Mitochondrion 12 (2012) 449–458

Contents lists available at SciVerse ScienceDirect

Mitochondrion

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

Recombinant mitochondrial transcription factor A protein inhibits nuclear factor of activated T cells signaling and attenuates pathological hypertrophy of cardiac myocytes

Takeo Fujino [a], Tomomi Ide [a,*], Masayoshi Yoshida [a], Ken Onitsuka [a], Atsushi Tanaka [a], Yuko Hata [a], Motohiro Nishida [b], Takako Takehara [a], Takaaki Kanemaru [c], Naoyuki Kitajima [b], Shinya Takazaki [d], Hitoshi Kurose [b], Dongchon Kang [d], Kenji Sunagawa [a]

[a] Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Department of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[c] Morphology and Core Unit, Kyushu University Hospital, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[d] Department of Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 27 March 2012
Received in revised form 6 June 2012
Accepted 11 June 2012
Available online 16 June 2012

Keywords:
Cardiac hypertrophy
Mitochondrial transcription factor A
Nuclear factor of activated T cells

ABSTRACT

The overexpression of mitochondrial transcription factor A (TFAM) attenuates the decrease in mtDNA copy number after myocardial infarction, ameliorates pathological hypertrophy, and markedly improves survival. However, non-transgenic strategy to increase mtDNA for the treatment of pathological hypertrophy remains unknown. We produced recombinant human TFAM protein (rhTFAM). rhTFAM rapidly entered into mitochondria of cultured cardiac myocytes. rhTFAM increased mtDNA and abolished the activation of nuclear factor of activated T cells (NFAT), which is well known to activate pathological hypertrophy. rhTFAM attenuated subsequent morphological hypertrophy of myocytes as well. rhTFAM would be an attractive molecule in attenuating cardiac pathological hypertrophy.

1. Introduction

Mitochondrial dysfunction has been reported in various forms of heart failure. Mitochondrial DNA (mtDNA) is decreased in the heart from post-myocardial infarction (MI) model in mice (Ide et al., 2001). In human as well, Karamanlidis et al. demonstrated that mitochondrial biogenesis is severely impaired in the myocardium from end-stage heart failure patients. They also showed that there was no significant change in the expression of the fibroblast marker in their sample, suggesting the decrease of mtDNA and mitochondrial biogenesis are not due to the fibrosis occurring under these conditions, changing the ratio of cardiac myocytes with a high amount of mitochondria to fibroblasts and other cell types with low amounts (Karamanlidis et al., 2010). mtDNA could be a major target for locally generated reactive oxygen species (ROS), and an intimate link among mtDNA damage and defects in the electron transport function might play an important role in the development and progression of cardiac remodeling and failure (Ide et al., 2001).

Mitochondrial transcription factor A (TFAM), a nucleus-encoded protein, binding upstream of the light strand and heavy strand promoters of mtDNA, promotes transcription of mtDNA. It also plays an important role in regulating mtDNA copy number (Kanki et al., 2004). TFAM is a high mobility group protein having DNA-binding properties, regardless of its DNA sequence (Parisi and Clayton, 1991). TFAM molecules are abundant enough to cover the entire mtDNA, and indeed most of them bind mtDNA, suggesting that mtDNA is packaged with TFAM (Alam et al., 2003; Kang et al., 2007). Disruption of the *tfam* gene in mice has been shown to cause depletion of mtDNA, loss of mitochondrial transcripts, loss of mtDNA-encoded polypeptides, and severe respiratory chain deficiency (Larsson et al., 1998). Moreover, targeted disruption of *tfam* in cardiac myocytes induced deletion of mtDNA and dilated cardiomyopathy (Li et al., 2000; Wang et al., 1999). In addition, a reduction in TFAM expression has been demonstrated in several forms of cardiac failure (Garnier et al., 2003; Ide et al., 2001; Karamanlidis et al., 2010). We have previously demonstrated in mice, in MI, that TFAM

