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Development of cell-based immunoassays to measure type I collagen in cultured fibroblasts

Brian Jones, Christine Bucks, Patrick Wilkinson, Michael Pratta, Francis Farrell, Pitchumani Sivakumar*

Immunology Research, Centocor Research & Development Inc., 145, King of Prussia Road, Radnor, PA 19087, USA

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ABSTRACT

Excessive deposition of type I collagen by activated fibroblasts is a hallmark of scarring and fibrotic pathologies. Quantitation of collagen I at the protein level is paramount to measure functionally relevant changes during pathological remodeling of the extracellular matrix. We describe two new cell-based assays to directly quantify the amount of collagen I incorporated into the extracellular matrix of primary human lung fibroblasts. Utilizing a monoclonal antibody specific to native human collagen I, we optimized conditions and parameters including incubation time, specificity and cell density to demonstrate dose-dependent induction of collagen I by transforming growth factor beta, as measured by in-cell enzyme linked immunosorbent assay. The results obtained by this assay were mimicked by an "In situ Quantitative Western Blot" on cultured cells using the same antibody. Results from these assays were comparable to those obtained with a commercial assay for collagen I N-propeptide, which is an index of collagen formation. These assays have been optimized for a 96-well format and provide a novel and useful approach for screening of anti-fibrotic agents in vitro. The assays described here also offer a significant improvement in throughput and specificity over conventional methods that primarily measure soluble collagen.

1. Introduction

Type I collagen (COL1) is the major fibrillar protein of the extracellular matrix (ECM) of connective tissues (Ricard-Blum and Ruggiero, 2005). Although the primary function of COL1 is to provide a structural scaffold to maintain tissue integrity (Nimni, 1983; Ottani et al., 2001), recent studies identified that the collagenous ECM could be dynamic rather than static (Kadler, 2004), and regulate multiple aspects of cell behavior including adhesion, migration and signal transduction (Heino, 2007; Vogel, 2001). Mutations in COL1 genes, and misregulated assembly and deposition of COL1 lead to several debilitating disorders of ECM metabolism, such as fibrosis (Biagini and Ballardini, 1989; Raghow, 1994), scarring (Shoshan, 1979; Zhang et al., 1995), osteogenesis imperfecta (Prockop et al., 1993), and Ehlers Danlos Syndrome (de Paepe, 1996).

To date, most collagen assay methods have measured changes in gene expression of collagen and/or transcriptional regulation of collagen through COL1 promoter-reporter assays (Rishikof et al., 2005; Yata et al., 2003). The biosynthetic pathway of COL1 is highly complex and involves several post- and co-translational modifications such as hydroxylation of proline and lysine residues, glycosylation of hydroxylysine residues and crosslinking of collagen monomers resulting in the deposition of collagen fiber bundles in the ECM (Davidson and Berg, 1981; Last et al., 1990; Myllyharju, 2003). Although steady state mRNA levels are a useful predictor of COL1 alterations, there is a significant lag between the transcription of COL1 and the production of functional COL1 protein, suggesting that measurement of gene expression may not correlate with protein production. Quantitation of COL1 at the protein level is paramount to measure functionally relevant changes during pathological remodeling of the ECM.

Conventionally, the colorimetric determination of hydroxyproline has been used as an index to measure COL1 protein (McAnulty et al., 1991). Additionally, in vitro biosynthesis of collagen can be quantitated by measuring the incorporation of tritiated proline into cells (Nacher et al., 1999). Yet, other studies have used a Sircol dye binding assay to quantitate soluble collagens in culture supernatants. However, all of these methods measure only soluble collagen. Moreover, these assays are time-consuming, offer less sensitivity and specificity and may require the use of radioactivity. Therefore, there is a need to develop alternate methods to quantify COL1 changes at the protein level.

Here, we describe two new cell-based assays to measure relative changes in COL1 in cultured fibroblasts. These assays were centered on the hypothesis that an antibody specific to native COL1

Abbreviations: ELISA, enzyme linked immunosorbent assay; NHLF, normal human lung fibroblasts; NHDF, normal human dermal fibroblasts; COL1, collagen type I; TGFβ, transforming growth factor beta; ECM, extracellular matrix; ICW, in-cell-Western.

* Corresponding author. Tel.: +1 610 240 5735; fax: +1 610 889 4525.
E-mail address: psivakum@its.jnj.com (P. Sivakumar).

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will bind to COL1 in the ECM of cultured cells, and such binding can be quantitated by an immunohistochemical method. We identified a commercially available antibody that bound to ECM-associated COL1 and specifically detected increased COL1 production induced by TGFβ, through an in-cell ELISA method. Specificity and selectivity of this antibody were confirmed through solid phase binding assays and immunohistochemistry. Secondly, a quantitative "in-cell-Western" detection method was utilized to confirm the ability of this antibody to measure the induction of COL1 protein with TGFβ. These cell-based quantitative assays, validated and optimized for a semi high throughput 96-well plate format, provide a novel method to screen and identify agents that modulate COL1 assembly in vitro.

2. Materials and methods

2.1. Proteins and antibodies

Monoclonal (mab6308) and polyclonal (pab292 and pab34710) anti-collagen I antibodies and mouse IgG1 isotype control were obtained from Abcam (Cambridge, MA). Rabbit IgG isotype control was obtained from Jackson Immunoresearch (West Grove, PA). Fibronectin and rat tail type I collagen were obtained from BD Biosciences (Bedford, MA). Human collagen types I, III and IV and porcine gelatin were purchased from Sigma Aldrich (St. Louis, MO). Collagen I was denatured by heating native collagen for 3 min, at 65 °C. TGFβ and anti-TGFβ antibody were purchased from R&D Systems (Minneapolis, MN). All cytokines were frozen at −20 °C at a working stock concentration of 10 μg/ml; fresh aliquots were used for each experiment.

