Human insulin-like growth factor-IA expression in transgenic mice promotes adenomatous hyperplasia but not pulmonary fibrosis


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Insulin-like growth factor-I (IGF-I) is a 70-amino acid growth factor that regulates development, growth, repair, and aging through its actions on multiple tissues and cell types (17, 18). IGF-I exhibits pleiotropic activities that include the stimulation of cell proliferation and the protection of cells against apoptosis (26, 27). In addition, IGF-I has been shown to stimulate collagen cell proliferation and the protection of cells against apoptosis (26, 27). Likewise, blockade of the IGF-I signaling cascade appears to represent a viable anticancer therapy for a number of adenocarcinomas, including non-small cell lung cancer (34). Most responses to IGF-I are induced by ligation of the IGF-I receptor, a receptor tyrosine kinase that stimulates signaling cascades involved in the regulation of the cell cycle and cell survival, including ERK and Akt (26, 27). Collectively, these findings suggest that IGF-I plays an important role not only in lung development but also in pulmonary injury, fibrosis, and carcinogenesis.

To further investigate the role of IGF-I in bleomycin-induced pulmonary fibrosis and inflammation and in lung cancer, we developed a line of transgenic mice in which human IGF-IA is overexpressed by alveolar type II epithelial cells under the control of the human surfactant protein C (SP-C) promoter. In this report, we show that transgenic overexpression of human IGF-IA in pulmonary epithelial lining fluid increased the incidence of premalignant epithelial adenomatous hyperplastic lesions in older mice but did not affect pulmonary inflammation or fibrosis either basally or in response to intratracheal instillation of bleomycin.

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**MATERIALS AND METHODS**

**Materials.** Recombinant human and mouse IGF-I were obtained from R&D Systems (Minneapolis, MN). Anti-phospho (Ser 473)-Akt antibody (no. 9277) and anti-Akt antibody (no. 9272) were purchased from Cell Signaling Technology (Beverly, MA). The anti-phospho-ERK antibody (no. V8031) was from Promega (Madison, WI), and the anti-ERK1/2 antibody (ERK1/2-CT, no. 06 –182) was purchased from Upstate Biotechnology (Lake Placid, NY). Bleomycin (Bleocin) was generously donated by Bristol Squibb Myers (Princeton, NJ).

**Construction of the fusion gene.** A 358-bp fragment of a human IGF-IA cDNA (11) corresponding to bases 180–538 of the coding region was inserted into the plasmid pNeoNut (a gift from Dr. A. Joseph D’Ercole, University of North Carolina at Chapel Hill) between a rat somatostatin signal peptide (corresponding to bases 495–615) (10) and a polyadenylation sequence derived from the human growth hormone gene (7). The mouse metallothionein promoter sequences (25) of the plasmid pNeoNut were replaced with a 3.7-kb SacI/Kpn1 human genomic fragment corresponding to the human SP-C promoter (13) (a gift from Dr. Jeffrey A. Whitsett, University of Cincinnati College of Medicine) to generate the plasmid SP-C/IGF-I, an IGF-IA expression plasmid under control of the surfactant-associated protein C promoter. The original pNeoNut plasmid driving IGF-I expression under control of the metallothionein promoter was previously shown to express IGF-IA in stably transfected Chinese hamster ovary cells (7). The SP-C/IGF-I plasmid was sequenced at the University of North Carolina Automated Sequence Facility using the dye terminator method (31) to ensure no mutations in the construct and the correct reading frame for translation.

**Generation of transgenic mice.** The human SP-C promoter/IGF-I fusion gene was excised from plasmid sequences by digestion with SacI and EcoRI restriction enzymes, isolated by gel electrophoresis in low melting temperature agarose (FMC Bioproducts, Rockland, ME), and purified from the agarose. The fusion gene was then precipitated with 2.5 vol of 95% ethanol at −20°C. After washing with 70% ethanol was completed, the DNA pellet was dissolved in TE buffer (10 mM Tris-HCl, pH 7.2, 1 mM EDTA). Microinjection of the fusion gene was performed at the Transgenic Mouse Facility of the Program in Molecular Biology and Biotechnology, University of North Carolina, using hybrid C3H/CS7B6f fertilized mouse eggs.

