Title

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Journal

Respiratory Physiology & Neurobiology, 132(3)

ISSN

1569-9048

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Publication Date

2002-09-01

DOI

10.1016/s1569-9048(02)00111-8

Peer reviewed

Respiratory Physiology & Neurobiology 132 (2002) 341–346

RESPIRATORY PHYSIOLOGY & NEUROBIOLOGY

www.elsevier.com/locate/resphysiol

Short communication

In vitro wounding of airway smooth muscle cell monolayers increases expression of TGF-β receptors

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Accepted 10 June 2002

Abstract

During an exacerbation of asthma, there is bronchial epithelial cell injury and influx of inflammatory cells. In these instances, the release of proteases and various cytokines could lead to injury of the airway smooth muscle cells (ASMCs). Airway remodeling is a characteristic finding in asthma but the role of ASMC injury in remodeling is unknown. Previously, we demonstrated that mechanical wounding of confluent monolayers of bovine ASMCs resulted in the release of biologically active transforming growth factor-beta1 (TGF-β1), which in turn, induced collagen I expression. In the present study, we demonstrate that after mechanical wounding, ASMCs had an increased expression of the signal transducing TGF-β receptors TβR-I and TβR-II as detected by flow cytometry and Western analysis. Corticosteroids are standard therapy in asthma and the presence of dexamethasone decreased wound-induced release of TGF-β1 and the expression of collagen I, fibronectin, and TβR-II. These results suggest that ASMC injury may play an important role in airway fibrosis mediated by TGF-β1, which can be prevented by the use of corticosteroids.

Keywords: Bronchi, epithelial cell injury; Disease, asthma; Mammals, cow; Mediators, TGF-β1; Receptor, TGF-β; Smooth muscle, airways; Upper airways, epithelial cell injury

1. Introduction

In asthma, remodeling of the airways is characterized by bronchial epithelial cell (BEC) shedding, goblet cell hyperplasia, fibrosis in the subepithelial region and the lamina propria, increase in airway smooth muscle cell (ASMC) mass and appearance of myofibroblasts (Bousquet et al., 2000; McKay and Sharma, 2002). The presence of myofibroblasts is usually observed in instances of repair after an injury (Morishima et al., 2001; Powell et al., 1999). Injury to the ASMCs could occur in vivo during airway inflammation and shedding of the bronchial epithelium since inflammatory cells as well as injured epithelial cells can release a number of proteolytic mediators, such as elastases, metalloproteinases, chymase, procollagenase, oxygen radicals and numerous other substances (Bousquet et al., 2000). However, little is known about the role of ASMC injury in airway

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1569-9048/02/$ - see front matter
PII: S1569-9048(02)00111-8

remodeling. Recently, we demonstrated that mechanical wounding of confluent monolayers of bovine ASMCs resulted in an increased release of biologically active transforming growth factor-beta1 (TGF-β1) (Coutts et al., 2001), a potent regulator of connective tissue synthesis (Khalil, 2001). TGF-β is secreted as a latent protein called latent-TGF-β which has no biological effect (Khalil, 2001). To have a biological effect TGF-β has to be in its active form and interact with the serine/threonine TGF-β receptors type I (TβR-I) and II (TβR-II) (Khalil, 2001). TβR-II is an 85 kDa protein that is constitutively phosphorylated and binds TGF-β, while TβR-I is a 55 kDa protein that cannot bind TGF-β, but is critical for signal transduction (Khalil, 2001). Upon binding TGF-β1, the TβR-II recruits and phosphorylates TβR-I, which initiates signal transduction (Khalil, 2001).

In the present study, we have demonstrated that injury to the ASMC monolayer leads to increased expression of TβR-I and TβR-II as well as connective tissue synthesis. Corticosteroids are standard therapy for asthma (Chan et al., 1998). When wounded ASMC monolayers were cultured in the presence of dexamethasone, there was a decrease in the release of active TGF-β1 and expression of TβR-II and connective tissue proteins.

2. Materials and methods

2.1. Cell culture

The ASMCs were isolated from bovine trachea cultured in alpha-minimum essential medium (αMEM) supplemented with 10% fetal calf serum and antibiotic–antimycotic reagents and incubated at 37 °C in a humidified atmosphere (5% CO_2-balanced air) as previously described (Coutts et al., 2001). The ASMCs were passaged with 0.05% trypsin/0.53 mM EDTA. All the cell culture reagents were purchased from GIBCO BRL (Burlington, ON, Canada).

