Irreversible Heavy Chain Transfer to Chondroitin*

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Background: Heavy chain transfer to hyaluronan oligosaccharides is irreversible.

Results: Chondroitin oligosaccharides are also irreversible heavy chain acceptors.

Conclusion: Heavy chain transfer to hyaluronan is preferred over chondroitin.

Significance: Irreversible heavy chain transfer is influenced by both the size and type of heavy chain acceptor.

We have recently demonstrated that the transfer of heavy chains (HCs) from inter-α-inhibitor, via the enzyme TSG-6 (tumor necrosis factor-stimulated gene 6), to hyaluronan (HA) oligosaccharides is an irreversible event in which subsequent swapping of HCs between HA molecules does not occur. We now describe our results of HC transfer experiments to chondroitin sulfate A, chemically desulfated chondroitin, chemoenzymatically synthesized chondroitin, unsulfated heparan sulfate, heparan sulfate, and alginate. Of these potential HC acceptors, only chemically desulfated chondroitin and chemoenzymatically synthesized chondroitin were HC acceptors. The kinetics of HC transfer to chondroitin was similar to HA. At earlier time points, HCs were more widely distributed among the different sizes of chondroitin chains. As time progressed, the HCs migrated to lower molecular weight chains of chondroitin. Our interpretation is that TSG-6 swaps the HCs from the larger, reversible sites on chondroitin chains, which function as HC acceptors, onto smaller chondroitin chains, which function as irreversible HC acceptors. HCs transferred to smaller chondroitin chains were unable to be swapped off the smaller chondroitin chains and transferred to HA. HCs transferred to high molecular weight HA were unable to be swapped onto chondroitin. We also present data that although chondroitin was a HC acceptor, HA was the preferred acceptor when chondroitin and HA were in the same reaction mixture.

Heavy chain (HC)2 transfer to hyaluronan (HA) is the only naturally occurring covalent modification of HA that has been reported. It is formed during both pathological (1–3) and developmental (4, 5) processes. The function of HC-HA is largely unknown, but it is clear that the transfer of HCs to HA, effectively, cross-links HA, stabilizing its tertiary structure between itself and other molecules of the extracellular matrix such as versican and pentraxin-3 (6, 7).

Iol is a serum-derived proteoglycan composed of a protease inhibitor (bikunin) and two HCs attached to a single chondroitin sulfate (CS) chain of bikunin via an ester linkage between a HC aspartate and the 6-OH of GalNAc on CS. In general, Iol is not synthesized locally but enters the tissue in serum through permeabilized vasculature. The enzyme TSG-6 is not constitutively expressed in tissues but can be induced by cytokines such as TNF-α and IL-1 (2). A variety of cell types produce TSG-6, including fibroblasts, peripheral blood mononuclear cells, and recently described mesenchymal stem cells (8–17). Induction of TSG-6 gene expression typically occurs in 2–4 h (8), and it is generally considered an acute phase protein.

The formation of HC-HA starts when TSG-6 removes an HC from inter-α-inhibitor to form a covalent HC-TSG-6 intermediate (18). In the presence of HA, TSG-6 rapidly transfers the HC to HA. In the absence of HA, or another HC acceptor, TSG-6 “shuffles” HCs between Iol molecules with two HCs and produces some forms with one HC or three HCs (19). TSG-6 is also known to shuffle HCs between larger HA molecules until an equilibrium is reached where the HCs are evenly distributed between individual HA molecules (20). Thus, HC transfer between larger HA molecules is a reversible event. In contrast, when TSG-6 transfers HCs to smaller HA molecules such as HA oligosaccharides 8–21 monosaccharides in length, this is an irreversible event where subsequent HC swapping cannot occur (20).

We and others (21) have reported that HC transfer to HA increases the avidity of leukocytes to HA. Little is known regarding the effect this has on leukocyte activation. There is evidence that CD44 “caps” (i.e. becomes more densely populated) on the surface of a mononcytic cell line in the region where it is bound to HC-HA (22). Subsequent HA degradation was observed by these cells, indicative of a phagocytic phenotype.