**Identification of transgenic mice.** Mice expressing the human IGF-IA transgene were identified via PCR of the IGF-IA insert. Tail clips were obtained from each experimental mouse and digested overnight at 37°C with proteinase K in a shaking water bath. Tail clips were obtained from each experimental mouse and digested overnight at 37°C with proteinase K in a shaking water bath. DNA was precipitated from the agarose. The fusion gene was then precipitated with 2.5 vol of 95% ethanol at −20°C. After washing with 70% ethanol was completed, the DNA pellet was dissolved in TE buffer (10 mM Tris-HCl, pH 7.2, 1 mM EDTA). Microinjection of the fusion gene was performed at the Transgenic Mouse Facility of the Program in Molecular Biology and Biotechnology, University of North Carolina, using hybrid C3H/CS7B6f fertilized mouse eggs.

**Western blotting.** We detected specific proteins in cell lysates using Western blot analysis. Primary mouse lung fibroblasts were plated at 2.5 × 10^4 cells/well in six-well tissue culture plates and allowed to adhere overnight. Cells were washed in serum-free medium and allowed to quiesce for 30 min before stimulation with mouse IGF-I, human recombinant IGF-I, or serum-free medium alone. Media were removed at the specified time points, and 400 μl of lysis buffer [50 mM Tris-HCl, pH 7.4, 136 mM NaCl, 10% (vol/vol) glycerol, 1% NP-40, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM NaF] were added to each well of the six-well plate. The cells were scraped off the plate and processed as described (6).

**Primary mouse lung fibroblast isolation.** Primary cultures of mouse lung fibroblasts were derived from 8- to 12-wk-old male mice. Mice were euthanized by carbon dioxide asphyxiation at 14 and 28 days after instillation. Mice were euthanized with carbon dioxide asphyxiation at 14 and 28 days after instillation. All animals were euthanized with 200 μl of intraperitoneally administered Avertin to unconsciousness. The skin overlying the trachea was cleaned with ethanol, and a single incision was made in the skin. Bleomycin (4 U/kg) was administered at a concentration of 1.6 × 10^3 U/ml in sterile saline or an equivalent volume of saline was then instilled intratracheally after orotracheal intubation with a 22-gauge gavage. Gavage location was removed in a sterile fashion, and the tissue was minced into 1- to 2-mm³ sections and placed into tissue culture in 10% FCS enriched tissue culture media on scored plates. After 5–10 days, pulmonary fibroblasts growing out of the tissue were passaged for routine tissue culture. All experiments were performed on early passage (passages 2–4) cells.

**Bleomycin instillation.** Mice between the ages of 8 and 12 wk were weighed, given an identification number, and instilled intra-tracheally with either bleomycin or an equivalent volume of saline. Tail clips were obtained, and the genotype of each mouse was subsequently determined (investigators were blinded to genotypic identity until data analysis). Mice were then euthanized at 14 or 28 days after instillation, and studies were performed as outlined below. All mice were anesthetized with 300 μl of intraperitoneally administered Avertin to unconsciousness. The skin overlying the trachea was cleaned with ethanol, and a single incision was made in the skin. Bleomycin (4 U/kg) was administered at a concentration of 1.6 × 10^3 U/ml in sterile saline or an equivalent volume of saline was then instilled intratracheally after orotracheal intubation with a 22-gauge gavage. Gavage location was removed in a sterile fashion, and the tissue was minced into 1- to 2-mm³ sections and placed into tissue culture in 10% FCS enriched tissue culture media on scored plates. After 5–10 days, pulmonary fibroblasts growing out of the tissue were passaged for routine tissue culture. All experiments were performed on early passage (passages 2–4) cells.

