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Altered TGF-β endocytic trafficking contributes to the increased signaling in Marfan syndrome

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ABSTRACT

The main cardiovascular alteration in Marfan syndrome (MFS) is the formation of aortic aneurysms in which augmented TGF-β signaling is reported. However, the primary role of TGF-β signaling as a molecular link between the genetic mutation of fibrillin-1 and disease onset is controversial. The compartmentalization of TGF-β endocytic trafficking has been shown to determine a signaling response in which clathrin-dependent internalization leads to TGF-β signal propagation, and caveolin-1 (CAV-1) associated internalization leads to signal abrogation. We here studied the contribution of endocytic trafficking compartmentalization to increased TGF-β signaling in vascular smooth muscle cells (VSMC) from MFS patients. We examined molecular components involved in clathrin- (SARA, SMAD2) and caveolin-1- (SMAD7, SMURF2) dependent endocytosis. Marfan VSMC showed higher recruitment of SARA and SMAD2 to membranes and their increased interaction with TGF-β receptor II, as well as higher colocalization of SARA with the early endosome marker EEA1. We assessed TGF-β internalization using a biotinylated ligand (b-TGF-β), which colocalized equally with either EEA1 or CAV-1 in VSMC from Marfan patients and controls. However, in Marfan cells, colocalization of b-TGF-β with SARA and EEA1 was increased and accompanied by decreased colocalization with CAV-1 at EEA1-positive endosomes. Moreover, Marfan VSMC showed higher transcriptional levels and membrane enrichment of RAB5. Our results indicate that increased RAB5-associated SARA localization to early endosomes facilitates its TGF-β receptor binding and phosphorylation of signaling mediator SMAD2 in Marfan VSMC. This is accompanied by a reduction of TGF-β sorting into multifunctional vesicles containing cargo from both internalization pathways.

1. Introduction

Marfan syndrome (MFS) is a multisystemic connective tissue disorder with autosomal dominant inheritance that affects between 1.5 and 17.2 in 100,000 births [1]. The disease is characterized by skeletal, ocular and cardiovascular manifestations and is caused by a mutation of the gene encoding for extracellular matrix (ECM) protein fibrillin-1 (FBN1) [2]. FBN1 is the principal component of microfibrils, which, together with elastin, constitute the elastic fibers of the ECM in connective tissues throughout the body. Approximately 3000 *FBN1* mutations with variable dysfunctionality have been found to date, and the progress of the disease is difficult to predict through its mutation [3,4]. The most severe problems arise from abnormalities of the cardiovascular system, such as decreased integrity of the tunica media of the ascending aorta, which consists of vascular smooth muscle cells (VSMC) and elastic fibers [5]. These aortic abnormalities are due to elastic fiber fragmentation and increased collagen deposition [6]. The severe effects of the mutation are often asymptomatic until a life-threatening aortic aneurysm or dissection occurs.

Transforming growth factor-β (TGF-β) has been suggested to be the molecular link between gene mutation and disease onset, and has been used as a predictive marker of aneurysm progression [7,8]. This paradigm has recently been challenged and a dual role of the cytokine has been proposed, however the stage of disease progression is crucial [9–11]. TGF-β is secreted into the extracellular environment as a large latent complex (LLC), which is covalently attached to the latent TGF-β

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binding protein (LTBP). LTBPs interact with FBN1 through their characteristic 8-Cys module, which is uniquely shared between the two proteins [12]. The result is regulatory linkage of mature TGF-β to microfibrils, which prevents uncontrolled activation of TGF-β. Under physiological conditions, TGF-β is maintained in an inactive state by controlled binding of LTBP to FBN1. In MFS, fragmentation of microfibrils caused by insufficient or dysfunctional FBN1 leads to reduced binding capacity of LTBP and increased active TGF-β in the extracellular environment [13]. TGF-β induces collagen production, tightly regulates ECM remodeling and leads to tissue fibrosis, which compromises the organ structure as well as its function [14,15].

TGF-β receptor internalization takes place through both clathrin-coated pits and lipid raft-associated caveolin-1- (CAV-1) positive vesicles, which have opposite effects on TGF-β signal transduction [16]. The ligand binds the constitutively active TGF-β receptor II (TBRII), which leads to TGF-β receptor I (TBRI; also named ALK5) recruitment and phosphorylation [17]. Receptor/ligand complexes internalized through clathrin-coated pits are targeted to early endosome associated protein-1- (EEA1) positive early endosomes, and initiate regulatory SMAD (R-SMAD) signaling through phosphorylation of SMAD2 and SMAD3. SMAD2/3 phosphorylation is dependent on the SMAD anchor for receptor activation (SARA), which is a bridging factor with binding sites for the TGF-β-receptor, SMAD2 and early endosomes, and is associated with TGF-β signal activation [18–20]. A third SMAD protein, the inhibitory SMAD7, can compete with SMAD2/3, target the receptor for degradation, and abrogate signaling. In contrast to clathrin-dependent endocytosis, receptor internalization through caveolae destines receptors for proteasome degradation by binding of SMAD7 and E3 ubiquitin ligase poly-ubiquitination, which terminates the signaling cascade [16,21].

