The influence of sulfated polysaccharides on the circulating levels of hyaluronan

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When chondroitin sulfate (CS) or dextran sulfate (DxS) was administered intravenously in rats the levels of circulating hyaluronan (HA) rapidly increased. 70 min after injection the levels were found to be about 10–20 times the initial values. Saline injections were without effect on HA levels. CS given intraperitoneally was found to give prolonged blocking of liver uptake of labeled HA and increased endogenous serum HA to about 10 times the initial level at 180 min. HA excretion in urine was dramatically increased by CS given intravenously, intraperitoneally as well as subcutaneously. Size-exclusion chromatography showed a mean MW of the circulating HA of around 50 kDa while urinary HA had a mean MW of about 10 kDa. Circulating HA has previously been shown to be very effectively cleared via receptor mediated endocytosis by reticuloendothelial cells, primarily liver endothelial cells. As CS and DxS bind to the same receptors and inhibits HA clearance, the effects of sulfated polysaccharides on inflammatory conditions and angiogenesis might be via HA, previously shown to affect these processes. Such a mechanism could also explain increased HA levels as a secondary event to increased CS and other sulfated biological polysaccharides in some physiological and pathological conditions.

Key words: hyaluronic acid/turnover/metabolism/glycosaminoglycans/angiogenesis

Introduction

Hyaluronan (hyaluronic acid; HA) is a polysaccharide consisting of repeating units of glucuronic acid and N-acetylglucosamine. It is found as a high molecular weight polymer in connective tissues such as skin and cartilage, in the vitreous body of the eye and in synovial fluid (Laurent and Fraser, 1989). The polysaccharide associates with several proteins in the extracellular matrix and interacts with some cell-surface HA-binding proteins (Knudson and Knudson, 1993).

Small amounts of “free” polysaccharide, about 20–40 mg/day in adult man (Tengblad et al., 1986), enters the general circulation via the lymph (Laurent and Fraser, 1981) after 80–90% is removed in lymph nodes before reaching the bloodstream (Fraser et al., 1988). The lymph node clearance is inhibited by chondroitin sulfate (CS) (Tzaicos et al., 1989).

The serum level of HA is normally very low (10–50 μg/l) due to rapid and efficient receptor mediated endocytosis by the liver (Fraser et al., 1981, 1986; Laurent and Fraser, 1992) in liver endothelial cells (LEC) (Erikkson et al., 1983; Fraser et al., 1985; Smedsrød et al., 1990; Smedsrød, 1991). Inhibition studies with LEC in culture (Smedsrød et al., 1984; Raja et al., 1988; McGary et al., 1989) as well as turnover and biodistribution studies in vivo (Fraser and Laurent, 1989; Gustafson and Björkman, 1997) show that the receptors recognize other ligands besides HA, such as CS and dextran sulfate (DxS). Heparin does not seem to inhibit the binding of high MW HA to LEC in vitro (Smedsrød et al., 1984; Smedsrød, 1991) or in vivo (Gustafson and Björkman, 1997).

CS and HA share not only the specificity for liver and lymph node receptors linked to intracellular degradation, but also for degrading enzymes (Roden et al., 1989). This lack of specificity in CS and HA catabolism gives a basis for ways of increasing the concentration of a polysaccharide in body fluids, without exogenous administration of the same polysaccharide.

The present study shows that systemically administered non-HA ligands for HA receptors such as CS and DxS can increase the circulating levels of HA.

Results

When the effect of CS on the turnover and biodistribution of intravenously administered labeled HA was studied in the rat, it was found that intravenous CS could effectively inhibit the receptor mediated liver uptake of 125I-T-HA at doses above 4 mg/kg body weight in short term experiments (Figure 1). However, when the liver uptake was studied by phosphoimaging in vivo over time, it was found that the inhibition caused by intravenous CS was lost after a few h, probably because the CS was rapidly lost from the bloodstream not only via liver uptake but primarily via kidney filtration as shown earlier (Gustafson and Björkman, 1997). When additional intraperitoneal injections of CS were given prior to the intravenous injections in order to achieve a slow release of CS into the bloodstream, the inhibition of HA liver uptake was significantly improved and very low liver uptake was seen at 24 h (Figure 2). CS given intraperitoneally about 1 h before injection of the 125I-T-HA was more effective than if given just before the injection of labeled HA and reduced liver uptake, as studied by phosphoimaging, to less than 20% of the control that only received CS intravenously (Figure 2).