In this study, we investigated the ability of different glycosaminoglycans (GAGs), and an anionic polysaccharide with GAG-like chemophysical properties, to function as HC acceptors. We also tested the ability of these GAGs to function as irreversible HC acceptors.

EXPERIMENTAL PROCEDURES

Reagents—Human serum was used as our source of Iol (donor 736; Equitech-Bio Inc., Kerrville, TX). Whole serum was used, and Iol was not purified from the serum. Recombinant...
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Human TSG-6 (2104-TS; R&D Systems, Minneapolis, MN) was resuspended at 0.005 mg/ml. Two independently sourced chondroitin preparations were employed in this study: (a) chemically desulfated (acid-methanol treatment) shark cartilage chondroitin (400640; Seikagaku, Japan) or (b) chemozymatically synthesized chondroitin (quasi-monomodisperse with average molecular mass, 40 kDa; method of Tracy et al. (23)). The former preparation contains some residual sulfate groups (~6% of the disaccharide units), but the latter preparation completely lacks sulfation due to the in vitro synthetic scheme. In addition, the chemical desulfation process results in chain fragmentation. Quasi-monomodisperse unsulfated heparosan (average molecular mass, 29 kDa; method of Sismey-Ragatz et al. (24)) and HA (average molecular mass, 41 kDa; method of Jing and DeAngelis (25)) were also made by chemoenzymatic syntheses in vitro. Quasi-monomodisperse HA 1,000 kDa in size (molecular mass/number average molecular weight = 1.007) was purchased from Hyalose, L.L.C. (HYA-1000K-1; Oklahoma City, OK). The HA14 oligosaccharide, 14 monosaccharides in length, was kindly provided by Seikagaku (lot 010618). Heparan sulfate was purchased from Seikagaku (400700-1A). Chondroitin sulfate A (sturgeon notochord) was also purchased from Seikagaku (400658-1A); its disaccharide composition was 6% ΔDi05, 24% ΔDi65, and 70% ΔDi45 (as determined by fluorophore-assisted-carbohydrate electrophoresis (FACE) analysis; data not shown). Two different alginites were used (A2033 and A2158; Sigma-Aldrich). The antibody against Iol was purchased from Dako (A0301, Dako North America, Inc. Carpinteria, CA). Streptomyces hyaluronidase (100740-1, Seikagaku, East Falmouth, MA) was used at 8 microunits/μL of reaction volume. An HA ladder of different sizes of HA oligosaccharides and polysaccharides was generated by limited digestion of HA (80190; 1.7 kDa; Lifecore Biomedical, Chaska, MN) with ovine and polysaccharides was generated by limited digestion of HA (80190; 1.7 kDa; Lifecore Biomedical, Chaska, MN) with ovine testicular hyaluronidase (109169; Roche Applied Science). This was done by incubating 1 μl of a 12.8 μg/ml stock of testicular hyaluronidase with 100 μl of sodium hyaluronate at 1 mg/ml in 100 mM ammonium acetate, pH 7.0 for 15.5 h at 37 °C.

Heavy Chain Transfer and Swapping Reactions—The reaction volumes were 25 μl of PBS containing 1 mM MgCl2, 5% serum supplemented with 1.25 μg of HA, chondroitin, CS, heparan sulfate, heparosan, alginate, and/or 0.005 μg TSG-6. TSG-6 was always added last and marked the beginning of the incubation period (time 0). EDTA was used to stop the reaction by adding 0.5 μl of a 0.5 M (pH 8.0) solution. In HC-swapping studies, HCs were first transferred to HA or chondroitin molecules, and after a specified time, equal mass amounts of HA or chondroitin were added to the same reaction mixture for a specified time. All experiments included negative and positive controls, as described for each figure. Most experiments included internal replicates (i.e. incubations at different time points). For those that did not, they were repeated for confirmation at least one time.

Fluorophore-assisted Carbohydrate Electrophoresis—FACE was used to assess the purity of the HA oligosaccharides used in the size range study. This method has been described previously (26, 27).