Abbreviations: mtDNA, mitochondrial DNA; ROS, reactive oxygen species; TFAM, mitochondrial transcription factor A; MI, myocardial infarction; NFAT, nuclear factor of activated T cells; GST, glutathione S-transferase; MTS, mitochondrial targeting signal; rhTFAM, recombinant human TFAM; ΔMTS-rhTFAM, rhTFAM without MTS; rhTFAM-ΔC, rhTFAM lacking C-terminal tail; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; COX I, cytochrome c oxidase I; COX III, cytochrome c oxidase III; SDHA, succinate dehydrogenase complex subunit A; NDUFA9, NADH dehydrogenase 1 alpha subcomplex subunit 9; ATIII, antithrombin III; RPL27, ribosomal protein L27; MCIP1, modulatory calcineurin interacting protein 1; HPRT, hypoxanthine guanine phosphoribosyl transferase; AngII, angiotensin II; ET-1, endothelin-1; BNP, brain natriuretic peptide.

* Corresponding author. Tel.: +81 92 642 5360; fax: +81 92 642 5374.
E-mail address: tomomi_i@cardiol.med.kyushu-u.ac.jp (T. Ide).

1567-7249/$ – see front matter © 2012 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2012.06.002

overexpression attenuated the decrease in mtDNA copy number, ameliorated pathological hypertrophy, and dramatically improved survival rate (Ikeuchi et al., 2005). In addition, the overexpression of TFAM in Hela cells reduced mitochondrial ROS generation (Hayashi et al., 2008). Taken together, these findings indicate that upregulating TFAM results in increasing mtDNA copy number and attenuates cardiac pathological hypertrophy. However, the effective way how to increase TFAM expression or mtDNA copy number in clinical situation remains unknown.

Recent study showed that exogenously administered recombinant TFAM engineered with an N-terminal protein transduction domain, followed by a matrix mitochondrial localization sequence, was recruited into mitochondria of cultured cells (Iyer et al., 2009). Therefore it is conceivable that exogenously administered recombinant TFAM manifests beneficial impacts on myocytes, and this method could be useful for the therapy of cardiac pathological hypertrophy. In the present study, we examined whether exogenous recombinant TFAM protein was recruited into cardiac myocytes and functioned to increase mtDNA copy number and attenuate hypertrophy of myocytes in vitro. The results indicated that recombinant TFAM protein inhibited nuclear factor of activated T cells (NFAT) signaling and prevented pathological hypertrophy of cardiac myocytes.

2. Material and methods

2.1. Preparation of human TFAM protein

We used a glutathione S-transferase (GST) gene fusion purification protocol to synthesize recombinant human TFAM protein. Two TFAM proteins, rhTFAM (recombinant human TFAM with mitochondrial targeting signal (MTS)) and ΔMTS-rhTFAM (recombinant human TFAM without MTS), were prepared. The nucleotide sequences corresponding to human TFAM and TFAM without MTS were cloned from human cDNA library, and were subcloned into pGEX-6P-1 (GE Healthcare). The constructs were transformed into competent cells (BL21 (DE3), Invitrogen). The transformed bacteria were cultured in LB medium (MP Biochemicals) supplemented with 100 μg/ml ampicillin (Wako), in the shaking incubator (Bio-Shaker BR-300LF, TAITEC). When the culture achieved an optical density of wavelength 600 nm to 0.5–0.7, isopropyl-β-D-thiogalactopyranoside (0.7 mM, nacalai tesque) was added to the medium and incubated for further 2 h. Growth and expression of the bacteria culture were performed at 37 °C with variable agitation and airflow. The bacteria were harvested and pelleted by centrifugation at 8000*g* and stored at −80 °C.

