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Uptake of hyaluronan in hepatic metastases after blocking of liver endothelial cell receptors

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To follow the biodistribution of exogenous hyaluronan in tumor-bearing animals, a total of seventeen inbred rats with hepatic metastases from a colonic adenocarcinoma received ^{125}I-labelled hyaluronan by intravenous injections. Group I received only labeled hyaluronan (25 μg), whereas group II received 2.5 mg chondroitin sulphate prior to labeled hyaluronan, to block receptor uptake in normal liver endothelial cells. Animals in group III received intravenous, as well as intraperitoneal chondroitin sulphate (2.5 mg), to see if a better and prolonged blocking could be achieved. Radioactivity was visualized by whole body autoradiography, using phosphorimaging and the average radioactivity determined as phosphoimaging density units of the total area of hepatic metastases, normal liver, and skeletal muscle by computer-based image analysis. At 5 h, tumors in groups II and III showed higher uptake (4.8 ±1.8, *P* = .01 and 3.6 ±1.1, *P* = .01, respectively), in comparison to group I (1.8 ±0.6), and the mean normal liver/tumor concentration ratio was reduced from 21.4 ±10.1 in group I to 5.7 ±2.7 in group II and 3.5 ±1.1 in group III (*P* = .008 and *P* = .01, respectively).

Our study shows that hyaluronan targets liver metastases of a colon adenocarcinoma. Furthermore, chondroitin sulphate pretreatment increases tumor uptake, while uptake at normal receptor sites is significantly reduced. The results also suggest that after blocking of normal hyaluronan/chondroitin sulphate receptors in healthy tissue, hyaluronan may be used to deliver drugs to specific hyaluronan receptor-positive sites of pathology.

Keywords: ***Hyaluronic acid, hepatic metastases, phosphoimager***

Introduction

The liver is one of the most common sites for recurrent disease following surgery for colorectal cancer [1]. Radical surgery of hepatic metastases is rarely possible, and most patients are treated with cytotoxic agents. 5-Fluorouracil (5-FU) alone or in combination with other drugs is frequently used to achieve a regression of the tumor [2]. Resistance to cytotoxic drugs is one important obstacle to successful treatment [3]. In addition, the drug must gain entrance into the tumor, where several properties, such as poor vascular supply [4] and high-osmotic pressure [5], prevent efficient uptake of chemotherapeutic agents into tumor tissue. A possible way to promote drug penetrance in tumors would be to alter its physical characteristics by using a carrier substance that specifically targets the tumor. Hyaluronan is an endogenous macromolecule that is cleared from the circulation via receptor mediated uptake in liver endothelial cells. The hyaluronan receptors in these cells are not downregulated after ligand binding and also recognize chondroitin sulphate [6, 7]. In addition to these "normal" receptors, we have found that hyaluronan has the ability also to accumulate in tumors [8–10] and that tumor cells derived from a chemically induced rat colorectal carcinoma carry saturable hyaluronan binding sites that do not bind chondroitin sulphate [8, 11].

The aim of this study was to analyze the uptake of labeled exogenous hyaluronan in hepatic metastases after inhibiting the rapid elimination of hyaluronan through saturation of the liver receptors by chondroitin sulphate.

Materials and methods

Animals

Thirty-six inbred female Wistar rats (Möllegaards Ltd., Denmark), weighing 170–210 g (mean 193 g), were given free access to a standard laboratory diet and water. A 10-day period of acclimatization preceded the first surgical procedure. The experiments were approved by the regional Ethics Committee for Animal Research, Uppsala, Sweden.

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Tumor cells

The experimental tumor was a colonic adenocarcinoma of rat origin [12]. The tumor cells were grown as a monolayer culture in a nutrient mixture composed of Ham's F-10 (Flow Laboratories, Swedish AB, Stockholm, Sweden) supplemented with 10% fetal calf serum, 2mM L-glutamine, penicillin (20 U/ml), and streptomycin (20 μg/ml). Tumor cell aggregates were achieved mechanically with a scraper (Cell Lifter, Costar Corporation, Cambridge, Massachusetts).

