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EXPERIMENTALLY INDUCED DISEASE

Distribution of Cells Labelled by a Novel Somatic Stem Cell-recognizing Antibody (A3) in Pulmonary Genesis and Bleomycin induced Pulmonary Fibrosis in Rats

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Summary

Stem cells play important roles in organogenesis and remodelling after tissue injury. A monoclonal antibody (A3) has been produced against rat somatic stem cells. The present study investigated the distribution of cells labelled by A3 in the lung of fetal, neonatal and adult rats, as well as in the lung of rats with bleomycin (BLM) induced pulmonary fibrosis. In developing fetal lungs, $A3^+$ interstitial cells were present around the bronchi/bronchioles and arterioles, while in neonatal and adult lungs, the A3 reactivity of the interstitial cells gradually disappeared and instead, vascular endothelial cells in alveolar capillaries and arterioles expressed A3. By double immunofluorescence labelling, the $A3^+$ interstitial cells also expressed vimentin (a mesenchymal marker) and CD34 (a marker of immature mesenchymal cells), indicating that the interstitial cells were immature mesenchymal cells concentrated in organs as precursors to cells of connective tissues. $A3^+$ endothelial cells were co-expressed RECA-1 (a marker of rat endothelial cells) and A3 was localized to the cell membrane and cytoplasm of these cells by immunoelectron microscopy. In BLM induced fibrotic lesions, there were many $A3^+$ cells, which also expressed vimentin or RECA-1 by dual immunofluorescence labelling. There were few $CD34^+$/$A3^+$ double positive cells. No cells co-expressed A3 and α-smooth muscle actin (a marker of well-differentiated myofibroblastic cells). Although the detailed properties of cells labelled by A3 remain to be discovered, A3 would appear to be a useful marker of immature mesenchymal cells and vascular endothelial cells in developing lungs and in pulmonary fibrosis.

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Introduction

Even in adults, stem cells are present in the bone marrow and viscera and they are regarded as possible progenitor cells for endothelial, mesenchymal and epithelial cells in remodelling of injured tissues (Toma *et al*., 2001; Rackwitz *et al*., 2012). Therefore, stem cell biology has become one of the most intensely studied areas of biomedical research. Stem cells isolated from the bone marrow and adipose tissue may be used as novel therapies for incurable chronic diseases such as pulmonary fibrosis (Tzouvelekis *et al*., 2011; Rackwitz *et al*., 2012).

Malignant fibrous histiocytoma (MFH) is the most common soft tissue tumour in human adults (Brooks, 1986; Hashimoto *et al*., 1990; Dei Tos, 2006). MFH is regarded as a high-grade undifferentiated pleomorphic sarcoma, of which the origin has been considered to be mesenchymal stem cells with pluripotential differentiation (Dei Tos, 2006; Tarkkanen *et al*., 2006;

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Matushansky *et al.*, 2007). In order to investigate the histogenesis of MFH, we have previously generated a MFH cell-specific antibody (A3) by using a rat MFH cloned cell line (MT-8) (Yamate *et al.*, 1991) as the antigen. Some properties of the antigen recognized by A3 have been described (Yamate *et al.*, 2007). A3 labels bone marrow stem cells, pericytes and immature mesenchymal cells in rat viscera (Yamate *et al.*, 2007). Pericytes are regarded as stromal stem cells (Hinz *et al.*, 2007; Crisan *et al.*, 2008) and immature mesenchymal cells may be capable of differentiating into connective tissue cells in organogenesis (Iwasaki *et al.*, 1987). Therefore, A3 would appear to be a useful marker of somatic stem cells.

The lung is ontogenetically derived from the lung bud of the endoderm. The lung bud differentiates into bronchi, bronchioles and alveoli, as well as interstitial connective tissues. The interalveolar septa consist of endothelial cells and epithelial cells (Hübner *et al.*, 2008; Ling *et al.*, 2008). Idiopathic pulmonary fibrosis is a lethal progressive fibrotic lung disorder (Hübner *et al.*, 2008; Tzouvelekis *et al.*, 2011). In hepatic fibrosis, bone marrow-derived mesenchymal stem cells may play important roles in the pathogenesis; the stem cells may be a source of collagen-producing fibroblastic or myofibroblastic cells (Zemel *et al.*, 2009; Kisseleva and Brenner, 2012).

