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Absorption, distribution and mechanism of action of SYSADOAS

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ABSTRACT

Symptomatic Slow Acting Drugs for Osteoarthritis (SYSADOA), such as hyaluronic acid (HA), chondroitin sulfate (CS) and glucosamine (GlcN) are natural compounds, composed of repeating disaccharides, used to treat patients with osteoarthritis (OA). Many questions about the kinetics and mechanism of action of SYSADOA remain poorly answered. This review examines the data supporting oral absorption and body distribution of SYSADOA, and discusses their mechanism of action. SYSADOA are absorbed in the small intestine with a bioavailability ranging from 5 to 45% and accumulate in articular tissues. The mechanism of action of HA and CS differs in several aspects from that of GlcN. Being large molecules, HA and CS do not penetrate into chondrocytes, synoviocytes, osteoblast, osteoclast and osteocytes, and so elicit the anti-inflammatory effect by engaging membrane receptors, e.g. CD44, TLR4, and ICAM1, with a resulting dual effect: impede the fragments of extracellular matrix engaging these receptors, cause of inflammatory reaction, and block the signal transduction pathways activated by the fragments and so diminish the nuclear translocation of pro-inflammatory transcription factors. GlcN penetrates into cells by means of glucose transporters. The primary effect of GlcN is associated to its ability to *O*-GlcNAcylate proteins and as a consequence, modulates their activity, e.g. decrease nuclear factor-κB nuclear translocation. GlcN may also affect the transcription of pro-inflammatory cytokines by epigenetic mechanisms. The characteristics of the mechanism of action support the use of CS combined with GlcN, and suggest that HA and CS shall be more effective in initial phases of OA.

Contents

1. Introduction . . . 0
2. Gastrointestinal absorption of SYSADOAS . . . 0
3. Body distribution of SYSADOAS . . . 0
4. Mechanism of action of SYSADOAS . . . 0
5. Conclusions . . . 0
Conflict of interest statement . . . 0
Uncited reference . . . 0
References . . . 0

Abbreviations: ADAMTS, aggrecanases; Agg-f, aggrecan fragments; Akt, serine/threonine protein kinase B; AP-1, activating protein 1; B2R, bradykinin 2 receptor; BK, bradykinin; CD44, cluster designation 44; CII-f, collagen II fragments; CS, chondroitin sulfate; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FN-f, fibronectin fragments; GlcN, glucosamine; GLUT, glucose transporter; HA, hyaluronic acid; HA-f, hyaluronic acid fragments; HAS2, hyaluronic acid synthase-2; HMW-HA, high molecular weight hyaluronic acid; Hyal, hyaluronidase; IκBα, inhibitor κBα protein; ICAM1, intercellular adhesion molecule 1; IKK, inhibitor κB kinase; IL-1R, interleukin 1β receptor; IRAK, IL-1R associated kinase; IRAK-M, IL-1 receptor associated kinase-M; JNK, c-jun NH2-terminal kinase; LMW-HA, low molecular weight hyaluronic acid; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MKK, MAPK kinase; MKP-1, mitogen activated protein kinase phosphatase-1; MMPs, matrix metalloproteinases; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor κB; NOS2, nitric oxide synthase 2; p105, inactive p50; p50, nuclear factor κB1; p65, proto-oncogene c-RelA; PgE_2, prostaglandin E_2; PI3K, phosphoinositide-3-OH kinase; Raf, proto-oncogene serine/threonine-protein kinase; RANKL, receptor activator of NF-κB ligand; Ras, proto-oncogene rat sarcoma; RHAMM, receptor for hyaluronan-mediated motility; RhoA, Ras homolog gene family member A; ROCK, RhoA/Rho kinase; PKC, protein kinase C; ROS, reactive oxygen species; TAB, TAK binding protein; TAK, transforming growth factor-beta-activated kinase; TGF-β1, transforming growth factor-β1; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; Tpl2, tumor progression locus 2; TRAF6, TNF receptor-associated factor 6.

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1. Introduction

This review will focus on three natural compounds, hyaluronic acid (HA), chondroitin sulfate (CS) and glucosamine (GlcN) considered as Symptomatic Slow Acting Drugs for Osteoarthritis (SYSADOA) with an effect size of 0.60, 0.75 and 0.58, respectively (Zhang et al., 2010). HA is a non-sulfated, high molecular weight glycosaminoglycan (>500 kDa) composed of repeating disaccharides, D-glucuronic acid and *N*-acetyl-D-glucosamine, linked by β(1 → 3) and β(1 → 4) bonds. Endogenous HA is widely distributed in the organism where it has multiple functions, such as space filling, hydration, lubrication of joints, facilitation of cell migration; moreover, HA is actively implicated in tissue injury/inflammation, tissue repair, and wound healing (Jiang et al., 2011). CS is a complex glycosaminoglycan formed by alternate disaccharide sequences of differently sulfated residues of D-glucuronic acid and N-acetyl-D-galactosamine; most frequent in human, are CS sulfated in position 4 or 6 of N-acetyl-D-galactosamine, generating chondroitin-4-sulfate (C4S or CSA) and chondroitin-6-sulfate (C6S or CSC), or sulfated in position 2 of D-glucuronic acid; CS molecular weight depends upon the source and varies between 10 and 40 kDa (Volpi, 2011). HA and CS are primarily located in the extracellular matrix (ECM) and are essential to the cellular–ECM interplay (Fig. 1). GlcN (2-amino-2-deoxy-β-D-glucopyranose) is an endogenous aminomonosaccharide (179 Da) synthesized from glucose and utilized for the biosynthesis of glycoproteins and glycosaminoglycans (Dahmer & Schiller, 2008).

In recent years, understanding of the kinetics and dynamics of HA, CS and GlcN has made considerable progress, however much of the information is scattered and unstructured. This review aims to integrate and harmonize the knowledge on intestinal absorption, joint distribution and mechanism of action of HA, CS and GlcN.

2. Gastrointestinal absorption of SYSADOAS

2.1. Hyaluronic acid

Due to its molecular weight, HA is poorly absorbed by the gastrointestinal tract and consequently, it has been administered locally, into the joint. However, there is evidence that in the horse oral HA reduces joint effusion (Bergin et al., 2006). Indirect and direct mechanisms may contribute to the response of oral HA. In mice, oral HA binds to Toll-like Receptor-4 (TLR4) in the luminal surface of the large intestine to up-regulate the suppressor of cytokine signaling 3, and diminish pleiotrophin expression, altogether resulting in the down-regulation of systemic pro-inflammatory cytokines (Asari et al., 2010). On the other hand, in the rat and dog the bioavailability of oral HA is around 5%, absorption mediated by lymphatic transport or paracellular pathway (Reed et al., 1992; Balogh et al., 2008; Hisada et al., 2008). Further studies are needed to explore whether oral HA is effective in patients with osteoarthritis (OA).

2.2. Chondroitin sulfate

With the everted rat gut sac model, in two hours 9% of a dose of ^{14}C–CS is absorbed from the small intestine, 17% from the cecum, and 23% from the colon (Barthe et al., 2004). In the rat and the dog, the bioavailability of CS is around 10%, and total bioavailability, e.g. CS and olygosaccharides derived from CS, is approximately 70%, and that independently of the molecular weight of CS (Palmieri et al., 1990; Conte et al., 1991, 1995; Ronca et al., 1998). In the horse, total bioavailability appears to be around 30% (Du et al., 2004).

In healthy volunteers, the bioavailability of CS is 12% (Table 1); although, the bioavailability of CS and derived olygosaccharides is around 22% (Conte et al., 1991; Ronca & Conte, 1993; Ronca et al., 1998). The absorption of CS occurs without lag-time and the time required to reach plasma CS peak concentration (t_{max}) is around 2–3 h, indicating that CS rate of absorption is rapid. In humans, the profile of oral bovine CS plasma concentrations as a function of time suggests that CS is absorbed in the proximal segments of the small intestine (Ronca et al., 1998).

