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Partially acetylated chitooligosaccharides bind to YKL-40 and stimulate growth of human osteoarthritic chondrocytes

Jon M. Einarsson [a,*], Sven Bahrke [b], Bjarni Thor Sigurdsson [c], Chuen-How Ng [a], Petur Henry Petersen [c], Olafur E. Sigurjonsson [d,f], Halldor Jonsson Jr. [e], Johannes Gislason [a], Finnbogi R. Thormodsson [c,g], Martin G. Peter [b]

[a] Genis hf, KÍM Medical Park, Vatnagarðar 18, IS-104 Reykjavik, Iceland
[b] Universität Potsdam, Institut für Chemie, Karl-Liebknecht-Str. 24-25, D-14476 Potsdam, Germany
[c] University of Iceland, Faculty of Medicine, Vatnsmyrarvegur 16, IS-101 Reykjavik, Iceland
[d] Landspitali University Hospital, The Blood Bank, Snorrabraut 60, 105 Reykjavik, Iceland
[e] Landspitali University Hospital, Orthopaedic Department, Fossvogur, 108 Reykjavik, Iceland
[f] Reykjavik University, School of Science and Engineering, Menntavegur, 101 Reykjavík, Iceland
[g] ValaMed ehf, KÍM Medical Park, Vatnagarðar 18, IS-104 Reykjavik, Iceland

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ABSTRACT

Recent evidences indicate that cellular kinase signaling cascades are triggered by oligomers of N-acetylglucosamine (ChOS) and that condrocytes of human osteoarthritic cartilage secrete the inflammation associated chitolectin YKL-40, prompting us to study the binding affinity of partially acetylated ChOS to YKL-40 and their effect on primary chondrocytes in culture. Extensive chitinase digestion and filtration of partially deacetylated chitin yielded a mixture of ChOS (Oligomin™) and further ultrafiltration produced T-ChOS™, with substantially smaller fraction of the smallest sugars. YKL-40 binding affinity was determined for the different sized homologues, revealing micromolar affinities of the larger homologues to YKL-40. The response of osteoarthritic chondrocytes to Oligomin™ and T-ChOS™ was determined, revealing 2- to 3-fold increases in cell number. About 500 μg/ml was needed for Oligomin™ and around five times lower concentration for T-ChOS™, higher concentrations abolished this effect for both products. Addition of chitotriose inhibited cellular responses mediated by larger oligosaccharides. These results, and the fact that the partially acetylated T-ChOS™ homologues should resist hydrolysis, point towards a new therapeutic concept for treating inflammatory joint diseases.

1. Introduction

Rheumatic diseases affect a large part of the human population, especially the elderly. Several forms of rheumatism manifest themselves as acute inflammatory arthritis or degenerative arthrosis that is caused by destruction of articular tissue, with no cure available. The search for biomaterials that can restore damaged tissue has demonstrated that chitosan promotes chondrocyte proliferation in cell culture [1,2]. Some 25 years ago chitohexaose and hexa-*N*-acetylchitohexaose was reported to inhibit the growth of sarcoma in mice [3]. Consequently, number of other biological activities has been observed in vertebrates [2,4,5], microorganisms [6,7], and plants [8]. Recently, it has been shown that binding of chitin or chitooligosaccharides induces phosphorylation of the chitin receptor CERK1 in the plant *Arabidopsis thaliana*, triggering downstream signalling by protein phosphorylation cascades [9].

Following our observation that mixtures of partially acetylated chitooligosaccharides (ChOS) can alleviate the symptoms of inflammatory rheumatoid joint disorders more efficiently than glucosamine (unpublished results), it was of interest to further investigate the molecular mechanism involved. Osteoarthritic chondrocytes secrete large amounts of the chitolectin or "chitinase-like" glycoprotein YKL-40 (also known as HC-gp39), an enzymatically inactive member of family 18 chitinases [10–13], that binds to heparin and to collagen types I, II, and III [14]. Besides osteoarthritic chondrocytes, YKL-40 is also secreted by human synovial cells, osteoblasts, osteocytes, macrophages and neutrophils, and is considered a marker of inflammation [15–20], including osteoarthritis [21]. YKL-40 is a growth factor, inducing cell proliferation through activation of protein kinase mediated signalling pathways [22], and is involved in stimulation of chondrocyte growth and synthesis of extracellular matrix proteoglycans [23,24]. However, YKL-

* Corresponding author.
E-mail address: jon@genis.is (J.M. Einarsson).