2.2. Scrape wounding of confluent monolayers ASMCs

ASMCs in passage 1–3 were grown to confluence, and then changed to serum-free αMEM containing antibiotics plus 1 g/l insulin, 0.67 mg/l sodium selenium, 0.55 g/l transferrin and 0.2 g/l ethanolamine (GIBCO BRL) for 10 days as previously described (Coutts et al., 2001). Each monolayer was mechanically wounded by 18 scrapes using a policeman, then washed with the serum-free αMEM and cultured with fresh serum-free αMEM in the absence or presence of dexamethasone (10^{-4} M) added to the culture at 1.5 h before, simultaneously, and 2, 4, 6 h after the wounding.

2.3. TGF-β1 assay by ELISA

Twenty-four hours after wounding, the overlying conditioned media (CM) was removed, stored in the presence of the protease inhibitors and frozen at −86 °C until ready for use (Coutts et al., 2001). DuoSet TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN) was used to determine TGF-β1 in neutral CM (representing active TGF-β1) or CM that was acidified and subsequently neutralized (representing total TGF-β1), according to the manufacturers instructions.

2.4. TGF-β1 receptor assay by flow cytometry

Twenty-four hours after wounding, ASMCs were removed from the culture dishes using a non-enzymatic cell disassociation solution (Sigma), and then stained with Fluorokine cytokine flow cytometry reagents (R&D Systems) as instructed by the manufacturer. Negative staining control for each sample was set by using identical cells stained with biotinylated negative control reagent and then avidin-fluorescein. ASMCs expressing the specific TGF-β1 receptors were analyzed using Coulter Epics XL-MCL Flow Cytometer and EXPO 32 Software.

2.5. Western blotting and immune detection

As described previously (Coutts et al., 2001), ASMCs were trypsinized, and whole cell protein was extracted on ice with triple-detergent lysis buffer, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane (BioRad). The membrane was blocked and then incubated with the primary antibodies, anti-TβR-I and TβR-II antibodies (Santa Cruz, CA), anti-collagen type I and anti-fibronectin antibodies (Cedarlane Laboratories, Hornby, ON, Canada), followed by secondary antibodies (Santa Cruz, CA). The proteins on the membrane were then immunodetected by the ECL system (Amersham, Arlington Heights, IL) according to the manufacturer's instruction. Relative absorbance was quantitated using the Quantity One imaging system (BioRad, Hercules, CA), normalized with data of un-wounded control and expressed as fold of control.

2.6. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). All P-values (2-tailed) were based on the Student t-test. Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Collagen I and fibronectin expression after wounding of ASMC monolayers

There was a significant increase in collagen I and fibronectin expression as detected by Western analysis after wounding ASMC monolayers. The increase in collagen I and fibronectin was inhibited at all time intervals after the addition of dexamethasone to wounded monolayers (Fig. 1A and B).