In Caco-2 cells, CS (15 kDa) is not transported from the apical to the basolateral membrane; although 3% of oligosaccharides of low molecular weight (3 kDa) derived from CS are transported across Caco-2 cells in 3 h (Cho et al., 2004). On the other hand, CS and its disaccharides cross the intestinal barrier via a paracellular pathway, passage inversely associated to the transepithelial electrical resistance (Sim et al., 2005; Jin et al., 2010b); the passage is increased by the presence of bile acids that alter the tight junction structure (Raimondi et al., 2008). Absorption through a paracellular pathway appears to be a common mechanism for saccharides, such as dextran (10 kDa) (Raimondi et al., 2008) and low molecular weight HA (<70 kDa) (Hisada et al., 2008).

The low bioavailability of CS does not appear associated to intestinal degradation since in vitro studies suggest that CS is not depolymerized by stomach and intestinal contents and/or by the gastro-intestinal tissue; however, there is a steady degradation of CS in the presence of cecum and colon contents (Barthe et al., 2004). Since CS is absorbed in proximal segments of the small intestine, saturation of the paracellular pathway, rather than intestinal degradation, may be an explanation for CS low bioavailability.

Bovine CS contains monosulfated disaccharides, of which 55% sulfated in position 4 (ΔDi-4S), 40% in position 6 (ΔDi-6S), and 5% of nonsulfated disaccharide (ΔDi-0S). In humans, oral administration of bovine CS increases primarily the plasma concentrations of ΔDi-4S and ΔDi-6S (Table 1) (Volpi, 2002). On the other hand, CS from shark is absorbed more slowly and by containing 31% of ΔDi-4S, 50% of ΔDi-6S, 17% of ΔDi-2,6S and 2% of ΔDi-0S generates lower concentrations of ΔDi-4S and ΔDi-6S than bovine CS (Volpi, 2003) (Table 1).

2.3. Glucosamine

In the rat, the bioavailability of D(+)-GlcN HCl ranges between 6% and 19% (Aghazadeh-Habashi et al., 2002; Ibrahim et al., 2012), and the bioavailability of ^{14}C-GlcN sulfate is 40% (Setnikar & Rovati, 2001). In the dog, GlcN HCl bioavailability is around 11% (Adebowale et al., 2002). In healthy volunteers, GlcN sulfate is absorbed rather rapidly with a t_{max} of around 3 h (Persiani et al., 2005), and a bioavailability of 44% (Setnikar & Rovati, 2001).

Using the everted rat gut model, it was shown that GlcN is primarily absorbed in the duodenum and much less in the jejunum, ileum and colon (Ibrahim et al., 2012). GlcN is a substrate of the glucose transporters GLUT1, GLUT2 and GLUT4; however, the uptake of GlcN is done primarily by GLUT2 since its affinity for GlcN is higher than that of GLUT1 and GLUT4 (Uldry et al., 2002). Enteric absorption of GlcN is primarily carried out by GLUT2 (Caccia et al., 2007; Ibrahim et al., 2012).

The incomplete oral bioavailability of GlcN might be explained by an intestinal first-pass metabolism. In the rat, oral bioavailability of GlcN is lower than following intraperitoneal and intravenous routes, supporting an intestinal first-pass metabolism (Ibrahim et al., 2012). Inside cells, GlcN is phosphorylated to form glucosamine-6-phosphate, which subsequently enters into the hexosamine biosynthetic pathway to form proteoglycans, glycolipids, and glycoproteins (Anderson et al., 2005). Moreover, in intestinal epithelial cells, exogenous GlcN may contribute to the formation of mucin (Forstner, 1970). Therefore, first-pass metabolism in the epithelial cells of the intestine may explain GlcN incomplete bioavailability.

When rats are given orally a combination of 100 mg/kg neomycin, 50 mg/kg tetracycline and 50 mg/kg bacitracin twice daily for 2 days, the percentage of the dose of GlcN recovered in feces increases from 0.1% in control to 11% in antibiotic-treated rats; in parallel, the percentage of GlcN dose recovered in 24 hour urine increases from 1.8 to 5.5% (Ibrahim et al., 2012); suggesting that the intestinal flora may also contribute to reduce GlcN oral bioavailability.

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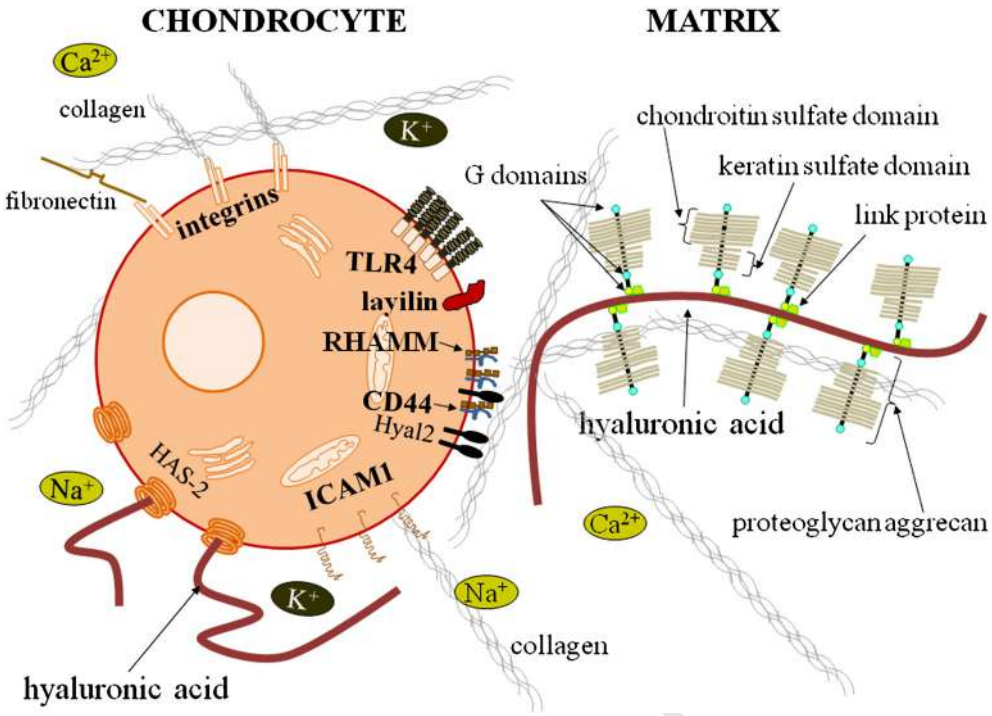

Fig. 1. Molecular organization of healthy articular cartilage. Pericellular and territorial matrix surrounding the chondrocyte contains collagen, hyaluronic acid (HA) and aggrecan. The proteoglycan aggrecan includes chondroitin sulfate and keratin sulfate domains; moreover, aggrecan core contains three globular domains (G domains). Aggrecans are anchored in the matrix by binding to hyaluronic acid through the link protein. The chondrocyte presents several surface receptors essential to the chondrocyte–pericellular interplay, such as CD44, TLR4, ICAM1 and RHAMM. In addition, the membrane encloses HA synthase-2 (HAS2) to synthesize HA, and hyaluronidase 2 (Hyal2) to cleave HA.

3. Body distribution of SYSADOAS

3.1. Hyaluronic acid

In the rat, following oral administration, HA distributes into the body, and 0.5% of the dose reaches the knee joint (Balogh et al., 2008). In the joint, HA reaches synovial membranes by simple diffusion, and the ECM of cartilage and subchondral bone through the lymph flow (Antonas et al., 1973; Liu, 2004).

Under physiological conditions, the turnover of HA in the ECM of the cartilage (Fig. 2), includes chondrocyte synthesis by membrane-bound hyaluronic acid synthase 2 (HAS2), release of high-molecular weight HA (HMW-HA) into the ECM, and degradation by extracellular enzymes, or receptor-mediated endocytosis, primarily conducted by the cell-surface glycoprotein cluster designation 44 (CD44) (Knudson et al., 2002). Endocytosis starts with the invagination of caveolin-rich membrane containing hyaluronidase 2 (Hyal2) to form endosomes, where Hyal2 will transform HMW-HA to oligosaccharides of around 20 kDa; these low molecular weight olygosaccharides are delivered to low pH lysosomes rich in Hyal1 that will further degrade the oligosaccharides to fragments of 0.2–0.8 kDa (Stridh et al., 2012).