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40 can also be an antigen resulting in autoimmune destruction of cartilage tissue [15]. Studies of the binding of fully acetylated chitin fragments to YKL-40 and protein crystallography of protein–ligand complexes [12,25,26], revealed high affinity binding that is associated with changes of the conformation of the protein [12], suggesting that ChOS could be involved in early steps of cellular signaling. Here we show that partially acetylated ChOS can bind with high affinity to YKL-40 and stimulate growth of chondrocytes in culture.

2. Materials and methods

2.1. Materials

N,N′,N″-Triacetylchitotriose ($\mathbf{A}_3$) was from IsoSep AB (Sweden). Chitohexaose ($\mathbf{D}_6$) and hexa-*N*-acetylchitohexaose ($\mathbf{A}_6$) came from Seikagaku (Tokyo, Japan). Hexasaccharides, $\mathbf{D}_2\mathbf{A}_4$ and $\mathbf{D}_3\mathbf{A}_3$, and the undecamer $\mathbf{D}_5\mathbf{A}_6$ were isolated by SEC (Biogel P4) and HP-CEC from a sample of Oligomin™. The isomer composition and sugar sequences of $\mathbf{D}_3\mathbf{A}_3$ and $\mathbf{D}_2\mathbf{A}_4$ were determined by MALDI-TOF MS^n (for details [27,28]). YKL-40 was purchased from Quidel (San Diego, CA, USA). All other chemicals were of highest purity as purchased from various suppliers.

SigmaStat and SigmaPlot were used for statistical and graphical analysis, applying ANOVA and *t*-tests.

2.2. Preparation of chitooligosaccharides

Q2 Chitin flakes (Primex Iceland), 2.5 kg, were deacetylated to about 50% DD in 50% aqueous NaOH (w/w). The chitosan slurry was washed with water and transferred to a 200 L blender, 150 L of water added, and the pH adjusted to 3.8 using 30% HCl. Chitinase from *Penicillium* species was added and extensive hydrolysis achieved by incubation for 22 h at 25 °C with stirring at 50 rpm. The chitinase concentration for maximal hydrolysis rate was previously optimised by monitoring the rate of the viscosity decline in 1.0% chitosan–HCl, pH 3.8 at 25 °C (Brookfield viscometer). The chitooligosaccharides were ultra-filtrated through a Helicon SS50 (PTGC, 10 kDa cut-off) spiral-wound membrane (Millipore, USA) using tangential flow in a Millipore PUF-200-FG pilot module. The resulting filtrate was desalted by nano-diafiltration through thin-film membranes, type DK4040F, 150–300 Da cut-off (Osmonics, Germany), using a semi-automatic pilot unit Type R apparatus (GEA Filtration, Germany). The retentate was subjected to spray-drying, using a rotary atomizing spray-drying unit (Nero, Denmark) with inlet and outlet air temperatures of 190 °C and 80 °C respectively. The yield was 2.08 kg of ChOS, termed Oligomin™.

In a different protocol a 1 kDa cut-off Helicon SS50 membrane in a Millipore PUF-200-FG pilot module was used in the second ultra-filtration step, greatly reducing oligosaccharides of $DP \leqslant 4$. During ultrafiltration, the volume of the retentate was kept constant by addition of water, until oligosaccharides of DP 1–3 were less than 10% of the total composition, as judged by HPLC. The spray-dried product (1.1 kg) was named Therapeutic Chitooligosaccharides (T-ChOS™).

Endotoxin was determined by the PyroGene Recombinant Factor C Endotoxin Detection System (Lonza, USA) according to the manufacturer's instructions and confirmed by Lonza (Belgium).

