Biochemical and Biophysical Research Communications 434 (2013) 298–304

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

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

ARTICLE INFO

Article history:
Received 20 February 2013
Available online 26 March 2013

Keywords:
Cell culture
Chitolectin
Chitooligosaccharides
Chondrocytes
High affinity binding
Rheumatoid arthritis
YKL-40

ABSTRACT

Recent evidences indicating 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, prompted 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-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

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

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.02.122

[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

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.

3. Results

3.1. Characterization of ChOS

Peak integration of HP-SEC chromatograms shows that Oligomin™ contains a mixture of approximately 41% ChOS with degree of polymerization (DP) 1–3; 53% ChOS of DP 4–12; and 6% of higher oligosaccharides (Fig. 1A). The average degree of acetylation of Oligomin™ is 63% (F_A 0.63). In the production process of T-ChOS™, mono-, di-, and trisaccharides are removed to a large extent by ultrafiltration. The main components (ca. 79%) are ChOS of DP 4–12 (Fig. 1B). MALDI-TOF MS confirms the presence of ChOS of DP 2–12 in Oligomin™ and of DP 4–10 in T-ChOS™ (Table 2; and Supplemental Figs. S1, S2, and Table S1). Both products are essentially free of endotoxin (0.38 ± 0.13 EU/mg). The commercial sample of hexamer $\mathbf{A}_6$ contains small amounts (⩽5%) of the tetra-, penta-, and heptamers, while hexamer $\mathbf{D}_6$ is contaminated with a trace of pentamer $\mathbf{D}_5$, as determined by MALDI-TOF MS (data not shown).

3.2. Determination of binding affinity of YKL-40

Table 3 shows the homologues, degree of polymerization (DP), isomer compositions and the dissociation constants (K_d) with YKL-40 for different hexamers of ChOS and one homologue of undecamers. The K_d of 13.7 for $\mathbf{A}_6$, confirms high affinity binding of the fully N-acetylated hexasaccharide (Table 3). Partially acetylated hexamers containing four or three *N*-acetyl groups, i.e. $\mathbf{D}_2\mathbf{A}_4$ and $\mathbf{D}_3\mathbf{A}_3$, are reasonably strong binders with K_d 40.6 and 51.1 μM, respectively; whereas the K_d of a mixture of partially acetylated isomeric undecamers $\mathbf{D}_5\mathbf{A}_6$ is closer to that of the fully acetylated hexamer $\mathbf{A}_6$. The corresponding hexamer of glucosamine ($\mathbf{D}_6$) has virtually no affinity for YKL-40.

3.3. RT-PCR

The phenotype and viability of human osteoarthritic chondrocytes in culture was probed by analysing the expression of four proteins, YKL-40, YKL-39 and collagen type II, and AMCase, that is expressed in macrophages. Fig. 2 shows mRNA of YKL-40, YKL-39 and collagen type II expressed over a time period of 14 days, while AMCase is absent, demonstrating the presence of typical monolayer chondrocyte cell cultures.

3.4. Growth stimulation of chondrocytes by partially acetylated ChOS

Oligomin™ stimulates proliferation of human chondrocytes in cultures of primary cells from osteoarthritic cartilage tissue from seven individuals (Fig. 3). The maximum effect is observed at 500 μg/ml of Oligomin™ while this stimulation is abolished at a concentration of 1000 μg/ml (Fig. 4A).

Application of T-ChOS™ results in maximal enhancement of cell counts at considerably lower concentrations of 50–100 μg/ml (Fig. 4B). Addition of 50–63 μg/ml of *N,N′,N″*-triacetylchitotriose ($\mathbf{A}_3$) to 100 μg/ml of T-ChOS™ results in complete inhibition of enhanced cell proliferation (data not shown).

4. Discussion

A mixture of chitooligosaccharides (ChOS) was prepared from chitin flakes by alkaline deacetylation, followed by an extensive chitinase digestion and ultrafiltration. After drying, the obtained

Table 1
The genes and primers used for RT-PCR.