Table 1
Oral bioavailability of CS in humans.

Pre-dose				Post-dose							Ref.
CS_{max} (μg/ml)	ΔDi-0S (%)	ΔDi-4S (%)	ΔDi-6S (%)	Dose (g)	F (%)	CS_{max} (μg/ml)	t_{max} (h)	ΔDi-0S (%)	ΔDi-4S (%)	ΔDi-6S (%)	
				0.5[a] i.v. 3[a] p.o.	13.2 24.3[b]	11.4 ± 3.7	3.2 ± 0.4				Balogh et al., 2008
				0.2[c] i.m. 1.2[c] p.o.	20.9[d]	4.6 ± 1.7	4.0 ± 0.4				Bassleer et al., 1998b
				0.8[e] p.o.	12 19[f]	6.6 ± 0.7	1.0 ± 0.3				Bandow et al., 2010
3.9 ± 0.9	60 ± 9	40 ± 9	NQ	4[g] p.o.	NA	12.7 ± 4.7	2.4 ± 1.4	23 ± 12	61 ± 11	17 ± 7	Bastow et al., 2008
2.1 ± 1.3	60 ± 9	40 ± 9	NQ	4[h] p.o.	NA	4.9 ± 2.1	8.7 ± 4.5	31 ± 21	52 ± 26	15 ± 17	Beier and Loeser, 2010

CS_{max}, peak concentration of CS; ΔDi-0S, non-sulfated disaccharide; ΔDi-4S and ΔDi-6S, disaccharide sulfated in positions 4 and 6, respectively; F, bioavailability; t_{max}, time required to reach CS_{max}.
NQ not quantifiable.
NA not available.
[a] Bovine CS, mw 16 kDa.
[b] CS + low molecular weight disaccharides in urine.
[c] Shark depolymerized CS, mw 7.5 kDa.
[d] CS + low molecular weight disaccharides.
[e] ^{131}I-labeled bovine CS.
[f] CS + low molecular weight disaccharides in urine.
[g] Bovine CS, mw 25–30 kDa.
[h] Shark CS, mw 45 kDa.

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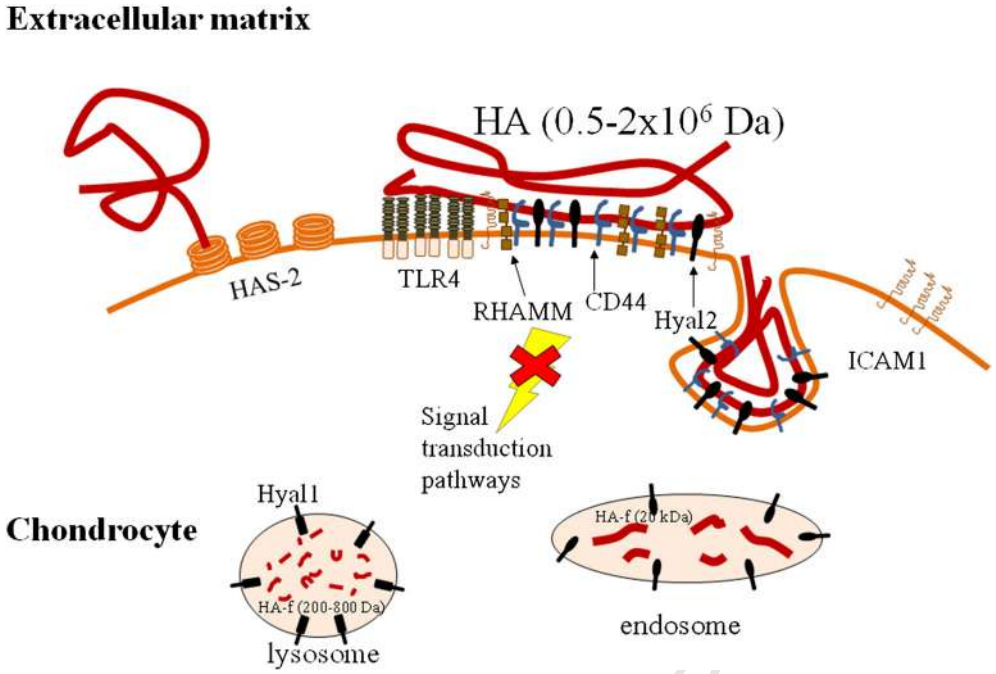

Fig. 2. Interplay between the chondrocyte and HA in the extracellular matrix. High molecular weight HA is synthesized by HAS2 and once released in the extracellular matrix it may engage and inactivate cell-surface CD44, TLR4, ICAM1 and RHAMM. Binding to CD44 may also initiate the endocytosis and degradation of HA by Hyal2 and Hyal1.

3.2. Chondroitin sulfate

In the rat, 6 h after the injection of ^{3}H–CS (3.2 mg/kg), the concentrations of CS plus derived olygosaccharides are 4.2 μg/g in articular cartilage and 3.6 μg/ml in synovial fluid (Ronca et al., 1998). In healthy volunteers, for the first 40 min after oral administration of ^{99m}Tc–CS, the concentrations of CS and derived olygosaccharides are greater in the thigh and in the calf as compared with knee tissues, but afterwards the radioactivity progressively increases in knee joint to become much higher than in the adjacent tissues (Ronca et al., 1998).

Incubation ^{99m}Tc–CS (24 kDa) with human rib cartilage, leads to a progressive accumulation of ^{99m}Tc–CS in the cartilage to amount 53% of the dose after 72 h (Sobal et al., 2009a). Following incubation of ^{99m}Tc–CS with human knee cartilage, 45% of ^{99m}Tc–CS uptake is found in superficial cartilage and 35% in subchondral layers (Sobal et al., 2013). On the other hand, using monolayers of chondrocytes and chondrocytes in suspension, the uptake of ^{99m}Tc–CS by the chondrocytes does not exceed 1% (Sobal et al., 2009a). These results strongly suggest that oral CS reaches the joint, distributes into the cartilage and subchondral layers, but the penetration into the chondrocyte is very limited.

3.3. Glucosamine

In the horse, both GlcN sulfate and GlcN HCl administered orally accumulate into the synovial fluid of radiocarpal joints (Laverty et al., 2005; Meulyzer et al., 2008). It is noteworthy that synovial fluid GlcN concentrations are four fold higher in presence of a LPS-induced joint inflammation (Meulyzer et al., 2009).

When ^{99m}Tc–GlcN is incubated with human rib cartilage, uptake of GlcN is around 85% (Sobal et al., 2009b). In *Xenopus* oocytes, the uptake of GlcN is primarily conducted by GLUT2, with a minor contribution of GLUT1 and GLUT4 (Uldry et al., 2002). Chondrocytes express GLUT1, 2, 3, 4, and 5 (Ohara et al., 2001), supporting that in human knee chondrocytes GlcN uptake is carried out by GLUT transporters (Mroz & Silbert, 2004; Shikhman et al., 2009).

It is improbable that HMW-HA and CS penetrate into synoviocytes, osteoblasts or osteoclasts. However, due to the presence of GLUT transporters across the cell membrane, GlcN may be able to penetrate into synoviocytes (Tai & Carter-Su, 1988; Tai et al., 1992), osteoblasts (Zoidis et al., 2011) and osteoclasts (Larsen et al., 2005).

4. Mechanism of action of SYSADOAS

Most ECM–chondrocyte interactions are mediated by membrane receptors, such as CD44, TLRs, intercellular adhesion molecule 1 (ICAM1), receptor for hyaluronan-mediated motility (RHAMM), integrins, and layilin (Fig. 1). These membrane receptors have in common that they bind not only HA, but also collagen and CS (except layilin), and that they modulate transcriptional factors such as nuclear factor-κB (NF-κB). Moreover, CD44, TLRs, ICAM1, integrins and RHAMM respond to fragments of components of the ECM, such as fragments of HA (HA-f), collagen II (CII-f), fibronectin (FN-f), and aggrecan (Agg-f) (Bobacz et al., 2007).

