Dysregulation of lung injury and repair in moesin-deficient mice treated with intratracheal bleomycin

Soshi Hashimoto,1 Fumimasa Amaya,1 Hiroki Matsuyama,1 Hiroshi Ueno,1 Shojiro Kikuchi,2 Masaki Tanaka,3 Yosihisa Watanabe,3 Masahito Ebina,4 Akitoshi Ishizaka,5 Sachiko Tsukita,6 and Satoru Hashimoto1

1Department of Anesthesiology and Intensive Care, 2Department of Surgery, and 3Department of Cell Biology, Research Institute for Geriatrics, Kyoto Prefectural University of Medicine, Kyoto; 4Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai; 5Pulmonary Division, Department of Medicine, Keio University School of Medicine, Tokyo; and 6Laboratory of Biological Science, Organismal Biosystems Laboratory, Graduate School of Frontier Biosciences/Department of Pathology, Graduate School of Medicine Osaka University, Osaka, Japan

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Hashimoto S, Amaya F, Matsuyama H, Ueno H, Kikuchi S, Tanaka M, Watanabe Y, Ebina M, Ishizaka A, Tsukita S, Hashimoto M. Dysregulation of lung injury and repair in moesin-deficient mice treated with intratracheal bleomycin. Am J Physiol Lung Cell Mol Physiol 295: L566–L574, 2008. First published July 25, 2008; doi:10.1152/ajplung.90250.2008.—Moesin belongs to the ezrin/radixin/moesin (ERM) protein family and participates in cellular functions, such as morphogenesis and motility, by cross-linking between the actin cytoskeleton and plasma membranes. Although moesin seems necessary for tissue construction and repair, its function at the whole body level remains elusive, perhaps because of redundancy among ERM proteins. To determine the role played by moesin in the modulation of pulmonary alveolar structure associated with lung injury and repair, we examined the morphological changes in the lung and the effect of bleomycin-induced lung injury and fibrosis in moesin-deficient (Msn−/−) and control wild-type mice (Msn+/+). Immunohistochemical analysis revealed that moesin was specifically localized in the distal lung epithelium, where ezrin and radixin were faintly detectable in Msn−/− mice. Compared with Msn+/+ mice, Msn−/− mice displayed abnormalities of alveolar architecture and, when treated with bleomycin, developed more prominent lung injury and fibrosis and lower body weight and survival rate. Furthermore, Msn−/− mice had abnormal cytokine and chemokine gene expression as shown by real-time PCR. This is the first report of a functional involvement of moesin in the regulation of lung inflammation and repair. Our observations show that moesin critically regulates the preservation of alveolar structure and lung homeostasis.

ezrin; radixin; lung inflammation; alveolar structure

AN ABNORMAL INFLAMMATORY REACTION and repair process may cause a variety of acute or chronic life-threatening disorders, including acute lung injury, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and cystic fibrosis. Studies in animals (2, 16) and clinical observations (27) have suggested that the integrity of the alveolar epithelium and its capacity to recover from injury are critical elements in the development of lung fibrosis. Therapeutic strategies aimed at preserving and repairing the alveolar epithelium and its capacity to recover from injury are critical elements in the development of lung fibrosis. Several studies have also suggested that, in mammalian cells, the ERM proteins participate in wound closure by reorganizing the actin cytoskeleton during wound healing (21, 33).

Although it has been hypothesized that moesin is a key factor in the preservation of alveolar structure and regulation of lung inflammation and repair, the involvement of ERM proteins in the maintenance of alveolar structure has not been studied in depth. Therefore, we have examined the pathophysiological function of moesin in the lung tissue of moesin-deficient mice. This is the first report of the functional involvement of moesin, an ERM protein predominantly expressed in the distal lung epithelium, in alveolar structure, and in the regulation of inflammation and tissue repair.
MATERIALS AND METHODS

Animal preparations. Our creation of moesin-deficient (Msn−/−) mice has been described previously (11). These genotypes have been backcrossed onto a C57BL/6 background for more than eight generations. All animals were bred and kept in a pathogen-free environment and were provided with food and water ad libitum. The moesin gene is located on the X chromosome. Male hemizygous (Msn−/−) and littermate wild-type (Msn+/+) mice, 8–10 wk of age, were used for all experiments. All animal procedures were carried out in accordance with a protocol approved by the institutional Animal Care and Use Committees of Kyoto Prefectural University, School of Medicine.

