

Respiratory syncytial virus augments production of fibroblast growth factor basic *in vitro*: Implications for a possible mechanism of prolonged wheezing after infection

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Respiratory syncytial virus (RSV) has been linked to the development of clinical asthma. Cellular mechanisms of this observation are not yet clearly elucidated. In chronic asthma, production of growth factors and remodeling are associated with prolonged wheezing. It was hypothesized that cells infected with RSV may produce excessive levels of fibroblast growth factor basic (FGFb), and epidermal growth factor (EGF). Airway epithelial cells were incubated with either: (i) virus, (ii) inactivated virus, or (iii) media only. The levels of FGFb and EGF were measured in the cellular supernatant fluid. The study demonstrated that by 24 h after RSV inoculation, or exposure to RSV-killed virus, cells are stimulated to produce significantly more FGFb, compared with non-infected/non-exposed control cells. FGFb is an important factor in remodeling and fibroblast activation in the airway. Using treatment with actinomycin D and cyclohexamide the effect of inhibiting translation or transcription in the infected cells, on FGFb production was demonstrated. There were no alterations in EGF production detectable. Based on the findings, the mechanism of FGFb secretion after RSV inoculation, appears to be regulated at the levels of both transcription and translation. The increased FGFb release potentially could contribute to fibroblast activation and remodeling in the airway, and thus provide another possible mechanism for prolonged wheezing after infection.

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Respiratory syncytial virus (RSV) is the most significant viral cause of lower respiratory tract infection (LRTI) and bronchiolitis in infants and children worldwide (1). In addition, significant adult disease has been associated with RSV infection (2). Many studies have established a link between asthma, transient wheezing and RSV infection (3). Chronic inflammatory changes associated with asthma leads to remodeling and structural airway pathology, even in younger patients (4). These structural changes are associated with increased reactivity and constriction of the airway. The process of remodeling is associated with the expression of growth factors in the airway (5). Among the growth factors

represented, fibroblast growth factor basic (FGFb), and epidermal growth factor (EGF), have been implicated in the repair phase of remodeling. Based on these associations, it was hypothesized that cells infected with RSV may produce excessive levels of FGFb and EGF, and thus contribute to the pathogenesis of prolonged wheezing after RSV infection.

Methods

Viral stock preparation

Frozen aliquots of RSV A Long strain, were prepared at concentrations of 7.35 log₁₀ PFU/ml

and stabilized in sucrose. On the day of inoculation, the stock solution was diluted in the appropriate growth media. Cells were infected with 200 PFU per well. In selected wells, some cells were treated with inactivated virus. Virus at the same concentration was inactivated by 30 min of exposure to UV light, using methods previously described (6).

Cell culture and RSV infection

Hep-2, a transformed epithelial cell line (ATCC, Rockville, MD, USA), was used as the host cell, by methods previously described (6,7). Experiments were repeated using A549 alveolar epithelial cells (ATCC). Briefly, the cells were grown in triplicate in sterile multi-well plates at 37°C, 5% CO₂ until 80% confluence. On the day of inoculation, the media [modified Eagle's minimum essential medium (EMEM), supplemented with 1% penicillin (PCN)/streptomycin, 1% L-glutamine, 5% fetal bovine serum] was aspirated, washed and the cells incubated with either: (i) virus, (ii) inactivated virus, or (iii) media only for 60 min, while gently shaking every 15 min to ensure distribution of the virus. The cells were then washed three times, with phosphate-buffered saline (PBS), and re-incubated with basal EMEM medium, with no added fetal calf serum. The media used for A549 cells was modified Hams F-12. Cell groups were exposed to: (i) live RSV (RSV), (ii) killed (irradiated RSV) (KV), and (iii) no RSV (NV).

The cell culture supernatants were then removed after every 24 h, until 120 h after the exposure to virus. The supernatants were aliquoted, clarified by low-speed centrifugation and frozen at -70°C for further analysis. Subculture of all cell groups was performed to verify state of viral replication, by methods previously described (6).