2.2. Fibroblast cultures

Normal human lung fibroblasts (NHLFs) and normal human dermal fibroblasts (NHDFs) were obtained from Lonza (CC-2512-NHLF, CC-2511-NHDFs) (Walkersville, MD). Cells were maintained in Fibroblast Growth Media (FGM-2 Bullet kit) (Lonza) per manufacturer's instructions, and were used between passages P3 and P6.

2.3. Cell-based ELISA protocol

NHLF and NHDF cells were cultured in inner wells of a 96-well plate, in either FGM-2 media or Dulbecco's Modified Eagle's Medium containing 5% FBS and 100 units/ml penicillin and 100 μg/ml streptomycin (5% DMEM). Cells were stimulated with TGFβ for 24-h, and then re-stimulated with TGFβ in fresh media containing 20 μg/ml ascorbic acid for an additional 24 h to promote collagen synthesis (Booth and Uitto, 1981). After stimulation, cells were washed three times in phosphate buffered saline (PBS), fixed in 95% ethanol for 10 min at room temperature, washed twice in PBS, and blocked in PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. To keep the cell monolayer intact, plates were inverted and gently tapped dry, followed by manual washing with a multi-channel pipette. Primary antibodies were added in the range 1:500 to 1:2000 (in blocking buffer) and incubated for 2 h at room temperature. Cells were then washed four times with PBS/0.05% Tween20. Secondary antibody (peroxidase conjugated anti-mouse IgG, 1:5000 in blocking buffer) (Jackson ImmunoResearch) was added and incubated for 1 h at room temperature. The plate was washed as before, developed for 20 min, in the dark, with TMB one solution (BD Biosciences). The assay was stopped by addition of 1N sulfuric acid, and the optical density (OD) was read at 450 nm using a Spectramax Plus plate reader.

2.4. In-cell-Western assay

NHLF cells were cultured and stimulated as described for the ELISA protocol. After stimulation, cells were washed three times in PBS and fixed in 95% ethanol for 20 min at room temperature. All washing and staining steps were performed using gentle agitation on plate rotator. Cells were permeabilized by washing 5 times (5 min each time) with PBS/0.1% Triton X-100. To keep the cell monolayer intact, plates were inverted and gently tapped dry, followed by manual washing with multi-channel pipette. Plates were blocked with 100 μl per well Odyssey Blocking Buffer (LI-COR biosciences, Lincoln, Nebraska), for 1.5 h at room temperature or overnight at 4 °C. Block buffer was removed, and primary antibody (mab6308) or IgG1 isotype control (used in the range 25–2.5 μg/ml, in blocking buffer) was added for 2.5 h at room temperature. Plates were washed 5-times (5-min per wash) with PBS-0.1% Tween20. Secondary antibody (goat anti-mouse-IR 800, LI-COR biosciences) was added for 1 h at room temperature. To discriminate between live and dead cells, DRAQ5 (LI-COR) was added during the secondary incubation period. The plate was washed as before, and then 100 μl PBS was added to each well prior to scanning the plate. Signal intensity of stained cells was acquired using the Odyssey Imager and Odyssey 2.1 software.

2.5. Immunofluorescence microscopy

NHLFs were seeded at 20,000 cells per well on 4-well chamber slides and allowed to attach overnight. Cells were stimulated for 24-h with TGFβ and then re-stimulated with TGFβ in the presence of 20 μg/ml ascorbic acid for an additional 24 h. After stimulation, cells were washed three times in PBS, fixed in 95% ethanol, and blocked for 2 h in PBS containing 1% donkey serum and 0.05% sodium azide. Cells were incubated with mab6308 (primary antibody, 1:1000 in blocking buffer) for 2 h at room temperature, Cells were then washed three times in PBS, and incubated for 1 h at room temperature with Cy3 conjugated donkey anti-mouse IgG–antibody (1:200 in blocking buffer) (Jackson ImmunoResearch, West Grove, PA). Washing was repeated as before, the chamber wells were removed, and cells were mounted in anti-fade media (9:1 glycerol:PBS, with 1% n-propyl gallate). Images were acquired with the Nikon Eclipse E800 Upright Microscope at 20× magnification.

2.6. Solid phase binding assays

96-Well plates were coated with antigens (collagens, gelatin or fibronectin) serially diluted in PBS, for 2 h at 37 °C. Plates were washed twice in PBS and blocked in PBS/0.5% BSA for 2 h at room temperature with gentle shaking. Blocking buffer was removed and plates were incubated with primary antibody (mab6308, diluted 1:2000 in blocking buffer) for 2 h at room temperature. Plates were washed four times with PBS/0.05% tween-20 and incubated with secondary antibody (peroxidase conjugated goat anti-mouse IgG, 1:5000 in blocking buffer) for 1 h at room temperature. Plates were washed four times as before, and TMB one solution was added and incubated for 15 min in the dark. The reaction was stopped with 1N sulfuric acid and plates were read at 450 nm in a Spectramax plus plate reader.

2.7. Radioimmunoassay for procollagen I N-propeptide

P1NP levels in NHLF supernatants were measured using a commercial Radio Immuno Assay Kit (Immunodiagnostic Systems, Scottsdale, AZ), per the manufacturer's protocol.