Polysaccharides

The hyaluronan used for labeling and turnover studies was of bacterial origin, supplied by Hyal Pharmaceutical Corporation (Toronto, Canada), and contained less than 0.2% protein. The mean molecular weight (Mw) was 400 kDa, with approximately 95% of the material sized between 100 kDa and 2000 kDa, as determined by size exclusion chromatography [13]. The hyaluronan was labeled with ^{125}I-tyrosine (T) by a method that does not change the Mw of the polysaccharide or its binding to cell surface receptors [14]. Chondroitin sulphate-A from bovine trachea was obtained from Sigma Chemical Company (St. Louis, Missouri; product number 8529). This batch contained 1.9 ng hyaluronan/μg chondroitin sulphate as determined by a specific radioassay for hyaluronan (HA-50, Pharmacia, Uppsala, Sweden). The mean Mw was found to be approximately 30000 Da, by gel filtration chromatography on Sephacryl S-1000 and S-300, calibrated with hyaluronan standards under conditions previously described [15].

Surgical procedures

The animals were anesthetized with fentanyl:midazolam:sterile water (1:1:2), 3.3 ml kg^{-1}, given intraperitoneally. The tumor inoculation was performed as previously described [16]. Briefly, a peripheral branch of the superior mesenteric vein was isolated, opened, and cannulated with a plastic catheter (Polyethylene Tubing, PP 380, Swevet AB, Stockholm, Sweden). A suspension containing 5×10^6 viable tumor cells in 100–200 μl saline was injected during a 20 sec period. After irrigation, the catheter was withdrawn and the vessel ligated. Postoperatively, the animal weights were registered daily. After 3 weeks, the rats were randomly allocated to three different groups, and treated with intravenous injection in the caudal vein (IV-inj) or intraperitoneally (IP-inj). In group I, the animals received IV-inj, 25 μg labeled hyaluronan [14]. Animals in group II received 2.5 mg chondroitin sulphate by IV-inj, 3 min prior to labeled hyaluronan. In group III, the animals received similar treatment as group II, with the addition of 2.5 mg chondroitin sulphate by IP-inj, 30 min after labeled hyaluronan. The animals were sacrificed after 30 min (n=7) or 5 h (n=9). One animal was killed approximately 1–2 min after injection of a high dose of unlabeled hyaluronan, followed by a tracer dose of labeled hyaluronan to observe the distribution of circulating hyaluronan when all hyaluronan binding sites were blocked to estimate tumor vascularity. During the injection of the radioactivity, distribution in the whole body was controlled by a handheld Geiger counter. At completion of the experiment, animals were killed in CO_2 chamber.

Phosphoimaging

After sacrifice, the rats were immediately frozen in hexane and cooled with dry ice to −78°C for 20 min. The frozen rats were then mounted in an aqueous gel of carboxymethyl cellulose, which was rapidly frozen around the animals. Sagittal whole-body sections, 10 or 20 μm thick, were attached onto a tape (No. 810, Minnesota Mining & Manufacturing Co., USA). The sectioning was performed at −20°C with a cryomicrotone (PMV Co, Stockholm, Sweden) as previously detailed [17, 18]. The sections were freeze dried and placed on a Bio-Rad phosphoimager screen. The screen was exposed for 10 days, and the image developed and analyzed on a Bio-Rad GS-525 Molecular Imager. The average radioactivity was determined as phosphoimaging density units. Between 30 to 40 sections of each rat were examined. The observer was not aware of the group to which the animal belonged.

Statistical methods

Individual metastases in the animals and the differences between groups were analyzed with Mann-Whitney U test. A P value of less than .05 was considered statistically significant.

Results

Metastatic growth

Sixteen rats had a total of 50 hepatic metastases (Table 1). Nineteen animals had no hepatic metastasis and were excluded from further analysis. The hepatic metastases were evenly distributed in the livers. When given after a high dose of unlabeled hyaluronan, the injected labeled hyaluronan was well distributed in the general circulation

Table 1. Number of rats and (hepatic metastases) in the three groups

Group I	*II*	*III*
4(9)	8(34)	4(7)

Group I: Not inhibited by chondroitin sulphate.
Group II: Inhibited by intravenous chondroitin sulphate.
Group III: Inhibited by intravenous and intraperitoneal chondroitin sulphate.

