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Preventive approach against drug-induced pulmonary fibrosis through the suppression of epithelial-mesenchymal transition

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Abstract: A number of drugs induce pulmonary injury and subsequently lead to serious lung diseases such as pulmonary fibrosis as the adverse drug reactions. However, an effective preventive approach against drug-induced pulmonary fibrosis has not been established due to poor understanding of common preventive targets in a variety of drugs showing pulmonary toxicity. Epithelial-mesenchymal transition (EMT), a cellular phenotypic change of the epithelial to mesenchymal state, contributes to the development of pulmonary fibrosis through the conversion of damaged alveolar epithelium into myofibroblasts. As several drugs with pulmonary toxicity have been reported to induce EMT, EMT serves as a bridge between the drugs and pulmonary fibrosis. Accumulated evidence supports the potential of EMT as a preventive target against drug-induced pulmonary fibrosis. Additionally, since there are mechanistic differences between the main pharmacological effect and EMT induced by the drug, prevention based on EMT suppression would be possible and would contribute to continuous clinical treatment with the drug to avoid EMT-mediated serious pulmonary fibrosis. Furthermore, targeting EMT seems to be adequate for exerting a preventive effect since EMT in damaged alveolar epithelial cells occurs prior to the development of the pathophysiological state of the whole lung in a bleomycin-induced lung injury rat model. This viewpoint deals with the benefits and perspectives of preventive approaches against drug-induced pulmonary fibrosis through the suppression of EMT, which has rarely been addressed.

Introduction

A number of drugs cause drug-induced lung injury (DILI), which can lead to serious lung diseases such as pulmonary fibrosis. Although the pathogenic process of DILI depends on the types and features of the drugs, two basic mechanisms are currently considered. The first is dose-dependent cytotoxic drug-induced damage to the alveolar epithelium, airway epithelium, and vascular endothelial cells like the case of bleomycin and amiodarone (Schwaiblmair *et al.*, 2012), while the second is dose-independent immune-mediated reactions like the case of both low (for rheumatoid arthritis) and high (for cancer) doses of methotrexate (MTX) (Barrera *et al.*, 1994; Jakubovic *et al.*, 2013). These mechanisms are likely affected by a variety of host and environmental factors, including age, genetic predisposition via the expression of drug metabolism- or immune-related genes, underlying pathological conditions in the lung, and interactions with concomitant drugs (Kubo *et al.*, 2013). Therefore, it seems quite difficult to establish a common preventive approach for lung injury triggered by different types of drugs. However, epithelial-mesenchymal transition (EMT) has been highlighted as a critical biological process contributing to idiopathic pulmonary fibrosis via phenotypical conversion of damaged alveolar epithelial cells into myofibroblasts that can produce extracellular matrix (Jolly *et al.*, 2018). Additionally, recent studies have demonstrated that EMT can be induced by several drugs with the potential to lead to serious pulmonary fibrosis (Kawami *et al.*, 2016; Weng *et al.*, 2020a, 2020b). Current knowledge regarding the relationship between drug-induced pulmonary fibrosis and EMT strongly indicates that EMT is an effective target for the prevention of drug-induced pulmonary fibrosis. To date, however, the application of targeting EMT to the preventive approach has been poorly discussed. This viewpoint focuses on the benefits and perspectives of preventive approaches against drug-induced pulmonary fibrosis through the suppression of EMT.

Main Text

Contribution of EMT to pulmonary fibrosis

EMT is a biological process in which epithelial cells downregulate epithelial characteristics and acquire a mesenchymal state, which

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was first proposed and characterized by Elizabeth Hay (Hay, 1995). During EMT, epithelial cells lose polarity and cobblestone morphology, while acquiring a spindle-shaped morphology with an increase in mesenchymal marker genes such as fibronectin, α-smooth muscle actin (α-SMA), and vimentin. Moreover, three types of biological situations were considered in the EMT process. In brief, type I EMT is observed during embryonic development, mainly during gastrulation, type II EMT is associated with wound healing and tissue regeneration during organ injury, and type III EMT contributes to cancer progression by enhancing metastasis and invasion of tumor cells (Zeisberg and Neilson, 2009). Thus, various physiological and pathophysiological reactions in the body are closely or partially linked to EMT.

