Bleomycin-induced pulmonary fibrosis is attenuated by a monoclonal antibody targeting HER2

Jihane A. Faress,1,4 David E. Nethery,1 Elizabeth F. O. Kern,2,4 Rosana Eisenberg,3 Frank J. Jacono,1,4 Chris L. Allen,1 and Jeffrey A. Kern1,4

Divisions of 1Pulmonary, Critical Care and Sleep Medicine, and 2Clinical and Molecular Endocrinology, and 3Departments of Internal Medicine and Pathology, University Hospitals Case Medical Center, Case Western Reserve University, and 4Louis Stokes Cleveland VA Medical Center, Cleveland, Ohio

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Bleomycin-induced pulmonary fibrosis is attenuated by a monoclonal antibody targeting HER2. J Appl Physiol 103: 2077–2083, 2007. First published October 4, 2007; doi:10.1152/japplphysiol.00239.2007.—The importance of HER2/HER3 signaling in decreasing the effects of lung injury was recently demonstrated. Transgenic mice unable to signal through HER2/HER3 had significantly less bleomycin-induced pulmonary fibrosis and showed a survival benefit. Based on these data, we hypothesized that pharmacological blockade of HER2/HER3 in vivo in wild-type mice would have the same beneficial effects. We tested this hypothesis in a bleomycin lung injury model using 2C4, a monoclonal antibody directed against HER2 that blocks HER2/HER3 signaling. The administration of 2C4 before injury decreased the effects of bleomycin at days 15 and 21 after injury. HER2/HER3 blockade resulted in less collagen deposition (362.8 ± 37.9 compared with 610.5 ± 27.1 µg/mg; P = 0.03) and less lung morphological changes (injury score of 1.99 ± 1.55 vs. 3.90 ± 0.76; P < 0.04). In addition, HER2/HER3 blockade resulted in a significant survival advantage with 50% vs. 25% survival at 30 days (P = 0.04). These results confirm that HER2 signaling can be pharmacologically targeted to reduce lung fibrosis and remodeling after injury.

bleomycin lung injury; HER2 blockade

RECURRENT INJURY OF THE ALVEOLAR EPITHELIUM and abnormal repair is critical in the pathogenesis of pulmonary fibrosis (26). The epidermal growth factor receptor family (EGFR) plays a central role in the recovery from lung injury and the development of fibrosis. Acute lung injury increases EGFR expression and upregulates the production of the EGFR ligand transforming growth factor (TGF)-α (18). Increased TGF-α production by the pulmonary epithelium leads to severe pulmonary fibrosis (15) with the degree of lung remodeling directly proportional to the level of TGF-α expression (9). Transgenic mice expressing both TGF-α and a mutant EGFR incapable of signaling in response to ligand, both placed under the control of the human SPC promoter, are protected against pulmonary fibrosis (8). Finally, TGF-α knockout mice have significantly reduced fibrosis after bleomycin lung injury (10). These data confirm the importance of EGFR signaling and the pulmonary epithelium in regulating fibrosis.

The EGFR family consists of four membrane-bound proteins (HER1/EGFR, HER2, HER3, and HER4) that homodimerize or heterodimerize with each other to form functional receptors.

HER2 also appears important in promoting epithelial recovery after injury. In vitro, disruption of a pulmonary epithelial cell monolayer resulted in HER2 activation (30). Inhibition of HER2 increased, whereas activation of HER2 decreased, time to monolayer reformation. These data led us to hypothesize that HER2 signaling plays an important role in recovery from lung injury in vivo. We developed a transgenic mouse strain expressing a dominant negative HER3 (DNHER3) under SPC promoter control to test this hypothesis (20). The DNHER3 has its intracellular domain deleted, a domain required for HER2 signaling (25), thus effectively inhibiting HER2 activation in the pulmonary epithelium. After bleomycin injury, the DNHER3 strain had decreased collagen deposition, preserved airspace and epithelial cell volume density, and a survival advantage compared with wild-type littermates (20). We concluded that inhibition of HER2/HER3 signaling in the pulmonary epithelium after lung injury decreased mortality through decreased fibrosis and lung remodeling.

However, the importance of HER2 signaling after lung injury in a wild-type genetic background and the potential for translation of these studies as a therapeutic strategy remain unclear. Therefore, we hypothesized that pharmacological blockade of HER2 signaling in wild-type mice would also decrease lung collagen deposition and improve survival after bleomycin-induced lung injury. We blocked HER2/HER3 signaling using the monoclonal antibody 2C4 directed against the extracellular domain of HER2. This antibody inhibits HER2 dimerization and activation (27). Our studies show that pharmacological inhibition of HER2/HER3 signaling in wild-type mice decreased lung collagen deposition, improved the pathological lung injury score, and improved survival after bleomycin lung injury.

