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Bleomycin-induced epithelial–mesenchymal transition in sclerotic skin of mice: Possible role of oxidative stress in the pathogenesis

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ABSTRACT

Epithelial–mesenchymal transition (EMT) derived myofibroblasts are partly responsible for the increased collagen synthesis and deposition that occur in tissue fibrosis; however EMT occurrence in skin fibrosis and its mechanism remain unknown. The aim of this study was to investigate whether epithelial cells undergo EMT and determine the role of oxidative stress in this process. BALB/c mice were subcutaneously injected with bleomycin (BLM) or phosphate buffer saline (PBS) into the shaved back daily for 2, 3, and 4 weeks. Skin collagen deposition was evaluated by histopathology and Western blotting. EMT characteristics in the skin were determined by histopathology and immunofluorescent staining for E-cadherin and vimentin, which were further evaluated by Western blotting and reverse transcriptase polymerase chain reaction (RT-PCR). To investigate the role of oxidative stress in EMT, the antioxidant N-acetylcysteine (NAC) was intraperitoneally (100 mg/kg body weight/day) injected daily for 3 weeks. The epithelial suprabasal cells were detached from the basement membrane zone (BMZ) in the sclerotic skin treated with BLM. Immunofluorescent staining indicated vimentin-positive epithelial cells frequently occurring in the thickened epidermis of BLM-treated mice. Western blotting and RT-PCR showed that the expression of E-cadherin was significantly decreased but that of vimentin significantly increased in the skin treated with BLM. NAC attenuated BLM induced oxidative damage, changes in E-cadherin and vimentin expressions and collagen deposition in the sclerotic skin of mice. This study provides the first evidence that BLM induces the EMT of the epithelial cells superficial to the basement membrane zone in the skin fibrosis. Oxidative stress may contribute, at least in part, to BLM induced EMT and skin fibrosis in mice.

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Introduction

Bleomycin (BLM) is an antineoplastic agent used in the treatment of Hodgkin's lymphoma, squamous cell carcinomas, testicular cancer, and malignant pleural effusion (Hecht, 2000). BLM is inactivated by a cytosolic cysteine proteinase enzyme, BLM hydrolase. The enzyme is widely distributed in normal tissues with the exception of the skin and lungs (Ibrahimi and Anderson, 2010). Thus, cutaneous changes including fibrosis, hyperpigmentation, alopecia, gangrene, edema, Raynaud's phenomenon, and "flagellate" erythema (scratch dermatitis) have been described (Yamamoto et al., 1998). Moreover, scleroderma is also reported to be developed in malignancy-bearing patients after BLM therapy (Kerr and Spiera, 1992). Although the mechanism of BLM induced skin fibrosis is not fully understood, a thickened dermis due to uncontrolled excessive deposition of collagen is the hallmark of the disease (Jinnin, 2010). Increased numbers of myofibroblasts are largely responsible for the augmented collagen synthesis and deposition that occur in skin fibrosis (Postlethwaite et al., 2004). The cellular origins of myofibroblasts that populate fibrotic tissue during skin fibrosis have not been well defined, but the potential sources include proliferation of resident fibroblasts, differentiation of progenitor cells from the bone marrow, circulating fibrocytes and pericytes, and transition of epithelial and endothelial cells to a fibroblast phenotype, a process termed epithelial–mesenchymal transition (EMT) (Kalluri and Neilson, 2003; Nakamura and Tokura, 2011a).

EMT is a highly regulated biological process, in which fully differentiated epithelial cells can undergo a transition to a mesenchymal phenotype. In addition to a role in forming primary mesenchymal cells in mesoderm from primitive epithelium during gastrulation in the early embryo, growing evidence from clinical observations and animal models suggest that EMT is associated with the chronic diseases such

Abbreviations: EMT, Epithelial–mesenchymal transition; BLM, Bleomycin; NAC, N-acetylcysteine; BMZ, Basement membrane zone; ROS, Reactive oxygen species; MDA, Malondialdehyde.

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as tumor progression and organ fibrosis (Kalluri and Weinberg, 2009). In fibrotic tissues, myofibroblasts have been thought to originate from the pathological activation of interstitial fibroblasts. However, elegant cell tracing studies have now shown that a significant portion of these myofibroblasts arise from the conversion of epithelial cells through an EMT process (Hinz et al., 2007). Following the initial demonstration of EMT in differentiated cells of renal tubules and ducts, it is now clear that lens epithelium, endothelium, hepatocytes, alveolar epithelial cells, and cardiomyocytes can all undergo EMT and contribute significantly to tissue fibrosis (Thiery et al., 2009). Increased numbers of fibroblasts and myofibroblasts have been recognized as the major effectors involved in the excessive deposition of extracellular matrix (ECM), especially collagen, in organ tissue under pathological conditions (Postlethwaite et al., 2004) and more evidence suggests that injured epithelial cells undergoing EMT are an important source of myofibroblasts in tissue fibrosis of kidney, liver, heart, lung, and intestine (Flier et al., 2010; Kim et al., 2006; Rastaldi et al., 2002; E.M. Zeisberg et al., 2007; M. Zeisberg et al., 2007). A most recent study suggested that part of cells in eccrine glands might undergo EMT and differentiate into the myofibroblasts, contributing to the skin fibrosis; however it is unclear whether and how the epithelial cells from other locations undergo EMT during the fibrosis of the skin (Nakamura and Tokura, 2011b).

