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Retinoic acid induced repair in the lung of adult hyperoxic mice, reducing transforming growth factor-β1 (TGF-β1) mediated abnormal alterations

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ABSTRACT

The aim of the study was to determine the effects of retinoic acid on lung alveolar repair in adult hyperoxic mice and to investigate the relationship between TGF-β1 and retinoic acid during the repair processes. Adult mice were divided into 4 groups. Two groups were given daily intraperitoneal injections of peanut oil/dimethylsulfoxide mixture and retinoic acid (50 mg/kg body weight, 50 μl of volume) dissolved in peanut oil/dimethylsulfoxide mixture for 12 days with a 2-day break on days 6 and 7. Following hyperoxia (100% oxygen) for 72 h the remaining two groups were treated in the same manner as already described: peanut oil/dimethylsulfoxide mixture and retinoic acid. Lung structure was investigated by light microscopy. TGF-β1 and Smad protein expressions in the lung were assayed by biochemical methods. Hyperoxic mice exhibited damage to the alveolar walls, increased cell proliferation and induced Smad3/TGF-β1 signaling. Smad2 and phospho-Smad2 protein expressions were unchanged in all groups. Retinoic acid administration improved the degenerative alterations caused by hyperoxia and helped in alveolar repair. This positive effect of retinoic acid resulted from the inhibition of Smad3/TGF-β1 signaling via reduced Smad4 mRNA and increased Smad7 protein expression. Retinoic acid also induced alveolarization and restricted Smad3/TGF-β1 signaling by decreasing Smad4 mRNA in healthy mice. Thus, retinoic acid helped repair Smad3/TGF-β1-induced lung damage in hyperoxic mice.

Introduction

Major damage is caused to airway epithelium and mesenchyme as a result of exposure to toxic gases, infectious agents, hypo- and hyperoxic conditions and gastric aspiration (Finkelstein and Johnston, 2004; Matute-Bello et al., 2008). The alveolar epithelium contains type I and type II epithelial cells (AECI and AECII) and is the primary target in acute and chronic lung injuries (Stone et al., 1992; Gropper and Wiener-Kronish, 2008). Since the alveolar epithelium provides gas exchange between inspired air and the blood, any damage to the alveolar epithelium originating from the insults described above is physiologically very important. Excessive injury and inadequate or incorrect repair of alveolar epithelium can play an important role in the development of lung diseases, such as pulmonary fibrosis, emphysema, and chronic obstructive lung diseases (Gropper and Wiener-Kronish, 2008; Matute-Bello et al., 2008). Currently, knowledge of the molecular mechanisms involved in alveolar repair is very limited. Consequently it is important to determine new and effective ways of establishing adequate and correct repair processes after alveolar epithelial injury.

Retinoic acid (RA) is an active form of vitamin A. It plays a role in lung development, alveolarization, lung growth and homeostasis in prenatal, neonatal and early postnatal stages. In addition, RA stimulates cell proliferation on primary AECII cultures derived from neonatal rats (Nabeyrat et al., 2000). RA can also induce alveolar repair in adult rats with elastase-induced emphysema, partially rescue emphysema in the tight-skin mouse mutants and improve dexamethasone-impaired alveologenesis in adult mice outside the temporally restricted period of alveologenesis (Massaro and Massaro, 1997, 2000; Maden and Hind, 2004). However, this effect of RA on alveolar repair has not been reported in several animal models with dexamethasone or elastase-impaired alveologenesis and cigarette smoke-induced pulmonary emphysema (Srinivasan et al., 2002; Lucey et al., 2003; Fujita et al., 2004; March et al., 2004, 2005). It is likely that the effects of RA on induced alveolar repair may depend on the dose, length of treatment and its pharmacokinetics.

Abbreviations: AECI, type I alveolar epithelial cell; AECII, type II alveolar epithelial cell; cDNA, complementary DNA; ELISA, enzyme-labeled immunosorbent assay; QRT-PCR, quantitative real-time polymerase chain reaction; pro-SPC, pro-surfactant protein C; RAGE, the receptor for advanced glycation end products; RA, retinoic acid; TGF-β1, transforming growth factor β1.

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Transforming growth factor β1 (TGF-β1) regulates cell proliferation, differentiation, and migration, depending on cell type. Various cell types secrete this cytokine in the lung, which acts in an autocrine and paracrine manner by Smad-dependent or non-Smad signaling pathways. Smads are proteins that mediate intracellular signaling of the TGF-β superfamily. Smad2 and 3 are phosphorylated by the TGF-β1 receptor and form complexes with Smad4 upon TGF-β1 stimulation. The heteromeric Smad complexes translocate into the nucleus, where they activate the expression of target genes. Smad6 and 7 are inhibitors of the Smad pathway, preventing phosphorylation and activation of Smad2 and 3 (Bhaskaran et al., 2007). Adenoviral-mediated transfer of TGF-β1 to the neonatal rat lung and its overexpression results in disrupted alveolar development (Gauldie et al., 2003; Vicencio et al., 2004). Blockage of TGF-β1 signaling by ablation of Smad3 generated a similar phenotype in mice. Smad3 deficiency caused abnormal lung alveolarization and developmental antecedents of centrilobular emphysema in mouse lung (Chen et al., 2005). In addition, it was shown that the transdifferentiation of AECII to AECI is modulated by Smad-dependent TGF-β1 pathway (Bhaskaran et al., 2007). On the other hand, TGF-β1 is known to play a role in directing correct or incorrect repair of alveolar epithelium under micro-environmental signals. TGF-β1 is an inducer of epithelial–mesenchymal transition in alveolar epithelial cells via Smad3 both in vitro and in vivo (Yao et al., 2004; Kasai et al., 2005; Willis et al., 2005). During epithelial–mesenchymal transition, the loss of E-cadherin expression promotes the ability of epithelial cells to adopt mesenchymal phenotypes in pulmonary fibrosis.

