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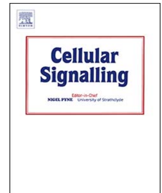

Tubulin beta 3 and 4 are involved in the generation of early fibrotic stages

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ABSTRACT

The endothelial-mesenchymal transition (EndMT) is a fundamental cellular mechanism that occurs under both physiological and pathological conditions and includes the fibrotic stages of numerous organs, namely, the skin, kidneys, heart, lungs and liver. Endothelial cells that undergo EndMT are one of the main source of (myo) fibroblasts in fibrotic tissues. A critical step in cellular transdifferentiation is morphological change, which is engineered by the reorganization of cytoskeletal elements such as microtubules. These dynamic structures consist of *αβ*-tubulin heterodimers that are also involved in cellular movement and intracellular trafficking, processes modulated during EndMT. One fundamental mechanism that underlies microtubule stabilization is the regulation of the levels of *α* and *β*-tubulin. However, little is known about the roles of specific tubulin isotypes in the development of EndMT-based diseases.

This study provides the first evidence that the upregulation of TUBB3 and TUBB4 is coupled with increased cell migration in EndMT-induced HMEC-1 cells. Immunochemical analysis reveals that these tubulins are upregulated in the early stages of EndMT, and siRNA analysis indicates that they are engaged in the generation of mesenchymal behavior *via* the enhancement of cell migration. This modulation seems to be especially important in wound healing. Finally, cell surface analysis reveals that TUBB3 and TUBB4 are necessary for the transport and proper localization of N-cadherin within the plasma membrane. We believe that our results will be valuable for the development of effective new anti-fibrotic therapies.

1. Introduction

Due to instability in cell contacts, cell-cell junctions become disorganized, and the cell acquires migration properties [1]. Endothelial cells undergo these processes during the development of different disorders, such as a cancer progression. As the outcome of pathological wound healing, fibrosis is another essential pathology where cell contact instability plays a major role due to the EndMT [2]. Physiological wound healing is a complex process that leads to rapid restoration of the mechanical integrity of the disrupted tissue. Wound healing comprises four overlapping phases: coagulation, inflammation, proliferation, and remodeling. First, recruited platelets form a blood clot and release multiple chemokines and growth factors (TGF-β1, PDGF) that recruit inflammatory cells. Platelets also participate in the recruitment of the endothelial cells that form new capillaries and the fibroblasts that deposit new extracellular matrix (ECM) components to replace the clot during scarring [3–5]. The remodeling phase is the final step of wound healing in physiological conditions. However, this phase can become uncontrolled and develop into fibrosis. Fibrosis is characterized by long-term inflammation that leads to an increased production and deposition of ECM components, which stiffen the tissue, and the accumulation of myofibroblasts (activated fibroblasts). It is well known that myofibroblasts are a heterogenic group of cells, and a main source of the myofibroblasts is the endothelial cell that has undergone transdifferentiation to a mesenchymal fibroblast-like cell during the phenomenon known as EndMT. Accumulative evidence demonstrates that EndMT is the critical process in the development of cystic, kidney, heart, dermal, pulmonary and intestinal fibrosis [6–11]. Additionally, the fibrotic phenotype has been described in systemic sclerosis [12], atherosclerosis [13], pulmonary hypertension [14], diabetic nephropathy [15], diabetic retinopathy [16], sepsis [17], and cerebral cavernous malformations [18]. According to US government data, approximately 45% of natural deaths can be associated with different fibrotic disorders [19]. However, knowledge of the molecular

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mechanisms that underlie these disorders remains lacking.

EndMT is mediated by many factors, but the superfamily of Transforming Growth Factor-β (TGF-β) plays a pivotal role in this process [20]. TGF-β stimulates modulation of the cytoskeletal composition and organization. TGF-β causes reorganization of cell morphology and results in a cell polarization change from an apical-basal organization to one resembling an elongated spindle. The cellular ability to form capillary tubes *in vitro* decreases [21]. Increased expression of Snail1, a zinc-finger transcription factor, during TGF-β1-stimulated EndMT may independently induce the loss of cell-cell adhesion and is correlated with down-regulation of the proteins involved in the formation of adherens junctions and tight junctions [22]. These alterations are acquired from changes in cell migration properties. During the mesenchymal transition, cells do not typically undergo complete EndMT. They might become arrested between the endothelial and mesenchymal phenotypes, thus allowing cell-cell contacts to be partially maintained to enable group migration rather than single cell migration [22–24]. Simultaneously, the basal lamina is reorganized and enriched by collagen that is secreted by the migratory cells, which stimulates cell motility.

Like microfilaments, microtubules (MTs) regulate integrin-mediated adhesion. They lead to the destabilization and increased turnover of focal adhesions (FAs), which regulate cell polarization and migration [25–28]. They contribute to the maintenance of cell shape and are involved in the intracellular transport of cellular organelles, vesicles, proteins or signaling molecules. They also play important roles in the organization of cellular junctions [29]. As an important element of the cytoskeleton, the microtubule forms highly dynamic polymers comprising αβ-tubulin dimers [30]. Vertebrates encode eight α-tubulins and seven β-tubulins. Although the functions of many tubulins remain unclear, different tubulin isotypes are known to have specific cellular, tissue and developmental distributions [31]. This expression and localization diversity of particular tubulins is postulated as a mechanism that regulates microtubule polymerization and depolymerization, alongside post-translational modifications and modulations in the microtubule–association protein (MAP) levels [32].