MATERIALS AND METHODS

Bleomycin lung injury. Male C57BL/6 mice, 6 wk old, were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in a pathogen-free environment. All procedures were performed under a protocol approved by the Case Western Reserve University Institutional Animal Care and Use Committee. 2C4, a monoclonal antibody targeting the extracellular domain of HER2 (7, 13), was a gift of Genentech (San Francisco, CA). 2C4 binds the HER2 ectodomain and blocks HER2 heterodimerization with other HER receptors. An isotype-matched monoclonal antibody (IgG1K) directed against an irrelevant epitope was from

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Sigma (15154-085K6113; St. Louis, MO) and was used as a control. Mice were loaded with 2C4 (30 mg/kg) by intraperitoneal (IP) injection, whereas control animals were injected with an equivalent dose of IgG1k. Seventy-two hours later, a maintenance dose of 2C4 (15 mg/kg) or IgG1k was injected IP followed by intratracheal injection of 0.04 U of bleomycin. Mice were anesthetized with an IP injection (0.2 ml) of a mixture of ketamine HCl (8.6 mg/ml), xylazine HCl (1.7 mg/ml), and acepromazine (0.29 mg/ml) in sterile PBS. A skin incision was made, and the trachea was exposed. Bleomycin (Sigma) was injected intratracheally (0.04 U) using a Tridak stepper (Indicon, Brookfield, CT) and a 30-gauge needle. Control animals were injected with an equal volume of PBS. The skin was then closed with surgical glue, and the mice were allowed to recover. The day of bleomycin injury was considered day 0. 2C4 and IgG1k maintenance injections were continued twice a week throughout the study.

Bronchoalveolar lavage and lung collection. At days 0, 3, 15, and 21, mice were killed with an IP overdose (0.2 ml) of ketamine HCl (43 mg/ml), xylazine HCl (8.5 mg/ml), and acepromazine (1.45 mg/ml) in sterile PBS. A skin incision was made from the neck to the abdominal cavity, the ribs were removed, and the trachea, heart, and lungs were exposed. The trachea was cannulated with a blunt 21-gauge needle, and the lungs were lavaged with three aliquots of sterile PBS (0.5 ml) with more than 90% fluid recovery. The bronchoalveolar lavage (BAL) fluid was centrifuged (1,500 rpm, 10 min, 4°C), and the cell pellet was separated from the supernatant. The cells were resuspended in PBS (250 μl) and counted on a hemocytometer. Nucleated cells (20,000) were applied to slides using a Cytospin centrifuge (ThermoShandon, Pittsburgh, PA). Slides were dried overnight and stained with modified Wright stain (Diff-Quik, Dade Behring, Deerfield, IL) for cell count and differential. The BAL supernatant was stored at −80°C until used for protein analysis. Following the BAL, a right ventricle flush with PBS was performed, and the lungs were dissected free of the chest. Lungs were homogenized in cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100) containing protease inhibitors (50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM EDTA, 0.4 mM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate) for protein and collagen analysis, using a Polytron homogenizer. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA).

Lung fixation and pathology scoring. The lungs of mice killed at days 15 and 21 were inflated with 10% formalin to 20 cmH2O pressure and fixed for 30 min for histological analysis. The lungs were removed en bloc and transferred to a cassette and paraffin embedded, and 5-μm sections were cut and stained with hematoxylin and eosin. Fibrosis was quantified using the Ashcroft scoring system (1) by a pathologist blinded to the treatment protocol. Sections of both lungs from two depths separated by 150 μm were examined from each animal at ×200 magnification. The entire lung was assessed for fibrosis. The degree of fibrosis was graded from 0 (normal lung) to 8 (severe distortion of structure, large fibrous areas, and honeycomb lesions). The mean score from all fields (average 50/animal) was taken as the fibrosis score.

Collagen quantification. Histology slides were stained with sirius red, and the collagen staining was quantified. Paraffin-embedded lung sections from mice killed at day 21 after bleomycin injury and stored at −20°C were brought to room temperature. Sections were deparaffinized with xylene, rehydrated through decreasing concentrations of alcohol, then equilibrated in PBS. Sections were stained with sirius red for 20 min at room temperature and mounted for microscopic analysis. Sections of whole lungs from three to four mice in each group were examined at ×200 magnification. Ten random fields from one depth were scored for collagen staining as follow: 0 for no staining identified, 1 for staining occupying <25% of the area of the field, 2 for 25–50%, and 3 for >50% staining. The numbers were then averaged and divided by 10. The scoring was done by two reviewers, and the mean score of each group was reported. In a separate experiment, soluble collagen in lung homogenates was measured by Sircol Assay (Biocolor, Newtownabbey, Ireland) following the manufacturer’s instructions. Collagen content was expressed as microgram of collagen per milligram of protein of the total lung homogenate.