Immunoblotting analysis. Rat monoclonal antibodies (M11, anti-mouse ezrin; R21, anti-mouse radixin; and M22, anti-mouse moesin) were provided by the Department of Cell Biology, School of Medicine, Kyoto University (25). The lungs were homogenized in lysis buffer (0.5% Triton X-100 in phosphate-buffered saline) containing protease inhibitors. The protein concentrations in lung homogenates were measured by the Bradford method using protein assay CBB solution (Nacalai Tesque, Kyoto, Japan). Equal amounts of protein (50 μg) were added to Laemmli sample buffer and boiled for 5 min. The protein extractions from each lung tissue were electrophoresed on 12.5% SDS-polyacrylamide gels, followed by transfer to a polyvinyl- idene difluoride membrane (Millipore, Bedford, MA). The membranes were soaked in blocking buffer for 30 min and incubated with anti-ezrin, -radixin, or -moesin antibodies at 4°C overnight. After being washed, the membranes were incubated with alkaline phosphatase-conjugated anti-rat antibody. The color reaction for alkaline phosphatase was captured, avoiding conducting airways, vasculature, poorly inflated regions, and the pleura. The average alveolar size was calculated. We used the Ashcroft scoring system for the quantitative histological analysis of fibrotic changes induced by bleomycin (4). The mean score of all fields examined was the fibrosis score of each animal.

Lung water content. The lung water content due to lung injury was measured using the lung wet-to-dry weight ratio as previously described (44). Briefly, the right lobe was dissected, weighed immediately after removal, and dried for 5 days in an oven at 60°C. The wet-to-dry weight ratio was calculated as the ratio of the wet weight to the dry weight.

Bronchoalveolar lavage fluid analysis. To determine whether the differences in leukocyte populations recovered from the bronchoalveolar lavage fluid were the consequence of lung injury in moesin-deficient mice, we counted the total cell number and cell differential in the fluid at 7 days after the instillation of bleomycin, when the inflammatory response is maximal (20), and in untreated mice (control). The total number of cells and the cell differential in bronchoalveolar lavage fluid were counted in each genotype, as previously described (26). The animals were killed by anesthesia with sevoflu- ran. The trachea was exposed by a midline incision and cannulated with a sterile polypropylene 20-gauge catheter. The lungs were lavaged four times with 0.8 ml of ice-cold PBS. In each animal, 90% of the total lavage volume was consistently recovered. After centrifugation of the bronchoalveolar lavage fluid at 400 g for 10 min at 4°C, the cell pellet was resuspended in 1.0 ml of PBS. The total number of cells in the lavage was counted with a hemocytometer. Cell differentials were counted on cytospin preparations stained with Diff-Quik (Sysmex, Kobe, Japan). At least 400 cells per sample were counted under light microscopy. The concentration of total protein in the bronchoalveolar lavage fluid was measured in the cell-free supernatant stored at −80°C with a BCA protein assay kit (Pierce Chemical, Rockford, IL).

Quantitative real-time RT-PCR. Total RNA was extracted from lung tissues using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Total RNA (2 μg) was reverse-transcribed to cDNA with SuperScript III first-strand synthesis for RT-PCR (Invitrogen Life Technologies). Primer sequences for tumor necrosis factor (TNF)-α, macrophage inhibitory protein (MIP)-2, transforming growth factor (TGF)-β1, procollagen type I α1, and 18S rRNA are available on
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request (Takara Bio, Tokyo, Japan). Real-time quantitative PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) and Platinum SYBR green qPCR SuperMix-UDG (Invitrogen Life Technologies). The amplification conditions were 50°C for 2 min, 95°C for 2 min, 40 cycle at 95°C for 15 s, and 60°C for 30 s, followed by a dissociation curve analysis to confirm the formation of specific product. The relative mRNA expression levels were calculated using the relative standard curve method and normalized to 18S rRNA.

