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Giantin is required for coordinated production of aggrecan, link protein and type XI collagen during chondrogenesis

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ABSTRACT

Extracellular matrix (ECM) constitutes a proper micro-environment for cell proliferation, migration and differentiation, as well as playing pivotal roles in developmental processes including endochondral ossification. Cartilage ECM is mainly composed of fibrous proteins, including collagen, proteoglycan, and hyaluronan. Because almost all ECM components are transported by intracellular vesicular transport systems, molecules that mediate vesicle transport are also important for endochondral ossification. Giantin, encoded by the *Golgb1* gene, is a tethering factor for coatomer 1 (COPI) vesicles and functions in the *cis-medial* Golgi compartments. An insertion mutation in the *Golgb1* gene, resulting in a lack of giantin protein expression, has been detected in *ocd*/*ocd* rats that exhibit a pleiotropic phenotype including osteochondrodysplasia. To reveal the function of giantin in chondrogenesis, the present study assessed the effects of loss of giantin expression on cartilage ECM and Golgi morphology. Giantin was expressed in normal, but not in *ocd*/*ocd*, chondrocytes in the epiphyseal areas of embryonic femurs, whereas GM130 was expressed in both normal and *ocd*/*ocd* chondrocytes. The staining intensities of safranin O and azan (aniline blue) were reduced and enhanced, respectively, in epiphyseal cartilage of *ocd*/*ocd* femurs. Immunostaining showed that levels of type II collagen and fibronectin were comparable in normal and *ocd*/*ocd* cartilage. Levels of type XI collagen were higher, while levels of aggrecan, link protein and hyaluronan were lower, in *ocd*/*ocd* than in normal cartilage, although semi-quantitative RT-PCR showed similar levels of type XI collagen, aggrecan and link protein mRNAs in normal and *ocd*/*ocd* cartilage. Isolated chondrocytes of *ocd*/*ocd* and normal rats showed similar immunostaining patterns for *cis*-, medial-, and *trans*-Golgi marker proteins, whereas monolayers of *ocd*/*ocd* chondrocytes showed reduced levels of aggrecan and link protein and increased level of type XI collagen in spite of similar transcripts levels. These findings suggest that giantin plays a pivotal role in coordinated production of aggrecan, link protein and type XI collagen in chondrocytes, and that loss of giantin causes osteochondrodysplasia with disturbance of these ECM components.

1. Introduction

Cartilage is primarily composed of chondrocytes and extracellular matrix (ECM) secreted by chondrocytes. Cartilage ECM consists of collagen, non-collagenous glycoprotein, proteoglycan, and hyaluronan. Collagens provide tensile strength, whereas proteoglycan and hyaluronan provide compressive resistance by expanding and contracting [1]. Because cartilage ECM also provides the microenvironment required for proper chondrocyte proliferation, arrangement, and differentiation, loss of or structural changes in cartilage ECM molecules could result in defects in chondrogenesis [2]. Aggrecan, a major proteoglycan in cartilage ECM, is composed of core protein and covalently attached glycosaminoglycans. Aggrecan binds to hyaluronan via link protein, forming hydrated gel-like structures [3,4]. Mutations in aggrecan and link protein genes can result in chondrodysplasia and dwarfism and

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may even be lethal [5–9].

Almost all ECM molecules are synthesized at the rough endoplasmic reticulum (ER) and transported by intracellular vesicular transport. Transport vesicles coated with coat proteins, including coatomers 1 (COPI) and 2 (COPII) and clathrin, are pinched off from the donor compartment and directed to the acceptor compartment. COPII-coated vesicles mediate transport from the ER to the ER-Golgi intermediate compartment (ERGIC), and COPI-coated vesicles mediate transport from the ERGIC to the Golgi apparatus, from the Golgi to the ER, and within the Golgi apparatus. Clathrin-coated vesicles are involved in transport between the *trans*-Golgi network (TGN) and the plasma membrane [10]. Before fusion, transport vesicles are tethered to membranes of the acceptor compartment by tethering factors, including Golgin, COG, TRAPPI TRAPPII and GRASP, with tethering factors belonging to the same family forming complexes to tether transport vesicles [11–13].

Osteochondrodysplasia (OCD) in rats is caused by a functional null mutation in the gene encoding giantin, and is transmitted in an autosomal recessive manner [14,15]. Affected rats (*ocd*/*ocd*) exhibit dwarfism, osteochondrodysplasia, cleft palate, dome-shaped skull, systemic edema, and nervous malfunction and die soon after birth [16–19]. Embryos of *ocd*/*ocd* rats show delayed endochondral ossification, decreased cartilage ECM, reduced chondrocyte proliferation, and progressive disarrangement of chondrocyte column [15,20,21]. Electron microscopic analysis indicated that chondrocytes of *ocd*/*ocd* rats were swollen and contained expanded vesicles [22]. Because giantin is involved in COPI vesicle transport into the *cis-medial* compartment of the Golgi apparatus [23,24], some characteristics of *ocd*/*ocd* cartilage and chondrocytes may reflect the impaired intracellular vesicular transport of ECM molecules. This study therefore examined the effects of giantin loss on cartilage ECM production and Golgi morphology.

