The International Journal of Biochemistry & Cell Biology 79 (2016) 69–79

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Inhibiting post-translational core fucosylation prevents vascular calcification in the model of uremia

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ARTICLE INFO

Article history:
Received 1 December 2015
Received in revised form 20 June 2016
Accepted 9 August 2016
Available online 10 August 2016

Keywords:
α-1,6-fucosyltransferase
Vascular calcification
TGFβ-R

ABSTRACT

Vascular calcification (VC) is an independent risk factor for cardiovascular disease and mortality in uremia. Post-translational core fucosylation is implicated in a number of pathological processes. First, we investigated the role of core fucosylation and key TGF-β1 pathway receptors in calcified arteries *in vivo*. To determine whether blocking core fucosylation effectively inhibited VC and TGF-β/Smad signaling pathway, we established an *in vitro* model of phosphate-induced calcification in rat vascular smooth muscle cells (VSMCs) to assess the role of core fucosylation in VC. Core fucose could be detected at markedly higher levels in calcified VSMCs than control cells. *Fut8* (α-1,6 fucosyltransferase), the only enzyme responsible for core fucosylation in humans, was significantly upregulated by high phosphate. Exposed to high phosphate media and blocking core fucosylation in VSMCs by knocking down *Fut8* using a siRNA markedly reduced calcium and phosphorus deposition and Cbfα1 expression (osteoblast-specific transcription factor), and increased α-Sma expression (smooth muscle cell marker). *Fut8* siRNA significantly inhibited TGF-β/Smad2/3 signaling activation in VSMCs cultured in high phosphate media. In conclusion, this study provides evidence to suggest core fucosylation plays a major role in the process of VC and appropriate blockade of core fucosylation may represent a potential therapeutic strategy for treating VC in end-stage renal disease.

1. Introduction

Vascular calcification (VC) is a common complication and the major cause of cardiovascular disease in patients with end-stage renal disease (Karohl et al., 2011). The prevalence of VC increases during the progression of chronic kidney disease (CKD), ranging from 40% among patients with stage 3 CKD to 80–99% in patients with end-stage renal disease on dialysis (Garland et al., 2008; Sigrist et al., 2006; Adeney et al., 2009; Chertow et al., 2002). However, the precise molecular mechanisms underlying VC still need to be clarified. VC is no longer regarded as a passive process and is considered an actively-regulated and complex process that is not yet completely understood. During VC, the specific, indispensable transcriptional regulator of osteoblastic differentiation core-binding factor subunit 1α (Cbfα1) is upregulated, while expression of the VSMC marker α-smooth muscle actin (α-SMA) decreases (Giachelli et al., 2005; Hruska et al., 2005).

A number of cytokines and signaling pathways have been demonstrated to stimulate the occurrence and development of VC, including TNF-α, osteonectin, osteocalcin, the BMP-2 signaling pathway, transforming growth factor-β (TGF-β) signaling pathway (Yetkin and Waltenberger, 2009; Wang et al., 2013) and the Wnt/β-catenin/OPG/RANKL/RANK axis (Evrard et al., 2015). These studies have provided important data regarding the mechanisms that underlie VC and helped to infer the process that occur during the progression of VC. Most of the above-mentioned studies have explored the roles of key proteins in VC by altering the expression of these genes or proteins. In fact, in addition to gene and protein expression levels, post-translational modifications of proteins can also have a major effect on protein function. Data increasingly indicates that post-translational modifications directly and definitively regulate protein function in a range of pathophysiological processes, and in some cases, this regulation is independent of the

Abbreviation: VSMCs, vascular smooth muscle cells; CKD, chronic kidney disease; *Fut8*, α-1,6 fucosyltransferase; cbfα1, core-binding factor subunit 1 α; α-SMA, α-smooth muscle actin; TGFβ1, transforming growth factor-β1; LCA-FITC, fluorescent *L. culinaris* agglutinin–fluorescein complex.

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http://dx.doi.org/10.1016/j.biocel.2016.08.015
1357-2725/

expression levels of the modified proteins (Kumar and Klein, 2004; Hao et al., 2011). Therefore, post-translational modifications may be critical regulators of protein function.

Glycosylation is a key type of post-translational modification and has significant effects on the regulation of various physiological processes, including cell growth, differentiation and migration (Takahashi et al., 2009; Zhao et al., 2008). Core fucosylation, which consists of an α-1,6 fucose substitution on the innermost *N*-acetylglucosamine (GlcNAc) of the pentasaccharide core of N-linked glycans, is a special protein glycosylation pattern associated with a number of biological and pathological functions (Ferrara et al., 2011; Wang et al., 2006a,b; Akasaka-Manya et al., 2008). Recent investigations reported that core fucosylated proteins are associated with various cancers, such as pancreatic cancer, lung cancer, ovarian cancer and prostate cancer (Noda et al., 1998a,b, 2003; Geng et al., 2004; Chen et al., 2013; Saldova et al., 2007; Tabarés et al., 2006; Okuyama et al., 2006; Ahn et al., 2014). Additionally, the core fucosylated α-fetoprotein (AFP-L3) has been approved by the Food and Drug Administration (FDA) for the early diagnosis of hepatocellular carcinoma (HCC).