Western blot analysis. Phosphorylated HER2 (p-HER2) was measured in lung tissue by Western blot technique as described previously (16). Equal protein amount of each sample was loaded and separated by electrophoresis on 7.5% SDS-PAGE gels (Bio-Rad) and electroblotted onto PVDF membranes. Nonspecific binding was blocked by incubating blots in 5% nonfat dry milk or 3% BSA in PBS-Tween 20 (PBS-T) at 4°C. Membranes were probed with a p-HER2 polyclonal antibody (Tyr 1248, 1:200 dilution, Santa Cruz Biotechnology). Following incubation with secondary antibodies conjugated to horseradish peroxidase (1:5,000 dilution, Santa Cruz Biotechnology), specific protein bands were detected by enhanced chemiluminescence autoradiography (Amersham, Piscataway, NJ). Blots were then stripped and reprobed with a monoclonal antibody directed against β-actin (clone Ac-74, Sigma). Relative amounts of individual protein bands were quantified by analysis of digitized images using National Institutes of Health Image software.

Survival. Mice were loaded with 2C4 and IgG1k as above. Seventy-two hours later, they were injured with 0.08 U of bleomycin intratracheally. Mice were allowed to recover and continued to receive IP 2C4 or IgG1k twice a week until death or the end of study (day 30).

Statistical analysis. Differences in measured variables between treatment and control group were assessed using the Student’s t-test. Data are expressed as means ± SD or SE. A P value of <0.05 was considered statistically significant. Survival analysis was performed by the method of Kaplan and Meier and analyzed using a log rank sum test.

RESULTS

2C4 inhibits HER2 activation in vivo. Our first study sought to verify 2C4 inhibition of HER2 activation in the lung and define an appropriate dose schedule. Weekly vs. twice weekly maintenance dosing (15 mg/kg) was tested to define maximal receptor inhibition. An IP loading dose of 30 mg/kg was used based on prior experience. Seventy-two hours after the loading dose was given, the animals were injected with 15 mg/kg IP every 3.5 or 7 days for 1 wk and killed just before the next scheduled dose. The lungs were dissected free from the chest and cut into 1-mm cubes and stimulated with neuregulin-1 (NRG-1) (10 nM), the HER2/HER3 ligand. At various time points after stimulation with NRG-1, the tissue was harvested and lysed. p-HER2 was determined in the protein lysates by Western blot for p-HER2 and HER2. As shown in Fig. 1A, NRG-1 stimulation of lung tissues not exposed to 2C4 induced a two- to threefold increase in p-HER2 at 5 min with peak receptor activation occurring at 15 min and returning to baseline at 60 min. Lung tissues from animals injected with 2C4 were also exposed to NRG-1, and HER2 activation was measured. Twice weekly injection of 2C4 inhibited HER2 activation at all time points (Fig. 1A), whereas 2C4 administered once a week failed to achieve complete HER2 inhibition (data not shown). Therefore, a twice weekly regimen was chosen for all subsequent studies. To verify that 2C4 blocks HER2 activation after bleomycin administration, mice treated with 2C4 or IgG1k per protocol were killed immediately before and 30 min, 8 h, and 24 h after bleomycin injury. Lung homogenates were subjected to Western blot for p-HER2 and HER2. As shown in Fig. 1B, HER2 phosphorylation increased sixfold 30 min after belomycin administration and was maintained up to 24 h. In the mice 2078 HER2 BLOCKADE IN BLEOMYCIN-INDUCED PULMONARY FIBROSIS

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treated with 2C4, there was total blockade of bleomycin-induced HER2 activation at all points. Thus 2C4 blocks bleomycin-induced HER2 activation in vivo.

HER2 inhibition does not affect bleomycin-induced acute lung injury. Having chosen a 2C4 dose and schedule that allowed us to study the effect of pharmacological HER2 inhibition on lung injury, studies were carried out as shown in Fig. 2. Acute lung injury results in disruption of the alveolar-capillary barrier and the initiation of an inflammatory cascade that is characterized by cellular influx and protein leak in the early phase. To study the effect of HER2 inhibition on early lung injury, mice were killed 3 days after bleomycin injury and BAL protein and inflammatory cell content were determined. As shown in Table 1, the BAL protein concentration increased almost 12-fold in injured mice without HER2 blockade (IgG1K injected) compared with uninjured mice (0.72 ± 0.56 μg/μl vs. 0.06 ± 0.03 μg/μl; P = 0.01). Treatment with 2C4 did not significantly affect bleomycin-induced protein leak. BAL protein concentration was similar in the 2C4-treated mice (0.85 ± 0.33 μg/μl) compared with the IgG1K-treated mice (P = 0.6).