Western Blot Analysis—Samples were electrophoresed on 4–15% mini-PROTEAN TGX gels (Bio-Rad) and blotted using the Bio-Rad nitrocellulose and Trans-Blot Turbo System. Samples of 25 μl with 1.25 μl of serum gave a strong HC signal on the blots with an Iol antibody (A0301, Dako North America, Inc. Carpinteria, CA; 1:8000 dilution). The molecular weight standard was purchased from Li-Cor (928-40000). The blots were blocked for 1 h with Li-Cor blocking buffer (927-40000; Li-Cor) and then probed with the Iol antibody (dilution 1:8000) in the blocking buffer with 0.1% Tween 20 for 1 h. The blots were washed 5× in PBS with 0.1% Tween 20 and probed with an IRDYE secondary antibody (Li-Cor; 926-32213) at 1:15,000 dilution in blocking buffer with 0.1% Tween 20 and 0.01% lauryl sulfate for 45 min. The blots were washed as described previously and imaged on an Odyssey Infrared Imaging System (Li-Cor).

RESULTS

Chondroitin and Hyaluronan Are Heavy Chain Acceptors, but Not Chondroitin Sulfate, Heparan Sulfate, Alginate, or Heparosan—We tested the ability of different GAGs to function as HC acceptors (Fig. 1). We also tested the ability of two different alginate preparations (described from the manufacturer as having low and medium viscosity) to function as HC acceptors. The rationale for testing alginate was that this polymer is a linear, anionic polysaccharide with GAG-like chemical properties. Human serum was our source of Iol (A, lane 1). We had two positive controls: (i) a HA oligosaccharide, 14 monosaccharides in length (HA14), that induces a gel shift when HCs are transferred from the chondroitin sulfate chain of Iol (250 kDa) to a single HC molecule (84 kDa) (lane 3), and (ii) the redistribution of the Iol band (250 kDa) to the top of the gel when HCs are transferred to a HA molecule with a mass of 1000 kDa (i.e. it is too large to enter the gel) (lane 4). Lane 10 portrays an important negative control in which Iol was incubated with TSG-6 alone, for the entire reaction period (4 h). In the absence of a HC acceptor, TSG-6 shuffles HCs between Iol molecules, producing Iol with an extra HC (i.e. three HCs), Iol lacking one of its two HCs (i.e. containing only one HC), and the release of free HCs. This has been previously demonstrated in an earlier study (20). At longer incubation times, such as 24 h, TSG-6 will disassemble Iol, completely releasing all HCs and bikunin (20). Furthermore, the doublet of bands at ~125 kDa is likely the covalently TSG-6–HC intermediate (18). Thus, in the present experiment, the Iol band profile will resemble the profile in lane 10 if the glycan added to the reaction mixture is not an HC acceptor. Thus, neither chondroitin sulfate (lane 6), heparan sulfate (lane 7), or the two alginate preparations (lanes 8 and 9), functioned as HC acceptors. In contrast, HC transfer to chemically desulfated chondroitin was observed as a cluster of bands (due to the polydisperse nature of this polymer), ranging from ~90–140 kDa (lane 5). We expanded this observation to include similar sizes of synthetic HA (41 kDa), chemozymatically synthesized unsulfated chondroitin (40 kDa), and unsulfated heparosan (29 kDa) (B). HA and chondroitin accepted HCs, but heparosan, a polysaccharide with the identical monosaccharide composition as HA but connected with different glycosidic linkages, did not.

FACE Analysis of Shark Cartilage Chondroitin—We used FACE analysis to confirm the disaccharide composition of our
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FIGURE 1. Chondroitin and hyaluronan are heavy chain acceptors, but not chondroitin sulfate, heparan sulfate, alginate, or heparosan. A, Western blot of human serum (lanes 2–10), incubated with TSG-6 and different glycans as listed in the table, and probed with a polyclonal antibody against Iαl (green). Molecular mass standards are shown in red. All reactions were for 4 h at 37 °C. Lane 1 is the molecular weight standard. Lane 2 shows intact Iαl (iii). In lane 3, all of the HCs from Iαl were transferred to an HA molecule 14 monosaccharides in length. In lane 4, all of the HCs from Iαl were transferred to 1000 kDa HA that is so large that it does not enter the gel (i). In lane 5, the HCs from Iαl were transferred to a mixture of different sizes of unsulfated chondroitin. In lanes 6–10, because the glycans in these lanes were not HC acceptors, HCs were shuffled among Iαl molecules, releasing free HCs (v) and forming Iαl with only one HC (iv) or three HCs (ii). Alginate 1 was the low viscosity preparation, and alginate 2 was the medium viscosity preparation. Equal amounts (μg) of glycans were added in each reaction condition. B, Western blot of human serum (lanes 2–9) incubated with TSG-6 and hyaluronan (41 kDa), chemoenzymatically synthesized chondroitin (40 kDa) and heparosan (29 kDa), as listed in the table, and probed with a polyclonal antibody against Iαl.