As CS should inhibit the uptake also of endogenous HA via CS/HA receptors, we studied the effects on the circulating levels of HA after CS administration. The serum HA levels increased rapidly from the normal levels of 50–100 ng/ml to 500–900 ng/ml (about 10 times the initial value) at 70 min after injection of 5 mg highly pure CS C (90% 6-sulfate, 10% 4-sulfate) (Figure 3). The increase in total plasma HA was between 3.8 and 8.1 μg at 70 min; and as the CS preparation contained only 60 ng HA/mg CS, only 0.3 μg could be attributed to injected HA. Furthermore, at 70 min the urine, normally with HA concentrations less than 10 μg/ml, was found to have increased levels of up to 70 μg/ml at the 70 min time point (Figure 4). As normal urinary output is about 0.3–0.4 ml/h in the rat, the increased levels indicate an increased excretion in 70 min of up to 20–25 μg.
Fig. 1. Effect of intravenous CS on the blood levels (circles) and liver uptake (triangles) of intravenous $^{123}$I-HA in rats. Three rats were given CS A (solid symbols) and three rats CS C (open symbols) at the indicated different amounts 30 s prior to labeled HA, and the radioactivity in blood and liver determined 12-15 min later. The liver uptake was also determined in rats receiving no CS. For details, see the Materials and methods section.

Fig. 2. Phosphoimage of three rats that 24 h prior to imaging received intravenous injections of 5 mg CS C followed 30 s later by $1 \text{mg}^{123}$I-HA. In addition, rat B received an intraperitoneal injection of 25 mg CS C in connection with the intravenous injections, and rat C received 25 mg intraperitoneally 1 h before the intravenous injections. The region over the liver contained 3576 phosphoimaging density units (PD) in rat A, 2110 PD in rat B and 650 PD in rat C. See Materials and methods for details.

Fig. 3. Effect of intravenous CS on the levels of HA in rat serum. 0.5 ml of 10 mg/ml CS in sterile phosphate-buffered saline was injected in a tail vein of Sprague-Dawley rats. Before the injection, time 0, a blood sample, -0.5 ml, was collected. Blood was then collected from the cannulated vein at 10 and 20 min, and a larger blood sample from the aorta at 70 min. Serum was prepared and analyzed for HA. Open squares represents a rat receiving CS A that also contained 10 $\mu$g HA. Solid triangles represent mean values ± SD of three rats receiving CS C with only negligible amounts of HA.

When 5 mg CS A (80% 4-sulfate and 20% 6-sulfate, also containing 2 $\mu$g HA/mg CS) was injected intravenously, it was found that HA blood levels increased from 1 min (when it was already high due to the injection of 10 $\mu$g HA present in the preparation) up to 10 min and then appeared to stay at a steady state level significantly higher than when CS was given with only minute amounts of HA (Figure 3).

When the serum from blood drawn prior to injection and at 70 min were subject to size-exclusion chromatography on a calibrated column of Sephacryl, HA could be detected over a

Fig. 4. MW distribution of HA in rat serum and urine. Rat serum from an initial blood sample (open circles), a blood sample taken 70 min after an intravenous injection of 5 mg CS (solid circles), and urine collected at 70 min (open squares), were subject to size exclusion chromatography on a 50 ml column of Sephacyl S1000 and S300 calibrated with HA standards of known MW values (three standards indicated). After separation the fractions were analyzed for HA, and the total amount of HA present in the serum and urine was calculated from the volume of urine collected and total blood volume estimated to be 6% of the body weight.
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wide MW range with most of the material eluting with a mean MW of about 50–60 kDa in both cases (Figure 4). The urinary HA was of relatively low MW with a mean MW of around 10 kDa (Figure 4).

The effects of subcutaneous and intraperitoneal CS C on HA serum levels and the daily excretion of HA in rat urine were studied with animals in metabolic cages. The intraperitoneal injections gave significantly increased blood levels at 2 and 7 h that were back to normal levels at 24 h after injection (Figure 5), and also significantly increased urinary HA excretion during 24 h (Figure 6).

Subcutaneous CS C caused moderate but significantly increased serum HA levels at 2, 7, and 24 h after injection (Figure 5). The urinary HA was significantly increased over 48 h (Figure 6).