Cell pellets were resuspended in sonication buffer (20 mM Tris–HCl, 500 mM NaCl, 250 mM, 2-mercaptoethanol 5 mM, 1% NP-40 and protease inhibitor cocktail (Complete Mini, Roche Diagnostics)) and mildly sonicated at 4 °C. The lysate was then clarified by centrifugation at 8000*g* for 30 min. The supernatant was mixed with Glutathione Sepharose 4B resin (GE Healthcare) for 2 h and applied to polypropylene columns (Thermo SCIENTIFIC). Resin absorbed with GST-TFAM protein was washed with wash buffer (20 mM Tris–HCl, 150 mM NaCl, 5 mM 2-mercaptoethanol, 0.1% NP-40) to isolate the vector protein. Then it was mixed with Turbo3C Protease (160 units/ml, Accelagen) and elusion buffer (20 mM Tris–HCl, 150 mM NaCl, 5 M 2-mercaptoethanol, 0.1% NP-40) at 4 °C overnight to remove GST from TFAM. The flow-through was collected and dialyzed with Slide-a-lyzer Dialysis Cassette (7 K MWCO, Thermo Scientific) in phosphate buffered saline (PBS). The solution was screened via sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) analysis for proper size and purity. The purity was above 98% by Coomassie Brilliant Blue (CBB) staining. Western blot analysis was also performed using anti-human TFAM specific antibody to verify the success of target protein purification (Fig. 1). The solution was stored at −80 °C and used as recombinant TFAM protein. We also performed the same procedure using empty vector without TFAM sequence and the product was also stored at −80 °C. In addition, we synthesized recombinant human TFAM lacking the C-terminal 25

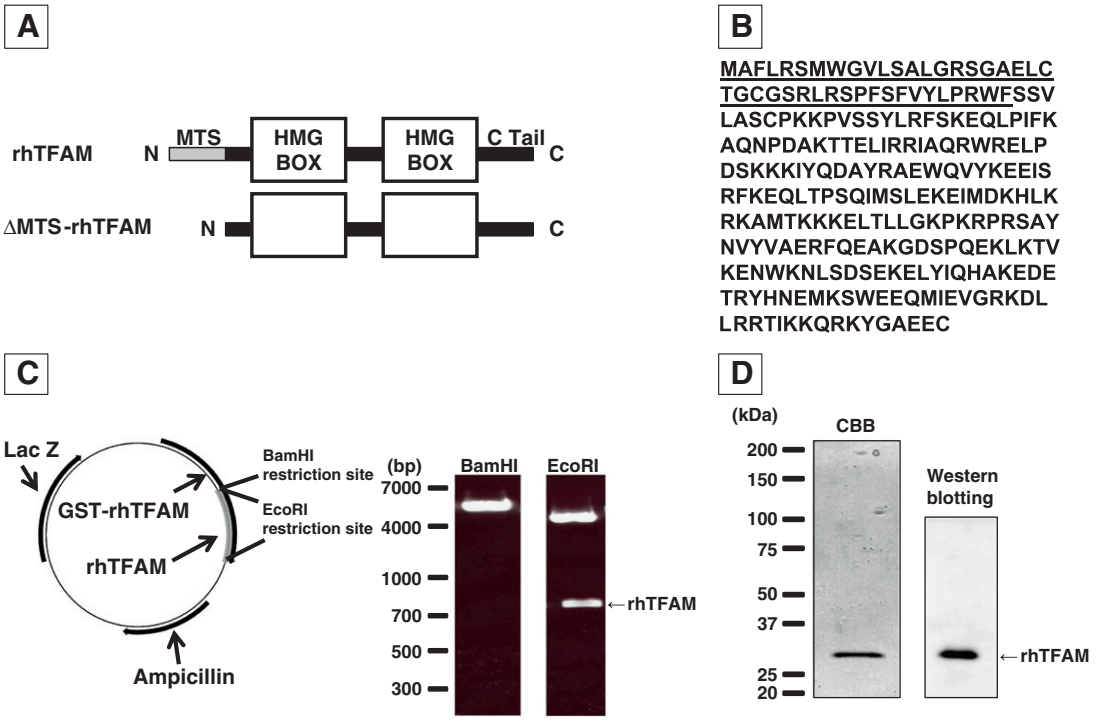

Fig. 1. Synthesis of recombinant human TFAM protein. (A and B) Schematic structure (A) and amino acid sequence (B) of human TFAM protein. Underlined part is MTS. (C) Schematic structure and electrophoresis of pGEX-6P-1-TFAM, the subcloned plasmid we constructed for synthesizing recombinant TFAM. TFAM sequence was inserted between the EcoRI restriction sites. The left lane shows linear whole plasmid (BamHI restriction), and the right lane shows electrophoretically separated pGEX-6P-1 and TFAM (EcoRI restriction). (D) SDS-PAGE analysis of the product. We confirmed the product as TFAM by CBB staining (left lane) and Western blotting using human TFAM -specific antibody (right lane).