Biochimica et Biophysica Acta 1840 (2014) 645–655

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Discovery of novel differentiation markers in the early stage of chondrogenesis by glycoform-focused reverse proteomics and genomics

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ARTICLE INFO

Article history:
Received 18 July 2013
Received in revised form 20 September 2013
Accepted 16 October 2013
Available online 24 October 2013

Keywords:
Osteoarthritis
Chondrogenic differentiation
Glycomics
Glycoform-focused reverse proteomics and genomics
Mass spectrometry

ABSTRACT

Background: Osteoarthritis (OA) is one of the most common chronic diseases among adults, especially the elderly, which is characterized by destruction of the articular cartilage. Despite affecting more than 100 million individuals all over the world, therapy is currently limited to treating pain, which is a principal symptom of OA. New approaches to the treatment of OA that induce regeneration and repair of cartilage are strongly needed.
Methods: To discover potent markers for chondrogenic differentiation, glycoform-focused reverse proteomics and genomics were performed on the basis of glycoblotting-based comprehensive approach.
Results: Expression levels of high-mannose type *N*-glycans were up-regulated significantly at the late stage of differentiation of the mouse chondroprogenitor cells. Among 246 glycoproteins carrying this glycotype identified by ConA affinity chromatography and LC/MS, it was demonstrated that 52% are classified as cell surface glycoproteins. Gene expression levels indicated that mRNAs for 15 glycoproteins increased distinctly in the earlier stages during differentiation compared with Type II collagen. The feasibility of mouse chondrocyte markers in human chondrogenesis model was demonstrated by testing gene expression levels of these 15 glycoproteins during differentiation in human mesenchymal stem cells.
Conclusion: The results showed clearly an evidence of up-regulation of 5 genes, ectonucleotide pyrophosphatase/phosphodiesterase family member 1, collagen alpha-1(III) chain, collagen alpha-1(XI) chain, aquaporin-1, and netrin receptor UNC5B, in the early stages of differentiation.
General significance: These cell surface 5 glycoproteins become highly sensitive differentiation markers of human chondrocytes that contribute to regenerative therapies, and development of novel therapeutic reagents.

1. Introduction

Human osteoarthritis (OA), the most common form of joint disease, is characterized by degeneration of the articular cartilage [1–3]. Currently, OA is recognized as a major disease that causes disability in the elderly; the prevalence of this disease is expected to increase dramatically over the next 20 years with population aging. Currently available pharmacological therapies are mainly used for pain relief, and include acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxgenase-2 inhibitors, glucocorticoids, and opioids. Other palliative drugs affecting various targets such as catabolic enzymes or cytokine-activated signaling cascades are currently in development, but a curative treatment to induce regeneration and repair of cartilage is still lacking.

Mesenchymal stem cells (MSCs) in various adult tissues, such as bone marrow, adipose tissue, and synovial fluid, have multipotency and self-renewal capabilities, including induction to undergo chondrogenic differentiation [4–8]. Especially, cells from the synovial membrane have the ability to differentiate into chondrocytes [6], indicating that they potentially have a physiological role in the repair and degeneration of the articular cartilage. Therefore, it is expected that medications induce proliferation and differentiation of MSCs *in vitro* and *in vivo* will contribute to repair and regeneration of damaged cartilage. Advent of new sensitive markers that contribute to development of novel therapeutic reagents to induce differentiation of MSCs are strongly required for improving quality of life of patients suffering OA.

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0304-4165/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbagen.2013.10.027

Protein glycosylation is one of the most important posttranslational modifications in cell surface proteins and extracellular matrix proteins, which is considered to have a variety of biological functions, including enhancement of protein stability, controlling cell-to-cell communication and adhesion [9]. Recently, it has been well documented that glycan modifications of proteins greatly contribute to the pathogenesis of many diseases [10,11]. One of the characteristics of cartilage is that chondrocytes exist in the extracellular matrix. It is also well known that glycoproteins are abundant on the cell surface and in cartilage extracellular matrix. Recently, we reported that some high-mannose type *N*-glycan levels are decreased significantly both in human OA cartilage and in degraded mouse cartilage [12], strongly suggesting an association between *N*-glycans of cell surface glycoproteins and the pathogenesis of OA. Pabst et al. [13] also demonstrated that the levels of glycophenotype of primary human chondrocytes were altered by inflammatory cytokines. These means clearly that proteins with such characteristic *N*-glycoforms might become novel chondrogenic cell markers. However, glycoproteins bearing high-mannose type *N*-glycans in cartilage and their functional roles in the process of cartilage degradation have not yet been uncovered. Glycoproteomics is a new potential strategy among focused proteomics approaches for the characterization of plasma membrane proteins [14]. This approach has facilitated comprehensive identification of cell surface glycoproteins with low abundance and insolubility by hydrazide chemistry [15,16] and lectin affinity chromatography [17]. However, determination of the total glycan structures from whole cellular glycoproteins had long remained to be a challenging and extremely difficult task due to the lack of general platforms for high throughput glycomics [18]. We have developed a standardized protocol of glycomics based on the simple chemical enrichment method, namely glycoblotting method [19] that allows for rapid and large-scale enrichment analysis of human serum glycans [20–25]. We also recently demonstrated the versatility of this glycoblotting protocol for the monitoring and characterization of the processes of dynamic cellular differentiation of mouse embryonic carcinoma cells and mouse embryonic stem cells into cardiomyocytes or neural cells [26]. In the present study, we discovered novel chondrogenic differentiation markers through high throughput glycoproteomics uncovering proteins displaying characteristic glycan structures, notably a combination of glycoblotting-based quantitative cellular *N*-glycomics and glycoform-focused reverse proteomics/genomics.