Our previous studies showed that TGF-βRII and ALK5 are modified by core fucosylation via a process dependent on *Fut8* (Shen et al., 2013; Lin et al., 2011), which is the only enzyme that catalyzes α1,6-linked core fucosylation in humans (Uozumi et al., 1996; Yanagidani et al., 1997). This data indicates the absence of core fucose on TGF-β receptors markedly dysregulated downstream TGF-β/Smad2/3 signaling and induces pathological changes in renal tubular cells, and more importantly, these effects were independent of the expression of TGF-βRII and ALK5. However, it remains unclear whether core fucosylation plays crucial role in VC.

The aim of the present study was to determine whether blocking core fucosylation effectively inhibits VC and over activation of the TGF-β/Smad signaling pathway in CKD. To test this, we investigated the role of core fucosylation and key receptors of the TGF-β1 pathway that participate in VC in the calcified arteries of rats with adenine-induced CKD and human patients with CKD. As phosphate loading is known to induce VC in animal models of CKD, and hyperphosphatemia is a significant risk factor for VC and cardiovascular mortality in patients with preexisting CKD (Adeney et al., 2009; El-Abbadi et al., 2009; Kestenbaum et al., 2005), we established an *in vitro* model of CKD using high phosphate culture medium. *Fut8 was* knocked down in rat aortic vascular smooth muscle cells (VSMCs) exposed to high phosphate media. This study suggests that inhibition of core fucosylation attenuates VC, and indicates that the ability to target post-translational modifications of key proteins may provide a novel and effective strategy for the treatment of VC.

2. Materials and methods

2.1. Ethical considerations and animal care

All experimental protocols were reviewed and approved by the Committee on Ethics of Animal Experimentation of Dalian Medical University. Animal experiments were conducted in accordance with the regulations set by the institutional committee for the care and use of laboratory animals, and approved by local authorities. Male Sprague-Dawley (SD) rats (250–300 g) were housed under a 12 h light/dark cycle with free access to food and water.

2.2. Reagents and antibodies

Fut8 small interfering (si) RNA fragments were purchased from Genepharma (Shanghai, China). Biotinylated *Lens culinaris* agglutinin (LCA-Biotin), fluorescein-labeled *L. culinaris* agglutinin (LCA-FITC) were purchased from Vector (Burlingame, CA, USA). Anti-*Fut8* antibody (sc-34629; Yanagidani et al., 1997), anti-TGFβ1 antibody (sc-146; Li et al., 2013), anti-TGF-βRII antibody (sc-17791; Recouvreux et al., 2011), anti-ALK5 antibody (sc-399; Velasco et al., 2008), anti-Smad 2/3 antibody (sc-8332; Zhou et al., 2010), anti-p-Smad2/3 antibody (sc-11769; Wang et al., 2012), anti-Cbfα1 antibody (sc-10758; Xiao et al., 2005), anti-β-actin antibody (sc-47778) and Protein G PLUS-Agarose (sc-2002; Ma et al., 2001) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-α-smooth muscle actin (SMA) antibody (ab5694; Ramani et al., 2010) was purchased from Abcam (Cambridge, UK). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). DMEM, FBS and pancreatic enzyme were purchased from Hyclone (UT, USA). RIPA was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). SYBR PrimerScript RT-PCR Kit and ISOPLUS were purchased from Takara (Takara, Otsu, Shiga, Japan). Horseradish peroxidase (HRP)-goat anti-rabbit antibody (ZB-2301), HRP-goat anti-mouse antibody (ZB-2305) and HRP-rabbit anti-goat antibody (ZB-2306) were purchased from Beijing Zhong Shan Golden Bridge Biological Technology (Beijing, China). FITC- conjugated goat anti-rabbit antibody (SA00003-2), FITC-conjugated goat anti-mouse antibody (SA00003-1) and TRITC-conjugated goat anti-rabbit antibody (SA00007-2) were purchased from Proteintech Group (Chicago, IL, USA) and the BCA protein assay kit from Pierce (Madison, WI, USA).

2.3. Human radial artery samples

Part of this study assessed two patients with upper limb trauma with normal renal function who had no signs of cardiovascular disease or diabetes (control group) and four patients with CKD who exhibited clinical calcification (CKD group). Radial arterial samples in control group with upper limb trauma operation and CKD group during arterial venous fistula plasty were obtained after the patients provided informed consent.

2.4. Rat model of CKD

Following acclimatization to a standard diet for 7 days, 10-week-old male SD rats were randomly subdivided into two groups and treated as follows for 6 weeks: (1) normal group ($n = 8$), rats were given 0.8% saline once a day by gavage; and (2) CKD group ($n = 8$), given 2% adenine by gavage once a day at 250–300 mg/kg/d. Six weeks later, the rats were humanely scarified and the abdominal aortas were collected and processed for protein analyses and to determine the calcium content.

2.5. Histology and immunohistochemistry

For histological analysis, artery tissues were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Sections (2 um thick) were prepared and deparaffinized, endogenous peroxidase activity was quenched in 3% H_2O_2 for 15 min, then the sections were washed in PBS, incubated with anti-Cbfα1, α-SMA, *Fut8*, TGFβ1, TGFβRII, ALK5 or p-Smad2/3 antibodies at 37° C for 1 h, then incubated with the appropriate biotinylated secondary antibodies, followed by treatment with 3,3′-diaminobenzidine (DAB) as a chromogen. Slides were counterstained with Mayer's hematoxylin and mounted in glycerol jelly. Quantification of the area of immunostaining (brown color) in the artery regions of each tissue section was conducted using computer-based morphometric analysis (Name software; Olympus, Tokyo, Japan).