EMT has been suggested to be involved in the development of pulmonary fibrosis, as evidenced by a variety of experimental animal models including the bleomycin (BLM)-induced lung injury model (mouse, rat, and hamster) (Moeller *et al*., 2008). The mechanism by which EMT promotes pulmonary fibrosis has been well-discussed, mainly focusing on EMT-mediated disruption of the normal lung tissue repair process. Normally, after lung injury, epithelial cells release inflammatory mediators and subsequently recruit leukocytes (neutrophils, macrophages, and T cells) into the lungs. The recruited leukocytes secrete profibrotic cytokines, such as transforming growth factor-β1 (TGF-β1), which then recruit myofibroblasts from resident fibroblasts to produce extracellular matrix (ECM) components. These ECMs essentially work in the tissue repair process, whereas the amount of ECMs is regulated by proteases such as matrix metalloproteinases to avoid excessive accumulation of the ECM. Conversely, EMT also recruits myofibroblasts from damaged epithelial cells and enhances the excessive production of ECM, which may lead to the pathophysiological state of the lung. Thus, EMT disrupts homeostasis in the production and degradation of ECM during tissue repair, while EMT-induced defects in the balance of ECM amounts can be a targetable process for the prevention of pulmonary fibrosis.

EMT induced by drugs showing pulmonary toxicity

There are a variety of drug categories with the potential to induce lung injury, including anticancer drugs, antirheumatic drugs, interferons (IFN), immunosuppressive agents, herbal drugs, antimicrobial drugs, and antiarrhythmic drugs (Schwaiblmair *et al*., 2012). In particular, anticancer drug-induced lung injury is frequently reported, and its incidence is increasing (Kubo *et al*., 2013). In contrast, we have demonstrated that several anticancer drugs, such as BLM, methotrexate (MTX), and paclitaxel (PTX), induced EMT-like phenotypical changes in the alveolar epithelial model cell line A549/ABCA3 cells (Yamamoto *et al*., 2019). Additionally, there are reports on the EMT-inducing effects of other pulmonary cytotoxic drugs, such as amiodarone (Weng *et al*., 2020b) and IFN-γ (Imai *et al*., 2019), indicating that EMT has a causal correlation with drug-induced pulmonary fibrosis. Accordingly, the EMT-inducing potency of the drugs may be added as a novel risk factor for drug-induced lung injury in parallel with advanced age, existing pulmonary lesions, history of pulmonary surgery, and decreased respiratory function. Thus, evidence showing a close relationship between EMT and drugs with pulmonary toxicity is increasing.

Conversely, EMT occurrence in pulmonary fibrosis under *in vivo* condition is currently controversial, as one lineage tracing study demonstrated no transition of labeled AT-II cells into myofibroblast (Rock *et al*., 2011). As there are differences in histological appearance and the progressive behavior of the pulmonary pathological state between murine and human (Izbicki *et al*., 2002; Peng *et al*., 2013), further studies are needed to fill the gap in the species differences of pulmonary fibrosis.

Current therapeutic approach against pulmonary fibrosis

Currently, there is no drastic remedy for pulmonary fibrosis triggered by drugs. When drug-induced lung injury is suspected according to several diagnostic criteria, withdrawal of the drug and/or treatment with corticosteroids are selected as only symptomatic therapy. However, two antifibrotic agents, pirfenidone and nintedanib, for the treatment of idiopathic pulmonary fibrosis are clinically available (Sathiyamoorthy *et al*., 2017). Notably, these agents are reported to show anti-EMT effect. Pirfenidone inhibits BLM-induced lung injury in rats and TGF-β1-induced EMT in human fetal lung fibroblasts (Lv *et al*., 2020), nintedanib suppresses EMT in alveolar epithelial A549 cells via the regulation of TGF-β/SMAD axis (Ihara *et al*., 2020). Thus, the anti-fibrotic mechanisms of these agents are associated with their anti-EMT functions, at least partially. However, there are not enough data showing the effects of pirfenidone and nintedanib on drug-induced pulmonary fibrosis.