Measurement of lung collagen. The lung collagen content was measured using the Sircol collagen assay kit (Biocolor, Belfast, Northern Ireland) according to the manufacturer’s instructions. Briefly, 1,000 μl of Sirius red dye reagent were added to 100 μl of the supernatant from lung homogenate, mixed for 30 min, and centrifuged at 13,000 g for 10 min. Pellets (bead-dye-collagen complex) were then dissolved in 1,000 μl of alkali reagent (0.5 M NaOH), and the amount of collagen was measured using a standard curve for collagen provided by the manufacturer.

Statistical analysis. Data are means ± SE. Simple comparisons were made using two-tailed Student’s t-test, and multiple comparisons by analysis of variance with the Newman-Keuls test. Survival curves were constructed using the Kaplan-Meier method and compared with the log-rank test. P values <0.05 were considered significant.

RESULTS

Expression and distribution of ERM proteins in the lung of mature wild-type and moesin-deficient mice. Since the expression patterns of ERM proteins in whole lung tissue have not been thoroughly studied, we first examined the expression levels and histological distributions of 3 ERM proteins in the lung of adult wild-type and Msn−/− mice by immunoblotting and immunohistochemistry with anti-ezrin, -radixin, or -moesin specific monoclonal antibodies. Immunoblotting of whole lung tissue revealed that ezrin, radixin, and moesin were present in the lung tissue of wild-type mice. The expression of moesin was not detected in the lung tissue obtained from Msn−/− mice, and no significant compensatory upregulation of ezrin or radixin was observed (Fig. 1A), an observation concordant with that made in a previous study using an anti-ERM polyclonal antibody that recognized all ERM family members (11). Immunohistochemistry showed that in the lung of Msn+/+ mice, the expression of moesin was specifically and prominently localized in the apical membrane of the arterioles adjacent to terminal bronchioles and in the distal alveolar wall. Unlike that of moesin, the expression of ezrin was limited to the apical membrane of the proximal airways, and the expression of radixin was low in the lungs of wild-type mice. In contrast to the low expression of ezrin and radixin in the distal lung tissue of wild-type mice, it appeared increased in the lung of Msn−/− mice (Fig. 1B), suggesting that moesin deficiency had caused a shift in the distribution of ezrin and radixin to the distal alveolar wall, and a slight increase in their expression, without repercussion on their expression in the whole lung. To identify the cellular source of moesin expression in the distal alveolar wall, we used double immunofluorescence staining with anti-moesin monoclonal antibody and a goat polyclonal antibody specific for RAGE to detect the epithelial cells and anti-CD31 polyclonal antibody to detect the endothelial cells. This staining showed that moesin was predominantly expressed in most epithelial cells that expressed RAGE in the distal alveolar wall. In contrast, moesin was less prominently expressed in the capillary endothelium that expressed CD31.

Fig. 1. Abundance and localization of ezrin/radixin/moesin (ERM) proteins in lungs from moesin-deficient (Msn−/−) and wild-type (Msn+/+) mice. A: immunoblotting analysis of isolated Msn+/+ and Msn−/− lung with anti-ezrin, -radixin, and -moesin specific monoclonal antibody. In Msn−/− lung, moesin became undetectable without significant upregulation of ezrin or radixin. B: immunohistochemical analysis of the lung tissues isolated from Msn+/+ and Msn−/− mice with ezrin-, radixin-, and moesin-specific antibodies. In the Msn+/+ lung tissue, moesin was highly concentrated in the distal alveolar wall and the pulmonary arterioles, where ezrin and radixin are weakly present. By contrast, in the lung tissues of Msn−/− mice, the concentrations of ezrin and radixin in the distal lung were significantly increased. Photomicrographs (top and bottom) of representative mice lungs are shown at ≈200 original magnification (bars, 50 μm). High-power microphotographs (middle) show pulmonary alveoli from Msn+/+ mice (bars, 10 μm).

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Thus, in the distal lung, the expression of moesin was mostly confined to the alveolar epithelium instead of the endothelium (Fig. 2).