desulfated shark cartilage chondroitin preparation and the size distribution of individual chondroitin chains within this preparation (Fig. 2). The unsulfated chondroitin disaccharide (ΔDi0S) comprised 94% of the total GAG, with 3% composition for sulfation on the 6-OH of N-acetylgalactosamine (ΔDi6S) and 3% for sulfation on the 4-OH of this hexosamine (ΔDi4S). We labeled the individual, undigested, chains of chondroitin with AMAC and ANTS (lanes 3 and 6, respectively) to measure the size distribution of the GAG. For comparison, we labeled a HA ladder, generated by a limited digest with testicular hyaluronidase, with AMAC and ANTS (lanes 4 and 7, respectively). The resolution of the chondroitin bands was good but not quite as sharp as for HA. Our interpretation is that some of the chondroitin chains contained 4S and 6S, thus somewhat affecting the banding pattern and creating a cluster of bands on the gel. That being said, it is clear from the AMAC labeled chondroitin preparation that oligonucleotides as small as HA4 and -6 were detectable. It should be noted that AMAC labeled HA oligonucleotides do not migrate in the predicted pattern (i.e. from largest to smallest). This phenomenon has been described previously (14). Regardless, from HA8 and larger, the banding pattern for AMAC-labeled oligonucleotides is as expected (i.e. smaller oligonucleotides migrate faster). Because eight monosaccharides are the minimum size of HA that can function as a HC acceptor, chondroitin chains eight monosaccharides and above are the most relevant to this study (28). By comparing lanes 3 and 4, it is clear that our preparation of shark cartilage chondroitin contains chains at least as small as eight monosaccharides and as large as 40 or greater. This finding is also confirmed in lanes 6 and 7 that portray ANTS-labeled GAG chains. In our gel system, ANTS is only able to resolve GAG chains 14 monosaccharides and above. For this reason, we ran HA14 as a standard in lane 8. By comparing lanes 6 and 7, it is clear that the chondroitin preparation contains chains at least 14 monosaccharides in length and greater, ranging 40 monosaccharides and above. It should also be noted that the disaccharide composition of the chondroitin sulfate preparation from Fig. 1, lane 6, was 6% ΔDi0S, 24% ΔDi6S, and 70% ΔDi4S (as determined by FACE analysis; data not shown).

Kinetics of Heavy Chain Transfer to Chondroitin—In a previous study, we showed that HC transfer to HA, under defined experimental conditions, was detectable as early as 30 min after the addition of TSG-6 and complete between 2–4 h (20). We tested the kinetics of HC transfer to chondroitin under these same experimental conditions (Fig. 3). The kinetics of HC transfer to chondroitin were nearly identical to the kinetics of HC transfer to HA (11). Interestingly, the earliest (30 min) HC transfer to chondroitin was to higher molecular mass chains of chondroitin, as indicated by a 120-kDa band (lane 4). Thirty minutes later, HC distribution among different sizes of chondroitin chains was observed (lane 5). After 2 h, the HC distri-
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**FIGURE 2. FACE Analysis of shark cartilage chondroitin preparation.** Lane 1 portrays an AMAC-labeled glycan standard. Lane 2 portrays AMAC labeled disaccharides from a chondroitinase ABC digest of shark cartilage chondroitin. Lane 3 portrays full-length, undigested, shark cartilage chondroitin chains labeled with AMAC. Lane 4 portrays a limited testicular hyaluronidase digest of high molecular mass HA labeled with AMAC. Lane 5 portrays another AMAC-labeled glycan standard showing the migration of HA oligosaccharides 4 (HA4) and 6 (HA6) monosaccharides in length. It also shows bands for glucose, and the HA and OS disaccharides. The sample in lane 6 is the same as in lane 3, except that the chondroitin in lane 3 was labeled with AMAC, whereas in lane 6, the chondroitin was labeled with ANTS. Similarly, the sample in lane 7 is the same as in lane 4 except the HA ladder in lane 4 was labeled with AMAC, whereas in lane 7, it was labeled with ANTS. Lane 8 shows a band, toward the bottom of the gel, showing the migration of an HA oligosaccharide, 14 monosaccharides in length, labeled with ANTS. These gel conditions do not resolve ANTS labeled HA oligonucleotides smaller than HA14.