The aims of the present study were (1) to determine whether the cells labelled by A3 participate in organogenesis by examining the distribution and characteristics of those cells in the lungs of fetal, neonatal and adult rats, and (2) to determine whether the cells labelled by A3 have a role in the tissue changes that occur in pulmonary fibrosis induced in rats by bleomycin (BLM).

Materials and Methods

Animals and Bleomycin Induced Pulmonary Fibrosis

All of the experimental protocols and animal housing conformed to the institutional animal care guidelines of the Osaka Prefecture University.

Pregnant F344/DuCrj rats (15 days of gestation) were obtained from Charles River Japan (Hino, Shiga, Japan). These animals were housed in an animal room maintained at 22 ± 3°C and with a 12 h light–dark cycle and were allowed free access to a standard commercial diet (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water.

Lung samples were obtained from fetal rats on day 20 of gestation, neonatal rats aged 4–11 days and adult rats >5 weeks of age. At each time point, three to four rats were killed and pulmonary tissues were collected.

For BLM treatment, 20 F344/DuCrj male rats aged 5 weeks (Charles River Japan) were used. These rats were kept under the same conditions described above. BLM (5 mg/ml; Nippon Kayaku, Tokyo, Japan) was diluted to 1 mg/ml with sterile phosphate buffered saline (PBS). 100 µl of BLM solution was injected subcutaneously each day for 5 weeks (Ferreira *et al.*, 2006; Yamamoto, 2006; Moeller *et al.*, 2008; Moore and Hogaboam, 2008). Lungs were taken from five rats each at 1, 3 and 5 weeks after the first injection. The remaining five rats were injected with PBS according to the same schedule and were killed at 5 weeks as controls.

Histopathology and Immunohistochemistry

Lung samples were fixed in 10% neutral buffered formalin or periodate–lysine–paraformaldehyde (PLP). Formalin-fixed specimens were processed routinely and embedded in paraffin wax. Sections (3 µm) were stained with haematoxylin and eosin (HE) and with the azan-Mallory stain for collagen deposition.

The PLP-fixed specimens were embedded in paraffin wax by the AMeX method (PLP-AMeX method; Suzuki *et al.*, 2002). Sections (3 µm) were dewaxed with xylene, rehydrated through graded ethanols and washed in distilled water. Sections were treated with proteinase K for antigen retrieval and then endogenous peroxidase was blocked using H_2O_2 3% in PBS for 10 min at room temperature. Tissue sections were incubated with A3 and antibodies specific for vimentin and α-smooth muscle actin (α-SMA) (Table 1) for 12–14 h at 4 °C. Thereafter, sections were washed three times with PBS and incubated for 30 min with the secondary antibody (Histofine Simple Stain MAX PO; Nichirei, Tokyo, Japan). Positive reactions were 'visualized' by use of 3, 3′-diaminobenzidine (DAB) and H_2O_2. Sections were counterstained lightly with haematoxylin. Negative controls

Table 1
Primary antibodies used for immunolabelling.

Specificity	*Type of antibody*	*Dilution*	*Source*
Rat-MFH	Monoclonal (A3)	1 in 2,000	TransGenic Inc., Kobe, Japan
Vimentin	Monoclonal (V9)	1 in 200	Dako, Carpinteria, California, USA
RECA-1	Monoclonal (HIS52)	1 in 100	Serotec, Oxford, UK
CD34	Goat polyclonal	1 in 500	R&D Systems, Minneapolis, Minnesota, USA
α-SMA	Monoclonal (IA4)	1 in 400	Dako

MFH, malignant fibrous histiocytoma; RECA-1, rat endothelial cell antigen-1; α-SMA, α-smooth muscle actin.

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