Genes	Primer sequences (Forward/Reverse)	Size (bp)	Accession No.
AMCase	F: ATGTCATGACCTACGACCTCCA R: AAAGTGCCAGTGAAGTCATCCA	490	AF290004.1
YKL-39	F: ATCTGCCAGTTCCTGAAAGGAG R: GTCAGGAAAGGCTTGGAAGAGA	383	NM_004000.2
YKL-40	F: CCTGCTCAGCGCAGCACTGT R: GCTTTTGACGCTTTCCTGGTC	488	NM_001276.2
Collagen type II	F: GCCATGAAGGTTTTCTGCAAC R: ACAGTCTTGCCCCACTTACC	419	NM_001844.4

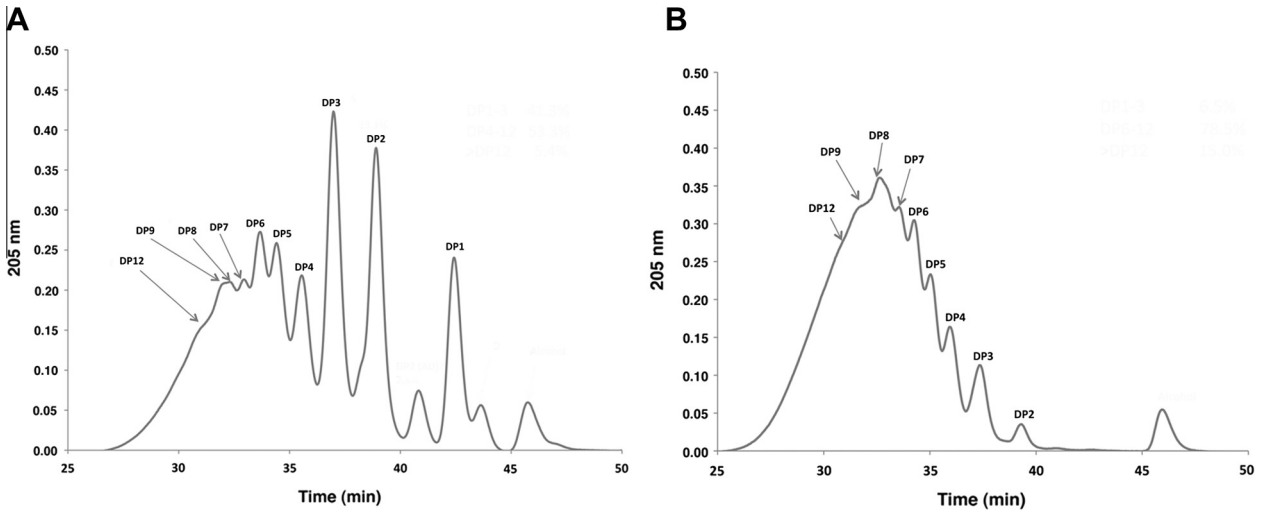

Fig. 1. HP-SEC of Oligomin™ on an TSK-Oligo column with assignment of peaks by degree of polymerization (DP) (**A**). HP-SEC chromatogram of T-ChOS™ on an TSK-Oligo column with assignment of peaks by DP (**B**).

Table 2
Main components present in the homologues of DP 3–10 of ChOS as observed by MALDI-TOF MS.

DP	3	4	5	6	7	8	9	10
Oligomin™	DA_2(F_A 0.66)	DA_3(F_A 0.75)	D_2A_3(F_A 0.60)	D_2A_4(F_A 0.66)	D_3A_4(F_A 0.57)	D_3A_5(F_A 0.63)	D_4A_5(F_A 0.56)	D_5A_5(F_A 0.50)
T-ChOS™		DA_3(F_A 0.75)	D_2A_3(F_A 0.60)	D_3A_3(F_A 0.50)	D_3A_4(F_A 0.57)	D_4A_4(F_A 0.50)	D_5A_4(F_A 0.44)	
							D_4A_5(F_A 0.56)	

Table 3
Equilibrium dissociation constants of ChOS with YKL-40.

