoptotic Bcl-2 family members as well as the up-regulation of antiapoptotic Bcl-2 family members (del Peso et al., 1997; Zhang et al., 2008b). PI3K has shown to increase Bcl-xL gene expression to prevent apoptosis in some cell types (Umeda et al., 2003). In addition, previous studies have shown that Akt, a kinase activated downstream of PI3K, exerts antiapoptotic effects through the phosphorylation of...
nucleolin (Barel et al., 2003; Borgatti et al., 2003). Previous reports indicate that ERK1/2 also increases cell survival by several mechanisms, including via the regulation of the phosphorylation state of antiapoptotic proteins (Horiiuchi et al., 1997; Ito et al., 1997). Both PI3K and ERK1/2 pathways are activated by HGF to decrease caspase-3 activation after serum starvation or UV irradiation, although it is not known whether these effects are direct or indirect (Xiao et al., 2001).

Our current findings indicate that ERK1/2 inhibition, but not PI3K inhibition, blocks HGF survival activity in the presence of Ang II. Our data indicate that activation of the ERK1/2 pathway is both necessary and sufficient to inhibit Ang II-induced apoptosis. Results from cell culture experiments using the overexpression of CA MEK1 demonstrate that CA MEK1 alone prevents degradation of antiapoptotic Bcl-xL protein and inhibits Ang II-induced apoptosis. Overexpression of DN Ras, upstream of ERK1/2 activation, blocks HGF inhibition of Ang II-induced apoptosis. These findings further suggest that other growth factors that sufficiently activate the MEK/ERK pathway will also inhibit Ang II-induced translocation of nucleolin to the nucleus and its dissociation from the Bcl-xL mRNA with subsequent down-regulation of Bcl-xL protein and apoptosis.

Our data also indicate that HGF promotes the binding of the protein nucleolin to the Bcl-xL mRNA to prevent Ang II-induced reduction of this mRNA. The multifunctional protein nucleolin binds to RNA and DNA and plays roles in proliferation, apoptosis, and differentiation (Sengupta et al., 2004). In the cytoplasm, nucleolin protects Bcl-2 and Bcl-xL mRNAs from degradation by binding to AU-rich regions of the 3' UTRs, thus contributing to cell survival by stabilizing the levels of these anti-apoptotic members of the Bcl-2 family (Sengupta et al., 2004; Otake et al., 2001). Previously, we found that Ang II caused a decrease in Bcl-xL mRNA half-life by reducing the nucleolin binding to the second AU-rich region of the UTR of the mRNA (Lee et al., 2010). We demonstrated that although nucleolin binds to both Bcl-xL and Bcl-2 mRNA in EC, Ang II treatment specifically reduces nucleolin binding to Bcl-xL but not to Bcl-2 (Lee et al., 2010). This was reflected in the reduction of Bcl-xL protein but not of Bcl-2 protein, suggesting Bcl-2 protein does not play a major role in Ang II-induced apoptosis.

Several studies have indicated that nucleolin protein might play a pivotal role in the balance between cell survival and apoptosis. Down-regulation of nucleolin, by small interfering RNA or by agents that cause its degradation, is sufficient to induce growth arrest and apoptosis (Kito et al., 2003; Ugrinova et al., 2007). Nucleolin can be regulated through proteolysis, methylation, ADP-ribosylation, and phosphorylation (Srivastava and Pollard, 1999). Specific phosphorylations affect nucleolin's stability and subcellular localization (Schwab and Dreyer, 1997). In the cytoplasm, nucleolin plays a role in cell survival but its translocation to the nucleus is associated with proapoptotic events (Schwab and Dreyer, 1997). In the cytoplasm, nucleolin can be phosphorylated by protein kinase C (PKC), cyclic AMP-dependent protein kinase (PKA), and ectoprotein kinase (Srivastava and Pollard, 1999). Nucleolin contains its own nuclear translocation signal, but nuclear translocation of nucleolin requires dephosphorylation (Schwab and Dreyer, 1997). In the nucleus, nucleolin can be phosphorylated on serine residues by the cell cycle-regulated kinases casein kinase II and cell division cycle 2 protein, resulting in its localization back to the cytoplasm (Peter et al., 1990; Bouche et al., 1994). We recently determined that Ang II-induced nuclear translocation of nucleolin and its dissociation from Bcl-xL mRNA
requires the activation of the protein phosphatase SHP-2 (Lee et al., 2010). We also determined that neither PKC nor PKA affected apoptosis by Ang II (Lee et al., 2010), suggesting that these two kinases are not involved in this pathway. The exact mechanism of the effect of ERK activation on nucleolin is not known. This issue is currently under investigation in our laboratory.

Our work also demonstrates that HGF inhibits Ang II-induced apoptosis in lung tissue ex vivo. Studies using the ex vivo method have indicated that alveolar cells divide and correctly differentiate in ex vivo lung slice cultures (Kinnard et al., 1994), and this technique has been used for apoptosis and cell survival studies (Li et al., 2003; Lang et al., 2007; Hackett et al., 2008). The in vivo mechanism(s) of HGF-induced cell survival is currently being investigated in our laboratory. The identification of key downstream signaling for the inhibition of cellular apoptosis during the progression of fibrosis may lead to the development of novel drug targets for this disease.

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