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Transforming Growth Factor-β Expression Induced by Rhinovirus Infection in Respiratory Epithelial Cells

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Abstract Rhinovirus infection of the lower airways is now a recognized disease, associated with bronchiolitis and asthma. The bronchial epithelial cells are the host cells when rhinovirus infection occurs in the airway. It was hypothesized that a pro-fibrotic growth factor response may occur in these infected cells, leading to production of a key transforming growth factor, TGF-β-1. Bronchial epithelial cells were inoculated with human rhinovirus and compared at day 1, 3 and 5 to control non-infected cells. Cell culture supernatant fluid and cellular RNA were isolated. The amount of released TGF-β protein was measured by enzyme-linked immunosorbent assay (ELISA). Expression of TGF-β at the level of transcription was measured by polymerase chain reaction (PCR) and gel electrophoresis. The results show that at all time points studied, TGF-β production is greater in the infected cells, as demonstrated by ELISA (P<0.05) and by semi-quantitative PCR analysis. It was concluded that bronchial epithelial cells infected with common cold virus and rhinovirus, showed higher levels of TGF-β. The production of TGF-β may be indicative of a normal repair mechanism to counter inflammation, or in the setting of persistent asthma, could potentially lead to increased fibrosis and collagen deposition.

Key words rhinovirus; TGF-β; bronchial; asthma; common cold

Rhinovirus infection, a common cause of upper respiratory infection, is also a significant viral cause of lower respiratory tract infection, and bronchiolitis in infants and children worldwide [1]. In addition, a significant adult lower respiratory disease has been associated with rhinovirus infection [2]. Many studies have established a link between asthma, transient wheezing and rhinovirus infection [3]. Chronic inflammatory changes associated with asthma lead to remodeling and structural airway pathology, even in younger patients [4]. These structural changes, termed remodeling, 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, transforming growth factor-β (TGF-β) has been implicated in the repair phase of remodeling [4]. Rhinovirus infection of the lower airways results in a pro-inflammatory response in both normal and asthmatic epithelial cells [6]. In addition, release of CXC chemokine epithelial-neutrophil activating peptide-78 by bronchial epithelial cells in response to Rhinovirus has been shown [7]. Based on the above associations, we hypothesized that cells infected with human rhinovirus (HRV) may produce TGF-β as a pro-fibrotic, anti-inflammatory regulator of epithelial repair.

Materials and Methods

Cell culture

Transformed BET-1A human bronchial epithelial cells (ATCC, Rockville, USA) were incubated and grown in small airways growth medium (SAGM) (Clonetics, Walkersville, USA) in 5% CO_2/95% air at 37 ºC, under sterile conditions. The epithelial cells were grown to approximately 75%–90% subconfluence in standard supplemented growth medium SAGM. The cells were then washed and incubated at 37 ºC with 5% CO_2 in serum-

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free and growth factor-free basal medium for varying time periods following viral inoculation. At the indicated times, culture supernatants were recovered and the cells were harvested, lysed and processed for isolation of RNA or protein analysis, as described below.

Viral infection of epithelial cells

Subconfluent monolayers of epithelial cells were washed three times with sterile HBSS. Human rhinovirus strain 16 (HRV-16), was added to the cells at a concentration of 10^3 $TCID_{50}$/ml HBSS. The cells were incubated with virus for 1 h, while gently shaking, and then washed three times [8]. The cells remained viable throughout the experiment and inoculation were confirmed by polymerase chain reaction (PCR). The cells were a gift from the lab of Dr. Sally SARAWAR. Control cells were not inoculated with virus.

Cell supernatant isolation

Cell culture fluids were collected at the time points studied, centrifuged for 5 min at 3000 rpm, and aliquoted for further use. The supernatants were frozen and stored at –80 ºC.

Quantitative ELISA for TGF-β-1

The levels of TGF-β-1 in the cell sample supernatants were quantitatively assessed by enzyme-linked immunosorbent assay (ELISA) using kits obtained from R&D systems (Minneapolis, Minnesota, USA) according to the manufacturer's instructions. The limit of detectability was 7 pg/ml. Any level of TGF-β below 7 pg/ml was considered to be zero for purposes of analysis.

