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Interleukin-6 is Required for Neuregulin-1 Induced HER2 Signaling in Lung Epithelium

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Abstract

A clear understanding of the mechanisms that regulate the alveolar epithelium's barrier is critical to develop new therapeutic strategies to mitigate lung injury. The HER2/HER3 receptor tyrosine kinase complex plays a central role in maintaining the alveolar-capillary barrier. This receptor complex is activated by its ligand, neuregulin-1 (NRG-1). Interleukin-6 (IL-6) is also known to induce HER2 signaling through HER2 transphosphorylation by the IL-6 receptor (IL-6R) complex (1). Due to this interaction, we hypothesized that NRG-1 and IL-6 cooperatively interacted to activate the HER2/HER3 complex

Studies were performed in cultured pulmonary epithelial cells measuring the HER2/IL-6/IL-6R/GP130 interaction and receptor activation by western blotting and confocal microscopy, IL-6 production by ELISA, and IL-6 inhibition using specific antibodies, small molecule inhibitors and shRNA.

We found that IL-6 was required for NRG-1 induced activation of HER2 in pulmonary epithelial cells. IL-6 inhibition led to a decrease in NRG-1 induced HER2 activation. The IL-6R and GP130, a subunit of the IL-6R complex, were physically associated with HER2 and were required for NRG-1 induced HER2 activation. Inhibition of GP130, the β -subunit of the IL-6 receptor decreased NRG-1 induced HER2 activation lower than control by 38%. Finally, HER2 activation increased IL-6 secretion more than two-fold over resting cells (526±131 vs 231±39.7 pg/ml), and inhibition of HER2 gene expression decreased basal IL-6 secretion over 80% (89±4.6 vs 1.3±0.8 pg/ml).

These findings identify a requirement for IL-6 and the IL-6R complex to allow NRG-1 mediated HER2 activation, and a HER2 driven IL-6 production feedback loop.

Keywords

Neuregulin-1; Interleukin-6; HER2; Tyrosine Kinase; Signaling; Interleukin-6 Receptor

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Introduction

The airway epithelium is the first barrier against inhaled insults and the final barrier against internal forces (hydrostatic, inflammatory, and ischemic) that disrupt water and solute movement across the epithelium. This barrier is formed by adhesion of airway and alveolar epithelial cells through the adhesive properties of junctional proteins. We have previously shown that the receptor tyrosine kinase (TK) HER2 is important in maintaining this epithelial cell barrier (2–6)

HER2 is a class I membrane bound receptor TK with four other family members; EGFR (HER1), HER3, and HER4. On ligand binding these receptors homo- or hetero-dimerize, and activate their intrinsic TK domains, resulting in auto-phosphorylation and downstream signaling (7, 8). The diversity of receptor combinatorial events is greatly simplified in the lung as HER1 (EGFR) and HER4 are expressed at lower levels relative to HER2 and HER3 (6, 9), and HER3 is the preferred dimerization partner for HER2 (10). Therefore, the HER2/HER3 heterodimer is the predominant signaling complex in the lung. However, HER2 has no known ligand (11, 12), and HER3 is catalytically inactive (10, 11). Therefore, in order to signal, the receptor components cooperate. Membrane bound NRG-1, the HER3 ligand, expressed at high levels in the pulmonary epithelium (9, 13), is shed by IL-1 β induced activation of ADAM17 (3). NRG-1 binds with HER3 in an autocrine fashion (14), and NRG-1/HER3 associates with HER2, activating HER2's TK domain. The NRG-1 and HER2/HER3 interaction is elegantly regulated in polarized epithelial cells by a spatially defined mechanism with segregation of the ligand apically, and the receptors laterally (6).

Activation of the HER2/HER3 signal pathway results in an alteration of the epithelial cell barrier through HER2 mediated phosphorylation of β -catenin, leading to dissociation of β -catenin from E-cadherin which results in decreased E-cadherin mediated cell adhesion and increased paracellular leak. In bronchoalveolar lavage (BAL) samples from patients with acute lung injury (ALI) requiring intubation and mechanical ventilation, BAL NRG-1 levels inversely correlated with ventilator free days, confirming shedding of NRG-1 during lung injury and indicating activation of this pathway in inflammatory ALI (15).