**Human IGF-I quantitation.** Bronchoalveolar lavage fluid (BALF) was thawed, and 500 μl of each sample were loaded into a Microcon YM-3 centrifugal filter device (Millipore, Bedford, MA). Samples were spun at 10,000 rpm in an Eppendorf benchtop centrifuge for 100 min. The concentrated samples were then assayed with a Quantikine human IGF-I immunoassay kit (R&D Systems) according to the manufacturer’s instructions. Duplicate samples were performed for all mice, and the mean result is reported. No cross-reactivity with mouse IGF-I was detected with either recombinant or endogenous mouse IGF-I.

**Hydroxyproline quantification.** Total lung hydroxyproline levels were used as a well-validated marker of bleomycin-induced fibrosis.
Mouse lung fibroblasts respond to human IGF-I. Before conducting in vivo experiments using human IGF-I in a mouse model, we first determined whether mouse lung cells responded to human IGF-I with the same efficacy as mouse IGF-I. Primary cultures of mouse lung fibroblasts were stimulated with either recombinant human IGF-I, recombinant mouse IGF-I, or medium alone. The cells were then lysed and analyzed for activation of ERK and Akt signaling by SDS-PAGE and Western blot analysis using phospho-Akt and phospho-ERK antibodies. Both Akt and ERK were phosphorylated to a similar extent in response to human IGF-I and mouse IGF-I but not in response to medium alone (Fig. 1). Densitometry revealed a 7.7-fold increase in phospho-Akt relative to total Akt at 60 min after stimulation with mouse IGF-I (100 ng/ml) and a 10.1-fold increase in response to human IGF-I (100 ng/ml). Similarly, there was a 2.0-fold and a 2.5-fold increase in phospho-ERK relative to total ERK at 15 min in response to mouse and human IGF-I, respectively. Thus the activity of human IGF-I and mouse IGF-I with respect to mouse cells appears equivalent.

Human IGF-I is expressed in the lungs of the transgenic animals. Creating mice that express human IGF-IA (rather than mouse IGF-I) under the control of the human surfactant protein C promoter allowed us to investigate the consequences of pulmonary overexpression of IGF-I (14) and to distinguish between endogenous and transgenic IGF-I production. We initially determined whether the transgenic mice expressed human IGF-I protein in their alveolar air spaces as would be predicted by their genotype. Bronchoalveolar lavage was performed on transgenic mice and wild-type littermate controls, and the levels of human IGF-I in the BALF were determined by ELISA. Figure 2 shows that the transgenic animals secrete human IGF-I into their air spaces, whereas the wild-type animals exhibit no detectable human IGF-I (P < 0.0001).

Effect of transgenic expression of human IGF-IA on lung histology, inflammatory cell accumulation, and collagen deposition. We investigated the effects of transgenic expression of human IGF-IA on gross lung histology, inflammatory cell numbers, and basal collagen matrix levels. Grossly, the transgenic mice were indistinguishable from their wild-type littermate controls. Baseline weights were not different (25.9 ± 1.5 g for wild-type animals vs. 25.5 ± 1.6 g for transgenic animals). Blinded review of lung histology by an expert lung pathologist revealed no appreciable differences between the transgenic mice and wild-type littermate controls, and both groups were characterized as normal mouse lung without atypical features or identifiable abnormalities (Fig. 3A). Quantification of the cellularity of the BALF revealed no significant differences between the human IGF-IA-expressing mice and wild-type littermate controls with regard to either total numbers of nucleated cells (P = 0.499) (Fig. 3B) or differential cell counts (Fig. 3C). In addition, basal levels of total lung collagen, as quantified by hydroxyproline levels, were not significantly different (P = 0.524) between the human IGF-IA-overexpressing mice and the wild-type littermates (Fig. 3D). Thus transgenic expression of human IGF-IA by alveolar type II epithelial cells had no demonstrable effect on basal measurements of gross lung pathology, bronchoalveolar lavage cellularity, or total lung collagen content.