TGF-β1 plays a crucial role in EMT via the SMAD signaling cascade pathway. TGF-β1 stimulates the TGF-β-type I/II receptor complex, which in turn leads to the phosphorylation of SMAD2/3. Phosphorylated SMAD2/3 can bind to SMAD4, and the subsequent complex regulates the transcription of EMT-related genes. Therefore, there are many TGF-β signaling antagonist agents under development at both the pre-clinical and clinical stages. For example, monoclonal antibodies against TGF-β1, antisense oligonucleotides (ASO), and small molecule TGF-β receptor kinase inhibitors are now considered for the treatment of fibrosis (Györfi *et al*., 2018). However, several failures in pre-clinical and clinical trials regarding monoclonal antibodies such as metelimumab (CAT-192) (Denton *et al*., 2007) and small molecule inhibitors such as SB431542 (Mori *et al*., 2004) are reported. Additionally, in our previous reports, we demonstrated that the TGF-β1 signaling pathway was partially associated with MTX-induced EMT in A549 cells (Kawami *et al*., 2016), whereas the underlying mechanism of TGF-β1-induced EMT was essentially different from that of the drugs (BLM, MTX, and PTX). We observed that cell cycle synchronization suppressed the EMT induced by the drugs, but not by TGF-β1 (Kawami *et al*., 2019a). Therefore, targeting TGF-β1 may not be applied to prevent drug-induced pulmonary fibrosis, and further efforts are required to identify preventive targets at the molecular level.

Benefits of preventive approach against drug-induced pulmonary fibrosis based on suppression of EMT

In general, compared to the main pharmacological effect of the drug, mechanistic studies on adverse drug reactions are less highlighted. As drug-induced pulmonary fibrosis is regarded as an adverse effect of the drug, preventive approaches against drug-induced pulmonary fibrosis have been less discussed.

Conversely, we are considering the benefits of a preventive approach against drug-induced pulmonary fibrosis based on the suppression of EMT from our experiences, and focusing on two possible discussion points as follows: EMT *vs.* main pharmacological effect in drug treatment, and EMT *vs.* fibrotic state in the onset and course of pulmonary fibrosis.

Drug-induced EMT is specific for pulmonary fibrosis but not for the main pharmacological effect

There have been several reports showing the relationship between drug-induced EMT and cytotoxic effects. For example, TGF-β1 independently induces both EMT and apoptosis in the cell cycle phases of G1/S and G2/M, respectively (Yang *et al.*, 2006). Additionally, suppression of EMT is known to enhance the cytotoxic effect of anticancer drugs, which is used to overcome drug resistance in cancer (Du and Shim, 2016). These findings support the hypothesis that EMT and cytotoxic reactions by the drugs are separately controlled in the cells, even though the factors regulating both events remain unclear.

Drug-induced pulmonary fibrosis is currently recognized as one of the side effects of the drug, while the relationship between drug-induced EMT and the main effect of the drug remains poorly elucidated. In fact, as we accumulated evidence concerning drug-induced EMT in A549 cells, especially in the case of MTX (Kawami *et al.*, 2016, 2018, 2019a), the induction of EMT by pulmonary toxic drugs such as MTX has been characterized without featuring the main pharmacological effect. Moreover, we recently found that knockdown of dihydrofolate reductase, a pharmacological target of MTX, did not influence MTX-induced EMT (Kawami *et al.*, 2019b), and MTX-induced increase in the expression of α-SMA, a representative EMT and fibrosis marker, was statistically independent of the pharmacological cytotoxic effect of MTX using a number of cloned A549 cells with different phenotypes (Ojima *et al.*, 2020). Furthermore, low mRNA expression level of plasminogen activator inhibitor 1 (PAI-1) was observed in A549 cells, while MTX treatment led to upregulation of PAI-1 mRNA/protein expression levels, followed by EMT induction (Yamagami *et al.*, 2020). Thus, PAI-1 could be the targetable factor, which does work as a critical EMT inducer under only drug treatment condition, but independent of main effect of the drug. These findings strongly suggest different underlying mechanisms between EMT and cytotoxic effects during MTX treatment (Fig. 1). Conversely, the regulation of complex signaling pathway cross talk network seems to be quite difficult, as evidenced by failure in development of inhibitors against TGF-β signaling pathway as described in the section of "***Current therapeutic approach against pulmonary fibrosis***". To establish a specific preventive approach against MTX-induced EMT, further efforts to identify molecular targets to separate EMT and cytotoxic effects of MTX are required.