It is noteworthy that CD44, TLRs, ICAM1, and integrins are also expressed in synoviocytes, osteoblasts and osteocytes, strongly suggesting that matrix–cell interactions in synovial membranes and in subchondral bone occur as do in cartilage. Effectively, synoviocytes express CD44 (Zhang et al., 2013), integrins (Pirila et al., 2001), ICAM1 (Hiramitsu et al., 2006), TLR4 (Gutierrez-Canas et al., 2006), and layilin (Murata et al., 2012). Osteoblasts express CD44 (Diaz-Rodriguez et al., 2012), integrins (Rubert et al., 2012), ICAM1 (Bloemen et al., 2009), and TLR4 (Kim et al., 2009; Bandow et al., 2010). Osteocytes express CD44, integrins (Rochefort et al., 2010), and may express TLR2 and 4 (Bidwell et al., 2008). Moreover, chondrocytes (Nishida et al., 1999), synoviocytes (Momberger et al., 2005), osteoblasts (Falconi & Aubin, 2007), and osteocytes (Bastow et al., 2008) express HAS2 and synthesize HA.

Whether initial events of OA occur in the cartilage, synovial membrane or subchondral bone is still under debate, although it is commonly believed that an early cause of OA is the alteration of the interplay between ECM components and chondrocytes. In normal resting cartilage, non-stressed situation, chondrocytes are quiescent cells, with little turnover of the cartilage matrix. Under physiological conditions, in the ECM, HA is primarily found as HMW-HA, e.g. >500 kDa (Fig. 2). HMW-HA promotes cell quiescence and tissue integrity by binding to

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CD44, ICAM1, TLR4 and RHAMM, thus blocking pro-inflammatory signaling pathways (Stern et al., 2006; Jiang et al., 2011; Campo et al., 2012a). On the other hand, CD44 contributes to the turnover of HA by carrying out HA internalization and degradation by Hyal (Knudson & Loeser, 2002; Jiang et al., 2011).

Early OA includes splitting of the ECM, increased cellular activity in cartilage and synovial membranes, increased tissue hydration, and remodeling of the subchondral bone (Guilak, 2011). Chondrocytes are activated, with cell proliferation, cluster formation, and increased production of both matrix proteins and matrix degrading proteins, such as Hyals, aggrecanases (ADAMTS), matrix metalloproteinases (MMPs), and chondroitinases, as well as enhanced formation of reactive oxygen species (ROS) (Loeser et al., 2012). As a result, there is an accumulation of HA-f, CII-f, FN-f, and Agg-f in the ECM (Sofat, 2009; Heinegard & Saxne, 2011; Loeser et al., 2012) that may bind and activate TLR4, CD44, ICAM1, RHAMM and integrins in chondrocytes (Sofat, 2009), synoviocytes (Sandell, 2012; Scanzello & Goldring, 2012) and osteoblasts (Falconi & Aubin, 2007; Woeckel et al., 2012).

The increase in HA-f enhances NF-κB nuclear translocation, and consequently, induces the expression of tumor necrosis factor-α (TNF-α), IL-6, MMP13, and nitric oxide synthase-2 (NOS2); the effects of HA-f are reverted by antibodies anti-CD44 and by HA binding protein (HABP) (Campo et al., 2012b). In addition, HA-f induce also NF-κB nuclear translocation by activating TRL4 (Campo et al., 2010a, 2010b) and ICAM1 (Stern et al., 2006). Similar effects are observed with FN-f and CII-f, e.g. both increase NF-κB nuclear translocation by activating CD44 and ICAM1 (Yasuda, 2010, 2011a, 2012).

The signal transduction pathways activated by engagement of ECM fragments to CD44 are depicted in Fig. 3, resulting in the increase of the nuclear translocation of NF-κB, p38MAPK, c-jun NH2-terminal kinase (JNK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), and activating protein-1 (AP-1) with up-regulation of target genes, such as ADAMTS, MMPs, Hyal2, IL-1β, and NOS2 (Slevin et al., 1998; Fitzgerald et al., 2000; Luo et al., 2005; Roman-Blas & Jimenez, 2006; Bourguignon et al., 2011; Prasadam et al., 2012; Roget et al., 2012). Less is known about ICAM1 signal transduction pathways, but may in part be redundant with CD44 intracellular signaling (Holland & Owens, 1997; Turowski et al., 2005; Hiramitsu et al., 2006; Yasuda, 2007; Appleton et al., 2010; Beier & Loeser, 2010; Yasuda, 2011b, 2012).

FN-f bind to integrin α5β1 and increase the expression of MMP13 via the activation of PKCδ, p38MAPK, JNK, ERK1/2, and probably NF-κB (Forsyth et al., 2002; Loeser et al., 2003). Moreover, FN-f engage CD44 and activate the PI3K/Akt pathway and enhance NF-κB nuclear translocation (Yasuda, 2011a).

The cytoplasmic regions of TLR4 and IL-1β receptor I (IL-1RI) are highly homologous, since both have a Toll/IL-1 Receptor (TIR) domain (Kariko et al., 2004). Engagement of TLR4 by HA-f causes receptor dimerisation and recruitment of the TIR domain contained in the adaptor protein myeloid differentiation factor 88 (MyD88), ultimately leading to the activation of NF-κB, p38MAPK, JNK, ERK1/2, and AP-1, and increase in expression of Hyals, ADAMTS, MMPs, chondroitinase, phospholipase A2 (PLA2), cyclooxygenase 2 (COX2), NOS2, and pro-inflammatory cytokines, as well as the formation of ROS (Fig. 3), that once released in the ECM will form additional fragments to sustain the inflammatory reaction (Kariko et al., 2004; Campo et al., 2009b; Vallieres & du Souich, 2010; Brown et al., 2011; Kim et al., 2012; Madry et al., 2012).

The signal transduction pathways triggered by CD44, ICMA1 and TLR4/IL-1RI in synoviocytes (Gutierrez-Canas et al., 2006; Hiramitsu et al., 2006; Wang et al., 2006), osteoblasts/osteocytes and osteoclasts (Bastow et al., 2008; Kim et al., 2009; Bandow et al., 2010; Kular et al., 2012; Schaffler & Kennedy, 2012; Yang et al., 2012) are similar to the