Effect of transgenic expression of human IGF-IA on bleomycin-induced pulmonary inflammation. We next investigated the effects of pulmonary overexpression of human IGF-IA on the development and progression of pulmonary inflammation in response to intratracheal instillation of bleomycin. Groups of human IGF-IA-expressing mice and wild-type littermate controls were instilled intratracheally with ~50 μl of bleomycin (4 U/kg) or saline vehicle. The mice were then subjected to bronchoalveolar lavage for analysis of BALF cellularity and IGF-I levels. All measurements were made at both 14 and 28 days after instillation. As can be seen in Fig. 4A, instillation of human IGF-IA-expressing mice with saline alone resulted in a significant decline in the level of human IGF-I detected in the BALF at 14 days (P < 0.0001). However, the level was restored to basal levels by 28 days. Bleomycin-instilled mice also showed a decline in BALF human IGF-I levels at 14 days compared with saline-instilled mice (P = 0.527). However, in
contrast to saline instillation, the reduction in IGF-I levels was sustained at 28 days in mice that had been instilled with bleomycin (\(P = 0.01\)) (Fig. 4A).

Figure 4B illustrates the changes in cellularity of the BALF in human IGF-IA-expressing mice and wild-type littermate controls in response to instillation of bleomycin or saline vehicle. Instillation of saline alone modestly increased the number of nucleated cells in BALF at 14 days but not at 28 days. In contrast, and as previously reported (23), intratracheal instillation of bleomycin into wild-type mice induced a significant increase in BALF cellularity at both 14 and 28 days (\(P < 0.0001\)). However, there was no significant difference (\(P = 0.869\)) in the BALF cellularity between the IGF-IA transgene-expressing mice and wild-type littermates (Fig. 4B). We also determined the differential cell counts from the BALF of all experimental conditions (Table 1). Marked increases in the numbers of foamy macrophages and lymphocytes were detected in response to instillation of bleomycin. However, no significant differences (all \(P\) values \(\leq 0.1\)) were detected in the number of these cells between human IGF-IA-expressing mice and wild-type littermate controls. Collectively, these data suggest that pulmonary inflammation in response to bleomycin is similar in human IGF-IA transgenic and wild-type mice.

Effect of transgenic expression of human IGF-IA on bleomycin-induced pulmonary fibrosis. Given that increased macrophage and alveolar epithelial cell expression of IGF-I has been detected in, and linked to, disease severity in IPF, we also investigated the effects of alveolar epithelial cell expression of human IGF-IA on the development of pulmonary fibrosis in the bleomycin model. As can be seen in Fig. 5A, instillation of bleomycin induced a significant increase (\(P < 0.0001\)) in total lung hydroxyproline levels in control wild-type littermates compared with either unstilled mice or mice that were instilled with saline alone. Similar levels of hydroxyproline were detected at both 14 and 28 days. Intratracheal instillation of bleomycin into human IGF-IA transgenic mice also produced an increase in total lung hydroxyproline content. However, there was no significant difference in the hydroxyproline levels of lungs from human IGF-IA-expressing mice compared with wild-type littermate controls (\(P = 0.637\)). Similarly, histological sections of lungs from both the human IGF-IA-expressing mice and wild-type littermate controls showed characteristic bleomycin-induced lung injury with patchy interstitial thickening accompanied by increased numbers of fibroblasts, collagen deposition, and inflammation. Pathological review revealed no differences between the two groups (Fig. 5B).

Transgenic pulmonary expression of human IGF-IA promotes adenomatous hyperplasia. No abnormalities, including early tumor formation, were identified on pathological review of 8- to 12-wk-old mice. We therefore “aged” nine transgenic animals and eight wild-type littermate controls to greater than 1 yr of age (13–16 mo) and submitted the lungs for blinded, expert histopathological review. The histology of the older transgenic animals revealed significantly increased foci of adenomatous hyperplasia compared with wild-type littermate controls. Of the transgenic mice, nine of nine exhibited adenomatous hyperplasia compared with only four of eight of the wild-type littermate controls (\(P = 0.03\)). These foci were

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Fig. 2. hIGF-I is present in bronchoalveolar lavage fluid (BALF) from transgenic mice expressing IGF-IA (IGF-IA\(^{TG}\)) but not in wild-type littermate controls. Bronchoalveolar lavage was conducted on euthanized mice, and hIGF-I was detected by ELISA.