Homologue	DP	F_A	Isomer composition[a]	K_d (μM)
$\mathbf{A_6}$	6	1.00	**AAAAAA**	13.7
$\mathbf{D_2A_4}$	6	0.67	**DDAAAA** (4%); **ADDAAA** (12%)	40.6
			DADAAA (43%); **AADDAA** (12%)	
			DAADAA (9%); **ADADAA** (19%)	
$\mathbf{D_3A_3}$	6	0.50	**DDADAA** (51%)	51.1
			DADDAA (49%)	
$\mathbf{D_5A_6}$	11	0.55	Not analysed	6.9
$\mathbf{D_6}$	6	0.00	**DDDDDD**	419.9

[a] For sequence analysis of $\mathbf{D_2A_4}$and $\mathbf{D_3A_3}$ [27,28].

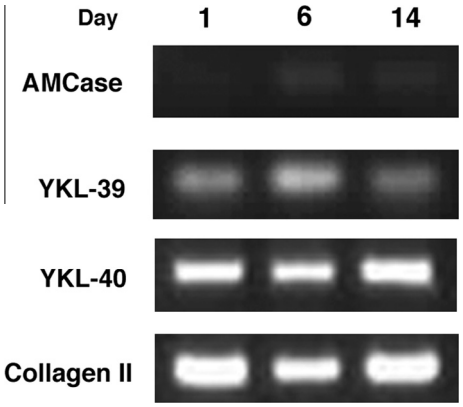

Fig. 2. RT-PCR of AMCase, YKL-39, YKL-40, and collagen type II. RNA was extracted at time zero (primary culture), and after 6 and 14 days. Conditions: RT-PCR 30 cycles, annealing temp. 58 °C, sample volume: 15 μl per lane, electrophoresis: 80 V for 35 min.

product was referred to as Oligomin™. Chromatographic analysis showed that 96% of the Oligomin™ is a mixture in the size range between monomers and dodecamers. A considerable fraction (41%) is made up of mono-, di- and trimers, leaving 53% within the range of DP 4–12.

Further ultrafiltration enriches the DP 4–12 fraction to 79%, leaving only trace amount of monomer, greatly reducing the three lowest oligomers in the ChOS mixture and lowering the degree of acetylation from 63% to 55%, indicating higher degree of acetylation in the lowest oligomers and the monomers. After drying, this refined product is referred to as T-ChOS™ (therapeutic chitooligosaccharides). High affinity of the chitinase-like glycoprotein (CLP) YKL-40 to both chitin and some fully N-acetylated ChOS is well established as evident by the reported values of 331 μM for tetra-*N*-acetylchitotetraose ($\mathbf{A_4}$) and 6.7 μM for hexa-*N*-acetylchitohexaose ($\mathbf{A_6}$) [11,12]. We confirmed this high affinity binding of YKL-40 to fully N-acetylated hexasaccharide ($\mathbf{A_6}$) and also showed reasonably strong affinity to partially acetylated hexamers containing four or three *N*-acetyl groups ($\mathbf{D_2A_4}$ and $\mathbf{D_3A_3}$). Moreover, partially acetylated isomeric $\mathbf{D_5A_6}$ has affinity similar to that of the fully acetylated hexamer ($\mathbf{A_6}$). This shows that full acetylation is not necessary for substantial binding of ChOS to YKL-40, although the fully deacetylated hexamer of glucosamine ($\mathbf{D_6}$) has virtually no affinity for YKL-40.