2. Experimental section

2.1. Cell culture and chondrogenic differentiation

ATDC5 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium (Sigma, St. Louis, MO) containing 5% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Life Technologies), 100 units/mL penicillin (Life Technologies), and 100 μg/mL streptomycin (Life Technologies). The cells were seeded into 6-well tissue culture plates at a density of 1.0×10^5 cells and cultured in the above medium supplemented with 10 μg/mL transferrin (Roche Diagnostics Co., Basel, Switzerland). For induction of chondrogenesis, 10 μg/mL bovine insulin (Sigma) was reacted to the sub-confluent cells. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every other day.

Human MSCs were obtained from Lonza Walkersville, Inc. (Walkersville, MD). Cell culture and chondrogenic differentiation of human MSCs were performed according to the manufacturer's protocols. Human MSCs were expanded in monolayer culture by passaging twice in MSC basal medium (MSCBM) with MSC growth supplement (MCGS), L-glutamine, and GA-1000. For induction of chondrogenic differentiation, human MSCs were suspended in chondrogenic basal medium with ITS supplement (containing insulin, transferrin, and selenite), dexamethasone, ascorbate, sodium pyruvate, proline, GA-1000, L-glutamine, and 10 ng/mL TGF-β3. The seeding cell number for human MSCs was 2.5×10^5 cells/15 mL polypropylene culture tube (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and the cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every other day.

2.2. Alcian blue staining

Accumulation of glycosaminoglycans associated with chondrocyte differentiation was assessed by staining with Alcian blue. Differentiating ATDC5 cells were rinsed with PBS (Life Technologies) and fixed with 95% methanol (Wako Pure Chemical, Osaka, Japan) for 20 min. They were then stained with 0.1% Alcian blue 8GX (Merck Chemicals, Darmstadt, Germany) in 0.1 M HCl (Nacalai Tesque, Kyoto, Japan) overnight.

Differentiating human MSCs were rinsed with PBS (Life Technologies) and fixed with 4% formaldehyde (Wako Pure Chemical). The formaldehyde-fixed cells were embedded in paraffin and sectioned. The paraffin-embedded sections were rinsed with *p*-xylene (Wako Pure Chemical), ethanol (Wako Pure Chemical), and 3% acetic acid (Wako Pure Chemical) and stained with 10 mg/mL Alcian blue 8GX in 3% acetic acid for 30 min.

2.3. Glycoblotting-based quantitative cellular N-glycomics

Release of total *N*-glycans was carried out directly using whole-cell lysates as follows. After inducing differentiation, ATDC5 cells were scraped in PBS containing 10 mM EDTA (Wako Pure Chemical) at various time points. The scraped cells were washed three times with PBS containing 10 mM EDTA and lysed by incubation with 1% Triton X-100 for 1 h on ice. The lysates were centrifuged at 15,000 rpm for 10 min at 4 °C, and the obtained supernatant was added to cold acetone (1:4) to precipitate proteins. The precipitates were collected by centrifugation at 12,000 rpm for 15 min at 4 °C followed by serial washing with acetonitrile. The resulting precipitates were dissolved in 50 μL of 100 mM ammonium bicarbonate containing 0.04% of 1-propanesulfonic acid, 2-hydroxyl-3-myristamido followed by incubation at 60 °C for 10 min. The solubilized proteinaceous materials were reduced by 10 mM DTT at 60 °C for 30 min followed by alkylation with 20 mM iodoacetamide in the dark at room temperature for 30 min. The mixture was then treated with 800 units of trypsin (Sigma-Aldrich, Cat. No. T0303, St. Louis, MO) at 37 °C overnight followed by heat inactivation of the enzyme at 90 °C for 10 min. After cooling to room temperature, *N*-glycans of glycopeptides were released from trypsin-digested samples by incubation with 2.5 units of peptide-*N*-glycosidase F (Roche Applied Science, Basel, Switzerland) at 37 °C overnight. The sample mixture was then dried by vacuum centrifugation and stored at −20 °C until use.

Glycoblotting of *N*-glycans was performed according to the procedure described previously [20]. BlotGlyco H beads (50 μL) (10 mg/mL suspension; Sumitomo Bakelite Co., Ltd.) were aliquoted onto the wells of a MultiScreen Solvinert filter plate (Millipore, Billerica, MA). Peptide-*N*-glycosidase F-digested samples were added with an internal standard (A2 amide glycan, $Hex_5(HexNAc)_4(NeuAcAmide)_2$, prepared from egg yolks) to the wells followed by addition of 360 μL of 2% acetic acid in acetonitrile. The plates were incubated at 80 °C for 100 min to specifically capture total glycans in sample mixtures onto beads via stable hydrazone bonds. The plate was washed with 200 μL of 2 M guanidine HCl in ammonium bicarbonate followed by washing with the same volume of water and 1% triethylamine in methanol (MeOH). Each washing step was performed twice. Unreacted hydrazide functional groups on beads were capped by incubation with 10% acetic anhydride in MeOH for 30 min at room temperature. Then, the solvent was removed under vacuum, and the beads were serially washed with 2 × 200 μL of 10 mM HCl, MeOH, and dioxane,