bution among the smaller sizes of chondroitin chains stabilized and did not change, significantly, for up to 8 h.

**Preferred Heavy Chain Transfer to Hyaluronan, Not Chondroitin**—Given the choice of HC transfer to HA or chondroitin, we wanted to determine whether TSG-6 had a GAG preference for HC transfer. To test this, we incubated human serum (i.e. our I/I source) with TSG-6 and combinations of HA14, HA1000K, and chondroitin (Fig. 4). When chondroitin and HA14 (lane 6) or chondroitin and HA1000K (lane 7) were mixed together with TSG-6 for 4 or 24 h (lanes 8 and 9, respectively), HCs from I/I (human serum) were preferentially transferred onto HA instead of chondroitin. Lanes 3–5 are controls where the GAGs were added separately. Lane 10 is a control where serum was incubated with TSG-6 alone to demonstrate the release of free HCs from I/I during a 24-h incubation period.

**Absence of Heavy Chain Swapping from Chondroitin to Hyaluronan**—Given the choice, TSG-6 prefers to transfer HCs to HA instead of chondroitin. We wanted to know whether HCs could be “swapped” from chondroitin to HA (Fig. 5). We transferred HCs to chondroitin for 24 h (lane 1) and then added HA14 from 1–24 additional hours (lanes 4–8). HCs transferred to chondroitin remained attached to chondroitin and could not be swapped onto HA14. Lane 9 is a control showing HC transfer to HA14 in the absence of chondroitin. Lane 10 is an end point control showing the stability of HC transfer to chondroitin in the absence of HA14 for 48 h.

**Irreversible Heavy Chain Transfer to Chondroitin**—The experiment portrayed in Fig. 6 is very similar to the one portrayed in Fig. 5 with the exception that HC swapping from chondroitin to HA1000K was also tested. In this experiment, HCs were transferred to chondroitin for 24 and 48 h to show the stability of the end product (lanes 3 and 4, respectively). After a 24-h incubation in which TSG-6 transferred HCs to chondroitin, HA14 was added to the reaction mixture for 4 and 24 additional hours (lanes 5 and 6, respectively). Similarly, after a 24-h incubation in which TSG-6 transferred HCs to chondroitin, HA1000K was added to the reaction mixture for 4 and 24 additional hours (lanes 5 and 6, respectively). As can be seen when comparing to controls showing complete HC transfer to HA14 and HA alone (lanes 7 and 10, respectively), once HCs were transferred to chondroitin, they could not be swapped onto HA.

Thus, we conclude that HC transfer to a certain size of chondroitin GAG chain is an irreversible event where subsequent swapping does not occur.