EndMT is typified by a loss of endothelial markers, a gain of mesenchymal markers, and an increase in the expression of transcription factors such as Snail and Slug [3]. Microtubules are involved in the translocation of newly expressed mesenchymal markers, one of which is N-cadherin [33]. The cadherin protein family comprises homophilic, transmembrane cell-cell adhesion molecules that are essential for the organization of cells into tissues during embryonic development. The cadherins also influence cell growth, migration and differentiation [34–36]. Regulation of cadherin localization to the plasma membrane, first observed during the epithelial-mesenchymal transition (EMT), is the mechanism that affects adherent junction assembly [37]. Previously, it has been shown that interactions between N-cadherin, catenins and the actin cytoskeleton are critical for cancer and fibrosis development [38–39]. Additionally, analyses of rat embryo fibroblasts and mouse C2 myoblasts have demonstrated that microtubule-dependent kinesin-driven transport is essential for N-cadherin transport and is required for cell-cell contact formation [33]. Nevertheless, the role of particular subunits of tubulins in the regulation of the microtubule-N-cadherin interaction in EndMT is unclear.

In this study, we examined an EndMT model that was generated using human microvascular endothelial cells (HMEC-1) that were stimulated with TGF-β1 or the zinc-finger transcription factor Snail. We provide the first indication that independently of Snail, TGF-β1, a well-known stimulant of the fibrotic phenotype, causes the upregulation of two of the five β subunits (TUBB3 and TUBB4) detected in the tested endothelial cell line. We also examine the roles of specific tubulin subunits in the generation of fibrotic behavior in a cellular model of EndMT. A siRNA analysis reveals that both of the tubulin isoforms examined here are involved in the stimulation of the cell movement observed during EndMT. Furthermore, it appears that TUBB3 and TUBB4 are the crucial elements of the microtubules that are responsible for efficient transport of N-cadherin to the cell membrane.

2. Materials and methods

2.1. Reagents

All standard tissue culture reagents, including the MCDB 131 medium, fetal bovine serum (FBS) and Penicillin-Streptomycin-Glutamine (100 ×) were purchased from Life Technologies, and the mouse monoclonal N-cadherin antibodies were purchased from Millipore. AlexaFluor®488-conjugated TGF-β1 antibodies, AlexaFluor®488-conjugated polyclonal sheep N-cadherin antibodies and AlexaFluor®543-conjugated LCK antibodies were obtained from R & D. All primers were synthesized by GenoMed. The Enhanced Chemiluminescence (ECL) Western blotting substrate, M-PER Extraction Reagents, NE-PER Nuclear and Cytoplasmic Extraction Kit, and Moloney murine leukemia virus reverse transcriptase originated from Fermentas (Thermo Scientific Pierce), and the siRNA oligonucleotides were obtained from Dharmacon. The goat anti-mouse antibodies, anti-rabbit antibodies and mouse anti-GAPDH conjugated with horseradish peroxidase were purchased from Santa Cruz Biotech. X-fect was purchased from Clontech. Bradford, a 30% Acrylamide/Bis 37.5:1 solution, ammonium persulfate (APS), 1,2-Bis(dimethylamino)ethane (TEMED), glycine, and blotting membranes were purchased from Bio-Rad. Matrigel™ was obtained from Corning. If not specified otherwise, all chemicals and solvents were of the highest analytical grade and were purchased from Sigma-Aldrich.

2.2. Cell culture

Human microvascular endothelial cells (HMEC-1), a gift from Kathryn Kellar at the Centers for Disease Control and Prevention, Atlanta, GA, were maintained in MCDB 131 medium supplemented with 10% (v/v) FBS, streptomycin (100 μg/ml), penicillin (100 units/ml), glutamine (2 mM), EGF (10 ng/ml) and hydrocortisone (1 μg/ml). The cells were cultured as a monolayer at 37 °C in a humidified atmosphere with 5% CO_2 and were routinely tested and confirmed to be mycoplasma-free.

2.3. EndMT induction

For EndMT induction, HMEC-1 cells were stimulated with TGF-β1 (5 ng/ml) for 48 h or transfected with an expression vector encoding *Snail* (Snail-pcDNA3.1) (gifts from Muh-Hwa Yang, Ph.D., Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan) or an empty vector (pcDNA3.1) as a control transfection using the X-fect reagent according to the manufacturer's protocol.

2.4. Cell transfection

To analyze the roles of TUBB3 and TUBB4 in EndMT-induced cells, siRNAs targeting human TUBB3 or TUBB4 and the negative control siRNA were used. The siRNAs were transfected into cells using the X-fect reagent according to the manufacturer's protocol. The siRNA treatment was performed 15 h after TGF-β1 stimulation, and the expression of the corresponding genes was silenced for 33 h. To induce TUBB1 or TUBB6 expression, cells were transfected with the pcDNA3.1 plasmids that expressed the tubulin isotypes or the pcDNA3.1 plasmids using the X-fect reagent. To express TUBB1 or TUBB6, total RNA was isolated from the HMEC-1 cells with the TriPure reagent and reverse transcribed using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's protocol. The *TUBB1* and *TUBB6* in-frame cDNA was cloned into pcDNA3.1. The TUBB1 and TUBB6 sequences were PCR-amplified using Pfu polymerase and corresponding primers, with one primer containing the EcorRI site and the other

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