Also, DxS with a mean MW of 500 kDa can bind to liver CS/HA receptors \textit{in vivo} (Gustafson and Björkman, 1997) and in the present study was found to cause increased circulating HA levels similar to CS when injected intravenously (Figure 7).

When another semisynthetic sulfated polysaccharide of low MW, pentosan polysulfate (PPS), was tested for its ability to interact with LEC CS/HA receptors, a slight inhibition of liver uptake of $^{125}$I-HA from the circulation was noted (not shown). PPS inhibited $^{125}$I-HA binding to isolated rat LEC in culture in a dose-dependent fashion (Figure 8). Chondroitin was found to be less effective in inhibiting binding of labeled HA to LEC in comparison to CS (Figure 8).

Discussion

The biological polysaccharides CS and HA show shared specificity for liver and lymph node receptors that normally clear a major part of circulating HA (Smedsrod et al., 1984; Fraser and Laurent, 1989). I propose that this is the basis behind the present findings of increased HA levels following CS given intravenously, intraperitoneally as well as subcutaneously (Figures 3, 5). When CS or DxS (Figure 7), which also interacts with the receptors \textit{in vivo} (Gustafson and Björkman, 1997), saturates the binding sites this leads to the accumulation of endogenous HA in the bloodstream. As the endogenous influx is estimated to be about 40–80 mg/d in an adult human under normal circumstances (Tengblad et al., 1986), inhibition of liver uptake would result in a massive accumulation of HA in plasma. However, during the circulation the high MW HA is broken down to smaller fragments by a saturable mechanism that is not significantly inhibited by CS (Gustafson and Björkman, 1997), filtered by the kidneys, and found in increased amounts in the urine (Figure 6). If the degradation of HA in the circulation could be inhibited, blocking for 1 day would result...
in a concentration of HA in plasma of about 20,000 ng/ml by the end of the day, compared with the normal level of around 50 ng/ml.

Systemic administration of HA has been shown to reduce leukocyte adherence to activated endothelial cells in vitro and in vivo (Seed et al., 1995a,b), to be anti-inflammatory in animal models (Ialenti and Di Rosa, 1994) and to reduce stenosis of arteries after experimental vascular damage (Ferns et al., 1995). Our present findings indicate that CS treatment would be equally effective due to its effect on the HA levels by inhibition of its binding to receptors and degrading enzymes. The CS could be given by intravenous as well as intramuscular or subcutaneous injections. An intramuscular or subcutaneous injection will give a prolonged effect on the HA levels (Figure 5) as the injected material will stay for some time as a depot in the tissues and slowly be released into the general circulation.

An optimal treatment strategy in connection with, e.g., balloon angioplasty could be to give CS in combination with HA to achieve momentary as well as prolonged therapeutic levels of HA without giving high doses of HA or continuous infusions.

Cartilage products, containing high amounts of CS, as well as purified CS, have been used in the treatment of inflammatory conditions such as osteoarthritis by systemic administrations and found to increase the level and MW of HA in the joints as well as reduce the inflammatory reaction (Collier et al., 1991; Conte et al., 1995). Bovine as well as shark cartilage, rich in CS, has also been found to have antiangiogenic properties on tumors (Langer et al., 1976; Lee and Langer, 1983). While short-term experiments on chicken wing buds failed to show an antiangiogenic effect of CS itself, high MW HA was found to be antiangiogenic (Feinberg and Beebe, 1983). The mechanism presented here could be the reason for the antiangiogenic properties of cartilage with the active substance being primarily CS working via inhibition of CS/HA binding sites linked to catabolism and thereby causing a secondary increase in high MW HA. The rate of catabolism at any given site would be the major factor that determines the rate of HA increase at this site and in the surrounding fluid. It is also possible that CS could knock off HA from CS/HA receptors not involved in uptake and degradation. The presence of CS/HA receptors and hyaluronidas will vary from tissue to tissue, but it is likely that, e.g., macrophages attracted to sites of inflammation or transformation will add to the catabolism of HA and such sites would therefore respond more to inhibitors than other, more healthy, sites. It is likely that systemic CS would be more effective as inhibitor than systemic high MW HA as the relatively low MW of natural CS, around 30 kDa, will penetrate out into the tissues and affect the HA levels locally, e.g., close to a tumor, in the joint, or in the retina. Low MW HA should be less effective as HA fragments have been reported to be angiogenic and HA with medium high MW around 30 kDa to be without effect on angiogenesis (Lees et al., 1995). The CS/HA receptors have also been shown to bind CS with a 3-fold higher affinity than HA of the same degree of polymerization (Laurent and Fraser, 1992), and exogenous CS will therefore be more effective than exogenous HA in inhibiting endogenous HA uptake and degradation.