Studies performed to establish a physiological relationship between TGF-β1 and RA have shown agonistic and mostly antagonistic interactions among these molecules in several tissues including lung and liver. The cumulative data indicate that TGF-β1 and RA play finely tuned and key roles in the alveolarization process, and in the maintenance of alveolar structure. However, there are no data reported on the interaction between TGF-β1 and RA during alveolar repair following alveolar epithelial injury in adult mice. Moreover, RA-Smads of TGF-β1 signaling crosstalk during alveolar repair has never been documented.

Hyperoxia is one of the best experimental models to induce alveolar epithelial injury (Matute-Bello et al., 2008). We used this experimental model to create alveolar epithelium injury in lungs of mice. This study aimed to investigate the relationship between Smad-dependent TGF-β1 signaling and RA during alveolar repair and to determine whether RA has an effect on alveolar repair in adult hyperoxic mice. These findings may lead to the possibility of a potential novel therapeutic approach for human lung repair in patients with lung disease characterized by alveolar epithelial damage.

Materials and methods

Animals

This study was conducted in accordance with the guidelines provided by the Animal Care and Use Committee of Istanbul University. Adult male C57BL/6J mice (aged 8–10 weeks, 20–25 g weight) were housed in humidity- and temperature-controlled rooms on a 12-h light/dark cycle and were allowed food and water ad libitum.

Experimental procedures

Mice were divided into the following experimental groups ($n = 10$ per group): Group 1: control mice treated with 1:1 peanut oil/dimethylsulfoxide (DMSO) mixture (Sigma–Aldrich, St. Louis, MO, USA); Group 2: mice treated with retinoic acid (RA) (Sigma–Aldrich) (50 mg/kg) dissolved in peanut oil/DMSO mixture; Group 3: mice treated with peanut oil/DMSO mixture following hyperoxia (100% oxygen); Group 4: mice treated with RA (50 mg/kg) dissolved in peanut oil/DMSO mixture following hyperoxia. Mice in groups 1 and 2 were kept in room air environmental conditions throughout the experiment. Mice in groups 3 and 4 were kept in hyperoxic conditions for 72 h and subsequently maintained in the room air conditions for 12 days. The treatments were given as a daily intraperitoneal injection of 50 μl for 12 days with a 2-day break on days 6 and 7. Continuous exposure to 100% oxygen was achieved using a flow-through system in a small research chamber (0.28 m^3) at 1 atmosphere pressure at the Department of Underwater and Hyperbaric Medicine, Istanbul Faculty of Medicine for 72 h. The oxygen level was monitored by means of an oxygen analyzer (Global MiniOx I Oxygen Monitor and Analyzer, Ohio Medical Corp., USA). Carbon dioxide was removed by absorption into soda lime. Mice were given free access to food and water during hyperoxia. At the end of the experiment, mice were killed by intraperitoneal injection of a 1:1 mixture of 40 mg/kg ketamine (Pfizer, Kirklareli, Turkey) and 10 mg/kg xylazin (Alfasan International BV, Woerden, The Netherlands) before harvesting the lungs for analysis. The lungs of the left side were immersed with fixative prior to microscopic analysis, while the lungs of the right side were used for enzyme-labeled immunosorbent assay (ELISA), Western blotting and quantitative real-time polymerase chain reaction (QRT-PCR).

Histology

The lungs ($n = 5$ per group) were fixed in Bouin's fluid for 24 h at room temperature. The tissues were dehydrated in alcohol, cleared in xylene, and embedded in paraffin wax. Lung sections 5 μm thick were cut and stained with hematoxylin-eosin and examined for histology under a light microscope (Nikon Eclipse Ti-U, Tokyo, Japan) fitted with a digital camera (Nikon LH-M100C-1 Camera, Tokyo, Japan).

Immunohistochemistry

The lungs ($n = 5$ per group) were fixed in 10% neutral buffered formalin for 24 h at 4 °C. Immunohistochemical reactions were performed by means of antigen–antibody reaction followed by visualization on 4 μm-thick serial paraffin sections. For antigen retrieval the sections were treated in a microwave oven 650 W for 15 min in a 20 mM citrate buffer pH 6.0. Endogenous peroxidase was eliminated by incubation in 3% hydrogen peroxide in methanol/phosphate-buffered saline (0.01 M; pH 7.4). The sections were treated with rabbit anti-Ki67 antibody (proliferation marker, Millipore, Molsheim, France) at 1:100 dilution for 1 h at room temperature and rabbit anti-pro-surfactant protein C antibody (AECII marker, pro-SPC, Millipore, Molsheim, France) at 1:1000 dilution overnight at 4 °C. The sections were then incubated in Histostatin Plus kit (Invitrogen, Paisley, Scotland) according to the manufacturer's instructions in a humidified chamber where the antibody reacted with the antigen, forming an immunocomplex. The peroxidase activity was demonstrated by means of a 3-amino-9-ethylcarbazole substrate kit (Thermo Scientific, Waltham, MA, USA). Slides were lightly counterstained with hematoxylin to reveal nuclei. One section was selected from each twenty-five sections (at intervals of 100 μm) and placed on slides. In five sections randomly selected per mouse, five microscopic fields were randomly selected from alveolar areas without bronchioles. Digital images of these fields were captured at 200× magnification and overlaid with transparent grids (1 mm × 1 mm) and then proliferative cells and AECIIs were counted in adjacent sections. The proliferation index was calculated as a percentage of Ki67 positive cells in the total number of cells counted in each section per animal. For negative