Blocking this pathway in animal models of lung injury decreased the extent of injury and improved survival. Inhibition of HER2/HER3 signaling in bleomycin lung injury models using either a transgenic mouse with a dominant negative HER3 incapable of HER2 activation or a monoclonal antibody that blocks HER2 activation, resulted in reduced pulmonary fibrosis and mortality (3–5).

A number of other inflammatory mediators are elevated in the alveolus and airways during the early phase of lung injury including Interleukin-6 (IL-6) (15, 16). IL-6 acts as a major pro-inflammatory mediator for the induction of the acute phase response (17), leading to a wide range of local and systemic changes including fever, leucocyte recruitment and activation. However, IL-6 is multi-functional, its effects context dependent and increased levels seen in lung injury can either contribute to or prevent organ injury (18). Some studies find a beneficial effect of IL-6 during lung injury (19–23). This is seen in IL-6KO mice that have more severe pulmonary inflammation and injury in response to LPS aspiration and

mechanical ventilation when compared to wild-type animals (23, 24). Treatment with exogenous IL-6 rescues the knock-out phenotype with decreased alveolar permeability, lung edema and reduced histological lung damage, and in wild type mice IL-6 decreased lung leukocyte infiltration following a LPS challenge or mechanical ventilation (23, 24). Others have reported a detrimental effect with circulating IL-6 mediating lung injury after acute kidney injury or pancreatitis, and promoting pulmonary fibroblast proliferation (25–27). Clearly, IL-6 plasma levels have been associated with higher morbidity and mortality (15, 28).

Previous studies have shown that HER2 is also involved in IL-6 expression and signaling. IL-6, HER2, and GP130, the beta-subunit of the IL-6 receptor (IL-6R), are physically associated and co-immunoprecipitate in response to IL-6 stimulation (1). IL-6 induced HER2 clustering to the GP130 complex, led to HER2 transphosphorylation, and HER2 activation of Shc/MAP kinase pathway (29). In addition, overexpression of HER2 in breast cancer cell lines led to increased IL-6 secretion which was decreased with HER2 inhibitors (30). These findings point to a critical interaction between HER2 and IL-6.

We hypothesized that a cooperative interaction existed between HER2 and IL-6 in the lung. To test this hypothesis, we studied the interaction of HER2 and IL-6 in airway epithelial cells. These studies confirmed that the IL-6R and GP130 were physically associated with HER2 and IL-6 induced HER2 transphosphorylation through GP130. Importantly, IL-6 and GP130 were also required for NRG-1 induced HER2 activation, and HER2 activation led to a significant increase in IL-6 production. These results identify an IL-6/IL-6R complex requirement for NRG-1 induced HER2 activation as well as a HER2 induced IL-6 production loop that could impact the inflammatory response during lung injury.

Materials and Methods

Chemicals and Reagents:

Antibodies were purchased following sources: HER2, p-HER2, HER3, pHER3, pEGFR, pSTAT3, STAT3, and NRG-1 from Cell Signal Technology, Inc., (Danvers, MA); β -Actin antibody from Sigma Chemical Co. (St. Louis, MO); neutralizing IL-6 antibody from R&D Systems (Minneapolis, MN), and an IgG isotype matched control antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture:

NuLi-1, BEAS2B and NCI-H292 cells were obtained from American Type Culture Collection Company, (Manassas, VA) and cultured in the media as recommended by the supplier. Forty-eight hours before use cells were subcultured at 70% density. Twenty-four hours before treatment cells were maintained in G₀-medium (0.5% FBS in DMEM).

Gene knock-down using shRNA:

Lentiviruses carrying gene specific shRNA targeting IL-6 (TRCN0000372667); IL6R (TRCN0000372728) and HER2 (TRCN00003329530) (Sigma Aldrich, St. Louis, MO), were obtained from the Functional Genomics Core; University of Colorado, Denver (<http://>

www.ucdenver.edu/academics/colleges/medicalschoo/centers/cancercenter/Research/sharedresources/FunctionalGenomics/Pages/FunctionalGenomics.aspx). Cells were infected with lentivirus (MOI=10) and 24 hours following infection, puromycin (10ug/ml) was added to select the infected cells. After 48h of selection, cells were maintained in low puromycin media (0.5ug/ml).