**Heavy Chains Cannot Be Swapped from Hyaluronan to Chondroitin**—In the experiment described in Fig. 4, we learned that, given the opportunity, TSG-6 prefers to transfer HCs to HA instead of chondroitin. In Figs. 4 and 5, we demonstrated that once the HCs are transferred to certain size of chondroitin, they cannot be swapped from chondroitin to HA (i.e. the process is irreversible). Previously, we have shown that TSG-6 can remove nearly all of the HCs from HC-HA1000K and swap them onto HA14 (20). This process was irreversible. In Fig. 7, we present data showing that, once transferred to HA, HCs cannot be swapped from HA to chondroitin. HC transfer to HA1000K for 24 h produces a HC-HA1000K complex that is too large to enter the gel (lane 3). During this incubation, a small portion of free HCs are released from HC-HA1000K, which appear as a faint 83-kDa band. After a 96-h incubation, more free HCs are released. This observation has been reported previously (20). Our interpretation is that, over time, TSG-6 will disassemble the HC-HA complex to release free HCs. When HA14 is added to the HC-HA1000K reaction mixture for 24 h after the HCs had been transferred to HA1000K, the intensity of the 83-kDa HC band increases, indicative of HC swapping from HC-HA1000K to HA14. After a 72-h incubation, the intensity of the 83-kDa HC band increases further. Comparison of the intensity of the HC-HA14 band in lane 6 with the HC-HA14 band in lane 7 (positive control) shows that HC swapping from HC-HA1000K to HA14 was nearly complete after 72 h. In contrast, the free HC bands in lanes 8 and 9, after a 24- and 72-h incubation with chondroitin, are much less than the HC-HA14 bands in lanes 5 and 6 (positive controls). The intensity of the free HC bands in lanes 8 and 9 more closely resemble the free HC bands in lanes 3 and 4 (negative controls). Lane 10 portrays how the hypothetical HC swapping to chondroitin would have appeared had it actually occurred. We conclude that, although HC swapping from HC-HA1000K to HA14 occurs, HC swapping from HA1000K to chondroitin does not.

**DISCUSSION**

The major conclusions of this manuscript are: (i) chondroitin, but not the structurally similar polyanionic carbohydrates heparosan, heparan sulfate, chondroitin sulfate, or alginate, is an HC acceptor; (ii) smaller sizes of chondroitin are irreversible HC acceptors; (iii) HA is a preferred HC acceptor compared
with chondroitin; and (iv) TSG-6 will not swap HCs from HC-HA to chondroitin. A schematic summary of our data is shown in Fig. 8.

Previous studies by Mukhopadhyay et al. (28) demonstrated that chondroitin, but not dermatan or chondroitin sulfate, was an HC acceptor. We have confirmed this observation and expanded it to exclude heparosan, heparan sulfate, and alginate, neither of which were found to be HC acceptors. Chondroitin and HA are identical in their chain linkage (1,3-N-acetylhexosamine linked 1,4 to GlcUA) with the only difference being that the hexosamines are C4 epimers: N-acetylgalactosamine for HA versus N-acetylglucosamine for chondroitin. Heparosan, an inactive GAG species, contains the same disaccharide composition as HA, but its linkage is different (1,4-GlcNAc linked to GlcUA).

It is intriguing that the axial position of the C4 hydroxyl of the hexosamine of chondroitin, and the different linkage of the heparosan disaccharide, has such a profound effect on HC transfer to these GAGs. In the latter instance, a different linkage structure of the same disaccharide unit in heparosan was sufficient to prevent HC transfer to this GAG. For chondroitin, the position of the C4 hydroxyl of GlcUA confers a preference of HC transfer from chondroitin to HA. This preference is most likely explained by increased affinity of TSG-6 for HA compared with chondroitin, a hypothesis that is supported by the study of Mukhopadhyay et al. (28). Although it is likely that the binding site of TSG-6 for HA (i.e. the link module) is the same as that for chondroitin, this has not been directly demonstrated.

Additionally, it is possible that a conformational change, induced by the formation of the TSG-6-HC intermediate,
might increase the affinity of TSG-6 for HA compared with chondroitin. In this model, the initial affinity of TSG-6 for chondroitin and HA might be roughly equivalent, but after TSG-6 removes a HC from I/H9251 I to form a TSG-6-HC intermediate, the affinity for HA, compared with chondroitin, may be increased.

The size of our shark cartilage chondroitin preparation ranged from 8 to >40 monosaccharides. This is significant because HA oligonucleotides 8–21 monosaccharides in length have been shown to be HC acceptors but not HC donors (11). In other words, HA oligonucleotides 8–21 monosaccharides in length are irreversible HC acceptors. Thus, we have shown that chondroitin can function as an irreversible HC acceptor (Fig. 6), and we conclude that this functionality is because HCs are being transferred or shuffled (Fig. 3) to chondroitin oligonucleotides, minimally, 8–21 monosaccharides in length as the upper size limit for irreversible HC transfer to HA oligosaccharides has not yet been determined.