EMT would occur prior to the development of drug-induced pulmonary fibrosis

The length of the latent period between drug exposure and the onset of drug-induced lung injury varies greatly, ranging from several weeks for MTX-induced pneumonia to several years for amiodarone-induced interstitial pneumonia (Kubo *et al.*, 2013).

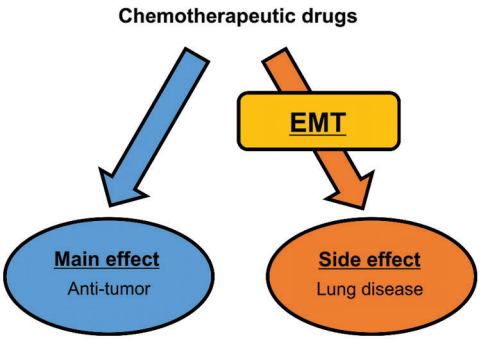

FIGURE 1. Hypothesized scheme on the induction of epithelial-mesenchymal transition by chemotherapeutic agents and the different mechanisms of their main pharmacological effects.

To prevent drug-induced pulmonary fibrosis, a preventive approach should be started prior to the development of fibrotic status in the lungs. However, as physical findings (cough, shortness of breath, and dyspnea), chest X-rays or computed tomography, and clinical laboratory tests (blood cell count, quantitative analysis of several cytokines, etc.) are currently used for the diagnosis of DILI, there are no effective predictive criteria for drug-induced pulmonary fibrosis. Recently, we found that EMT in purified alveolar epithelial type II (ATII) cells occurred before the development of fibrosis in the entire lung tissue in a rat model of BLM-induced pulmonary fibrosis rat model (Kawami *et al.*, 2021). Considering these results, as described in Fig. 2, the hypothesized scheme that BLM administration into the rat leads to a pathological state of pulmonary fibrosis through the transition state of EMT in ATII cells would be proposed. Accordingly, EMT in ATII cells is a key event in the mechanism underlying drug-induced pulmonary fibrosis, and EMT may be an effective preventive target prior to the development of a serious pathophysiological state of the lung. This concept is supported by another report that the population of α-SMA positive cells in the lungs of BLM-administered mice increased intermittently and returned to normal levels (Tsukui *et al.*, 2013).

Perspectives of preventive approach against drug-induced pulmonary fibrosis based on the suppression of EMT

As described above, a preventive approach against drug-induced pulmonary fibrosis targeting EMT would have two advantages. First, EMT would have high specificity for pulmonary fibrosis, but not for the main pharmacological effect. Another possibility is that EMT occurs prior to the development of pulmonary fibrosis mediated by drugs. According to these concepts, targeting molecules highly specific to EMT and starting the method at an early stage of drug treatment should be important factors to establish a preventive approach against EMT and drug-induced pulmonary fibrosis. Therefore, further study for identification of the targetable molecule is needed, and timing of the initiation of preventive approach in parallel with the drug treatment should be conducted.