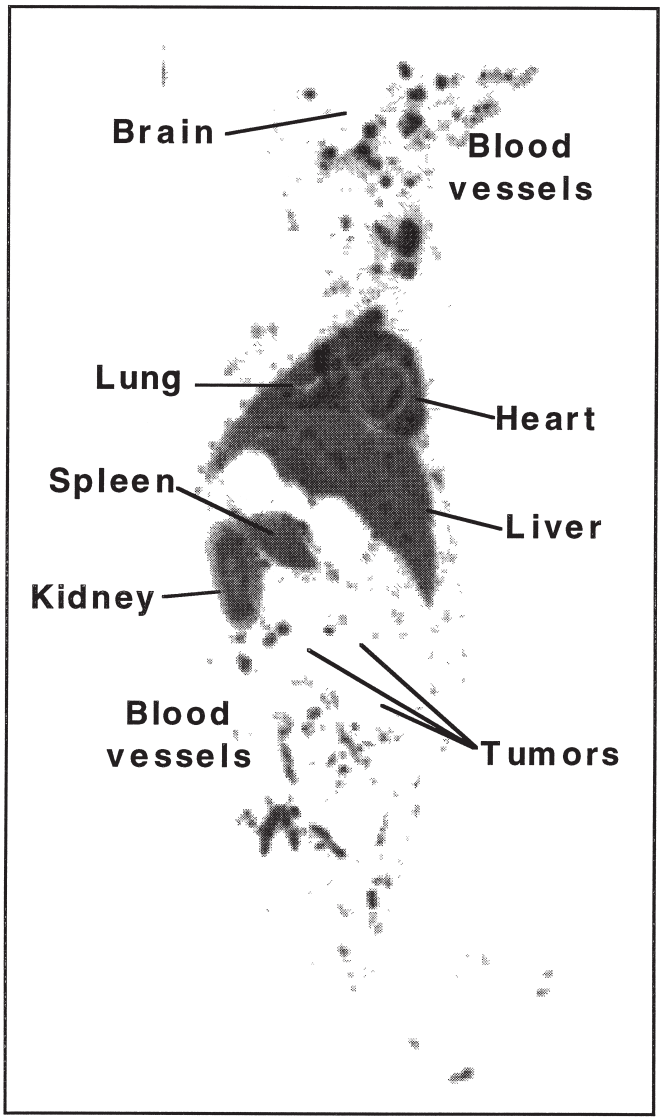

Figure 1. Autoradiograph of a whole-body section of a rat receiving 5 mg unlabeled hyaluronan and 30 sec later 20 μg labeled hyaluronan. Approximately 2 min later, the rat was killed and frozen.

a few minutes after injection, and the activity over the tumors was low (Figure 1).

Patterns of metastatic and hepatic uptake

After 30 min, there was no clear difference in tumor uptake between the groups (seven rats; data not shown). At 5 h, the radioactivity in the hepatic metastases was higher in group II than in group I (Table 2, $P = .01$), and was clearly visible by autoradiography (Figure 2). This difference was also present in group III compared with group I (Table 2, $P = .01$).

The liver/tumor ratio was higher in group I than in group II after 5 h (Table 3, $P = .008$). The liver/tumor ratio was also higher in group I compared with group III (Table 3, $P = .01$). Group II showed slightly higher liver/tumor ratio compared with group III (Table 3, $P = .05$).