BAL cellularity increased two- to threefold after bleomycin lung injury with a significant increase in neutrophils. The absolute number of total cells and neutrophils in the BAL of 2C4-treated mice was the same as that of IgG1K-treated mice. These data indicate that lung injury was similar between the IgG1K- and 2C4-treated groups. Thus 2C4 treatment does not appear to have a role in preventing or attenuating the acute injury response.

HER2 inhibition ameliorates lung remodeling and decreases collagen deposition. Next we examined the late effect of HER2 blockade on lung morphology and collagen deposition after bleomycin lung injury. C57BL/6 mice were killed at days 15 and 21, and their lungs were removed. Microscopically, mice with HER2 inhibition had less lung remodeling after bleomycin injury than animals with intact HER2 signaling. As seen in Fig. 3, significant morphological changes were evident in lung sections from bleomycin-injured/IgG1K-injected animals at 15 and 21 days after injury. Significantly fewer changes were noted in the bleomycin-injured/2C4-injected animals at both time points. The degree of injury and fibrosis was quantified using an Ashcroft injury score on hematoxylin and eosin-stained lung sections. Uninjured mice had an Ashcroft score of 0 at all time points. The bleomycin-injured/IgG1K-injected mice had an Ashcroft score of 3.84 ± 0.34 (day 15) and 3.90 ± 0.76 (day 21), whereas the bleomycin-injured-2C4-treated mice had an injury score of 3.78 ± 1.94 (day 15) and 1.99 ± 1.55 (day 21). Although the injury score was the same at day 15, it was significantly lower at day 21 (P = 0.04).
Our laboratory and others have previously shown the importance of the HER2/HER3 axis in modulating pulmonary fibrosis in mice. (51) In the present study, we show that pharmacological blockade of HER2 in vivo, which is exclusively expressed in the epithelium by KGF when administered before lung injury, was protective in several in vivo lung injury models, including hyperoxia, acid instillation, bleomycin, bacterial pneumonia, and bone marrow transplantation (19, 28, 31–33). This protective effect was attributed to increasing alveolar type II cell proliferation in vivo (21, 23). KGF receptor activation also stimulated alveolar type II cell proliferation and decreased Fas-induced apoptosis in vitro (2). These proliferative and antiapoptotic effects were mediated through the PI3K/AKT and the MAPK/ERK signaling pathways (22, 23). However, the protective effect of KGF in vivo cannot be solely explained by its alveolar type II cell proliferative properties since KGF was protective against hyperoxia-induced injury and fibrosis in mice without increasing epithelial cell proliferation (3).

In the EGFR, pharmacological inhibition of EGFR in vivo has effects on collagen production and fibrosis that vary in different injury models. AG1478, an EGFR-specific inhibitor, reduced collagen accumulation in a vanadium pentoxide lung injury model in rats (24). Gefitinib, another EGFR-TKI, produced conflicting results in vivo. It attenuated bleomycin-induced pulmonary fibrosis in C57BL/6 mice (12), whereas it worsened bleomycin-induced pulmonary fibrosis in ICR mice (29). In addition, gefitinib has been linked to the development of lung injury and fibrosis in patients with nonsmall cell lung cancer (30) and type II cell proliferation (6, 16, 17). Our prior work in a transgenic mouse strain incapable of signaling through HER2/HER3 due to expression of a dominant negative HER3 (DNHER3) in type II pneumocytes showed that the DNHER3 strain had less fibrosis, less lung remodeling, and a survival advantage compared with wild-type littermates after bleomycin injury (20). Our current data extend these findings by showing that pharmacological blockade of HER2 in wild-type mice has the same impact as the genetic alterations, suggesting its potential therapeutic utility.

2C4 is a humanized monoclonal antibody that targets a specific epitope in the extracellular domain of HER2. 2C4 binding directly inhibits HER2 dimerization with other family members and blocks any further downstream signaling. 2C4 is thought to be specific to the human HER2. Two residues in domain II of HER2 that have significant contact with 2C4 differ between rodent and human HER2. Although this difference in the amino acid sequence eliminates detectable binding between 2C4 and rodent HER2 in vitro, this does not appear to be the case in vivo. We have shown inhibition of HER2 activation, and 2C4 is able to immunoprecipitate HER2 from murine lung, although at low levels. This low-affinity binding, and potentially higher tissue level, appears to be sufficient for the biological effects seen in our injury model. Furthermore, it suggests that a more pronounced result may occur when translated into a system with higher affinity binding between 2C4 and HER2.

