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Role of insulin like growth factor axis in the bleomycin induced lung injury in rats

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ABSTRACT

Background: Alveolar epithelial cell injury has been proposed as a causative factor for the onset and progression of pulmonary fibrosis. However, the role of type II alveolar epithelial cells (AECs) in the epithelial mesenchymal transition (EMT) is controversial.
Aims: The present study performed in rats instilled with bleomycin investigated a) the expressions of the insulin growth factor (IGF-1) and insulin growth factor binding protein 5 (IGFBP-5) and transforming growth factor (TGF-β1) in the type II AECs, b) the role of type II AECs in EMT and extracellular matrix (ECM) formation and, c) the effect of pioglitazone on all the above parameters.
Methods: Male Wistar rats were divided into three Groups: Group I (saline control), Group II (Bleomycin, given as a single intratracheal instillation, 7 U/kg) and Group III (Bleomycin + Pioglitazone (40 mg/kg/day orally, starting 7 days post bleomycin instilled as in Group II). From lung tissues, the protein expressions of IGF-1, IGFBP-5, TGF-β1, surfactant protein C (SP-C, as a marker for type II AECs) and α-smooth muscle actin (α-SMA, as a marker for EMT), were determined on day 7 in Groups I and II and on days 14, 21 and 35 in all the three groups.
Results: IGFBP-5 and IGF-1 expressions were reduced significantly and TGF-β1 expression increased significantly in type II AECs in Group II from day 7 till day 35 as compared to Group I. An increase in SP-C and α-SMA expression and their co-localization were seen in the type II AECs undergoing EMT from day 7 till day 35. A concomitant remodeling and laying down of ECM was observed also. In Group III, with pioglitazone, there was a reversal with significant up-regulation in IGFBP-5 and IGF-1 expressions and down-regulation of TGF-β1 in the type II AECs along with a significant decrease in the solid area fraction, EMT and ECM in the lung tissue.
Conclusions: IGFBP-5, IGF-1 and TGF-β1 in the type II AECs play a key role in lung injury caused by bleomycin and pioglitazone attenuates the lung injury/fibrosis by restoring IGFBP-5 and IGF-1 and decreasing TGF-β1 expressions in the type II AECs.

1. Introduction

Pulmonary fibrosis is a progressive, degenerative complex lung disease (Oruqaj et al., 2015) the exact pathophysiological mechanisms for the development of which are not fully understood. There is a general consensus that this disease is due to the combination of alveolar epithelial cell injury which is followed by the release of proinflammatory/fibrotic cytokines, inflammatory cell infiltration, epithelial mesenchymal transition (EMT) and excessive deposition of extracellular matrix in the lung (Serrano-Mollar, 2012). Presently in idiopathic pulmonary fibrosis (IPF), combination therapies comprising of immunosuppressive agents, anti-oxidants and anti-inflammatory agents are often prescribed to the patients and have also been reported to be partially successful (Rafii et al., 2013). One possibility for the decrease in efficacy of these drugs whether alone or in combination may be because the treatment is started at a later stage when pulmonary fibrosis has already set in. An effective line of treatment may be targeting the primary factor namely the alveolar epithelial cells (AECs) which undergo apoptosis, alterations in the cytokines and the growth factors in the AECs and the interlink between them and the fibroblasts which ultimately result in pulmonary fibrosis (Sakai and Tager, 2013). In fact, it has been proposed that the aim of future therapy should be to increase the alveolar epithelial regeneration (Rafii et al., 2013) as well as to reverse the process of EMT (Kagalwalla et al., 2012). The present study makes an attempt to investigate this proposal in the bleomycin induced experimental model of lung injury in rats and following up the lung pathology in a sequential manner.

Growth factors such as transforming growth factor beta1 (TGF-β1), connective tissue growth factor (CTGF), fibroblast growth factor (FGF),

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insulin-like growth factor-1 (IGF-1) and platelet derived growth factor (PDGF) have been shown to regulate the growth and differentiation of the AECs (Allen and Spiteri, 2002). There is increasing evidence that the IGF axis may play a significant role in pathogenesis of pulmonary fibrosis (Hung et al., 2013; Ahasic et al., 2012; Yasuoka et al., 2006; Krein and Winston, 2002; Aston et al., 1995). In patients with IPF, IGF-1 expression has been shown to be increased in the bronchoalveolar lavage (BAL) fluid (Frankel et al., 2005) suggesting that an imbalance in the IGF axis may play a pivotal role in the progress of this disease. An increase in the IGF-1 has been reported in the BAL cells after bleomycin induced lung injury in the murine model also (Maeda et al., 1996). Such an increase in IGF-1 in the BAL fluid as well as in BAL cells is understandable as IGF-1 has been shown to be increased in the EMT cells, macrophages and myofibroblasts (Hung et al., 2013) and is generally considered to be profibroblastic (Hung et al., 2013). When attempts have been made to determine IGF-1 in AECs from IPF patients, it has been observed that as seen in BAL fluid and BAL cells, there is an increase (Maeda et al., 1996) in IGF-1 in the AECs in the early stage IPF when there is diffuse alveolar damage. However, a decrease in IGF-1 occurs in them in the later stage when there is interstitial fibrosis and honeycombing (Homma et al., 1995). Another study while confirming the increase has not found a decrease in IGF-1 staining of AECs in IPF patients with extensive collagen deposition and honeycombing (Maeda et al., 1996). Thus, even though the function of IGF-1 in the interstitial cells has been defined, there is no definite information on the role of IGF-1 in AECs in pulmonary fibrosis.