Distal air space enlargement due to moesin deficiency. We next carried out a detailed histological examination of hematoxylin and eosin-stained lung tissue sections from Msn<sup>−/−</sup> mice. Compared with those of wild-type littermates, the air spaces of untreated adult Msn<sup>−/−</sup> mice were markedly enlarged, without signs of inflammation or alveolar destruction. Furthermore, the proximal airways and pulmonary vessels of Msn<sup>−/−</sup> mice appeared normal (Fig. 3A). However, morphometric measurements revealed a significant increase in size of the distal alveoli of Msn<sup>−/−</sup> mice compared with their control littermates. The mean linear intercept values (Fig. 3B) and mean size of individual alveolar air spaces (Fig. 3C) were both significantly increased in 10-wk-old Msn<sup>−/−</sup> mice.

Weight loss and increased mortality of moesin-deficient mice after bleomycin instillation. There was no significant difference in the baseline body weight of age-matched Msn<sup>+/+</sup> (25.1 ± 1.3 g) compared with Msn<sup>−/−</sup> mice (24.8 ± 1.2 g). The Msn<sup>−/−</sup> mice lost weight rapidly and progressively and, 14 days after bleomycin instillation, had lost a significant amount of body weight compared with the wild-type mice (Fig. 4A). Furthermore, the 14-day survival rate of Msn<sup>−/−</sup> mice was 20%, compared with 100% for the wild-type littermates (Fig. 4B). These differences in loss of body weight and survival observed between the two genotypes were attributable to more severe lung injury in the Msn<sup>−/−</sup> mice.

Enhancement of lung injury by bleomycin in moesin-deficient mice. Histological examinations at 7 days after instillation of bleomycin showed massive infiltration of the alveolar interstitium with inflammatory cells in Msn<sup>−/−</sup> mice and extensive alveolar destruction with hemorrhage and pulmonary edema. In contrast, the lungs of wild-type mice examined at the same time after bleomycin administration showed minimal histological changes (Fig. 5A). Furthermore, 7 days after the administration of bleomycin, the extravascular water content in the lungs of moesin-deficient mice was significantly increased (Fig. 5B). After the instillation of bleomycin, the concentration of total protein in the bronchoalveolar lavage fluid (an indication of lung permeability) rose earlier in Msn<sup>−/−</sup> than in wild-type mice (Fig. 5C), suggesting that the dysfunction of alveolar epithelial and endothelial barrier in response to bleomycin occurs rapidly in presence of moesin deficiency. The total cell count in the bronchoalveolar lavage fluid recovered from the animals at 7 days after bleomycin treatment was higher in Msn<sup>−/−</sup> than in wild-type mice (Fig. 6A). The relative percentages of macrophages, lymphocytes, and neutrophils were similar in both genotypes (Fig. 6B).

Abnormal cytokine and chemokine expression in moesin-deficient mice. We measured, using RT-PCR, the levels of TNF-α, MIP-2, TGF-β1, and type I procollagen mRNA, which contribute to the inflammatory, angiogenic, and fibrotic responses in bleomycin-induced lung injury. We compared the gene expression for TNF-α, MIP-2, TGF-β1, and procollagen type Iα1 in lung tissue from treatment-matched Msn<sup>−/−</sup> compared with wild-type mice. The baseline amounts of TNF-α and MIP-2 mRNA were greater in Msn<sup>−/−</sup> than in wild-type mice. Furthermore, 7 days after the instillation of bleomycin, the levels of TNF-α and MIP-2 mRNA were higher in Msn<sup>−/−</sup> than in wild-type mice. In contrast, the level of TGF-β1 mRNA was significantly lower at baseline in the Msn<sup>−/−</sup> than in the wild-type mice.