Vertebrate hyaluronidas are generally active also on other GAGs like CS, dermatan sulfate and heparin (Afify et al., 1993). Increased hyaluronidas activity has been associated with prostate cancer progression (Lokeshwar et al., 1996), probably related to increased production of low MW HA fragments supporting tumor neovascularization (Rooney et al., 1995). Substrate inhibition of the enzyme by, e.g., heparin should reduce angiogenesis in this type of tumors. However, heparin does not seem to be recognized by CS/HA receptors in vivo (Gustafson and Björkman, 1997) and has in several studies been found to be angiogenic (Folkman et al., 1983). This has been explained by the binding, stabilization, and tissue release of angiogenic growth factors by heparin. Such binding and activation of growth factors has not been found for HA or CS, and PPS, with anticoagulant activity, has been shown to block angiogenesis induced by heparin binding of growth factors and to inhibit tumor growth in vivo (Zugmaier et al., 1992). CS, acting on both receptor and enzyme, and lacking potentially hazardous anticoagulant activity, therefore appears to be an excellent choice to reduce HA breakdown and increase HA concentration and mean MW in the body.

CS is a natural polysaccharide that is constantly present in higher animals and its activity and biocompatibility in humans and animals will be assured if properly purified and prepared under pharmaceutical control. A high MW will give better binding to the CS/HA receptors (Laurent and Fraser, 1992). However, to achieve the effect also on the lymph nodes and in peripheral tissues, a medium high MW (20–40 kDa) is preferred, as these molecules will distribute also in the interstitial fluid and lymph. As CS has been shown to reach high levels in blood between 2 and 24 h after oral administration of 0.8 g in humans (Conte et al., 1995), oral CS could be a convenient way of sustaining a high HA level, e.g., in the treatment of inflammatory conditions or after balloon angioplasty. However, as some CS will be broken down and desulfated before reaching the bloodstream, and such low MW material will bind with less affinity to the receptors (Figure 8), oral treatment will have to be further tested for the effect on HA levels and MW distribution at several different doses and formulations.
probably have limited use due to toxicity and immunological reactions.

Another novel finding was the high urinary clearance of HA found in the rat (Figure 6). It should be pointed out that the lymphatic HA normally entering the circulation has a very high MW in the order of several million Da (Tengblad et al., 1986). When CS is given it will distribute in interstitial fluid and lymph where it can inhibit the uptake of HA by lymph nodes (Tziaicos et al., 1989). This will result in increased amounts of high MW HA bypassing the nodes and entering the general circulation in comparison to the normal situation. The saturable mechanism breaking down HA in the bloodstream (Gustafson and Björkman, 1997) therefore seems to have quite a high capacity, at least in the rat, as most of the low MW HA found in urine most likely was produced in the circulation from high MW material. The importance of this mechanism needs further study in other species, but could be important in polysaccharide metabolism and in understanding and treating diseases of the urinary tract as well as in the use of polysaccharides in drug delivery (Gustafson, 1997).

The CS inhibition of HA catabolism can also, at least in part, explain some phenomena in HA metabolism observed under pathological conditions that previously have been hard to explain or been explained by effects on HA synthesis. In studies where the kidneys are blocked surgically the serum levels of HA increase 5- to 10-fold in 60 min (Engström-Laurent and Hellström, 1990). The effect was explained as a previously unrecognized uptake and degradation mechanism in the kidney. However, in view of the present results another plausible explanation is that the increase is secondary to CS blocking of the liver receptors. The blocking of CS excretion via the kidney, which is the predominant way of clearance from the circulation of this polysaccharide (Gustafson and Björkman, 1997), will give accumulation of CS in plasma, extensive binding of CS to the liver CS/HA receptors and a subsequent inhibition of HA binding and uptake by LEC. This could also be the explanation behind the increased blood levels of HA seen in uremia (Laurent et al., 1996). More studies on the interactions between polysaccharide binding sites and their ligands are needed in order to understand more fully the biological role of polysaccharides and optimize their use in medicine.