Insulin-like growth factor binding proteins (IGFBPs 1–10) are a family of proteins that bind IGFs with high affinity. The IGFBPs vary in their tissue expression and also in their response and regulation by other growth factors. The IGFBP-5 is the most conserved of all the IGFBPs. The IGFBPs have been suggested to be involved in the initiation and/or perpetuation of fibrosis by inducing the production of ECM components such as collagen type I and fibronectin (Pilewski et al., 2005). The IGFBPs bind and regulate the access of IGF to its receptor and thereby regulate the biological activity of IGF-1 on target cells. Depending on content, IGFBPs can potentiate or inhibit the biological effects of IGF-1 in a given tissue (Pilewski et al., 2005). There has been no investigation on the expression of IGFBP-5 in AECs and their interaction with IGF-1 expressed in AECs either in samples from IPF patients or in animal models administered with bleomycin.

Based upon these reports, for the present study, it has been hypothesized that in the rat, during bleomycin induced lung injury, there will be a decrease in the expression of IGF-1 in the type II AECs. This decrease predisposes to the injury of the type II AECs as IGF-1 is responsible not only for cell proliferation and tissue differentiation but also for protection from apoptosis (Laviola et al., 2007). Reduced IGF-1 expression will hamper the differentiation of type II AECs into type I cells and prevent parenchymal repair (Ghosh et al., 2013; Narasaraju et al., 2006). It has been hypothesized further that along with IGF-1, there will be a decrease in the expression of IGFBP-5 in the type II AECs. Since IGFBP-5, by increasing the epithelial production of the basement membrane protein, laminin, protects the epithelium from injury, its decrease will promote pulmonary fibrosis (Sureshbabu et al., 2011). Additionally, its decrease will make the pulmonary fibrosis worse by causing apoptosis of AECs by reducing the bio-availability of IGF-1.

To prove the hypothesis, after intratracheal instillation of bleomycin, lung sections have been examined at various time intervals. In them, the IGF axis has been assessed by determining the expressions of IGF-1 and IGFBP-5 in the type II AECs (SP-C is used as a marker for type II AECs), EMT has been studied by α-SMA expression and the ECM is determined by measuring the solid area fraction as well as by Masson's trichrome staining for collagen. Besides IGF axis, TGF-β1 expression has been investigated in the type II AECs. Subsequently, to assess whether there is an attenuation of the observed changes by a drug, pioglitazone has been administered. Thiazolidinediones (TZDs) like pioglitazone are the synthesized ligands for the peroxisome proliferator-activated receptorγ (PPARγ) and in vascular smooth muscle cells, PPARγ agonists have been reported to up-regulate IGF-1 and its receptor (Higashi et al., 2010). Its role in up-regulating the IGF-1 in the type II AECs is yet to be investigated.

2. Materials and methods

2.1. Chemicals and reagents

Bleomycin sulphate was purchased as Bleocip from Cipla Limited, Mumbai, India, and Pioglitazone tablets (Pioglar) were purchased from Ranbaxy laboratories Limited, New Delhi, India. The rat antibodies of Surfactant Protein C (SC-13979), IGFBP-5 (SC-13093), IGF-1 (SC-9013) were purchased from Santa Cruz Biotechnology, California, U.S.A. Antibodies of TGF-β1 (SAB4502954) and α-SMA (A5228), and the secondary antibody kits (Extra-2, 3 kit), for immunohistochemistry were purchased from Sigma Chemicals, Missouri, U.S.A. Secondary antibodies for immunofluorescence, goat anti rabbit Alexa Fluor 488 (A11008) and goat anti mouse Alexa Fluor 555 (A21422) were purchased from Molecular probes, Invitrogen, Carlsbad, U.S.A. Hoechst 33,258 dye (TC225) was purchased from Himedia laboratories, Mumbai, India. All other chemicals used were of the highest commercial grade available.

2.2. Animals

Seven weeks old male Wistar rats were obtained from Vallabhbhai Patel Chest Institute animal house. The animals were quarantined for minimum of 1 week prior to use. Rats weighing approximately 260 ± 20 g were used in this study. Proper precautionary measures were followed to minimize any suffering during the animal experimentation. Animal experiments were performed with the regulations specified by the Institute's Animal Ethical Committee and conform to the National guidelines on the care and use of laboratory animals, India.

2.3. Rat model for lung fibrosis

The animals were randomly divided into three Groups with six rats in each group: Group I: Saline control, Group II: Bleomycin and Group III: (Bleomycin + Pioglitazone). In Group II, induction of lung injury was done by a single intratracheal instillation of bleomycin sulphate (7 U/kg) in a volume of 100 μl saline (0.9%). Group I rats received a single dose of intratracheal saline (100 μl of 0.9% saline). In Group III, the pioglitazone drug treatment was given orally starting from 7 days after instillation of bleomycin and administered daily till the completion of the study. For intratracheal instillation, rats were anesthetized with ketamine hydrochloride (50 mg/kg, intra muscular). After inducing anesthesia, the skin overlying the trachea was cleaned with ethanol, and a small incision was made in the skin under local anesthesia (1% xylocaine). The trachea was exposed and using a 1 ml sterile syringe and 24 gauge needle, bleomycin was instilled. An equivalent volume of sterile saline was instilled intratracheally for the saline control Group. For better dispersion of the instillation into the lung, the animal's head was kept at an angle of 30 °C from the dissection table. The wound was closed with proper suturing of the skin and muscles. Betadine was applied to the wound until it healed. Pioglitazone was given orally as gavage in the conscious rat. For this purpose, the pioglar tablets were powdered and dissolved in distilled water and then administered daily for a duration of 28 days at a dose of 40 mg/kg/day.

Histopathology, morphometric changes, immunohistochemistry, immunofluorescence, Masson's trichrome were performed and assessed with image analysis software NIS-Ar elements in all Groups - on day 7 in Group I and Group II and on days 14, 21 and 35 in Groups I, II and III.