Recently, a number of studies have indicated that reactive oxygen species (ROS) also function as second messengers in signal transduction pathways for a variety of cellular processes, including proliferation, differentiation, and migration (Cannito et al., 2010). The role of ROS as intracellular mediators of EMT induction comes from the observation that treatment of mammary epithelial cells with repeated low doses of hydrogen peroxide, a protocol to mimic the chronic inflammation common to many human diseases, leads to a fibroblast-like phenotype (Mori et al., 2004). Moreover, several lines of evidence indicate that ROS also mediate transforming growth factor-beta (TGF-β), hepatocyte growth factor (HGF) and matrix metalloproteinase-3 (MMP3)-induced EMT, which may not only be due to its critical impact on signaling pathways but also due to its oxidative modifications of structural proteins (Lee and Nelson, 2012). However, it remains unclear whether ROS are involved in EMT induction in the process of BLM-induced skin fibrosis. Our previous study has indicated that BLM could induce oxidative stress and NAC attenuates BLM-induced oxidative stress in skin fibrosis (Zhou et al., 2013).

In the present study, we hypothesized that oxidative stress mediated EMT is an important contributor to the population of the effector's fibroblasts during BLM-induced skin fibrosis. We show here that EMT occurs in BLM-induced skin fibrosis, and oxidative stress inhibitor can attenuate the EMT and its associated collagen deposition.

Materials and methods

Chemicals and reagents. Bleomycin (BLM) was purchased from Nippon Kayaku (Tokyo, Japan) and N-acetylcysteine (NAC) from Sigma Aldrich (St. Louis, MO). Antibodies against E-cadherin and TGF-β were obtained from Cell Signaling Technology (Beverly, MA), anti-α-SMA antibody was obtained from Sigma Aldrich (St. Louis, MO), anti-Col1A1 and Col3A1 antibodies were obtained from Abcam Company (Cambridge, MA) and antibodies for vimentin and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phycoerythrin (PE) conjugated donkey anti-rabbit IgG, fluorescein isothiocyanate (FITC) conjugated donkey anti-goat IgG, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and HRP conjugated donkey anti-goat IgG were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Diamidino-2-phenylindole dihydrochloride was from Sigma Aldrich (St. Louis, MO). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). TRI reagent was from Molecular Research Center, Inc. (Cincinnati, Ohio). RNase-free DNase was from Promega Corporation (Madison, WI). All the other reagents were from Sigma or as indicated in the specific methods.

Animals and treatments. Specific pathogen-free (SPF), female BALB/c mice of six-week old were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. The animals were allowed free access to food and water at all times and were maintained on a 12:12 h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use. Mice were subcutaneously injected with 100 μl of BLM (1 mg per ml) or PBS into the shaved back daily for 2, 3, and 4 weeks. To investigate the protective effects of NAC on BLM-induced oxidative stress, EMT and collagen deposition, forty mice were divided into four groups randomly. In BLM alone group, all mice were subcutaneously injected with BLM (1 mg per ml) for 3 weeks. In the NAC + BLM group, in order to provide enough antioxidative material GSH before BLM treatment, mice were intraperitoneally injected with NAC (100 mg/kg body weight/day), beginning 3 h before BLM (1 mg per ml) for 3 weeks, which reference from Hagiwara et al research (Hagiwara et al., 2000). In the NAC alone group, mice were intraperitoneally injected with NAC (100 mg/kg body weight/day) for 3 weeks. PBS treated mice served as controls. In each group, 10 mice were killed by cervical dislocation on the day after the final injection for that week. Full-thickness punch biopsy specimens weighing 50 mg were obtained from each animal and stored at −80 °C for later analysis. All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Anhui Medical University.

Histopathology. The skin specimens were fixed in 10% formaldehyde phosphate buffer and embedded in paraffin. Five-micrometer thick sections were cut and mounted on glass slides. Sections were deparaffinized and the slides re-hydrated as follows with a 5-minute wash for each step: Xylene (twice), 100% ethyl alcohol (EtOH), 95% EtOH, 70% EtOH, 30% EtOH, 1× PBS, and distilled water (twice) and stained with hematoxylin and eosin to evaluate histopathological changes. Dermal collagen deposition was evaluated with Sirius red. Direct red 80 and Fast green FCF (color index 42053) were provided by Sigma-Aldrich. The sections were incubated for 2 h at room temperature with an aqueous solution of saturated picric acid containing 0.1% Fast green FCF and 0.1% Direct Red 80. The sections were covered with aluminum foil during the incubation. Stained slides were washed slowly under running distilled water for 6 min, dehydrated (3 min for each step), mounted, and examined by light microscopy. For Sirius red, quantitation was based on determining the optical densities of the chromogen. The methodology was similar to that previously described (Canbay et al., 2003).

Immunofluorescence. The skin specimens were snap-frozen in OCT compound. Five micrometer thick cryostat sections were prepared on poly L-lysin-coated slides, and fixed with cold acetone for 10 min and then air-dried. The sections were blocked with 5% fetal bovine serum (Thermo Scientific HyClone), and incubated with primary goat polyclonal anti-vimentin antibody at 1:50 or rabbit monoclonal anti-E-cadherin at 1:200 overnight 4 °C. Some sections were incubated with PBS alone in place of primary antibody as a negative control. After incubation, the sections were washed three times with PBS containing 0.1% Tween 20 before exposure to the secondary antibody. To detect primary antibodies, sections were incubated with FITC-conjugated anti-goat-IgG secondary antibody at 1:200 (for vimentin) and PE-conjugated anti-rabbit-IgG secondary antibody at 1:400 (for E-cadherin) for 45 min at room temperature. Diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg/ml) was used to stain nuclei for 15 min at room temperature. The sections were washed three times with PBS. Coverslips were mounted using the Molecular Probes Antifade Kit.

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