Fig. 2. Cellular source of moesin expression in the Msn<sup>−/−</sup> distal alveolar wall. Double-labeling immunofluorescence for moesin, the alveolar epithelial marker RAGE (receptor for advanced glycation end products), and the capillary endothelial marker CD31 is shown in lung of Msn<sup>−/−</sup> mice. Alveolar epithelial cells, labeled by RAGE (red), are lining the capillary endothelial cells, labeled by CD31 (green). The red and green signals are clearly localized in the distal alveolar wall. Double staining of moesin (green) and RAGE (red) reveals that several epithelial cells that line the alveolar walls express an orange immunofluorescence, indicating the colocalization of moesin and RAGE. Moesin-positive alveolar macrophages are not RAGE immunoreactive (arrow). By contrast, double staining of moesin (green) and CD31 (red) shows the near absence of moesin signal at the pulmonary capillary level. The nuclei are blue, due to the 4,6-diamidino-2-phenylindole counterstain. Bars, 20 μm.
From wild-type and ally, perivascular regions (Fig. 8), lesions were observed mostly in the subpleural and, occasionally, after the intratracheal instillation of bleomycin, focal fibrotic age-matched Msnβ mice. In wild-type mice, 2 wk bleomycin in moesin-deficient mice. (Fig. 7). Wild-type mice at 7 days after the instillation of bleomycin (Fig. 8A, thin bands, immediately next to the large vessels and airways present in the lungs of untreated animals was detectable only in Msnβ mice and areas of the distal alveoli are means ± SE from 6 mice. *P < 0.001 vs. age-matched Msnβ mice.

The present study documented the expression of moesin in the alveolar epithelium and the apical membrane of pulmonary arterioles in wild-type mice. Moesin deficiency caused the enlargement of the distal air space without apparent alveolar destruction and, following treatment with bleomycin, an enhanced inflammatory response and prominent pulmonary fibrosis. These observations indicate that 1) moesin plays a key role in the regulation of alveolar integrity and lung homeostasis and that 2) its deficiency causes the dysregulation of alveolar inflammatory response to bleomycin and subsequent repair.

The ERM proteins are a family of cytoskeletal regulatory proteins that share a 70–80% sequence identity of amino acids (28). These widely expressed proteins preserve the structure and function of actin-rich cell membrane extensions, such as lamellipodia, filopodia, and microvilli associated with cell motility and shape (3, 13, 19). In addition, the deficiency (39) or inactivation (17) of the single ERM protein moesin in wild-type mice and was similar in both genotypes after treatment with bleomycin. Like the expression of TGF-β1 mRNA, the baseline level of type I procollagen mRNA was lower in the Msn−/− mice, although it was significantly higher than in wild-type mice at 7 days after the instillation of bleomycin (Fig. 7).

Exacerbation of lung fibrosis and collagen deposition by bleomycin in moesin-deficient mice. In wild-type mice, 2 wk after the intratracheal instillation of bleomycin, focal fibrotic lesions were observed mostly in the subpleural and, occasionally, perivascular regions (Fig. 8A, left). In Msn−/− mice, however, bleomycin caused a massive disruption of the alveolar architecture and the development of cystic air spaces, abundant hemorrhage, and increased cellularity of the alveolar septa (Fig. 8A, right). The Masson trichrome-stained collagen present in the lungs of untreated animals was detectable only in thin bands, immediately next to the large vessels and airways (Fig. 8B, top). In Msn−/− mice, 7 days after the instillation of bleomycin, the lung architecture was abnormal and collagen had accumulated in the fibrotic interstitial tissues (Fig. 8B, middle right), whereas at 14 days, large areas of lung parenchyma were replaced by dense bands of collagen (Fig. 8B, bottom right). In wild-type mice, the same dose of bleomycin caused the deposition of considerably less and less dense abnormal stainable collagen in the lung (Fig. 8B, middle and bottom left), suggesting that moesin deficiency promotes the deposition of bleomycin-induced interstitial collagen in the lung. The average fibrotic scores determined using the Ashcroft method were significantly higher in Msn−/− than in wild-type mice at 7 and 14 days after bleomycin instillation (Fig. 9A). This microscopic observation was confirmed by measurements of the collagen content, which at 7 and 14 days after the instillation of 1 mg/kg of bleomycin were significantly higher in Msn−/− than in wild-type mice, whereas in untreated mice, the collagen content was similar in both genotypes (Fig. 9B). These observations indicate that the fibrotic response to bleomycin was both earlier and more severe in Msn−/− than in wild-type mice.