Fig. 3. Effect of transgenic expression of hIGF-IA on baseline lung histology, inflammatory cell accumulation, and collagen deposition. A: hematoxylin and eosin staining of sections of whole mouse lung preparations from IGF-IA transgene-expressing animals (IGF-IA\(^{TG}\)) and wild-type littermate controls. B: total numbers of nucleated cells in BALF. C: differential cell counts. PMN, polymorphonuclear neutrophils. D: basal levels of total lung collagen.

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**L.808 TRANSGENIC PULMONARY EXPRESSION OF IGF-I IN MICE**

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characterized by hyperplastic, hypertrophic airway epithelium lining small stretches of alveolar septae, and small airways (Fig. 6A). With regard to spontaneous pulmonary adenoma formation, six adenomas were identified among the nine transgenic animals for an incidence of 0.67 tumors/animal, whereas three were identified among the eight wild-type animals for an incidence of 0.375 tumors/animal (Fig. 6B). However, this difference did not achieve statistical significance (P = 0.454). The adenomas were characterized by nodular foci of hyperplastic and crowded epithelial cells (Fig. 6C). The nuclei of these cells were monotonous and without evidence of atypia or mitotic figures. There were focal clusters of macrophages within the air spaces of the larger adenomas. No appreciable differences in the histological type of tumor were noted between the transgenic mice and the wild-type littermate controls. Thus, in older mice, the overexpression of human IGF-IA appears to promote premalignant adenomatous hyperplastic lesions, precursors of adenomas.

DISCUSSION

Increased expression of IGF-I has been shown to be associated with prenatal lung development and alveolarization (21). IGF-1 has also been strongly implicated in the pathophysiology of both IPF and non-small cell lung cancer (18, 35). To investigate the function of increased expression of IGF-I in alveolar air spaces, we created transgenic mice in which human IGF-IA was expressed under the control of the alveolar type II epithelial cell-specific SP-C promoter. We demonstrated that the mice had the expected genotype and characterized the effect of pulmonary expression of human IGF-IA on baseline pulmonary pathology. In addition, we investigated the effect of human IGF-I expression on the development of lung injury and fibrosis in response to intratracheal delivery of bleomycin. Pulmonary expression of human IGF-IA did not alter either baseline lung characteristics in young (8- to 12-wk-old) mice or the severity of bleomycin-induced lung injury or fibrosis. However, in older mice (>1 yr), the expression of human IGF-IA was found to augment the development of adenomatous hyperplasia, a precursor of adenoma and adenocarcinoma development.

In view of the extensive in vitro and in vivo data supporting a role for IGF-I in the development of pulmonary fibrosis, the lack of effect of pulmonary overexpression of human IGF-IA on bleomycin-induced pulmonary fibrosis was unexpected. We have considered four plausible explanations for these observations. First, IGF-I activity and availability are regulated by insulin-like growth factor binding proteins (IGFBP), which predominantly inhibit, but in the case of IGFBP-1, -3, and -5, may also enhance IGF-I activity (2). In particular, when cells are preincubated with IGFBP-3, IGF-I-dependent proliferation is enhanced, whereas the concurrent administration of IGFBP-3 with IGF-I results in diminished IGF-I effects (2). Moreover, IGFBP-3 and -5 have well-characterized IGF-I-independent effects (2). For example, IGF-I-independent,
IGFBP-3-dependent induction of apoptosis is well described (2). Specifically as regards pulmonary fibrosis, IGFBP-2 and IGFBP-3 have been shown to be elevated in BALF in patients with IPF (1, 24). Therefore, bleomycin-induced changes in the levels of IGFBPs, especially IGFBP-2 and -3, could inhibit or mask the effect of IGF-I overexpression through decreased bioavailability of IGF-I or by IGF-I-independent mechanisms, as in the case of IGFBP-3.

