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Transforming growth factor-β enhances connective tissue growth factor expression in L6 rat skeletal myotubes

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Abstract

Transforming growth factor (TGF)-β plays an important role in fibrosis of various organs and tissues. TGF-β1 stimulates fibroblastic cells to form extracellular matrix (ECM), and regulates all critical events in wound healing. Connective tissue growth factor (CTGF), a TGF-β-inducible molecule, has recently been reported to promote fibroblast proliferation, migration, adhesion and extracellular matrix formation, both in vivo and in vitro. In this study, we demonstrated that TGF-β1 enhances CTGF mRNA and protein levels in L6 rat skeletal muscle myotubes. TGF-β might, therefore, play a role in fibrosis of skeletal muscle by stimulating CTGF expression in the muscle tissue itself.

Keywords: TGF-β; CTGF; Myotube; Fibrosis

1. Introduction

Transforming growth factor (TGF)-β plays an important role in fibrosis of organs such as the liver, kidney, skin and lung. TGF-β1 stimulates fibroblastic cells to proliferate, migrate and form extracellular matrix (ECM) during the critical events of wound healing [1]. TGF-β stimulates fibroblastic cells to promote production of ECM and suppresses synthesis of matrix metalloproteinases in order to induce fibrosis of organs, and is known to play a crucial role in fibrosis of the liver [2]. Following liver injury resulting from a toxin or viral infection, hepatic stellate cells promote production of TGF-β, which causes overproduction of ECM.

In skeletal muscle, TGF-β1 was reported to be elevated in Duchenne/Becker muscular dystrophy [3,4], myotonic dystrophy [5], congenital muscular dystrophy [6], and inflammatory myopathies [7,8]. TGF-β1 thus appears to be involved in the pathogenesis of fibrosis in chronic muscular disorders.

In 1991, Bradham et al. first used the term 'Connective tissue growth factor (CTGF)' to describe a novel growth factor secreted by human endothelial cells in culture [9]. CTGF is a member of the ctgf/cyr61/nov (CCN) gene family and is expressed in various human tissues and organs, including the kidney, heart, placenta, lung, liver and pancreas. CTGF acts to promote fibroblast proliferation, migration, adhesion, and ECM formation [10,11]. CTGF is mainly induced by TGF-β in cells and tissues, although thrombin [12], VEGF [13] and dexamethasone [14] have also been known to stimulate CTGF induction. In vitro, TGF-β promotes CTGF expression in fibroblastic cells, endothelial cells, epithelial cells, vascular smooth muscle cells, and neuronal cells [10,11]. In skeletal muscle cells, however, TGF-β induction of CTGF expression has not been widely studied. In this study, we therefore examined whether TGF-β is able to enhance CTGF expression in skeletal muscle using the L6 rat skeletal myotubes.

2. Materials and methods

2.1. Materials

L6 rat skeletal myoblasts were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

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Recombinant human TGF-β1 was purchased from Sigma (St Louis, MO, USA). Anti-human CTGF-terminal peptide antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other materials and chemicals were obtained from commercial sources.

2.2. Cell culture

L6 rat skeletal muscle myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Subconfluent cells were harvested with trypsin/ethylenediaminetetraacetic acid (EDTA) and were seeded into 100-mm dishes (1.8×10^4 cells/cm^2). Differentiation of L6 myoblasts into myotubes was achieved by growing the cells to confluence and changing the serum supplementation from 10% FBS to 2% (differentiation medium). Myogenic differentiation to myotubes was confirmed both morphologically and biochemically, as described previously [15,16]. After 72 h of exposure to differentiation medium, cells were cultured in serum-free DMEM containing different concentrations of TGF-β1 (0, 0.1, 1.0, 5.0, or 10.0 ng/ml). The effect of 5.0 ng/ml TGF-β was further studied at 0, 3, 6, 12, 24, or 48 h.

2.3. Northern blot analysis

Total RNA was isolated with an RNA isolation kit (NucleoSpin RNA II; Machery-Nagel, Düren, Germany). Total RNA (10 μg/lane) was subjected to electrophoresis on 1% agarose gels containing formaldehyde and was then transferred to Nylon hybridization transfer membrane. The membrane was fixed with UV-light (FUNA-UV-LINKER; Funakoshi, Tokyo, Japan). Membranes were hybridized with cDNA probes using the Gene Images AlkPhosDirect labeling and detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). A 0.23-kb cDNA fragment contained within the open reading frame of CTGF was used as a probe. Membranes were finally visualized using the CDP-Star detection reagent for chemiluminescent detection of alkaline phosphatase (Amersham). Membranes were then exposed to Hyperfilm ECL (Amersham) for 1 h at room temperature.

2.4. Western blot analysis

L6 myotubes were lysed in radioimmunoprecipitation (RIPA) buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), complete protease inhibitor mixture, 1% Triton X-100, and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 12,000 *g* for 20 min at 4 °C. Protein quantification was performed using BCA protein assay reagent (Pierce, Rockford, IL, USA). Equal amounts of supernatant were subjected to 10% SDS-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred to polyvinylidene difluoide. Blots were blocked with Tris-buffered saline (TBS; 20 mM Tris–Hcl [pH 7.5] and 137 mM NaCl) containing 0.1% Tween 20 and 3% dried milk powder, and were then incubated with anti-CTGF antibody at 4 °C overnight. Primary antibody was used at a 1:500 dilution. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Labs, West Grove, PA, USA), and were finally visualized using an ECL kit (Amersham).

3. Results

3.1. Northern blot analysis

Fig. 1 shows the effects of TGF-β1 on the expression of CTGF mRNA in L6 myotubes. Enhancement of CTGF mRNA was observed from 3 to 48 h after stimulation with 5 ng/ml TGF-β1, with peak expression seen at 6 h, as shown in Fig. 1(a). Expression of CTGF mRNA increased in a dose-dependent manner within a range from 0.1 to 10 ng/ml TGF-β1, as shown in Fig. 1(b). Enhancement of CTGF mRNA expression primarily occurred at 5–10 ng/ml TGF-β1.

3.2. Western blot analysis

Fig. 2 shows the effects of TGF-β1 on the expression of CTGF protein in L6 myotubes. The time course of CTGF protein expression induced by TGF-β1 is shown in Fig. 2(a). Stimulation with 5 ng/ml TGF-β1 increased CTGF protein levels in L6 myotubes from 3 to 48 h after treatment, with peak expression seen at 12 h, as shown in Fig. 2(a).

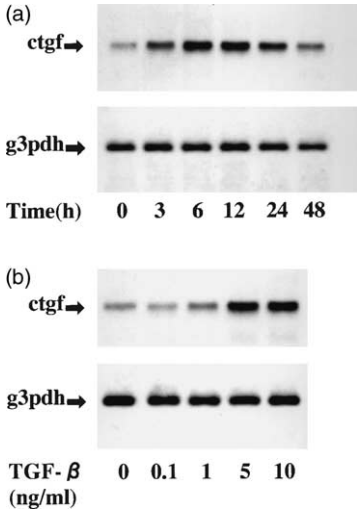

Fig. 1. (a) Time course of CTGF mRNA expression in L6 myotubes stimulated with 5 ng/ml TGF-β1. Enhancement of CTGF mRNA was observed from 3 to 48 h after stimulation. (b) Dose-dependent effects of TGF-β1 on CTGF mRNA in L6 myotubes. Enhancement of CTGF mRNA expression primarily occurred at 5–10 ng/ml TGF-β1. Equal loading was confirmed against G3PDH.