### DISCUSSION

Fig. 4. Increased susceptibility of moesin-deficient mice to intratracheal bleomycin. Msn−/− mice and wild-type littermates, 8–10 wk of age, received 1 mg/kg intratracheal bleomycin. A: the survival of each genotype was recorded throughout a 14-day period of observation and plotted according to the Kaplan-Meier method. Comparison of survivals to 14 days revealed a greater susceptibility to 1 mg/kg bleomycin in the group of Msn−/− mice (n = 10 for each genotype, *P < 0.001). B: the body weight of each genotype was recorded immediately before the administration of bleomycin and daily throughout the experimental period. Moesin deficiency increased significantly the bleomycin-induced loss of body weight. Data for each genotype are means ± SE of 6–7 mice. *P < 0.001 vs. bleomycin-treated Msn−/− mice at the same time point.

The present study documented the expression of moesin in the alveolar epithelium and the apical membrane of pulmonary arterioles in wild-type mice. Moesin deficiency caused the enlargement of the distal air space without apparent alveolar destruction and, following treatment with bleomycin, an enhanced inflammatory response and prominent pulmonary fibrosis. These observations indicate that 1) moesin plays a key role in the regulation of alveolar integrity and lung homeostasis and that 2) its deficiency causes the dysregulation of alveolar inflammatory response to bleomycin and subsequent repair.

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Drosophila causes the loss of epithelial characteristics and the adoption of invasive migratory behavior, indicating that ERM proteins are also responsible for the maintenance of epithelial integrity. The knock down of each ERM protein in vitro failed to cause phenotypic changes, indicating a redundancy of ERM protein functions at the cellular level (40). By contrast, the expression of individual ERM proteins in vivo is topologically diverse, suggesting specific functions of each protein in different tissues (7). Radixin-deficient mice are deaf (25) and develop mild liver injury beyond 8 wk of life (24). A lack of ezrin, the only isoform expressed in part of the polarized intestinal epithelia, results in abnormal villus morphogenesis and a disrupted terminal web in these cells. As a consequence, neonate mice lacking ezrin cannot survive past weaning (35). However, moesin-deficient mice develop normally and, compared with wild-type mice, are similar in weight, size, and reproductive ability up to 12 mo of age (11).

We observed that in adult wild-type mice, the expression of moesin was limited to the alveolar epithelium of the distal lung tissue and was different from the expression of ezrin or radixin. As observed previously (18), ezrin was expressed in the apical membrane of the proximal airways, whereas the expression of radixin was merely detected in lung parenchyma. Although the
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In the expression of ezrin and radixin in the lungs of mice, changes were associated with immunohistochemical changes in the amounts of ezrin or radixin, these morphological immunoblotting examinations showed no significant increases spaces causes abnormalities in alveolar structure. Although our mouse, ezrin and radixin proteins were both induced in the distal lung, where moesin was detected abundantly in wild-type mice, suggesting that the distribution of ezrin and radixin had changed in response to moesin deficiency. These observations suggest also that loss of function due to moesin deficiency was not overcome by the compensatory changes in the distribution of radixin and ezrin and that moesin is needed for the maintenance of a normal lung structure via the regulation of epithelial cell differentiation, control of lung inflammation, or both.

In our experiments, the distal air space enlargement observed in Msn<sup>−/−</sup> mice was accompanied by increased concentrations of inflammatory mediators. Real-time PCR showed increases in the expressions of TNF-α and MIP-2 genes in untreated lungs from Msn<sup>−/−</sup> compared with wild-type mice. Transgenic mice that overexpress TNF-α in the lung develop elements of emphysema and pulmonary fibrosis (12, 29, 30). In addition, these observations might indicate a greater activation of alveolar macrophages in Msn<sup>−/−</sup> mice. However, the absence of prominent lung inflammation in the Msn<sup>−/−</sup> mice and the importance of moesin in maintaining epithelial integrity indicate that alveolar macrophages are not a likely primary source of increased mediators.