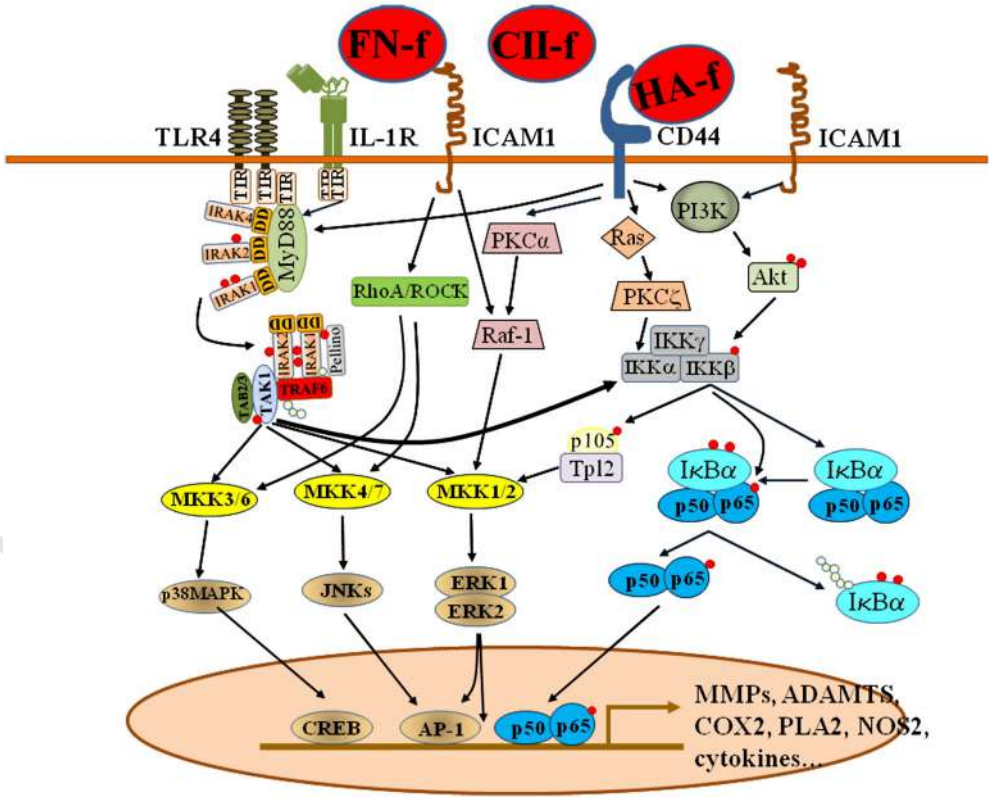

Fig. 3. Schematic representation of signal transduction pathways of activated surface receptors, CD44, TLR4, ICAM1 and IL-1R, resulting in increase of ADAMTS, Hyal2, MMPs, cyclooxygenase 2 (COX2), phospholipase A2 (LPA2), NOS2, and pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, etc. Where DD is death domain; CREB is cAMP-responsive element binding protein. Chains of open circles indicate ubiquitination and sites for proteasomal degradation; red circles indicate phosphorylation sites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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ones triggered in the chondrocyte. For instance, HA-f activates the osteoblast by engaging the CD44/RHAMM complex and activating the Raf-1 pathway (Hatano et al., 2012).

In osteocytes and osteoblasts (Fig. 4), mechanical stress, ECM fragments, prostaglandin E_2 and cytokines, increase both membrane-bound and soluble receptor activator of NF-κB ligand (RANKL), as well as osteoprotegerin (OPG), a soluble decoy receptor able to antagonize RANKL. Free RANKL binds the RANK receptor on osteoclast, quiescent and precursors, to promote osteoclastogenesis, essentially by activating NF-κB nuclear translocation, via the MyD88/TRAF6 signaling pathway, and AP-1 (Lacey et al., 2012). NF-κB and AP-1 enhance the transcriptional activity of nuclear factor of activated T cells cytoplasmic1 (NFATc1) that regulates the expression of osteoclastogenic-specific genes, such as cathepsin K, calpain, MMP13 and MMP14 (Delaisse et al., 2003; Cawston & Young, 2010; Nakahama, 2010; Braun & Schett, 2012).

4.1. High molecular weight hyaluronic acid

There is compelling evidence that HMW-HA reverses the pro-inflammatory effects of ECM fragments via CD44, TLR4 and ICAM1. In chondrocytes, by engaging CD44 and TLR4, HA-f increase NF-κB nuclear translocation and enhance IL-1β, IL-6 and TNF-α expression (Campo et al., 2010b, 2012a), response partially reversed by HMW-HA (Campo et al., 2012a). In chondrocytes stimulated with FN-f, HMW-HA reduces p38MAPK phosphorylation, effect mediated by the binding of HA to CD44 and ICAM1 (Yasuda, 2010, 2011a).

It has been proposed that the mechanism underlying the effect of HMW-HA is a simple effect of competition, where HMW-HA binds to CD44, TLR4 and ICAM1 and impedes the binding of ECM fragments or LPS (Campo et al., 2010a, 2010b). This may be the case when activation of NF-κB is elicited by LPS, via TLR4 (Campo et al., 2010a). However, in macrophages, HMW-HA diminishes LPS-induced increase in NF-κB nuclear translocation, and IL-1β, IL-6 and TNF-α expression, effect almost totally reversed by specific antibodies anti-ICAM1 (Yasuda, 2007). Since LPS binds to TLR4, these results suggest that HA elicits an anti-inflammatory effect by other mechanisms than simple competition. Moreover, CII-f enhance MMP13, increase partially prevented by HMW-HA by engaging ICAM1; an antibody anti-ICAM1 did not impede the CII-f-induced increase in MMP13, implying that the response to HMW-HA is not elicited by competing with CII-f, but rather by binding to ICAM1 and triggering an anti-inflammatory response (Yasuda, 2012).

Further supporting a direct anti-inflammatory effect, HMW-HA diminishes IL-1β-induced p38MAPK phosphorylation and MMP13 expression in a dose-dependent manner, effect mediated by CD44 (Julovi et al., 2011). The fact that the response to IL-1β depends upon its binding to IL-1R and activation of the MyD88/IRAK signaling pathway, and that HMW-HA does not bind IL-1R, supports the notion that HMW-HA can achieve an anti-inflammatory effect directly, without competing for the binding sites of the agent inducing the expression of MMPs.

In human osteoarthritic chondrocytes, Yatabe et al. (2009) have shown that HMW-HA blocks IL-1α-induced increase in ADAMTS4 by increasing the expression of IL-1 receptor associated kinase-M (IRAK-M), a negative regulator of IRAK1, effect mediated by CD44 and possibly ICAM1. On the other hand, HMW-HA, following engagement of TLR4, also leads to the over-expression of IRAK-M (del Fresno et al., 2005).

Incubation of human chondrocytes with HMW-HA prevents the increase of expression of MMP1, MMP3 and MMP13 triggered by IL-6 and soluble IL-6R. HMW-HA by a mechanism CD44-dependent activates MAPK phosphatase-1 (MKP-1) that dephosphorylates ERK1/2 at threonine and tyrosine residues (Hashizume & Mihara, 2010). Moreover, MKP-1 also dephosphorylates p38MAPK and JNK (Comalada et al., 2012; Wancket et al., 2012).

In summary, in chondrocytes, the anti-inflammatory effect of HMW-HA is secondary to the engagement to TLR4, CD44 and ICAM1, preventing the binding of ECM fragments, and increasing the expression of IRAK-M and MKP-1.

In the rabbit with OA, following intra-articular injection, HMW-HA accumulates in the cartilage and subchondral bone. At 10 weeks, compared with the control group, in the HMW-HA treated group, the gross morphological OA score is significantly improved, and MMP13 and IL-6 are reduced in cartilage and in subchondral bone (Hiraoka