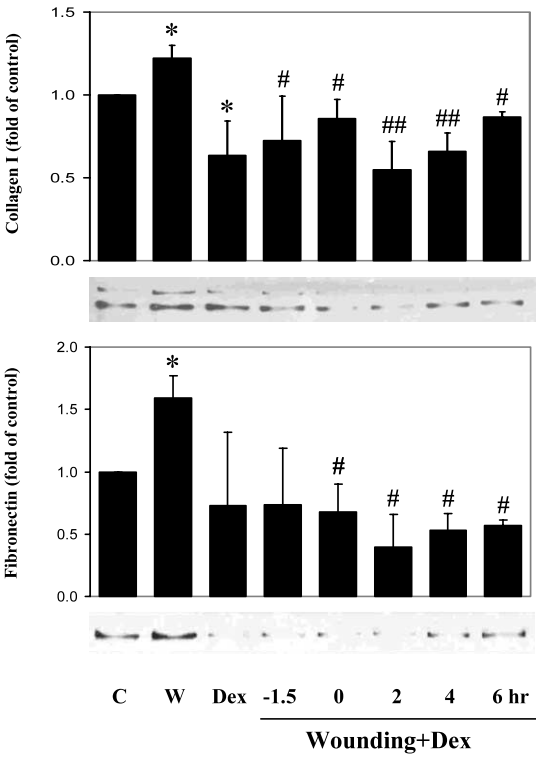

Fig. 1. Dexamethasone regulation of collagen I and fibronectin in wounded ASMC. Confluent ASMC monolayers were mechanically wounded in the absence or presence of dexamethasone (10^{-4} M) added at 1.5 h before, simultaneously, and 2, 4, 6 h after the wounding. Whole cell protein was extracted, SDS-PAGE and Western blotting were performed to detect the expression of collagen I (A) and fibronectin (B). $^{*}P < 0.05$ compared to un-wounded control; $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ compared to wounding.

3.2. Release of TGF-β1 by wounded ASMC monolayers

Consistent with our previous report (Coutts et al., 2001), there was a small amount of TGF-β1 in the CM of the confluent ASMC cultured with serum-free medium. Twenty-four hours after wounding, the active TGF-β1 and total TGF-β1 significantly increased by 24 and 45%, respectively, as compared to the culture without wounding. However, in the presence of dexamethasone, the increase in the release of TGF-β1 was totally abrogated (Table 1). In the absence of wounding, dexamethasone had no effect on the level of TGF-β1 in the CM (data not shown).

3.3. Expression of TβRs by wounded ASMC monolayers

Significant increases in TβR-I and TβR-II after wounding were detected by Western Blot (Fig. 2).

Table 1
Effects of wounding and/or dexamethasone on TGF-β1 release by ASMC monolayer (mean ± SEM)

	Active TGF-β1	Total TGF-β1
Control	53.43 ± 2.66	262.75 ± 0.67
Wounding	66.28 ± 2.24*	381.87 ± 16.92**
W + D	23.20 ± 10.36***	248.59 ± 7.34****

W + D: wounding in the presence of dexamethasone.
* $P < 0.05$, compared to control.
** $P < 0.005$, compared to control.
*** $P < 0.02$, compared to wounding.
**** $P < 0.002$, compared to wounding.

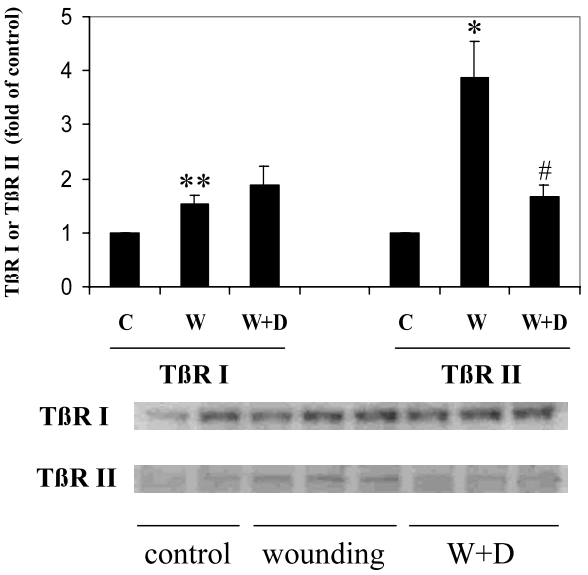

Fig. 2. Wounding-induced TGF-β1 and TβR-II expression. Confluent ASMC monolayers were mechanically wounded in the absence or presence of dexamethasone (10^{-4} M) for 24 h prior to protein extraction, SDS-PAGE and Western blotting for detection of TGF-β1 and TβR-II. *$P < 0.05$, **$P < 0.01$ compared to un-wounded control; $^{\#}P < 0.05$ compared to wounding.

The enhanced TβR expression was confirmed by flow cytometric analysis with a 1.33-fold of increase in the density of TβR, as compared to monolayers without wounding. In the presence of dexamethasone, wounding still induced an increase in TβR-I (Fig. 2). However, the wounding-induced increase in TβR-II was significantly inhibited by dexamethasone (Fig. 2).