Immunocytochemistry and confocal microscopy:

Cells were grown on collagen treated coverslips for 48 hours, and switched to G₀ media for 24h before use. After treatment with cytokines, cells were fixed with 2% paraformaldehyde in PBS for 1h. Cells were permeabilized with 1% Triton-X-100 in PBS for 10 min., and blocked with 1% BSA in PBS. Cells were incubated with primary antibodies in blocking media overnight at 4°C. The next day cells were washed with PBS-T (0.1% Tween-20 in Phosphate Buffered Saline, pH 7.5) four times then incubated with fluorescent secondary antibodies (Donkey anti-rabbit IgG-A488 and Donkey anti-mouse IgG-A555) for 1h. After washing, cells were mounted and photographed using a Zeiss microscope and photography system.

Western Blotting:

Ten µg of total protein was loaded on 4–12% NuPage Bis-Tris gradient gels (Invitrogen Inc., Grand Island, NY). Resolved proteins were transferred on to nitrocellulose membranes and then blocked and probed with primary antibody in blocking buffer overnight at 4°C in 1% milk in TBS-T. The next day, after washing with TBS-T (0.1% Tween-20 in Tris Buffered Saline, pH 7.5), the membrane was incubated with the secondary HRP-labeled-antibody for 1h. After washing with TBS-T and incubating with chemiluminescent substrate, images were captured using a FluorChem Q photography system from Protein Simple Inc. (Santa Clara, CA).

Immunoprecipitation:

Cells were lysed in immuno-precipitation assay buffer (RIPA buffer) containing protease- and phosphatase-inhibitors. One hundred µg of total protein was incubated with 20µg of primary antibody for 1h and then with Protein-A Agarose for another hour. The Protein-A Agarose-antibody complex was collected by centrifugation (1,000g × 3m), washed 3 times with TBS-T, denatured in 1X gel loading buffer and loaded on denaturing gel for further analysis.

ELISA:

Levels of secreted IL-6 in cell media were determined by using the corresponding DuoSet ELISA kit (R&D Systems Inc. Minneapolis, MN). A 4-Parameter curve fit was used to determine levels in the samples.

Statistics:

Values are expressed as means ± SE. Groups were compared using Student t-test and P< 0.05 was considered significant.

Results

IL-6 inhibition abolishes NRG-1 and IL-6-induced HER2 phosphorylation:

Quiescent NuLi-1 cells were stimulated with NRG-1 and IL-6 (Figure 1A, B) resulting in rapid HER2 phosphorylation as we, and others, have previously described (1, 3). IL-6 induced HER2 phosphorylation was abrogated by IL-6 inhibition with a neutralizing antibody, as expected (Figure 1B). However, NRG-1 induced HER2 phosphorylation was also abolished with the IL-6 antibody indicating a need for extracellular IL-6 for NRG-1-induced HER2 activation (Figure 1A). The requirement of IL-6 for NRG-1 induced signaling was not limited to NuLi-1 cells, or to specific culture conditions. The IL-6 requirement for NRG-1 induced signaling was also seen in other cell lines of lung origin, BEAS2B and NCI-H292 (Figs 1C and 1D) and in cells grown at an air-liquid interface (data not shown), suggesting a generalized IL-6 requirement for NRG-1 signaling. Thus, IL-6 alone leads to HER2 phosphorylation, yet there is a requirement for IL-6 in NRG-1 induced HER2 activation (Fig. 1B).

HER2 interacts with IL-6R/GP130 receptor complex:

The IL-6 induced transphosphorylation of HER2 and the inhibition of NRG-1-induced HER2 activation by an IL-6 neutralizing antibody suggested that HER2 activation required interaction with an activated IL-6R/GP130 receptor complex. To define a physical interaction between members of the IL-6R complex and HER2, the IL-6R and GP130 were immunoprecipitated from the NuLi-1 cell lysates and probed for HER2. Immunoblotting identified HER2 was associated with both constituents of the IL-6R complex, IL-6R and GP130, in resting cells and in IL-6 and NRG-1 stimulated cells (Fig. 2A). However, there was no significant difference in the degree of the association after IL-6 or NRG-1 exposure.

To confirm the functional involvement of IL-6R complex in HER2 activation, we inhibited GP130 activity using the GP130 specific inhibitor SC144 (31). NuLi-1 cells were stimulated with IL-6 in the presence of varying concentrations of SC144. Western blot analysis demonstrated that 10uM effectively inhibited the IL-6-induced HER2 phosphorylation (Fig. 2B). In addition, SC144 inhibited NRG-1 induced HER2 activation, further confirming the need for IL6R axis in HER2 activation (Fig. 2C).