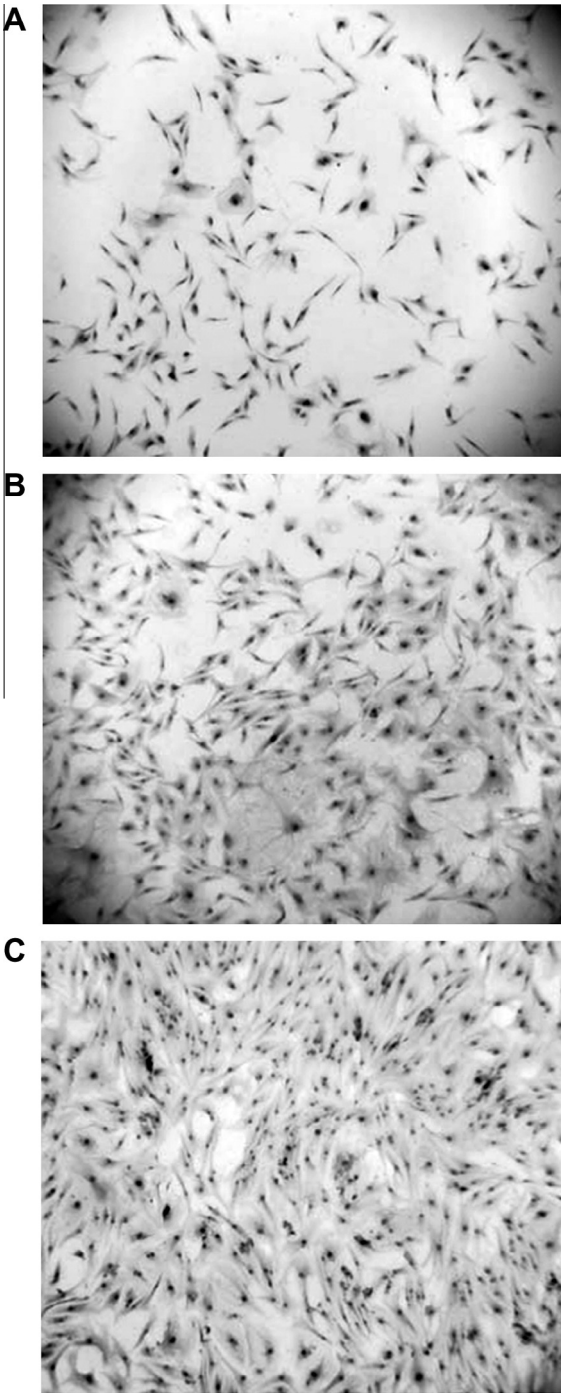

Fig. 3. Primary cell culture monolayers of osteoarthritic human chondrocytes, grown in the presence of 0 (A); 100 (B); and 500 μg/ml (C) of Oligomin™. Cell staining with Haematoxylin–Eosin, Magnification 200×.

A comparison of identified protein structures for family 18 chitinases and YKL-40, including their interactions with fully acetylated ChOS, may yield an explanation for this observation.