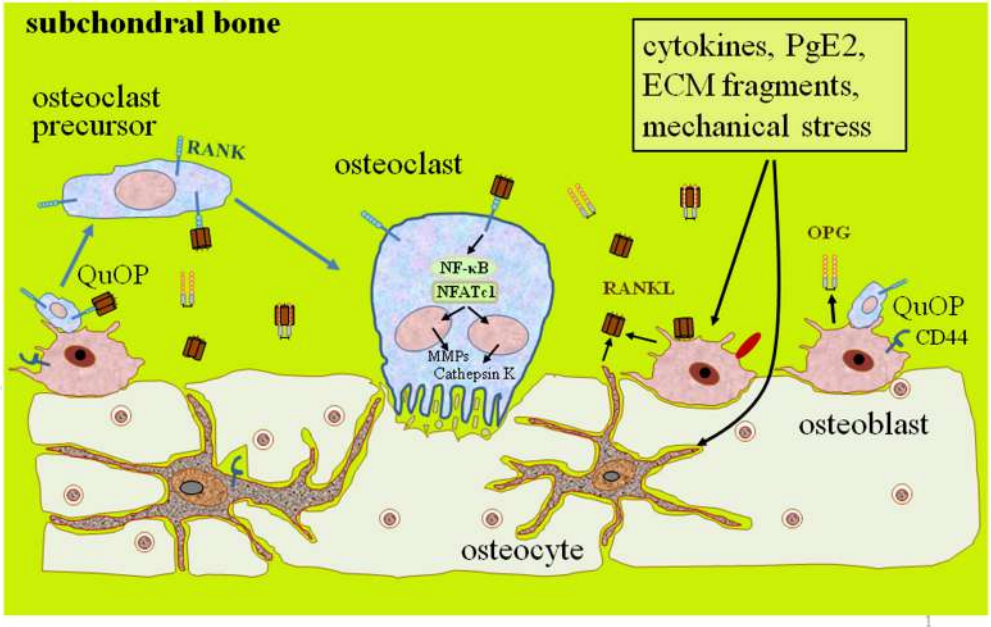

Fig. 4. Schematic representation of the osteoclastogenesis in the subchondral bone. Matrix (ECM) fragments, cytokines and prostaglandins (PgE_2) activate osteoblasts and osteocytes to enhance membrane-bound and soluble RANKL, as well as osteoprotegerin (OPG), a soluble decoy receptor able to antagonize RANKL. Soluble RANKL will bind to its receptor RANK on the quiescent osteoclast precursor (QuOP) to initiate its maturation to osteoclast precursor and to mature osteoclasts, e.g. osteoclastogenesis. Differentiation of osteoclast is driven internally by RANKL-induced activation of NF-κB and nuclear factor of activated T cells cytoplasmic1 (NFATc1). Mature activated osteoclasts bind the surface of osteocytes and release cathepsin K and MMPs, primarily MMP9, to initiate bone resorption.

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et al., 2011). The same authors incubated human osteoblasts with IL-6 and soluble IL-6R, with and without HMW-HA, and reported that HMW-HA reduces dose-dependently MMP13 expression, response probable mediated by the binding of HMW-HA to CD44 (Hiraoka et al., 2011).

Osteoclast differentiation and bone resorption are increased dose-dependently by low molecular weight HA (LMW-HA); moreover, LMW-HA potentiate the effect of RANKL on osteoclast differentiation and bone resorption, in part by increasing p38MAPK and ERK1/2 phosphorylation, and enhancing RANK receptor expression. The effects of LMW-HA are prevented by a CD44 function-blocking monoclonal antibody. In contrast, HMW-HA (2000 kDa) reduces the effect of RANKL on osteoclast differentiation (Ariyoshi et al., 2005). On the other hand, in mouse bone marrow stromal cells ST2, as model of osteoblasts, co-cultured with mouse monocyte RAW 264.7 cells, HMW-HA (2500 kDa) down-regulates RANKL expression and diminishes osteoclastogenesis of RAW 264.7 cells (Ariyoshi et al., 2013).

In mouse synovial fibroblasts, HA-f activate TLR-4 and CD44, and enhance the inflammatory response (Campo et al., 2012c). In cultured rheumatoid synovial fibroblasts, FN-f bind to α5β1 integrin, activate ERK, P38MAPK and JNK, and up-regulate the expression of MMP1, MMP3 and MMP13 (Yasuda et al., 2003). In contrast, in human fibroblast-like synoviocyte (HFLS) cells, HMW-HA (2500 kDa) inhibits IL-1β-induced ADAMTS4 expression by engaging CD44 and reducing p38MAPK and JNK activation (Kataoka et al., 2013). Moreover, in HFLS from patients with OA, HMW-HA prevents IL-1β-induced expression of TNF-α, IL-8 and NOS2, effect mediated by CD44 (Wang et al., 2006). In synovial fibroblasts from rheumatoid synovial tissues, IL-1β activates p38MAPK, JNK and NF-κB and up-regulates MMP1 and MMP3 expression; HMW-HA by reducing p38MAPK, JNK and NF-κB phosphorylation diminishes the expression of MMP1/3, effect mediated by ICAM1 (Hiramitsu et al., 2006).

These reports strongly support that HMW-HA diminishes osteoblast/osteoclast and synoviocyte activation by mechanisms similar to those described for the chondrocyte, e.g. primarily by engaging CD44 and ICAM1.

4.2. Chondroitin sulfate

The primary effect of CS as a SYSADOA appears to be associated with the inhibition of NF-κB nuclear translocation by reducing the phosphorylation of ERK1/2 and p38MAPK (Campo et al., 2008; Jomphe et al., 2008). Several mechanisms contribute to the effect of CS (Fig. 5).

In humans with knee OA, treated for ten days with 800 mg/day of CS, HA concentration and intrinsic viscosity of synovial fluid increase significantly (Ronca et al., 1998). In rabbit knee synovial membranes, CS increases HA synthesis (Nishikawa et al., 1988). The mechanisms underlying CS-induced synthesis of HA include the up-regulation of HAS1 and HAS2 (Itano et al., 1999; David-Raoudi et al., 2009). This effect may be explained by engagement of CS to β1-integrin (Wu et al., 2002), and up-regulation of transforming growth factor-β1 (TGF-β1) (Legendre et al., 2008; Gonzalez-Ramos et al., 2013) and of HAS2 (Meran et al., 2011; Foley et al., 2012). Therefore, the inhibition of NF-κB activation by CS is in part be mediated by an increase in HMW-HA synthesis.

In chondrocytes, LPS up-regulates gene and protein expression of TNF-α, IL-1β, IL-6, IFN-γ, MMP1, MMP13, and iNOS secondary to the

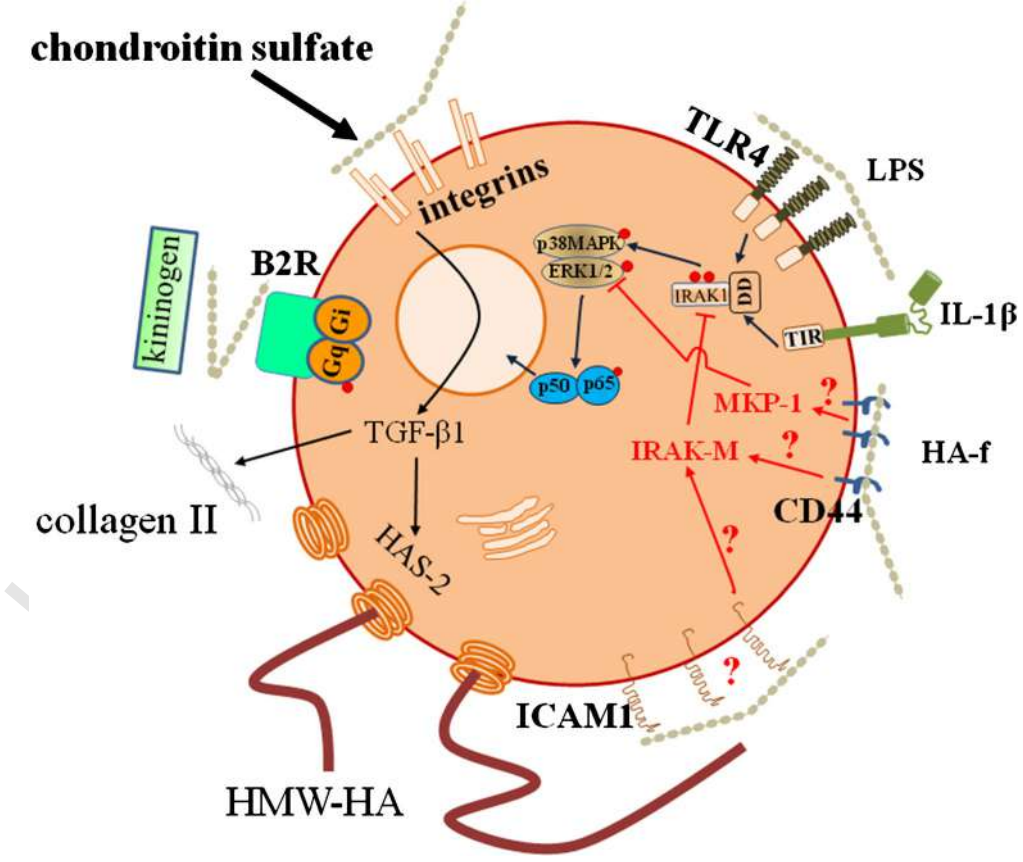

Fig. 5. Mechanism of action of CS. CS engages CD44, TLR4 and ICAM1 and so impedes their activation by HA-f, and possibly FN-f, Agg-f and CII-f. CS by engaging CD44 and ICAM1 may promote the release of IRAK-M, an inhibitor of IRAK, or the release of MKP-1, that will dephosphorylate p38MAPK and ERK1/2. These effects will reduce the nuclear translocation of NF-κB and the inflammatory reaction. In addition, CS engages integrins and increases TGF-β1 expression that will foster the synthesis of HMW-HA and of collagen II. Finally, CS diminishes the proteolysis of kininogen to BK and induces the desentization and internalization of B2R.