2. Materials and methods

2.1. Animals

OCD rats were bred and genotyped as described [18]. Timed pregnant heterozygous (*ocd*/+) female rats mated with heterozygous males were sacrificed by CO_2 gas or pentobarbital sodium overdose, and their embryos were dissected. All animal experiments and care were performed in accordance with the Guidelines of the Animal Care and Use Committee of Nippon Veterinary and Life Science University.

2.2. Histochemical and immunohistological analyses

For immunohistological determination of giantin and GM130 expression, the hind limbs of normal (+/+, +/*ocd*) and affected (*ocd*/*ocd*) embryos at embryonic day (E) 18.5 were dissected, mounted in OCT compound (Sakura Finetek, Tokyo, Japan), and frozen in liquid nitrogen. Fresh frozen sections (8 μm) were dried in air, fixed in ice-cold acetone for 10 min at −20 °C, and incubated with primary antibodies against giantin (1:300, ab37266, Abcam, Cambridge, MA) and GM130 (1:300, EP892Y, Abcam) overnight at 4 °C. After thorough washing, the sections were incubated for 1 h at room temperature (RT) with Alexa Fluor 488 and 568 conjugated secondary antibodies (Life Technologies, Carlsbad, CA) and mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies).

For ECM staining, the hind limbs of embryos were fixed in 4% paraformaldehyde/PBS at 4 °C, embedded in paraffin and cut into 5 μm sections. The sections were dried, deparaffinized and used for histological and immunohistological analyses. Staining with azan and safranin O was according to standard protocols. For detection of aggrecan, link protein, types II and XI collagens, and fibronectin, the sections were incubated with 25 mg/ml hyaluronidase for 30 min at RT, followed by incubation with primary antibody for 3 h at RT or overnight at 4 °C. ECM components were detected using antibodies against aggrecan core protein (1:1000, clone 1-C-6, Millipore, Temecula, CA), link protein (1:1000, clone 8-A-4, Millipore), fibronectin (1:1000, Chemicon International, Inc., Temecula, CA), type II collagen (1:1000, AB2036, Millipore; LB-1297, LSL, Tokyo, Japan), and type XI collagen (1:1000, LB-1110, LSL). After thorough washing, the sections were incubated with Alexa Fluor 488 conjugated secondary antibodies for 1 h at RT and mounted with ProLong Gold Antifade Reagent with DAPI. For hyaluronan detection, sections were incubated with chondroitinase ABC (1 U/ml) for 37 °C and stained as described previously [25]. All images were obtained by optical/fluorescence microscopy (Biozero; Keyence, Osaka, Japan).

2.3. Semi-quantitative RT-PCR

Total RNA was extracted from the femurs of E18.5 +/+ and *ocd*/*ocd* embryos and isolated chondrocytes using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Contaminating DNA was removed by digestion with DNase I (TaKaRa, Shiga, Japan), and cDNA was synthesized from 5 μg aliquots of each RNA using ReverTra Ace and random primers according to the manufacturer's instructions (Toyobo, Osaka, Japan). Sequences corresponding to *Col11a1* (encoding type XI collagen α chain), *Acan* (encoding aggrecan), and *Hapln1* (encoding hyaluronan and proteoglycan link protein 1) mRNAs were detected by semi-quantitative RT-PCR, with each normalized relative to expression of *Gapdh* mRNA, encoding glyceraldehyde-3-phosphate dehydrogenase, in the same sample.

2.4. Chondrocyte isolation, culture, and immunofluorescence of Golgi-associated proteins and ECM

Primary chondrocytes were isolated from knee joints and rib cages of normal and affected embryos at E21.5 as described [26]. Chondrocytes (0.5×10^5) were seeded onto 12 mm cover slips, cultured for two days for staining of Golgi-associated proteins after seeding or cultured for seven days for staining of ECMs after confluent. Media were changed each other day. Cells were fixed at RT for 10 min with PTEMF buffer (0.1% Triton X-100, 100 mM PIPES pH 6.8, 1 mM $MgCl_2$, 10 mM EGTA and 4% formalin) for Golgi-associated proteins or for 20 min with 4% PFA for ECMs. Golgi-associated proteins were detected as described [27]. Other primary antibodies were directed against ECM components, as above, as well as against giantin (1:200), GM130 (1:400), mannosidase II (1:400, 53FC3), and golgin 97 (1:200, sc-73619, Santa Cruz Biotechnology, Santa Cruz, CA). The cells were subsequently incubated with Alexa Fluor 488 and 568 conjugated secondary antibodies (1:2000, Life Technologies) and imaged by fluorescence microscopy. Semi-quantitative RT-PCR analysis for *Acan, Hapln1* and *Col11a1* was also performed in the primary culture of isolated chondrocytes as described above.

2.5. BrdU incorporation and TUNEL assays

BrdU positive cells were detected as described [28], with slight modifications. TUNEL assays were performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostic, Tokyo, Japan), according to the manufacturer's instructions.

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