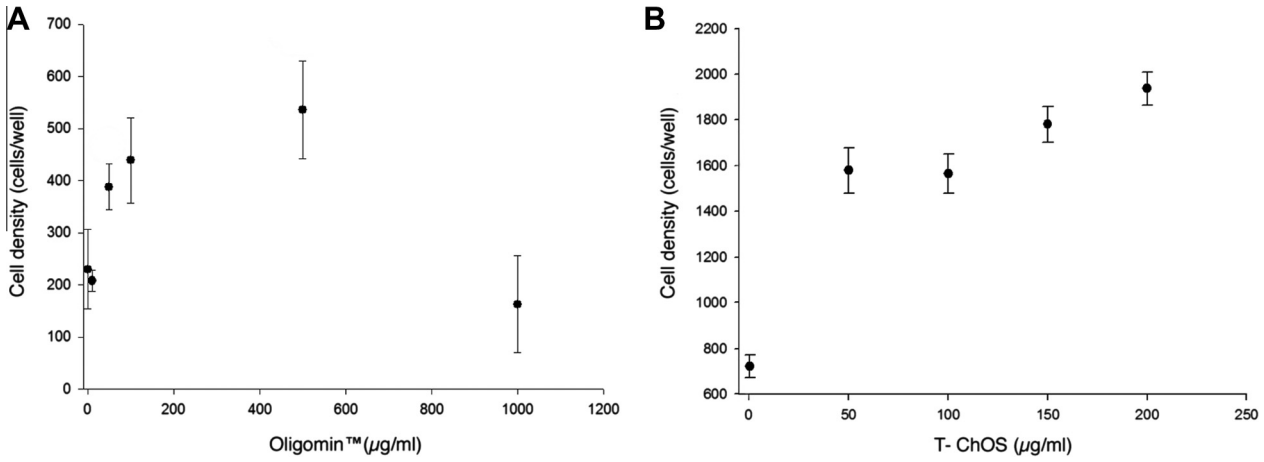

Fig. 4. The dose–response of chito-oligomers on chondrocyte cell growth. Human chondrocytes where cultured in 96 well microplates, supplemented with different concentrations of (A) Oligomin™ (0, 10, 50, 100, 500 and 1000 μg/ml). After 5 weeks of incubation, the cells were fixed in −20 °C methanol and HE stained. Cell density per well was counted on photographs and the mean (n = 8, SEM) compared to 0 μg/ml of Oligomin™. The difference was highly significance by t-test ($p < 0.001$). The effect of T-ChOS™ on chondrocyte was tested in similar manner and the results of T-ChOS™ concentration (0–200 μg/ml) is shown (B) after 10 days in culture. The mean cell density (n = 8, SEM), determent as above, was compared to 0 μg/ml of T-ChOS™ by t-test and was highly significant ($p < 0.001$).

N,N'-diacetylchitobiose occupies distal −5 and −6 sugar binding sites of YKL-40 [13], whereas $\mathbf{A}_6$ binds to the −3 to +3 and to the −4 to +2 sites [12,13,26]. In family 18 chitinases, the free energy of binding ($\Delta\Delta G°$) of an **A**-residue of a fully acetylated ChOS is usually negative at subsite −2 (ca. −3 to −5 kcal/mol) because of favoured hydrophobic interactions. However, due to substrate-assisted distortion necessary for catalysis [29], $\Delta\Delta G°$ is positive at subsite −1 (ca. +3 to +4 kcal/mol) [30–33], (Supplementary Table S2). Replacement of an **A**-residue with a less hydrophobic D-unit at site −2 will result in higher (or less negative) values of $\Delta\Delta G°$, meaning a less favoured contribution to the total free energy of binding. On the other hand, replacement of **A** with **D** at site −1 will impair substrate assisted distortion and therefore result in an improved binding at the −1 site. Although $\Delta\Delta G°$ values of binding to individual subsites of YKL-40 are unknown, we may assume that these considerations will also be valid for a family 18 chitolectin, because of its homology with family 18 chitinases. Thus, we propose a qualitative model for the alignment of partially acetylated ChOS at the sugar binding sites of YKL-40 (Table 4). Consider for example the binding of the major isomer **5** of $\mathbf{D}_2\mathbf{A}_4$, i.e. **DADAAA** in the table. Alignment at sites −3 to +3 shows four favoured hydrophobic interactions at −2, +1, +2, and +3, one less favourable interaction at site −1, and one disfavoured at −3, whereas alignment at sites −4 to +2 is favoured at −3, disfavoured at −2 and −1, and favoured at +1 and +2. Thus, we would expect that $\mathbf{D}_2\mathbf{A}_4$ bind preferentially with high affinity to sites −3 to +3. Analysis of the possible alignments of the other isomers of $\mathbf{D}_2\mathbf{A}_4$ or the isomers of $\mathbf{D}_3\mathbf{A}_3$ explains why hexamers bearing only 4 or 3 *N*-acetyl groups still show appreciable affinity for YKL-40.

The phenotype and viability of human osteoarthritic chondrocytes in culture was further examined by analysing the expression of four genes, three that are characteristic for chondrocytes, i.e. the two enzymatically inactive family 18 glycosyl hydrolase chitolectins YKL-40 and YKL-39 [34–37], and collagen type II [14,24], while the fourth, ACMase, an enzymatically active family 18 glycosyl hydrolyse, is absent from condrocytes but expressed in macrophages [38]. YKL-39 that is closely related to YKL-40, but it is not a glycoprotein and does not display any heparin binding [39], has recently been showed to have micromolar affinity for chitin oligosaccharides [40]. As depicted in Fig. 2, mRNA of the chitolectins YKL-40 and YKL-39, as well as of collagen type II, is expressed over the monitored time period of 14 days, while AMCase is absent, indicating osteoarthritic chondrocytes.

Oligomin™ stimulates proliferation of human chondrocytes in primary cell cultures from osteoarthritic cartilage tissue. We observed maximum effect at 500 μg/ml of Oligomin™ that is abolished at higher concentration. The more refined T-ChOS™ results in similar enhancement of cell counts, but at considerably lower concentrations (50–100 μg/ml), correlating with the lower ratio of smaller ChOS, i.e. predominantly **A**, $\mathbf{A}_2$ and $\mathbf{A}_3$ (41% in

Table 4
Model for alignments of partially N-acetylated hexamers $\mathbf{D}_2\mathbf{A}_4$ and $\mathbf{D}_3\mathbf{A}_3$ at binding sites −3 to +3 and −4 to+2 of family 18 chitinases and homologous YKL-40. Favoured and less disfavoured binding interactions ate sites −2 and −1 are highlighted in grey.