Materials and methods
Polysaccharides

The HA used for labeling and uptake- and turnover-studies was supplied by Hyal Pharmaceutical Corporation (HPC), Toronto, Canada. The molecular weight distribution of the HA was determined by chromatography on a calibrated column of Sephacryl HR with porosities noted as 400, 1000, and 2000 MW (Pharmacia, Uppsala, Sweden) in 0.25 M NaCl, 0.05% chlorobutanol (Lebel et al., 1989). The HA content in each fraction was monitored by determination of the absorbance at 214 nm. Radioactivity was measured by gamma-counting on a Packard auto-gamma gamma-counter.

Chondroitin sulfate A from bovine trachea, pentosan polysulfate, and chondroitin sulfate C from shark cartilage were from Sigma Chemical Co., St. Louis, MO. CS A contained 1.9 ng HA/mg and given to cultures of 100,000-250,000 liver endothelial cells/cm² in fibronectin-coated dishes with a diameter of 16 mm. The cultures were kept under standard culturing conditions in RPMI medium supplemented with L-glutamine (2 mM), gentamicin (50 µg/ml) and, in the case of parenchymal cells, 10% (v/v) fetal calf serum. Liver endothelial cells were cultured entirely without serum. All cells were cultivated overnight before the start of the experiments.

Uptake studies with cells in culture

In vivo studies
Sprague-Dawley rats, weighing 200–300 g, were fed standard rat chow and water ad lib. In order to repeatedly collect urinary samples for studies on the excretion of low MW HA, some experiments were performed with the animals in metabolic cages for up to 96 h. For intravenous injections and blood sampling from the tail vein, the rats were anesthetized with an intraperitoneal injection of a combination of midazolam (3 mg/kg body weight (bw)), fentanyl (0.2 mg/kg bw), and fluanisone (6 mg/kg bw).

In turnover studies of labeled polysaccharide, the animals received an injection in the tail vein of 125I-T-HA (8–15 × 10⁶ c.p.m., in 0.8–1.0 ml 0.15 M NaCl, 10 mM NaH₂PO₄, pH 7.4. In some studies the rats received unlabeled CS intraperitoneally and intravenously prior to the labeled polysaccharide. Liver uptake and urinary excretion of labeled HA was followed by scintigraphy. In some cases the rats were killed and a large blood sample taken from the aorta as well as a sample of liver tissue. The samples were assayed for radioactivity as previously described (Gustafson and Björkman, 1997). Total blood volume was estimated as 6% of the body weight.

Size exclusion chromatography of serum and urine

Two hundred microliter samples of serum or urine were subjected to size exclusion chromatography on a 50 column of Sephadex S1000 and S300 calibrated with HA standards of known MW values. After separation the fractions were analyzed for HA, and the total amount of HA present in the serum and urine was calculated from the volume of urine collected and total blood volume estimated to be 6% of the body weight.

Scintigraphic studies

The rats were anesthetized and injected as described above. In dynamic studies the injections were made with the rats placed on a Bio-Rad phosphoimage screen with a high resolution brass collimator between rat and screen. The screen was exposed for 10 min and the image developed and analyzed on a Bio-Rad GS-525 phospho-imager using the Molecular Analyst software on a Macintosh 7200/90 computer.

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cetylpyridinium chloride (Lindahl et al., 1965) was a kind gift from professor Ulf Lindahl, University of Uppsala, Sweden.

Labeling of HA

The HA was labeled with DL-tyrosine (Sigma Chemical Co.) as described previously (Gustafson et al., 1994), after CNBr-activation of the polysaccharide. The specific radioactivity was 2000–6000 d.p.m./ng. The 125I-T-HA kept a high molecular weight-profile upon gel filtration chromatography with a mean MW of around 0.5 × 10⁶ Da, and was found to be cleared from the circulation with the kinetics and organ distribution reported for biosynthetically labeled HA of high MW.

Cells

A single cell suspension was prepared from the liver of Sprague Dawley rats, weighing 200–300g, by collagenase perfusion for 10 min at 37°C. Liver endothelial cells, Kupffer cells, and parenchymal cells were purified by Percoll centrifugation and selective adherence as described by Pertoft and Smedsrod (1987), giving ~95% pure cells.

Monolayer cultures were maintained under standard culturing conditions in RPMI medium supplemented with L-glutamine (2 mM), gentamicin (50 µg/ml) and, in the case of parenchymal cells, 10% (v/v) fetal calf serum. Liver endothelial cells were cultured entirely without serum. All cells were cultivated overnight before the start of the experiments.
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References


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