To further define the HER2 and IL-6R interaction, confocal microscopy was performed on NuLi-1 cells stained for IL-6R or HER2. Co-localization of IL-6R and HER2 were identified on the cell surface of resting cells (Fig. 3A) and intracellularly (Fig. 3B). In IL-6-stimulated cells HER2/IL-6R co-localization increased and appeared to be predominantly intra-nuclear (Fig. 3C). A similar trend with increased intensity of co-localization and intra-nuclear translocation was seen in cells stimulated with NRG-1 (Fig. 3E). Interestingly, co-localization was abrogated when the cells were exposed to IL-6 neutralizing antibody along with IL-6 or NRG-1 (Fig. 3D&F).

HER2 Activation induces IL-6 production:

Overexpression of HER2 has been linked with overexpression of IL-6 (32). In addition, our data has shown a requirement for IL-6 in NRG-1 induced HER2 signaling. Therefore, we

next asked whether NRG-1 induced HER2 activation led to IL-6 production and secretion, potentially setting up a positive feedback loop for NRG-1 induced HER2 signaling. To test this hypothesis, NuLi-1 cells were stimulated with NRG-1 and IL-6 measured in the culture media. NRG-1 stimulation of cells increased IL-6 secretion over two-fold from baseline of 231 ± 39.7 pg/ml to 538 ± 131 pg/ml at 24h ($P < 0.05$) (Fig. 4A). To confirm the role of HER2 and the IL-6R in IL-6 induction, specific gene expression was knocked down using shRNAs targeting different regions of each gene's transcripts. shRNAs were transduced into the cells using lentivirus, and the level of secreted IL-6 in each condition assayed. IL-6 secreted into the media of control cells was 89 ± 4.6 pg/ml. As expected, the shRNA targeting of IL-6 transcripts caused a significant drop in IL-6 to 14.4 ± 2.9 pg/ml ($P < 0.05$) and cells exposed to IL-6R shRNA also had a significant decrease to 32.7 ± 3.8 pg/ml ($P < 0.05$) (Fig. 4B). However, IL-6 levels were below the detection limits of the assay in media from cells with HER2 targeted shRNA, (Fig. 4B). These data demonstrated that HER2 expression was required for increased IL-6 secretion and HER2 activation by NRG-1 increased IL-6 secretion, potentially acting as a positive feedback loop to further enhance NRG-1 induced HER2 activation.

Discussion

Lung inflammation leading to ALI and acute respiratory distress syndrome (ARDS) are major problems that carry a high morbidity and mortality. Estimates based on the National Heart Lung and Blood Institute's ARDS Network studies found an incidence of ~150,000 cases/year of ALI and ARDS in the U.S. (33). In mechanically ventilated patients, 10–20% develop ALI or ARDS (34). Associated with these diagnoses are mortality rates reported between 35% to 60%. Understanding how the inflammatory process is regulated in the lung could be a key to designing new therapeutic strategies to prevent or minimize these processes.

In large clinical trials biomarkers to predict the outcome of lung injury have found IL-6 plasma levels to be predictive of higher morbidity (15) and mortality (28). In addition, circulating levels of IL-6 are predictors of ARDS severity in sepsis and acute pancreatitis (35, 36). NRG-1 in BAL has also been shown to be a predictor of morbidity in ARDS, and the NRG-1/HER2 axis has a role in regulating the alveolar capillary barrier (2, 3, 15, 37). Importantly, the NRG-1/HER2 and the IL-6/IL-6R signaling axes have been shown to be interconnected in skeletal muscle (38), endothelial cells (39), prostate and breast carcinoma (1, 32), and we provide new data in these studies to include pulmonary epithelial cells showing that IL-6 leads to HER2 transphosphorylation and HER2 activation. Importantly, we have found that in pulmonary epithelial cells the HER2 and IL-6 receptor complexes are physically associated with IL-6R, GP130 and HER2 co-immunoprecipitating and localized together on the cell surface and intracellularly. In addition, the presence of IL-6 and GP130 are required for NRG-1 signaling through HER2. Thus, the coordination of IL-6 and NRG-1 is essential for activation of the HER2 signaling pathway. Further, NRG-1 and HER2 significantly increased the amount of IL-6 released by pulmonary epithelial cells. The increase in IL-6 seems to be due to both an increase in gene expression as evidenced by the effect of shRNA mediated transcript knockdown, and potentially by increased membrane shedding. MMP1, MMP3, and MMP10 are induced by IL-6, and MMP7 is induced by