Clearly, the interaction of TSG-6 with HA is a dynamic event in which the enzyme binds and then releases HA. Otherwise, it would be difficult to explain how HCs can be shuffled between HA molecules (19). This mechanistic point is important because it would be a misconception to conclude that, in the presence of chondroitin and HA, TSG-6 remains bound to HA. If so, then it is not clear how TSG-6 would release HA to remove a HC from the CS chain of I/H9251 I. Thus, our interpretation is that there is a dynamic equilibrium where, in a mixture of GAGs, TSG-6 is in a constant state of flux, repetitively binding and releasing HA.

Our studies and others imply that, in the absence of HA, TSG-6 shuffles HCs between I/H9251 I molecules, transferring an
additional HC to Iol, thereby producing some Iol molecules with only a single HC (Fig. 1) (20). In addition, whereas TSG-6 swaps HCs between Iol molecules, sometimes it makes a “mistake” and releases a free HC. In our reaction conditions, these free HCs appear between 2–4 h. After a 24-h incubation, so many mistakes have been made that all of the HCs are released. It is interesting that, within 2–4 h, TSG-6 had transferred all of the HCs from Iol/IH9251I onto chondroitin. In other words, the shuffling of HCs between Iol molecules (as shown in Fig. 1) did not occur when chondroitin was present. HC transfer appeared to be unidirectional, transferring HCs from Iol to chondroitin, but not the reverse. This observation is similar to what occurs with HA, and the kinetics were identical to our previously reported kinetic study of HC transfer to HA (20). It is not entirely clear to what extent “irreversible HC transfer” to chondroitin oligonucleotides contributed to this unidirectional HC transfer to chondroitin, in the absence of HA. It is not known whether the HCs could not be transferred back to the CS chain of Iol because they had been irreversibly transferred to chondroitin oligonucleotides, analogous to irreversible HC transfer to HA oligonucleotides (20).

Although the kinetics of HC transfer to chondroitin oligonucleotides, compared with larger chondroitin chains, was not examined in this work, it is unlikely that it would differ from the kinetics of transfer to a similar study performed on HA (20). In this study, identical HC transfer kinetics were observed when TSG-6 transferred HCs from Iol (human serum) to 1000 kDa HA and HA eight monosaccharides in length when an equal mass of GAG was used. That being said, the data in Fig. 3 demonstrates a time-dependent distribution of HCs on, what appears to be, higher molecular mass chondroitin chains onto smaller ones. It would be reasonable to predict that TSG-6 might, more frequently, come into contact with a larger chondroitin chain than several smaller chains due to the former molecules larger hydrodynamic size. However, this prediction was not observed as a difference in kinetics between HC transfer to HA1000K and HA8 (20). Our explanation for the different molecular mass distribution of HCs between different sizes of chondroitin chains is due to the irreversible HC transfer to smaller chondroitin chains. In this scenario, we explain the kinetic HC distribution to the lower molecular mass chondroitin chains as occurring because the HCs were being swapped off of higher molecular mass chondroitin chains onto smaller ones in the mixture of chondroitin chains.

It is somewhat surprising that CS was not a HC acceptor, considering that, under normal conditions, the HCs are attached to an undersulfated CS chain of Iol (29, 30). It would be reasonable to speculate that the CS preparation used in our study is too highly sulfated to allow HC transfer and that partial desulfation might permit partial HC transfer to our CS. It should be noted that, even though HCs are attached to an undersulfated CS chain of Iol, the sulfation of this chain is primarily present on the reducing terminus of the GAG, leaving the larger proportion of the non-reducing end unsulfated (29, 30).

Our data show that, although irreversible HC transfer to chondroitin occurs, HC swapping from HC-HA to chondroitin did not occur (Fig. 5). This was in contrast to HC swapping from HC-HA to HA14. Thus, unlike HA oligonucleotides, there seems to be little therapeutic potential for chondroitin as an artificial HC substrate for the removal of HCs from HC-HA (20). Nonetheless, HC transfer from Iol to chondroitin, and swapping between chondroitin chains, has already provided important insights into the mechanisms of TSG-6-mediated...
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TSG-6

A. 30 min

B. 4 hrs

C. 4 hrs

D. 4 hrs

E. 4 hrs

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