4. Discussion

The mechanisms important for airway remodeling in asthma are likely to be multifactorial and complex (Bousquet et al., 2000; Morishima et al., 2001; Coutts et al., 2001; and our unpublished data). Using a model emulating ASMC injury (Coutts et al., 2001), the present study demonstrates for the first time that in mechanically wounded monolayers of ASMCs, there is an increase in expression of TβR-I and TβR-II. In this model, ASMC injury results in a concomitant release of increased quantities of a biologically active form of TGF-β1 (Coutts et al., 2001). The increase in the expression of TβR-I and TβR-II would render these ASMCs to be highly responsive to synthesize connective tissue proteins by the TGF-β1 released from the same cells. It has been demonstrated that during exacerbation of asthma there is an influx of activated inflammatory cells and BEC damage (Bousquet et al., 2000; McKay and Sharma, 2002). Both activated inflammatory cells and injured BECs release proteolytic enzymes such as elastases, chymase, metalloproteinases, oxygen radicals and other potentially injurious cytokines (Bousquet et al., 2000). The release of these substances could result in ASMC injury in vivo and may account for the increase in myofibroblasts (Powell et al., 1999; Morishima et al., 2001) and subsequent fibrosis in the subepithelial region and in the lamina propria of the airway wall (Bousquet et al., 2000; Morishima et al., 2001). Although examples of ASMC injury models are uncommon, there are many studies using models of vascular smooth muscle (VSM) injury. For example, after VSM injury of the rat carotid artery, there was an induction of TβR-II expression by the VSM (Smith et al., 1999). The induction of TβR-II was directly responsible for collagens type I and III synthesis as a response to injury (Smith et al., 1999). In similar models of VSM injury it has been demonstrated that plasmin, a serine protease, regulates the release biologically active TGF-β1 (Khalil, 2001). We had demonstrated that wounding of monolayers of ASMCs generates plasmin, while the presence of aprotinin and alpha 2-antiplasmin, both inhibitors of plasmin not only neutralized plasmin but

also abrogated the release of active TGF-β1 and collagen synthesis by ASMCs (Coutts et al., 2001). The VSM findings are similar to the ASMC injury model, suggesting a common pathway in the pathogenesis of remodeling seen not only in the walls of arteries but also airways. These findings provide compelling evidence that in the event of ASMC injury in asthma there is likely release of TGF-β1, induction of TβR-I and TβR-II with a resultant increase in connective tissue synthesis mediated by TGF-β1.

The mechanisms that regulate TβRs are very poorly understood. We have recently demonstrated that granulocyte macrophage-colony stimulating factor (GM-CSF) induces ASMCs to express increased amounts of TβR-I and TβR-II in a dose dependent manner which, in turn, results in an increase in TGF-β mediated signal transduction and connective tissue synthesis (Chen et al., unpublished). ASMCs have been reported to release GM-CSF (McKay and Sharma, 2002) and it is then possible that mechanical wounding in this model could lead to an increase in GM-CSF release, which in turn may induce TβR expression. Irrespective of the mechanism involved, a consistent biological observation is that in models of wounding and injury TβRs are increased in expression by fibroblasts, ASMCs and VSM (Gold et al., 1997; Khalil et al., 2002; Smith et al., 1999).

The importance of TGF-β1 release and TβR expression by ASMCs in connective tissue synthesis was further confirmed in experiments using dexamethasone. The presence of dexamethasone in ASMC monolayers with wounds greatly decreased the generation of active TGF-β1 and TβR-II. Since both TβR-I and TβR-II are required for signal transduction, the reduction of TβR-II along with the reduction in TGF-β1 released is likely to have resulted in decreased connective tissue synthesis. These findings support the use of corticosteroids in asthma for the prevention of irreversible airway fibrosis and obstruction. However, many individuals with asthma develop irreversible airway remodeling despite use of adequate steroids (Chan et al., 1998). The current observations suggest that the mechanism of steroid unresponsiveness may not be related to expression of TGF-β1 and its receptors.

In conclusion, we have provided evidence that in the event of injury to the smooth muscle of airways there is a release of biologically active TGF-β1 and induction of TβR-I and TβR-II. These alterations are associated with increase in connective tissue synthesis. Furthermore, corticosteroids are likely to be of benefit in preventing TGF-β1 mediated airway fibrosis.

Acknowledgements

The work in this paper was supported by CIHR-R & D (GlaxoSmithKline). We thank Ms Valerie Romanchuk in preparing the manuscript.

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