HER2. Our prior studies have shown that metalloproteinases are required for membrane bound NRG-1 shedding (3, 40) and are also required for IL-6 membrane shedding. This sets up an autocrine/paracrine feedback loop consisting of an IL-6 requirement for NRG-1 induced HER2 signaling, leading to increased IL-6 secretion/shedding that may further allow NRG-1 as well as IL-6 induced signaling. Our prior studies showing that the NRG-1/HER2 signaling axis is injurious in the lung (3, 5, 37) and correlates with injury in patients (15), in conjunction with the current data showing an IL-6 requirement for NRG-1 induced HER2 activation, clearly shows an active NRG-1/HER2/IL-6 signaling loop in ALI/ARDS.

Similar feedback loops and enhanced effects have been identified between IL-6/IL-6R and HER2 signaling in cancer. HER2 overexpression in breast cancer resulted in elevation of IL-6 gene expression, HER2 induced secretion of IL-6, autocrine STAT3 signaling, and enhanced HER2 mediated transformation (30). This autocrine feedback loop is similar to what we report here and the mechanisms build on our previous work showing NRG-1 signals through STAT 3 (29). Thus, a constitutive requirement for IL-6 in NRG-1 induced HER2 signaling, and the resultant HER2 induced IL-6 secretion and STAT3 signaling provide a possible molecular basis for the pathologic inflammatory markers seen in lung injury and the association with disease severity. This suggests that IL-6 targeted therapies could have significant impact on lung disease marked by HER2 activation as we have shown in ARDS (15).

In summary, we show that IL-6 is a critical component of the HER2 mediated inflammatory process. IL-6 is required to initiate NRG-1 induced HER2 signaling, and NRG-1 induced HER2 activation amplifies IL-6 signaling. These findings suggest that therapeutic targeting of IL-6-induced signaling pathway would decrease both HER2 and IL-6 mediated inflammatory events and possibly decrease extent of pulmonary injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- IL-6 induces HER2 phosphorylation in cells
- HER2 interacts with constituents of IL-6R and Inhibition of GP-130 abolishes IL-6-induced HER2 phosphorylation
- HER2 signaling leads to increased IL-6 levels.

Figure 1: IL-6 is required for NRG-1-induced HER2 phosphorylation in cells of lung origin. Quiescent NuLi-1 cells were stimulated with (A) NRG-1 or (B) IL-6 in the absence or presence of IL-6 neutralizing antibody. Cell lysates were then analyzed for phospho-HER2. Similar findings were recorded in BEAS2B cells (C) and NCI-H292 cells (D).

Figure 2: HER2 is associated with the IL-6R complex and GP130 is required for IL-6 and NRG-1-mediated HER2 phosphorylation.

(A) NuLi-1 cells were stimulated with IL-6, NRG-1, or control and IL-6R or GP130 were immunoprecipitated from NuLi-1 cell lysates. Immunoprecipitates were subjected to western blotting and probed for HER2. IP=Immunoprecipitation target. IB=Immunoblotting target; (B) NuLi-1 cells were stimulated with IL-6 (C) or NRG-1 in the presence of the GP130 inhibitor SC144 (10 μ M) and the levels of pHER2 identified.

Figure 3: IL-6R and HER2 are co-localized in the cell.

NuLi-1 cells grown on glass coverslips were stained for IL-6R (red) and HER2 (green) and imaged by confocal microscopy **(A)** Resting cells Red rectangle=Z-axis view of Y-plane, Green rectangle=Z-axis view of X-plane(z-Axis, 400X) **(B)** Resting cells (200X). **(C)** Cells stimulated with IL-6 alone and **(D)** with IL-6 antibody. **(E)** Cells stimulated with NRG-1 alone and **(F)** with IL-6 antibody.

Figure 4: HER2 is required for IL-6 secretion.

(A) NuLi-1 cells were stimulated with NRG-1 for 8 and 24 h, and IL-6 secreted in the media assayed. (B) HER2, IL-6 and IL-6R expression were knocked down in NuLi-1 cells using lentivirus expressing specific shRNA. Secreted IL-6 was measured after 24h. Knock down was verified by Western blotting. (NT=non-targeting shRNA) *=P<0.05.