Isomer No.	Rel. amount	Binding at sites −3 to +3						Binding at sites −4 to +2					
		−3	−2	−1	+1	+2	+3	−4	−3	−2	−1	+1	+2
		Sign of $\Delta\Delta G°$						Sign of $\Delta\Delta G°$					
		(−)	(−)	(+)	(−)	(−)	(−)	(−)	(−)	(−)	(+)	(−)	(−)
D_2A_4													
1	(12%)	A	D	D	A	A	A	A	D	D	A	A	A
2	(12%)	A	A	D	D	A	A	A	A	D	D	A	A
3	(19%)	A	D	A	D	A	A	A	D	A	D	A	A
4	(4%)	D	D	A	A	A	A	D	D	A	A	A	A
5	(43%)	D	A	D	A	A	A	D	A	D	A	A	A
6	(9%)	D	A	A	D	A	A	D	A	A	D	A	A
D_3A_3													
1	(51%)	D	D	A	D	A	A	D	D	A	D	A	A
2	(49%)	D	A	D	D	A	A	D	A	D	D	A	A

Oligomin™ vs. 6.5% in T-ChOS™). Addition of 50–63 μg/ml of *N,N′,N″*-triacetylchitotriose (**A**$_3$) to 100 μg/ml of T-ChOS™ results in complete inhibition of the T-ChOS™ enhanced cell proliferation (Supplementary Fig. S3), indicating that low DP ChOS act as antagonists of the higher DP ChOS and that stimulation of cell proliferation of chondrocytes is caused by higher ChOS.

The high-affinity binding of partially acetylated ChOS to YKL-40 and stimulation of chondrocyte proliferation suggests a novel therapeutic approach in treating inflammatory rheumatoid diseases. Fully acetylated ChOS may be even better ligands in this respect though rapid degrading by family 18 chitinases present in human serum, especially under pathological conditions, is likely. We have shown earlier that partially acetylated ChOS are resistant to hydrolysis by a family 18 chitinase, when high-affinity binding to the enzyme results in alignment of a **D** residue at the −1 sugar binding site.[41–43]. Therefore, such partially acetylated ChOS are likely to have longer half-life in human tissues than their fully acetylated homologs.

Acknowledgments

We would like to thank the orthopedic surgeons for their generous work contribution in providing cartilage for this study. Funded by Icelandic Centre for Research, grant number 081204010; Technology Development Fund.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.122.