Second, mature IGF-I is produced by proteolysis of either IGF-IA or IGF-IB. However, a recent study by Bloor et al. (4) showed that the expression of IGF-IA mRNA was decreased in bronchoalveolar cells from IPF subjects compared with normal controls, whereas the expression of IGF-IB transcripts was increased. Although this may initially seem inconsequential in terms of the production of mature IGF-I, studies by Siegfried et al. (32) have shown that the 77-residue E peptide produced by proteolysis of IGF-IB exhibits intrinsic mitogenic activity. In contrast, the E peptide from IGF-IA was inactive (32). It is therefore possible that the fibrogenic activity of IGF-I is specific to the IGF-IB splice variant and is not associated with IGF-IA.

Third, the SP-C promoter results in luminal secretion of transgenic IGF-I. Previous studies have shown that, in IPF, IGF-I is localized to CD68-positive alveolar and interstitial macrophages as well as to alveolar epithelial cells. However, associations between increased IGF-I expression and disease severity were only observed for IGF-I localization to interstitial macrophages (35), suggesting that IGF-I expression by interstitial macrophages may be functionally important in the development or progression of IPF. Likewise, studies in an asbestosis model in sheep have shown that transforming growth factor-β expression is restricted to alveolar macrophages and epithelial cells, whereas IGF-I expression was also detected in interstitial cells (16). Similar compartment-specific expression patterns exist in the developing lung in which IGF-I, IGF-II, and IGF receptor levels vary considerably between mesenchymal, epithelial, and vascular tissues (29). Thus it is possible that air space delivery of human IGF-IA does not provide an appropriate localization for IGF-I to induce or increase pulmonary fibrosis.

Fourth, the fibrotic response is dependent on a large number of genes, cytokines, growth factors, and choreographed cellular responses, and thus it is possible that overexpressing a single gene product may not be sufficient to significantly alter the development of pulmonary fibrosis. Lastly, with regard to the lack of a significant difference in bleomycin-induced fibrosis...
between the two groups of mice, it is possible that the control animals have already achieved a peak effect with endogenous mouse IGF-I such that additional human IGF-I does not detectably alter the degree of observed fibrosis.

The decline seen in IGF-I levels in the BALF after bleomycin instillation was expected, as bleomycin has previously been shown to produce pronounced pulmonary inflammation and epithelial cell injury and apoptosis. However, the degree and extent of the declines in IGF-I levels after saline instillation were unexpected. Although we are not aware of any previous studies showing saline-induced effects on pulmonary inflammation, others have found that saline instillation induces a transient airway and/or air space inflammation lasting for several days (P. M. Henson, personal communication).

In contrast to its lack of effect on the development of pulmonary fibrosis, transgenic expression of human IGF-IA was found to increase premalignant adenomatous hyperplasia in old but not in young mice. Although there was a trend toward increased adenoma formation in the aged transgenic mice, this increase was not statistically significant. However, similar findings have been reported by DiGiovanni et al. (8) who examined the effects of overexpression of human IGF-I in mouse prostate epithelium. They found that overexpression of human IGF-I alone in the prostatic epithelium was sufficient to induce a step-wise progression of preneoplastic changes progressing from atypical hyperplasia to frank adenocarcinoma formation (8). Moreover, similar to the findings in our study, adenocarcinoma formation occurred exclusively in mice aged >6 mo and preferentially in mice aged >9 mo. Another recent study by Ng et al. (22) showed that exogenous administration of growth hormone and IGF-I to aging primates resulted in mammary gland hyperplasia, with four- to fivefold increases in IGF-I/H11022 expression in patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. Am J Respir Cell Mol Biol 21: 693–700, 1999.


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