Table 2. Concentration of radioactivity in hepatic metastases and skeletal muscle after 5 h. Figures are mean (SD) in the experimental groups and individual animals in phosphoimaging density units

	Tumor	*Muscle*
Group I	1.8 (0.6)	0.1 (0.1)
Animal 1	1.4 (—)	0.3 (—)
Animal 2	1.9 (0.7)	0.1 (—)
Group II	4.8 (1.8)	0.5 (0.2)
Animal 1	5.7 (2.7)	0.7 (—)
Animal 2	4.3 (1.8)	0.5 (—)
Animal 3	4.5 (—)	0.2 (—)
Group III	3.6 (1.1)	0.7 (0.4)
Animal 1	2.8 (0.4)	0.4 (—)
Animal 2	3.9 (0.8)	0.5 (—)
Animal 3	4.6 (1.3)	1.2 (—)
Animal 4	2.4 (—)	0.4 (—)

For definition of groups, see Table 1.

Discussion

We reported earlier that exogenous hyaluronan targets experimental tumors in animals [9, 10]. In those studies, the

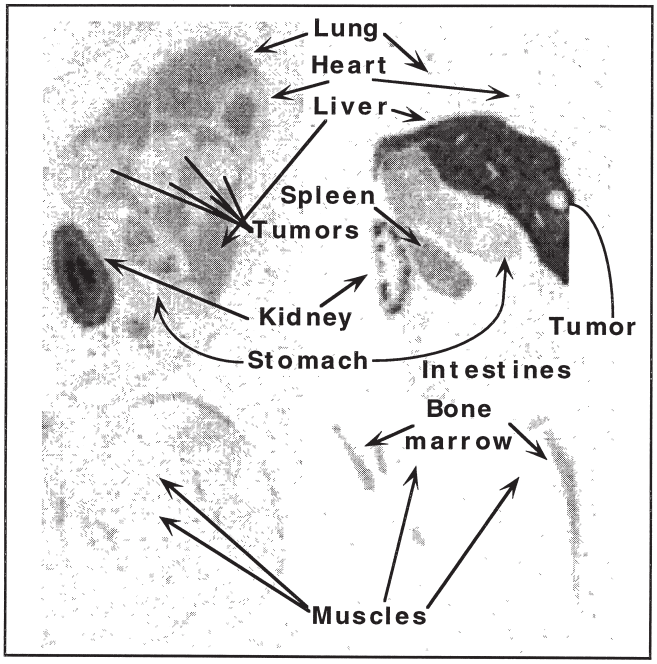

Figure 2. Autoradiographs of whole-body sections of two rats. The left autoradiograph is of a rat receiving chondroitin sulphate prior to labeled hyaluronan, and the autoradiograph to the right is of a rat receiving only labeled hyaluronan.

Table 3. Liver uptake and liver/tumor ratio of the radioactivity concentration at 5 h. Figures are mean (SD) in experimental groups and individual animals. Liver uptake figures are in phosphoimaging Density units. See Table 2 for tumor uptake

	Liver uptake	*Liver/tumor ratio*
Group I	35.9 (13.1)	21.4 (10.1)
Animal 1	16.3 (—)	11.5 (—)
Animal 2	42.4 (—)	24.7 (—)
Group II	24.9 (11.3)	5.7 (2.7)
Animal 1	37.6 (—)	7.4 (3.6)
Animal 2	15.8 (—)	4.3 (2.1)
Animal 3	35.7 (—)	7.9 (—)
Group III	11.6 (1.3)	3.5 (1.1)
Animal 1	12.4 (—)	4.5 (0.7)
Animal 2	9.7 (—)	2.6 (0.5)
Animal 3	12.5 (—)	2.9 (0.8)
Animal 4	11.7 (—)	4.8 (—)

For definition of groups, see Table 1.