Additionally, pharmaceutical insights may be considered as a preventive approach. EMT occurs in both physiological and pathological conditions, thereby preventing EMT, which may affect EMT-related normal events, such as tissue repair. Accordingly, a preventive approach limited to the pathophysiological state of the lung, such as intratracheal

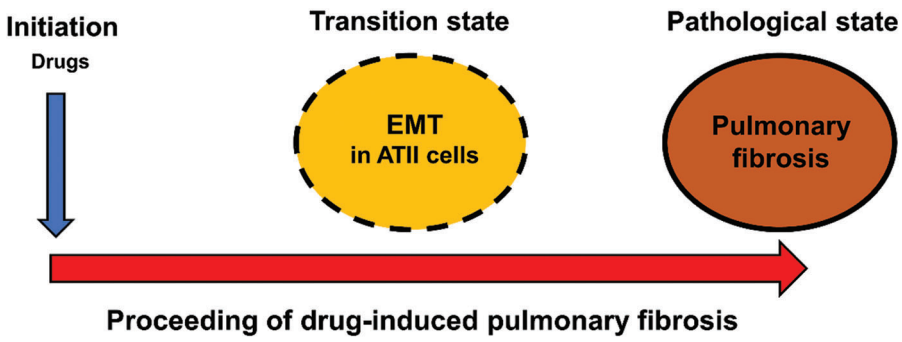

FIGURE 2. Hypothesized scheme of bleomycin-induced pulmonary fibrosis through epithelial-mesenchymal transition in ATII cells at the transition state.

administration, may be recommended to avoid undesired reactions via EMT inhibition in organs other than the lung. In the case of MTX-induced EMT, folic acid, an antagonist of MTX, suppressed MTX-induced EMT in A549 cells (Kawami *et al.*, 2018), although there is no available information regarding the inhibitory effect of folic acid on MTX-induced lung injury in clinical practice. Our previous report demonstrated that MTX would be distributed into the lung via reduced folate carrier (RFC) localized at the basal side of alveolar epithelial cells (Kawami *et al.*, 2019c), and differences in affinity between folic acid (Ki value; ~200 μM) and MTX (Km range; 1–7 μM) for RFC may lead to poor distribution of folic acid in the lung, resulting in less information concerning the clinical effect of folic acid on MTX-induced lung injury. Therefore, a pharmaceutical approach targeting the lung through intratracheal administration would be useful to realize efficient pulmonary drug delivery and effective inhibition against EMT, even though further elucidation is needed.

Furthermore, it should be also important point to consider the EMT categories. Among the EMT categories, type II EMT is mainly associated with pathogenesis of organ fibrosis (Marconi *et al.*, 2021). Additionally, pulmonary fibrosis is closely linked with type II EMT event (Salton *et al.*, 2019), which assumes that type II EMT would cause drug-induced pulmonary fibrosis. Therefore, specific suppressive approach against type II EMT would have an advantage in keeping type I EMT-mediated physiological reactions. However, there is currently not enough evidence to focus on the contribution of the drugs to the categories of EMT, and many growth factors/signaling pathways are overlapped between the types I-III EMT, which makes it quite difficult to separate the EMT types at molecular level. To apply EMT categories to drug-induced EMT, further investigations are needed.

Once the drug-triggered lung injury occurs, the deterioration of pulmonary structures and functions proceeds, leading to high mortality and poor prognosis. However, the mechanistic study on drug-induced lung injury using the clinical samples is currently not highlighted due to the low frequency and low productivity as just side effect, but not main effect of the drug. When monitoring of lung status during the clinical use of the pulmonary toxic drugs, liquid biopsy materials like blood and BALF can be possible to use in the EMT-related study. In particular, the changes of several biological factors independent of the main effect of the drug at the early stage of the treatment should be scientifically evaluated considering whether EMT is associated or not. Thus, this Viewpoint may inspire clinical researchers to focus on EMT-associated clinical findings during pulmonary toxic drug treatment, followed by an innovative progress in the research field of preventing drug-induced lung injury.

Conclusion

Drug-induced pulmonary fibrosis has not yet been fully characterized. Additionally, no effective preventive approach has been established for a variety of drugs that show pulmonary toxic reactions. This viewpoint focuses on EMT as a possible biological event for preventing drug-induced pulmonary fibrosis, considering the advantages of targeting EMT proposed from our experiences and knowledge. However, the detailed mechanism underlying drug-induced EMT remains unclear. Further understanding of the EMT process would be of great help for the establishment of a preventive approach against drug-induced pulmonary fibrosis based on the suppression of EMT.

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