References

[1] A. Denuziere, D. Ferrier, O. Damour, et al., Domard, chitosan–chondroitin sulfate and chitosan–hyaluronate polyelectrolyte complexes: biological properties, Biomaterials 19 (1998) 1275–1285.
[2] R.A.A. Muzzarelli, Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone, Carbohydr. Polym. 76 (2009) 167–182.
[3] K. Suzuki, T. Mikami, Y. Okawa, et al., Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose, Carbohydr. Res. 151 (1986) 403–408.
[4] S.K. Kim, N. Rajapakse, Enzymatic production and biological activities of chitosan oligosaccharides (COS): a review, Carbohydr. Polym. 62 (2005) 357–368.
[5] H. Yin, Y.G. Du, J.Z. Zhang, Low molecular weight and oligomeric chitosans and their bioactivities, Curr. Top. Med. Chem. 9 (2009) 1546–1559.
[6] X.B. Li, S. Roseman, The chitinolytic cascade in Vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase, Proc. Natl. Acad. Sci. USA 101 (2004) 627–631.
[7] K.L. Meibom, X.B.B. Li, A.T. Nielsen, et al., The Vibrio cholerae chitin utilization program, Proc. Natl. Acad. Sci. USA 101 (2004) 2524–2529.
[8] P. Vander, K.M. Vårum, A. Domard, et al., Comparison of the ability of partially N-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves, Plant Physiol. 118 (1998) 1353–1359.
[9] E.K. Petutschnig, A.M.E. Jones, L. Serazetdinova, et al., The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation, J. Biol. Chem. 285 (2010) 28902–28911.
[10] B.E. Hakala, C. White, A.D. Recklies, Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family, J. Biol. Chem. 268 (1993) 25803–25810.
[11] G.H. Renkema, R.G. Boot, F.L. Au, et al., Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologs of family 18 glycosyl hydrolases secreted by human macrophages, Eur. J. Biochem. 251 (1998) 504–509.
[12] D.R. Houston, A.D. Recklies, J.C. Krupa, et al., Structure and ligand-induced conformational change of the 39-kDa glycoprotein from human articular chondrocytes, J. Biol. Chem. 278 (2003) 30206–30212.
[13] F. Fusetti, T. Pijning, K.H. Kalk, et al., Crystal structure and carbohydrate-binding properties of the human cartilage glycoprotein-39, J. Biol. Chem. 278 (2003) 37753–37760.
[14] H.F. Bigg, R. Wait, A.D. Rowan, et al., The mammalian chitinase-like lectin, YKL-40, binds specifically to type I collagen and modulates the rate of type I collagen fibril formation, J. Biol. Chem. 281 (2006) 21082–21095.
[15] B. Volck, P.A. Price, J.S. Johansen, et al., YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils, Proc. Assoc. Am. Phys. 110 (1998) 351–360.
[16] B. Volck, K. Ostergaard, J.S. Johansen, et al., The distribution of YKL-40 in osteoarthritic and normal human articular cartilage, Scand. J. Rheumatol. 28 (1999) 171–179.
[17] B. Volck, J.S. Johansen, M. Stoltenberg, et al., Studies on YKL-40 in knee joints of patients with rheumatoid arthritis and osteoarthritis. Involvement of YKL-40 in the joint pathology, Osteoarthritis Cartilage 9 (2001) 203–214.
[18] J. Connor, R. Dodds, J. Emery, et al., Human cartilage glycoprotein 39 (HC gp-39) mRNA expression in adult and fetal chondrocytes, osteoblasts and osteocytes by in-situ hybridization, Osteoarthritis Cartilage 8 (2000) 87–95.
[19] J.S. Johansen, J.R. Kirwan, P.A. Price, et al., Serum YKL-40 concentrations in patients with early rheumatoid arthritis: relation to joint destruction, Scand. J. Rheumatol. 30 (2001) 297–304.
[20] E. Vignon, Is glycoprotein YKL40 a new marker for joint disorders?, Joint Bone Spine 68 (2001) 454–456
[21] K. Huang, L.D. Wu, YKL-40: a potential biomarker for osteoarthritis, J. Int. Med. Res. 37 (2009) 18–24.
[22] A.D. Recklies, C. White, H. Ling, The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase beta-mediated signalling pathways, Biochem. J. 365 (2002) 119–126.
[23] F. De Ceuninck, S. Gaufillier, A. Bonnaud, et al., YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes, Biochem. Biophys. Res. Commun. 285 (2001) 926–931.