Reverse transcription-PCR

Total RNA was isolated using RNA Stat60 (Tel-test Inc., Friendswood, USA) according to the manufacturer's instructions. RNA was quantified fluorimetrically using SYBR Green II (Molecular Probes, Carlsbad, USA). The isolated RNA (1 μg) was then reversely transcribed using a 20 μl volume reaction, which consisted of 10 U of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, USA), 4 μl of 5×reverse transcription (RT) buffer (Gibco BRL), 1 μl of 10 mM dNTP (Pharmacia, Uppsala, Sweden), 2 μl of 100 mM dithiothreitol (DTT), 0.5 μl of random primer $pd(N)_6$ (Pharmacia), 0.5 μl of RNasin (Promega), 1 μl of DEPC H_2O and 10 μl of total RNA. The reaction mixture was incubated for 1 h at 37 ºC. Expression of human TGF-β-1 mRNA transcripts was semi-quantitatively measured by PCR (anneal temperature, 60 ºC, for 35 cycles), and compared to expression of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene transcripts. The primers used for these experiments were: 5′-gcgacaccaacccgagttca-3′ (forward) and 5′-cccatactacacgggtgtcc-3′ (reverse) (Genset, La Jolla, USA). The PCR products were then separated on a 2% agarose gel. The isolated product of 203 bp as expected was detectable after ethidium bromide staining. A negative control without the addition of RNA and the positive control with total RNA derived from lung fibroblasts were processed otherwise similarly.

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

Quantitative ELISA for TGF-β-1

The results of ELISA analysis of the bronchial epithelial cell culture supernatants indicate that, compared with control at day 1, 3 and 5, there is significantly more TGF-β-1 production following HRV-16 inoculation. At day 1, 3 and 5, the mean levels of TGF-β-1 in the cell supernatants were elevated compared with control: 1076 pg/ml versus 46 pg/ml; 433 pg/ml versus 10 pg/ml; and 1904 pg/ml versus 73 pg/ml, respectively ($P<0.05$) (**Table 1**).

Reverse transcription-PCR

Semi-quantitative PCR analysis using the cellular mRNA

Table 1 Enzyme-linked immunosorbent assay (ELISA) results comparing the transforming growth factor-β (TGF-β) levels in cell supernatants from rhinovirus cells versus control cells

Day	Mean level in rhinovirus cells (pg/ml)	Mean level in control cells (pg/ml)	Standard deviation
1	1076	46	<10%
3	433	10	<8%
5	1904	73	<10%

from the cells under the conditions described showed a distinct amplicon of 203 bp for human TGF-β-1 in the inoculated cells at day 1, 3 and 5, respectively. In contrast, the non-infected control cells grown in serum-free medium did not show TGF-β-1 expression. Cellular mRNA from HRV-14 inoculated airway epithelial cells also demonstrated the transcript from day 1 to 5 (data not shown). The relative amount of TGF-β-1 is comparable at day 5 to HRV-16-inoculated cells. Positive and negative controls yielded expected results. The findings are shown in **Fig. 1(A)**. The corresponding *GAPDH* housekeeping gene expression for HRV-16 and control cells are shown in **Fig. 1(B)**.

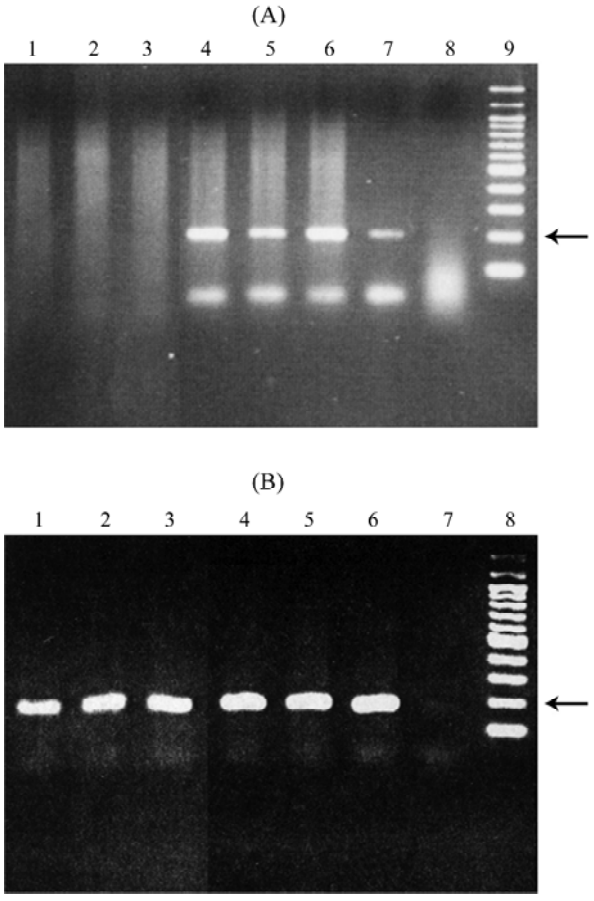

Fig. 1 Semi-quantitative polymerase chain reaction (PCR) analysis of transforming growth factor-β-1 (TGF-β-1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene expression