The role of TGF-β in MFS has thus far mostly been investigated in murine models. To date, the importance of TGF-β in MFS patients is supported by evidence of increased signaling, partly associated with epigenetic modification of the SMAD2 promotor, as well as by the beneficial effects of TGF-β-receptor blockers and neutralizing TGF-β antibodies on the progress of aortic aneurysms in murine models [11,22,23]. However, in MFS, altered TGF-β endocytic trafficking in human VSMCs is still to be elucidated. Here we examine the significance of TGF-β signal regulation by its endocytic compartmentalization at endogenous levels in primary human VSMC from aortic aneurysm tissue of Marfan patients. We show that TGF-β internalizes through the clathrin- and CAV-1-associated pathways at equal levels in VSMC from MFS and controls. However, increased SARA recruitment, facilitated by RAB5 to early endosomes, favors spatial interaction of the TGF-β receptor complex with SMAD2, thus contributing to increased TGF-β signaling in MFS.

2. Material and methods

2.1. Human tissue collection, ethics statements and subjects

Healthy ascending aortic tissue was collected from heart donors through the organ donation organization at the Hospital Clínic i Provincial (Barcelona, Spain) and Hospital de Bellvitge, L'Hospitalet de Llobregat (Barcelona, Spain). The age and gender of heart donors were unknown as the Spanish law protects organ donors' personal information. Ascending aortic aneurysm samples were collected from both male and female MFS patients undergoing aortic aneurysm repair surgery between 23 and 59 years of age. All patients fulfilled Marfan syndrome diagnostic criteria according to Ghent nosology [24]. From each patient, we obtained a sample of approximately 3 × 3 cm from the dilated ascending aneurysmal zone. Aortic tissue was maintained in cold saline or cardioprotective solution during delivery to the laboratory. Human tissues were collected with the required approval from the Institutional Clinical Review Board of Spanish clinical centers, and the patients' written informed consent conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

2.2. Human vascular smooth muscle cells culture

Human VSMC were isolated from healthy and Marfan aortae as previously reported [22]. Briefly, ascending aortic tissue was first cleaned of fatty tissue and separated from intima and adventitia layers, leaving just the tunica media. Subsequently, the tunica media was cut into 1–2 mm cubes, which were transferred to 100 mm culture plates. After adhesion at 37 °C for 45 min in the incubator, the small cubes of aortic media were gently covered with 4 ml of 231 culture medium (Gibco) supplemented with 25 ml of Smooth Muscle Growth Supplement (SMGS) from Gibco, 100 mg/ml streptomycin, 100 U/ml penicillin and plasmocin. Cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Explants were left undisturbed for 4 days to prevent detachment, and half the medium was replaced every 4 days. VSMC migrate out from the explants within 1–2 weeks. Then, after removing the explants from the flask surface, the cells were trypsinized, used as P1 stage cells and routinely subcultured. Primary cultures have limited expansion and were used for experiments between passages P3 to P8.

2.3. Antibodies and reagents

Rabbit polyclonal antibodies to SARA (sc-9135), RhoGDI (sc-359), RAB5 (sc-309) and mouse monoclonal antibody to SMAD7 (sc-101,152) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat polyclonal anti-TBRII (AF-241-NA) was from R&D Systems (Minneapolis, MN). Mouse monoclonal antibody to SMAD2 (3103S) and rabbit monoclonal antibody to pSMAD3 (Ser423/425; 9520) were from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody to EEA1 (610456) was from BD Biosciences (Franklin Lakes, NJ, USA). Mouse monoclonal antibody to the transferrin receptor (ref. 13-6800) was from Invitrogen (Carlsbad, CA, USA). Secondary peroxidase-conjugated antibodies to IgG mouse, rabbit and goat (W402B, W401B and V805A respectively) were from Promega (Madison, WI, USA). Alexa Fluor 647-conjugated goat anti-rabbit IgG (A21245) and Alexa Fluor 488-conjugated goat anti-rabbit (A-11070) were from Invitrogen, Cy3-conjugated goat anti-mouse IgG (115.167.003) and goat anti-human IgG (109.005.088) were from Jackson ImmunoResearch (West Grove, PA, USA). Recombinant human TGF-β was from Merck Millipore (Darmstadt, Germany). Human TGF-β 1 Biotinylated Fluorokine Kit (NFTG0) was from R&D Systems. Protein A-coated Dynabeads (10001D) were from Invitrogen. Water-soluble mounting medium DAPI-Fluoromount G (0100-20) was from Southern Biotech (Birmingham, AL, USA).

2.4. Membrane and cytosol fractionation of VSMC

Cells were rinsed twice in PBS and scraped into lysis buffer (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.5) supplemented with protease inhibitors. Extracts were mechanically lysed with 30 strokes of a 30G syringe, and subjected to 90 min of ultracentrifugation at 45,000 rpm using an S140-AT rotor (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C. The resulting supernatant was the cytosolic fraction. The membrane fraction containing pellet was resuspended in 100 µl radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS 140 mM NaCl), supplemented with protease inhibitors. Twenty micrograms of each subcellular fraction were analyzed by 12% (v/v) SDS-PAGE and blotted as described previously [22]. Cytosol and membrane protein bands were quantified and relativized against their respective fraction markers RhoGDI and transferrin receptor. Membrane enrichment was calculated by normalization with the corresponding cytosolic fraction, and membrane enrichment in Marfan patients was normalized against controls. Statistical analysis