2.3 Characterization of chitooligosaccharides

High pressure size exclusion chromatography (HP-SEC) was performed with a Beckman Gold system and TSK-oligo column (TosoHaas, Japan). The eluent was 5 mM ammonium hydroxide, pH 10.0 at a flow rate of 0.5 ml/min. UV detector: 205 nm. Twenty microliters of a 10 mg/ml solution of oligosaccharide mixtures were injected. Beckman Gold analysis software was used for peak analysis.

2.4. Determination of binding affinity of YKL-40 (cf. Houston et al. [12])

For the binding assays 50 µl of 1.00 µM YKL-40 and 50 µl of different concentrations of the oligosaccharides, both made up in 25 mM Tris–HCl buffer, pH 7.4, containing 1 mM dithiothreitol and pre-warmed to 25 °C for 15 min in a shaking water bath, were incubated for 7 min at 25 °C. The fluorescence (excitation 295 nm, emission 340 nm) was read in a Perkin-Elmer LS 50B fluorescence spectrometer. The affinity was calculated, from the means of triplicate experiments, using the equation:

$$F - F_0 = B_{max} \times c/(K_d + c)$$

where B_{max} is the fluorescence intensity of YKL-40 under saturation conditions and K_d is the equilibrium dissociation constant.

2.5. Cell culture

Human chondrocytes were isolated from osteoarthritic cartilage from seven non-identifiable patients. Cartilage explants (1–2 g) were placed in 10 ml of HBSS in Petri dishes and cut into cuboids of 1.0–1.6 mm edge length, then transferred into 20 ml of F12 complete media [F-12 + GlutaMax nutrient Mixture (Ham; Gibco, USA) with 10% foetal bovine serum (FBS), 100 U/ml penicillin/streptomycin and 1 µg/ml fungizone], containing 1 mg/ml of collagenase IA (Sigma, USA), and digested at 37 °C for 16–24 h on a rotary shaker (80 rpm). The cell suspensions were filtered through 30 µm filters (Miltenyi Biotec, Germany), washed twice in F12 complete media and cultured in 25 cm^2 Falcon flasks at 37 °C and 5% CO_2. After attaining ca. 20% confluence (6–7 days) the cells were reseeded into 96 well culture plates (Nunc, Denmark) and supplemented with sterile filtered Oligomin™ or T-ChOS™, in culture media, 5–6 days later. The media were replaced every 3–7 days. Finally, cells were washed two times with 200 µl PBS, stained with Crystal Violet (2.5 mg/ml) for 10 min, washed four times with 250 µl of distilled water, photographed and counted per 0.04 mm^2 area. The plates were then dried and 100 µl of a 33% aqueous acetic acid solution was added to each well. After shaking, the absorption was recorded at 570 nm, using a 96-well optical densitometer (SpectraMax, USA). Alternatively, cells were fixed in methanol (−20 °C) for 10 min, Haematoxylin–Eosin stained and counted.

For competition experiments with *N,N′,N″*-triacetylchitotriose and T-ChOS™, cells were grown for 7 days with a change of the media after 3 or 4 days. Each concentration of the chitotriose was tested in 6 wells.

2.6. RNA isolation and RT-PCR analysis

Total RNA was extracted (RNeasy kit, Qiagen, USA) and quantified using a NanoDrop ND-1000 spectrophotometer. First-strand cDNA synthesis was performed with RevertAid H Minus First strand cDNA Synthesis Kit (Fermentas, USA) with a random hexamer primer supplied with the kit. PCR was performed with appropriate primers (Table 1) following a protocol for real time PCR with a Taq DNA Polymerase kit (Fermentas, USA) in a GeneAmp PCR System 9700 PE (Applied Biosystems, USA). The reaction mixture contained 1 µl of the cDNA solution, 38.7 µl H_2O, 5.0 µl 10× Taq buffer, 1.0 µl dNPT, 1.0 µl of a primer, 3.0 µl of $MgCl_2$ and 0.3 µl Taq Polymerase. A characteristic run was 30 cycles (94 °C for 224 s, 58 °C for 45 s, 72 °C for 60 s, 72 °C for 300 s, the cooling to 4 °C). The annealing temperature was 58 °C in all cases.

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