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activation of the MyD88/IRAK4/TRAF6 signaling pathway and NF-κB nuclear translocation (Campo et al., 2008, 2009a). C4S and C6S diminish dose-dependently LPS-induced MyD88 and TRAF6 expression, and NF-κB activation, and consequently reduce IL-1β, TNF-α, iNOS and MMP13 increment, effect abrogated by an anti-TLR4 antibody (Campo et al., 2009a, 2009b). Therefore, like HMW-HA, C4S and C6S reduce LPS inflammatory reaction by interacting with TLR4.

There is some evidence supporting that C4S oligosaccharides and ΔDi-4S modulate TLR4 as do C4S and C6S. In macrophages stimulated by LPS, C4S oligosaccharides and ΔDi-4S inhibit the up-regulation of IL-6 by preventing IRAK1 activation (Jin et al., 2010a, 2011). This effect may be explained by the fact that C4S oligosaccharides and ΔDi-4S bind to the cysteine-rich motifs bordering the N- and C-terminal sides of leucine-rich repeats of TLR4, and so could impede LPS activation of TLR4 (Jin et al., 2011). Alternatively, it cannot be excluded that C4S oligosaccharides and ΔDi-4S may inhibit IRAK1 activation via the activation of CD44 and ICAM1 and release of IRAK-M.

CS reverses IL-1β-induced activation of chondrocytes (Jomphe et al., 2008), synoviocytes (Imada et al., 2010; Lambert et al., 2012), and osteoblasts (Pecchi et al., 2012); since CS does not bind IL-1βRI or IL-1βRII, it implies that CS blocks the IL-1β-induced activation of cells by a mechanism possibly involving CD44 and/or ICAM1.

There is evidence that CS directly and/or indirectly modulates CD44 anti-inflammatory effects. CS engages CD44 (Aruffo et al., 1990; Fujimoto et al., 2001; Naor & Nedvetzki, 2003), and is internalized by CD44 (Lo et al., 2013). Versican, a proteoglycan with up to 23 chains of CS, binds to CD44 (Kawashima et al., 2000), and inhibits HA-f-induced CD44 activation and ERK1/2 phosphorylation (Suwan et al., 2009). On the other hand, CS by increasing CD44 sulfation facilitates HA binding to CD44 (Esford et al., 1998).

Bradykinin (BK) and BK_2 receptors (B2R) in synovial membranes and chondrocytes modulate pain and inflammation in the joint with OA (Meini & Maggi, 2008). In human chondrocytes, BK increases the release of IL-6 and IL-8 via p38MAPK and NF-κB activation (Meini et al., 2011). The BK system is modulated by CS in two ways; on the one hand, CS binds to BK large precursor protein kininogen and impedes its proteolysis to BK (Renne et al., 2005); on the other hand, CS induces the desensitization and internalization of B2R by promoting G-protein-coupled receptor kinase phosphorylation of B2R (Shimazaki et al., 2012).

The integrity of the ECM is maintained by CS by reducing the expression of MMP1, MMP3, MMP13, ADAMTS1 and ADAMTS2, hence preventing the degradation of collagen, proteoglycans and aggrecan (Legendre et al., 2008). On the other hand, C4S and C6S increase the production and release of proteoglycans by human chondrocytes (Bassleer et al., 1998a; Nishimoto et al., 2005). Since CS increases TGF-β1 expression (Wu et al., 2002; Legendre et al., 2008), and TGF-β1 enhances collagen II synthesis (Gouttenoire et al., 2004; Patil et al., 2011), we may speculate that CS increases the synthesis of collagen II through TGF-β1.

Clinical trials have shown that CS diminishes synovitis (Clegg et al., 2006) and subchondral lesions (Wildi et al., 2011). In vitro, CS reduces IL-1β-induced inflammatory reaction in the subchondral bone (Tat et al., 2007; Pecchi et al., 2012), and in synovial membranes (Largo et al., 2010). We may speculate that the mechanism of action underlying the effect of CS in subchondral bone and in synoviocytes implicates CD44 and perhaps ICAM1. Indeed, further studies are required to better characterize how in vivo CS reduces synovial inflammation and reduces the progression of subchondral lesions.

4.3. Glucosamine

The SYSADOA effect of GlcN is associated with its ability to reduce NF-κB nuclear translocation (Largo et al., 2003; Lin et al., 2008), and that through several mechanisms.

Once in the cell, GlcN is subjected to the hexosamine biosynthetic pathway (Fig. 6) to generate UDP-N-acetylglucosamine (UDP-GlcNAc)

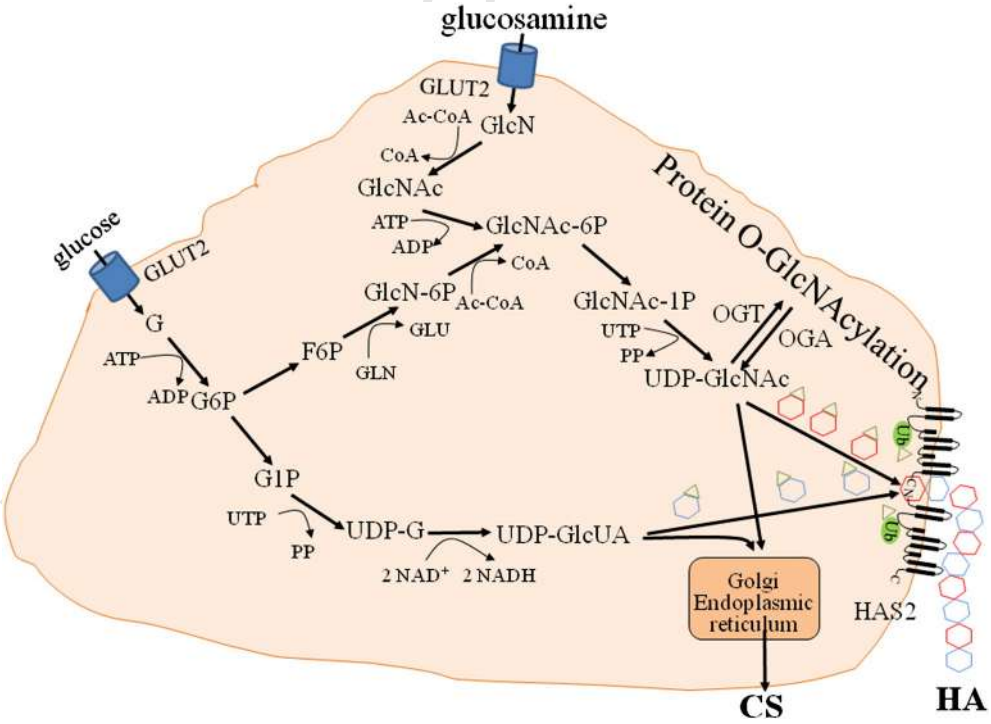

Fig. 6. Transmembrane transport of glucose and GlcN is facilitated by GLUT to be incorporated into the hexosamine biosynthetic pathway to finally generate uridin diphospho (UDP △) glucuronic acid (UDP-GlcUA ○) and UDP-N-acetylglucosamine (UDP-GlcNAc ○), saccharides required for the synthesis of HA and CS. UDP-GlcNAc is the substrate for protein *O*-GlcNAcylation, reaction catalyzed by *O*-linked N-acetylglucosaminyl transferase (OGT); protein deacylation is carried out by *O*-GlcNAc hydrolase (OGA). Where G6P is glucose-6-phosphate; F6P is fructose-6-phosphate; GlcN6P is glucosamine-6-phosphate; GlcNAc6P is N-acetylglucosamine-6-phosphate; GlcNAc1P is N-acetylglucosamine-1-phosphate; G1P is glucose-1-phosphate; UDP-G is UDP-glucose; ATP is adenosine triphosphate; ADP is adenosine diphosphate; UTP is uridin triphosphate; Ac-CoA is acetyl-coenzyme A; GLN is glutamine; GLU is glutamate; PP is pyrophosphate; Ub is ubiquitin.