Cyclohexamide/actinomycin D treatment of cells

In a separate set of experiments, the effects of cyclohexamide and actinomycin D were each assessed in triplicate. The Hep-2 cells were grown to 80% confluence, RSV infected, and incubated with or without, cyclohexamide (Sigma, St Louis, MO, USA) 10 µg/ml or actinomycin D (Sigma) 1 µg/ml for 24 h. The levels of growth factors was also assayed in these cell supernatants.

Assessment of cell viability. Cell viability was determined by trypan blue exclusion assay (Sigma), according to standard methods as per manufacturer's direction.

Quantitative ELISA assay for FGFb and EGF

The levels of FGFb and EGF in the cell sample supernatants were quantitatively assessed by enzyme-linked immunosorbent assay (ELISA) using kits, according to manufacturer protocols (R & D systems, Minneapolis, MN, USA). The lower limits of detectability was 0.7 pg/ml.

Statistical analysis

Statistical analysis was completed with either ANOVA or Student's *t*-test as appropriate, with the level of significance set at $p < 0.05$.

Results

Cell viability and RSV replication

The cells remained viable for the duration of the experiments as confirmed by trypan blue exclusion assay (data not shown). RSV replication in the infected group was confirmed by subculture. Non-replication was confirmed in the inactivated virus and control groups.

Quantitation of FGFb and EGF production

At baseline, at the start of the experiment, the levels of FGFb were low and not significantly different, in all groups ($p = \text{NS}$). The control cells showed a mean level of 6.4 ± 6.3 , with the levels in the KV, and RSV groups below 2 pg/ml. By 24 h of exposure, however, the levels in the RSV group were the highest at 917 ± 78 pg/ml, and in the KV group at 794 ± 122 pg/ml. The levels in the NV group increased, but were significantly lower with a mean value of 329.3 ± 0.6 pg/ml ($p < 0.01$). By 72 h the levels were non-detectable in all groups. EGF ELISA (R & D Systems) was also performed. The results indicated that for all time points and conditions studied, no detectable levels of EGF were measurable. The results were confirmed in the transformed airway A549 cells, in which RSV inoculation and KV exposure led to enhanced production of FGFb, but not EGF (data not shown).

The effect of actinomycin D and cyclohexamide on FGFb production in the RSV infected Hep-2 cells

In a separate set of experiments, the effects of actinomycin D (Act D) and cyclohexamide (CHX) on production of growth factors by RSV-infected cells was determined at time points of 24 and 48 h. The RSV group by 48 h, produced more FGFb, than the treated cells

($p < 0.05$). At 24 h, in the Act D- or CHX-treated cells, there was no significant difference in the FGFb levels observed ($p = \text{NS}$). The non-treated cells, had a mean FGFb level of 917 ± 55 pg/ml, and the CHX-treated cells had a mean level of 783 ± 10 pg/ml. At 24 h, in the presence of Act D, the treated cells produced 631 ± 20.7 pg/ml. At 48 h, the treated cells demonstrated significantly less FGFb production compared with control ($p < 0.01$). The Act D group mean value was 448 ± 1 pg/ml vs. 812 ± 14 pg/ml in the control cells. The CHX group on day two demonstrated even lesser production compared with control, with a mean of 182 ± 1 pg/ml ($p < 0.0005$).

Discussion

The study demonstrates that by 24 h after RSV inoculation, or exposure to KV, cells are stimulated to produce significantly more FGFb, compared with non-infected/non-exposed control cells. FGFb is an important factor in remodeling and fibroblast activation in the airway. Using treatment with Act D and CHX we demonstrated the effect of inhibiting transcription or translation in the infected cells, on FGFb production. The mechanism of enhanced FGFb production appears to be regulated at the levels of both transcription and translation, and does not require viral replication. This study indicates that cells exposed to RSV viral proteins could lead to fibroblast proliferation, and potentially airway remodeling by overproduction of FGFb.