(A) The PCR product of human TGF-β-1 was identified as the expected 203 bp band in the day 1, 3 and 5 human rhinovirus-16 (HRV-16) cells (lanes 4–6). The non-inoculated cells did not express detectable TGF-β-1 at day 1, 3 and 5 (lanes 1–3). The positive and negative controls are shown in lanes 7 and 8 respectively. Lane 9 is DNA marker. The arrow showed the PCR product of TGF-β-1. (B) The results of *GAPDH* housekeeping gene expression are shown for control (lanes 1–3), and HRV-inoculated cells (lanes 4–6). Lane 7 is a negative control. Lance 8 is DNA marker. The arrow showed the PCR product of *GAPDH* gene.

Discussion

This study demonstrates that human bronchial epithelial cells are induced to express TGF-β-1 following inoculation with human rhinovirus, both at the level of transcription and translation. Our findings show that TGF-β-1 is found in elevated amounts in the supernatant fluids of cells inoculated with HRV, compared to control cells. The results of PCR show the semi-quantitative transcripts were easily detectable in HRV-inoculated cells, but not in control cells. Levels of TGF-β were similar at day 1 and 5, with perhaps a slight decrease on day 3, using semi-quantitative PCR and ELISA analysis. The results of protein quantitation by ELISA indicated that on all days studied, the HRV-infected cells produced significantly more TGF-β-1 than the control cells. Levels were elevated, but lower at day 3. One possibility to explain the day 3 mild decline is that a feedback mechanism exists to stem the release of TGF-β following rhinoviral infection of airway epithelial cells. This observation will be further investigated. At day 5, the expression of TGF-β is comparable to day 1 level. HRV infection of bronchial epithelial cells may be an important factor contributing to pro-fibrotic responses, based on our study.

In both normals and asthmatics, rhinoviral infection can result in increases in airway reactivity, lower respiratory tract symptoms and cough, which can persist for weeks. In one study, HRV infection induces inflammation and enhanced production of 5-LO pathway proteins [9]. TGF-β can directly activate eicosanoid pathway enzymes. Based on our study, HRV can induce TGF-β release, which in turn could potentially activate eicosanoid pathway enzymes. This may provide one mechanism for the cellular inflammation associated with lower tract infection by rhinovirus in both normal and atopic subjects.

While in normal subjects, rhinovirus is a self-limited acute infection, 40%–85% of asthma exacerbations are associated with upper respiratory tract infections, and 60% of these are rhinoviral infections [10,11]. Some researchers suggest that rhinovirus infections have been an under-estimated cause of bronchiolitis, which is associated with prolonged wheezing and the development of childhood wheezing [12]. Respiratory epithelial cells are the host cells for rhinovirus replication, and important cells in the development of remodeling changes in asthma [13].

In the asthmatic airway, there is evidence that the bronchial epithelium is structurally and functionally abnormal. Studies have shown that in addition to proinflammatory mediators, injury to the epithelium releases

fibroproliferative factors including TGF-β [14]. TGF-β in particular leads to differentiation of lung fibroblasts to myofibroblasts, and the subsequent secretion of collagen. Cell signals under the control of TGF-β are transmitted from the epithelium to deeper submucosal layers [15].

Our study has shown that bronchial epithelial cells infected with the common cold virus HRV-16 are a potentially important source of TGF-β in the airway. These findings, along with other studies linking rhinovirus to asthma, suggest that TGF-β has a pathophysiologic role in the development of asthma and remodeling. Some researchers have suggested that TGF-β release may be a compensatory mechanism resulting in less inflammation and reactivity of the airway, while over time leading to fibrosis [16].

Rhinovirus infection can trigger the release of IL-1, IL-5, IL-6, IL-8, IL-11, RANTES and eotaxin, which can in turn recruit eosinophils, monocytes and neutrophils to the lung. Since in most patients, rhinovirus is a self-limited acute illness, TGF-β may be important in limiting the potential damage caused by these cytokines [9].

In summary, this study indicates that HRV-infected airway cells exhibit greater release of TGF-β compared to control cells. TGF-β may be an important cellular response to HRV infection.

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