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(Tammi et al., 2011; Vigetti et al., 2012). UDP-GlcNAc is a substrate for protein acylation through *O*-linked GlcNAcylation (*O*-GlcNAc). Proteins can be modified reversibly at serine or threonine residues by attachment of a GlcNAc molecule, reaction catalyzed by *O*-linked N-acetylglucosaminyl transferase (OGT). *O*-GlcNAc modification of proteins may reduce or increase their activity (Ozcan et al., 2010).

Several mechanisms contribute to GlcN-induced decline in NF-κB nuclear translocation. *O*-GlcNAcylation of IKKα inhibits its activity and hence reduces IκBα phosphorylation (Scotto d'Abusco et al., 2010); moreover, *O*-GlcNAcylation of IκBα impedes its phosphorylation and degradation by the proteasome (Zou et al., 2009). On the other hand, increased concentrations of GlcNAc inhibit ubiquitin-activating enzyme E1 (Guinez et al., 2008), and reduce proteasome activity and consequently, diminish IκBα degradation (Zhang et al., 2003; Liu et al., 2011).

GlcN is in part mediated by HA and CS. *HAS2* promoter contains functional response elements for different transcription factors, including SP1 (specificity protein 1) and YY1 (Yin-Yang 1), both repressors of HAS2 expression. GlcN promotes the *O*-GlcNAc modification of SP1 and YY1, and reduces their binding to *HAS2* promoter and as a result, upregulates HAS2 and enhances HA synthesis (Jokela et al., 2011). Moreover, UDP-GlcNAc induces the *O*-GlcNAcylation of HAS2 on serine 221, modification that not only prevents HAS2 degradation by the ubiquitin–proteasome system, but also increases its activity and enhances HA synthesis (Vigetti et al., 2012).

UDP-GlcNAc in the inner surfaces of the endoplasmic reticulum and Golgi vesicles is converted to N-acetylgalactosamine (GalNAc) by UDP-galactose 4′-epimerase and sulfated in positions 4 and 6 by sulfo-transferases to initiate the synthesis of CS (Nigro et al., 2009; Vigetti et al., 2012). In these vesicles, GalNAc residues with sulfate linked to 4- and/or 6-hydroxyl positions, are bound to D-glucuronic acid (GlcUA) to form C4S and C6S (Silbert & Sugumaran, 2002).

Contributing to GlcN anti-inflammatory properties, GlcN prevents COX2 N-glycosylation and as a consequence, increases COX2 degradation by the proteasome (Jang et al., 2007; Park et al., 2013).

There is growing evidence that epigenetics is a contributing mechanism to the progression of OA. DNA methylation of clusters of cytosine–phosphate–guanine (CpG) dinucleotides in the promoter represses gene transcription. In chondrocytes from patients with OA, DNA methylation is reduced and consequently, there is an epigenetic derepression of the genes coding for MMP3, MMP9, MMP13, ADAMTS4, and IL-1β; in addition, CpG islands of the DNA of superoxide dismutase-2 (SOD-2) and of bone morphogenetic protein-7 (BMP-7) are hypermethylated, with decreased transcription and expression of SOD-2 and BMP-7 proteins (Goldring & Marcu, 2012; Im & Choi, 2013).

In human chondrocytes, around 65% of CpG in −256 site of *IL-1β* promoter are methylated; incubation of chondrocytes with IL-1β/oncostatin decreases the methylation to 36% by reducing DNA methyl transferase-1 and consequently, gene expression of *IL-1β* is enhanced. GlcN increases −256 CpG methylation to 44%, and reduces IL-1β/oncostatin-induced *IL-1β* expression (Imagawa et al., 2011). Based on the evidence that NF-κB binding to the promoter of target genes reduces CpG methylation (Kirillov et al., 1996), Imagawa et al. proposed that GlcN enhances *IL-1β*-256 CpG methylation by reducing NF-κB nuclear translocation and binding to *IL-1β* promoter (Imagawa et al., 2011).

The effect of GlcN on epigenetics is probably substantial since OGT may *O*-GlcNAcylate methyltransferases, the carboxy-terminal domain of RNA polymerase II, the co-repressor switch-independent 3A–histone deacetylase, and core histones of the nucleosome and as a consequence, alter their activities (Hanover et al., 2012; Deplus et al., 2013).

In human osteoarthritic articular cartilage, GlcN does not appear to activate the synthesis of collagen II or of aggrecan, but may increase the release of proteoglycans (Bassleer et al., 1998b; Uitterlinden et al., 2006).

5. Conclusions

Oral bioavailability of HA is around 5% and that of CS of the order of 20%. HA and CS absorption is mediated by lymphatic transport and/or paracellular mechanisms and the low bioavailability might be secondary to the saturation of these processes. GlcN bioavailability approximates 45% and is mediated by membrane GLUT. The incomplete bioavailability of GlcN is due to an extensive first-pass effect through the hexosamine pathway in the epithelial cells of the intestine. Orally administered SYSADOA accumulate in the joints.

HMW-HA does not penetrate into the cell, except for its internalization by CD44 into chondrocytes and synoviocytes, and subsequent degradation by Hyal. HMW-HA prevents the inflammatory response in chondrocytes, osteoblasts, osteoclasts and synoviocytes triggered by ECM fragments and IL-1β, primarily by engaging TLR4, CD44 and/or ICAM1. Binding of HMW-HA to these membrane receptors impedes the binding of ECM fragments and the subsequent activation of the receptors. Moreover, HMW-HA binding to CD44 and ICAM1 promotes the release of IRAK-M and MKP-1 that block signal transduction pathways leading to the activation of transcription factors such as NF-κB.

Cell penetration of CS and its oligosaccharides is very limited; therefore, CS inhibits NF-κB nuclear translocation and the inflammatory reaction by engaging TLR4, CD44 and perhaps ICAM1, and preventing ECM fragments to trigger the inflammatory reaction. Moreover, by binding to TLR4, CD44 and perhaps ICAM1, CS may foster the release of IRAK-M and MKP-1. On the other hand, CS promotes HMW-HA synthesis and facilitates HMW-HA binding to CD44. In addition, CS contributes to maintain ECM integrity by increasing the expression of TGF-β1 and the synthesis of HA and collagen II. Finally, CS reduces the effect of BK.

The major effect of GlcN derives from its capacity to *O*-GlcNAcylate proteins, primarily IKKα and IκBα; as a consequence, GlcN reduces NF-κB nuclear translocation and abrogates the transcription of proteolytic and pro-inflammatory target genes. Moreover, diminished NF-κB activation increases the methylation of the promoter of pro-inflammatory genes and reduces their transcription. *O*-GlcNAcylation of ICAM1 increases its proteolysis by the proteasome. Finally, GlcNAc induces the synthesis of HA and CS. The great majority of studies aimed at elucidating GlcN mechanism of action have been conducted with GlcN-hydrochloride, therefore it cannot be assumed that the SYSADOA effect of GlcN-sulfate implicates identical mechanism of action.

The differences in mechanism of action between CS and GlcN explain why the combination of CS and GlcN is more effective than each individual drug. Moreover, based on the mechanism of action of SYSADOA, we may postulate that HA and CS will be more effective in early phases of OA when ECM fragments trigger the inflammatory reaction. On the other hand, GlcN should be more effective in more advanced phases of OA.

Conflict of interest statement

The author declares that there are no conflicts of interest

Uncited reference

Loeser, 2002

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