Respiratory syncytial virus has been closely linked to the development of prolonged wheezing by several clinical studies (8). The long-term respiratory consequences of RSV infection include the symptoms of asthma, increased bronchial reactivity, abnormalities in pulmonary function and lower arterial oxygen tension (9). Most, if not all, clinical studies regardless of design, have concluded that post-RSV infection, patients had a significantly higher frequency of bronchial obstructive symptoms, diminished lung function, or increased bronchial reactivity to histamine challenge or exercise than controls (10). These abnormalities of lung function have been noted to last for 7–10 years following initial infection, in most patients (10).

The cellular mechanisms of this clinical observation are not yet fully elucidated. RSV replicates exclusively in the respiratory epithelium (11, 12). Infection results in increased mucus production, a peribronchiolar mononuclear infiltrate, necrosis of ciliated bronchiolar epithelium, and epithelial regeneration can take weeks (13).

These cytologic changes contribute to airway obstruction. Other proposed mechanisms which have been studied, include models in which the damaged epithelium exposes sensory nerves, making reflex bronchoconstriction likely (14). Many groups have studied immunologic mechanisms, which have shown that many inflammatory mediators are released during RSV infection, which can cause bronchoconstriction directly or indirectly. There are several studies exploring the connection between RSV infection, eosinophil degranulation, β -chemokine release, and type 2 helper (Th2) lymphocyte-derived cytokine production (14). Each of these pathways is thought to contribute to the pathogenesis of asthma. There is still incomplete data to support the hypothesis that RSV infection alters allergen-specific immunoglobulin E production.

It is very likely that RSV infection also differentially alters the production of other growth factors by epithelial cells. As a result, the airway could become structurally altered and subepithelial deposition of collagen and fibroblast overproliferation could occur. This study confirms that cells infected with RSV, or exposed to non-replicating virus are induced to produce more FGFb than control cells. In a study of vascular endothelial growth factor (VEGF), the authors have shown that RSV infection potentially alters vascular remodeling (15). This process may explain the increased vascular permeability observed in the disease that is partly responsible for airway thickening as a result of edema. Other studies have shown that endothelin-1 production is modulated by RSV infection of epithelial cells (16, 17). This study shows the differential production of FGFb, which is directly associated with airway fibrosis, a central feature of chronic asthma, after RSV infection.

In studies of airway remodeling associated with asthma, there is repair by regeneration of injured cells, and replacement by connective tissue, leading to subepithelial scar formation and prolonged contraction of the airway (18). FGFb is a fibrogenic growth factor, that is released following epithelial cell injury. It can regulate fibroblast proliferation and migration (19). Following allergen challenge, FGFb has been shown to increase in the lung lavage obtained from subjects (20). FGF is also found in high levels in the tracheal aspirates of infants who developed bronchopulmonary dysplasia (21). FGFb modulates repair after lung injury, and the expression of FGF following RSV infection may reflect epithelial cell injury. However, if the effect was a generalized cellular injury

response, EGF would also have been expected to increase.

Epidermal growth factor, in contrast, showed no increase after RSV infection. EGF, while having some fibrogenic properties, promotes healing by stimulating the proliferation and migration of epithelial cells and increasing synthesis of fibronectin (22). In studies of growth factor distribution in the lungs of asthmatic patients, EGF was found mainly in the sub-mucosal layer, rather than in the epithelium, and did not correlate with the number of fibroblasts (22). Our study did not demonstrate any specific effect of RSV cellular inoculation on EGF production *in vitro*. The expression of EGF does not correlate with fibrosis patterns in the lung (23), but is important in the healing phase of repair.

We have shown for the first time, that cells infected with RSV or exposed to non-replicating RSV produce higher levels of pro-fibrotic growth factor FGFb. The mechanism of FGFb secretion appears to be regulated at the levels of both transcription and translation. The increased FGFb release potentially could contribute to fibroblast activation and remodeling in the airway, and thus provide another mechanism for prolonged wheezing after infection. In contrast, EGF, which is important in the repair of the epithelium, and not significantly fibrogenic, was not differentially expressed during infection or exposure to RSV. Further *in vivo* studies will be performed to expand on these findings.

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