In contrast to the changes observed with TNF-α and MIP-2, the expression of TGF-β1 and type I collagen gene was decreased in untreated Msn<sup>−/−</sup> compared with that in wild-type mice. TGF-β1 is needed to promote the reorganization of the actin cytoskeleton and the migratory phenotype in epithelial cells (8). Although the involvement of TGF-β1 in the pathogenesis of emphysema remains elusive, recent genetic studies in knockout mice with emphysematous phenotype have revealed that an impairment of its activation and signaling is the cause of progression of age-related emphysema (32, 45). Since the alveolar structure of Msn<sup>−/−</sup> mice resembles the morphological changes often observed in patients suffering from emphysema, and since the expression of the moesin gene is increased in emphysematous lungs (15), moesin is probably involved in the pathogenesis of emphysema and protects

Msn<sup>−/−</sup> mice seemed to grow normally (11), our detailed histological examinations showed that the distal air spaces were markedly enlarged. Our observations also showed unequivocally that a deficiency of moesin in the distal alveolar spaces causes abnormalities in alveolar structure. Although our immunohistochemical examinations showed no significant increases in the amounts of ezrin or radixin, these morphological changes were associated with immunohistochemical changes in the expression of ezrin and radixin in the lungs of Msn<sup>−/−</sup> mice. In Msn<sup>−/−</sup> mice lung, ezrin and radixin proteins were both induced in the distal lung, where moesin was detected abundantly in wild-type mice, suggesting that the distribution of ezrin and radixin had changed in response to moesin deficiency. These observations suggest also that loss of function due to moesin deficiency was not overcome by the compensatory changes in the distribution of radixin and ezrin and that moesin is needed for the maintenance of a normal lung structure via the regulation of epithelial cell differentiation, control of lung inflammation, or both.
against alveolar destruction. On the other hand, moesin may be involved in cellular differentiation, and vigorous attempts should be made in future studies to define the developmental ontology of the phenotype observed in Msn−/− mice through maturation.

To examine whether moesin deficiency exacerbates the pathological changes in inflammatory lung disease and subsequent alveolar repair, we induced lung injury and fibrosis with bleomycin (22). We instilled a low dose, since in a preliminary study we observed a nearly 100% mortality of Msn−/− mice with the experimental doses generally used (31). Whereas this smaller dose of bleomycin induced a mild inflammatory response and fibrosis in wild-type mice, the same dose instilled in Msn−/− mice caused a severe inflammatory reaction and prominent lung fibrosis associated with marked loss of body weight and high death rate. The administration of bleomycin initially caused epithelial/endothelial injury and alveolar inflammation, followed by a fibroproliferative process (22). An efficient alveolar epithelial repair process can inhibit the development of pulmonary fibrosis, since the presence of an intact pulmonary epithelial layer suppresses the proliferation of fibroblasts and the deposition of matrix (1). An enhanced fibroproliferative response caused by a delayed alveolar epithelialization after lung injury has been confirmed in several animal models (2). In previous observations, we showed that the targeted disruption of the moesin gene did not affect stress fiber, focal contact formation, and migration rate in fibroblasts, suggesting that a deficiency in moesin has minimal effects on the activity of fibroblasts (11). We observed an increased response of proinflammatory cytokine and chemokine after bleomycin treatment in Msn−/− mice. TNF-α and MIP-2 play a role in the pathogenesis of experimental lung injury, and blocking their action has a distinct protective effect against the development of lung injury and fibrosis caused by bleomycin (23, 34). Therefore, an exacerbated lung fibrosis induced by bleomycin in moesin-deficient mice is likely to be caused by an increased inflammatory reaction associated with abnormal alveolar epithelial function.

Recent studies have shown that ERM proteins are involved not only in the organization of the cytoskeleton but also in the signaling pathway. They were found to bind directly to the cytoplasmic domains of CD44, which were integrated by intracellular osteopontin (48). CD44-deficient mice suffer a high mortality from lung injury caused by bleomycin, suggesting a role played by CD44 in the resolution of pulmonary inflammation (41). Osteopontin-deficient mice treated with bleomycin, like moesin-deficient mice, develop lung fibrosis characterized by dilated distal air spaces (6). These observations suggest that moesin is capable of regulating both the inflammatory response and the repair process in the lung tissue via the CD44-osteopontin signaling pathway, although further studies are needed to confirm this relationship.

In conclusion, our study provides the first evidence of a functional involvement of moesin, a protein from the ERM family, in lung injury and its repair process. We found moesin to be crucial in the preservation of alveolar structure and integrity in distal lung tissue. Future studies of the mechanisms by which cytoskeletal moesin is involved in lung injury and repair might open the way toward new endogenous therapies protective against this fatal lung disease.