The mechanism by which HER2 blockade protects against fibrosis in the setting of bleomycin lung injury is not completely clear. It is possible that HER2 activation modulates the expression of profibrotic cytokines and growth factors that alter epithelial and fibroblast proliferation, differentiation, epithelial mesenchymal transition (EMT), and exaggerated extracellular matrix deposition. Specific growth factor receptor tyrosine kinases have been shown to modulate lung injury and fibrosis. Activation of the keratinocyte growth factor (KGF) receptor, which is exclusively expressed in the epithelium by KGF when administered before lung injury, was protective in several in vivo lung injury models, including hyperoxia, acid instillation, bleomycin, bacterial pneumonia, and bone marrow transplantation (19, 28, 31–33). This protective effect was attributed to increasing alveolar type II cell proliferation in vivo (21, 23). KGF receptor activation also stimulated alveolar type II cell proliferation and decreased Fas-induced apoptosis in vitro (2). These proliferative and antiapoptotic effects were mediated through the PI3K/AKT and the MAPK/ERK signaling pathways (22, 23). However, the protective effect of KGF in vivo cannot be solely explained by its alveolar type II cell proliferative properties since KGF was protective against hyperoxia-induced injury and fibrosis in mice without increasing epithelial cell proliferation (3).

**DISCUSSION**

In the present study, we show that pharmacological blockade of HER2 signaling before injury attenuates fibrosis and improves survival in a murine bleomycin lung injury model. HER2 inhibition did not affect the early injury phase since both 2C4- and IgG1K-treated groups had equal amounts of protein leak and neutrophil infiltration in their BAL 3 days after injury. Despite the same degree of acute injury, 2C4 ameliorated the long-term effects of bleomycin when given before injury, evidenced by less collagen deposition and fewer morphological changes at days 15 and 21 after injury. When exposed to a higher bleomycin dose that results in life-threatening pulmonary injury and fibrosis, 2C4 treatment before injury resulted in a survival advantage. These results confirm the role of the HER2/HER3 axis in modulating pulmonary fibrosis in mice. Our laboratory and others have previously shown the importance of the NRG-1/HER2 axis in recovery from lung injury.
Fig. 3. HER2 inhibition decreases pathological changes. Representative photomicrographs of sections from lungs of saline-injured/PBS-injected (saline/PBS; A), bleomycin-injured/2C4 and bleomycin-injured/IgG1K-injected mice at days 15 (B and C) and 21 (D and E) after injury. Lungs were inflated (20 cmH₂O pressure) and fixed with 10% formalin. Magnification = ×200 (left) and ×400 (right). Hematoxylin and eosin was used as stain.
cancer (11), a more frequent complication in the Japanese population and in those with underlying pulmonary fibrosis (5). Thus a clear role for growth factor receptor tyrosine kinases in recovery from injury has not been established. Although these different growth factor receptors have overlapping downstream signaling pathways, their activation or inhibition lead to different outcomes in vivo that may be partially explained by differences in the injury model and the organ involved.

HER2 belongs to the EGFR family and exerts similar proliferative and differentiation effects on the pulmonary epithelium as EGFR. In addition, HER2/HER3 signaling was implicated in EMT in cardiac and mammary tissue (4, 14). Its role, if any, in EMT in the lung is not known. Blocking EMT is an attractive strategy to decrease pulmonary fibrosis and is certainly a potential mechanism by which HER2 blockade may protect against pulmonary fibrosis. The beneficial effect of HER2 inhibition was seen when bleomycin-injured mice were pretreated with 2C4. Although bleomycin-induced lung injury in rodents is an extensively studied fibrosis model, differences in the pathogenesis of the human and murine fibrosis diseases undoubtedly exist. It remains to be explored whether HER2 blockade is beneficial in preventing or treating other lung injury models with less acute response such as radiation-induced lung injury or human pulmonary fibrosis.

In summary, we have shown that pharmacological inhibition of HER2 signaling attenuates pulmonary fibrosis and improves survival when given before bleomycin-induced lung injury in wild-type mice. This study provides insight into the pathogen-
esis of pulmonary fibrosis, which is currently thought to be an epithelial-driven fibrotic process rather than an ongoing chronic inflammation, and highlights the important role of HER2 in this process.

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