[24] F. Badariotti, M. Kypriotou, C. Lelong, et al., The phylogenetically conserved molluscan chitinase-like protein 1 (Cg-Clp1), homologue of human HC-gp39, stimulates proliferation and regulates synthesis of extracellular matrix components of mammalian chondrocytes, J. Biol. Chem. 281 (2006) 29583–29596.
[25] F. Fusetti, H. von Moeller, D. Houston, et al., Structure of human chitotriosidase – implications for specific inhibitor design and function of mammalian chitinase-like lectins, J. Biol. Chem. 277 (2002) 25537–25544.
[26] P. Zaheer-ul-Haq, N.N. Dalal, Aronson, et al., Family 18 chitolectins: comparison of MGP40 and HUMGP39, Biochem. Biophys. Res. Commun. 359 (2007) 221–226.
[27] S. Bahrke, Mass spectrometric analysis of chitooligosaccharides and their interaction with proteins. PhD Thesis, University of Potsdam, Germany. <http://opus.kobv.de/ubp/volltexte/2008/2017/>, 2007.
[28] S. Haebel, S. Bahrke, M.G. Peter, Quantitative sequencing of complex mixtures of heterochitooligosaccharides by vMALDI-Linear ion trap mass spectrometry, Anal. Chem. 79 (2007) 5557–5566.
[29] D.M.F. van Aalten, D. Komander, B. Synstad, et al., Structural insights into the catalytic mechanism of a family 18 exo-chitinase, Proc. Natl. Acad. Sci. USA 98 (2001) 8979–8984.
[30] T. Fukamizo, C. Sasaki, E. Schelp, et al., Kinetic properties of chitinase-1 from the fungal pathogen *Coccidioides immitis*, Biochemistry 40 (2001) 2448–2454.
[31] C. Sasaki, A. Yokoyama, Y. Itoh, et al., Comparative study of the reaction mechanism of family 18 chitinases from plants and microbes, J. Biochem. 131 (2002) 557–564.
[32] T. Taira, M. Fujiwara, N. Dennhart, et al., Transglycosylation reaction catalyzed by a class V chitinase from cycad, Cycas revoluta: a study involving site-directed mutagenesis, HPLC, and real-time ESI-MS, Biochim. Biophys. Acta 2010 (1804) 668–675.
[33] T. Ohnuma, T. Fukamizo, Chitin/chitosan oligosaccharides: effective substrates for functional analysis of chitinases/chitosanases, in: S.-K. Kim (Ed.), Chitin, chitosan, oligosaccharides and their derivatives, CRC Press, Boca Raton, FL, USA, 2010, pp. 295–324.
[34] J.-I. Tsuruha, K. Masuko-Hongo, T. Kato, et al., Autoimmunity against YKL-39, a human cartilage derived protein, in patients with osteoarthritis, J. Rheumatol. 29 (2002) 1459–1466.
[35] H. Du, K. Masuko-Hongo, H. Nakamura, et al., The prevalence of autoantibodies against cartilage intermediate layer protein, YKL-39, osteopontin, and cyclic citrullinated peptide in patients with early-stage knee osteoarthritis: evidence of a variety of autoimmune processes, Rheumatol. Int. 26 (2005) 35–41.
[36] T. Knorr, F. Obermayr, E. Bartnik, et al., YKL-39 (chitinase 3-like protein 2), but not YKL-40 (chitinase 3-like protein 1), is up regulated in osteoarthritic chondrocytes, Ann. Rheum. Dis. 62 (2003) 995–998.
[37] E. Steck, S. Breit, S.J. Breusch, et al., Enhanced expression of the human chitinase 3-like 2 gene (YKL-39) but not chitinase 3-like 1 gene (YKL-40) in osteoarthritic cartilage, Biochem. Biophys. Res. Commun. 299 (2002) 109–115.
[38] R.G. Boot, E.F.C. Blommaart, E. Swart, et al., Identification of a novel acidic mammalian chitinase distinct from chitotriosidase, J. Biol. Chem. 276 (2001) 6770–6778.
[39] B. Hu, K. Trinh, W.F. Figueira, et al., Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family, J. Biol. Chem. 271 (1996) 19415–19420.
[40] M. Schimpl, C.L. Rush, M. Betou, et al., Human YKL-39 is a pseudo-chitinase with retained chitooligosaccharide-binding properties, Biochem. J. 446 (2012) 149–157.

[41] M.C. Letzel, B. Synstad, V.G.H. Eijsink, et al., Libraries of chito-oligosaccharides of mixed acetylation patterns and their interactions with chitinases, Adv. Chitin Sci. 4 (2000) 545–552.
[42] F. Cederkvist, A.D. Zamfir, S. Bahrke, et al., Identification of a high-affinity-binding oligosaccharide by (+) nanoelectrospray quadrupole time-of-flight tandem mass spectrometry of a noncovalent enzyme–ligand complex, Angew. Chem., Int. Ed. 45 (2006) 2429–2434.
[43] F.H. Cederkvist, M.P. Parmer, K.M. Varum, et al., Inhibition of a family 18 chitinase by chitooligosaccharides, Carbohydr. Polym. 74 (2008) 41–49.