tumors originated from cells not known to bind hyaluronan by any high-affinity mechanism *in vitro*. The hyaluronan was found to localize mainly in tumor vessels or over cells close to vessels [9, 10]. When studying hyaluronan binding to tumor cells *in vitro* to find and characterize high-affinity receptor mechanisms, we observed that a cell line from a chemically induced tumor in Wistar rats (NGW) had the ability to bind hyaluronan with high affinity. A specific, Mw-dependent and saturable binding with a Kd of about 1 nM for a 450 kDa polysaccharide was observed [11]. Therefore, we used this cell line to produce metastases in rats for the present *in vivo* study, which indicates that the cells have accessible hyaluronan binding sites also *in vivo*. In contrast to the receptors on liver endothelial cell, the receptors on the tumor cells are more specific for hyaluronan, and the present results show that the specific hyaluronan tumor targeting is not significantly reduced even at high chondroitin sulphate doses (Table 2), indicating that the majority of the hyaluronan binding sites in the tumor do not recognize chondroitin sulphate *in vivo*. The chondroitin sulphate blocking—C-4-S and C-6-S are equally efficient [15]—will cause an accumulation of endogenous hyaluronan in the circulation by time [15], resulting in saturation of the more specific hyaluronan binding sites on the tumor cells. It is therefore important to administer the labeled material as quickly as possible after the chondroitin sulphate bolus dose while the binding sites are still largely unoccupied [19]. The duration of the chondroitin sulphate blocking is also rather short due to the fact that the Mw of the chondroitin sulphate is low enough to allow filtration out of the cirulation and an elimination via the kidneys [15]. It is therefore necessary to give repeated or slow-release doses of chondroitin sulphate until only small amounts of the labeled hyaluronan remains in the circulation to avoid uptake in liver endothelial cell by time [15] (Table 3). The increase in radioactivity seen in normal muscle after chondroitin sulphate blocking (Table 2) is most likely due to the formation of low, Mw-labeled hyaluronan generated by cleavage of the polymer in the circulation and subsequent redistribution into the interstitial space [7]. This will give an unfavorable tumor/skeletal muscle concentration ratio at the time points studied; however, the overwhelming part of these low Mw fragments will be excreted via the kidneys into the urine over 24 h [15], resulting in very low activities in skeletal muscle and other sites where no specific uptake occurs. The activity in the receptor positive tumors will, however, only be marginally affected as the hyaluronan is more firmly associated with this tissue, and the tumor/muscle concentration ratio therefore is likely to be more favorable at 24 h than 5 h. This has also been shown for tumors of the same origin but growing as solid tumors and studied 20 h after injection [8, 19]. That the tumor/muscle concentration ratio was higher (approximately 16) for the solid tumors could also be due to the facts that the labeled hyaluronan could be given as early as 30 sec after chondroitin sulphate and also in a lower dose [8, 19]. This should result in better blocking of binding sites in normal tissue and less competition from endogenous hyaluronan during the first minutes after injection. The blood supply also appears to be poor in the metastases, where it is low (Figure 1).

Our study shows that differences in specificity between hyaluronan binding sites can be used to block uptake of the polysaccharide in healthy tissue without reducing targeting to metastases originating from tumor cells with specific hyaluronan receptors. It is possible that targeting of hyaluronan and hyaluronan/drug complexes to accessible, unoccupied, specific hyaluronan binding sites can be used for more efficient drug treatment with reduced side effects. The affinity between 5-FU and hyaluronan is low, indicating limited possibilities to direct 5-FU to hepatic metastases. Further studies are needed to test the possibility of targeting other cytotoxic agents to hepatic metastases.

Acknowledgments

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References

1 Gilbert JM, Jeffrey I, Evans M, Kark AE (1984) *Br J Surg* **71**: 203–5.

2 Köhne-Wömpner CH, Schmoll HJ, Harstrick A, Rustum YM (1992) *Semin Oncol* **19**: 105–25.

3 Csóka K, Larsson R, Tholander B, Gerdin E, de la Torre M, Nygren P (1994) *Gynecol Oncol* **54**: 163–70.
4 Jain RK (1988) *Cancer Res* **48**: 2641–58.
5 Jain RK (1994) Sci Amer: 58–65.
6 Laurent TC, Fraser JRE (1992) *FASEB J* **8**: 2397–404.
7 Gustafson S, Björkman T. (1997) *Glycoconjugate J* **14**: 561–8.
8 Gustafson S (1997). *In The Chemistry, Biology and Medical Applications of Hyaluronan and Its Derivatives* (Laurent TC ed). London Portland Press.
9 Gustafson S, Björkman T, Forsberg N, Lind T, Wikström T, Lidholt K (1995) *Glycoconjugate J* **12**: 350–5.
10 Gustafson S, Björkman T, Wikström T, McCourt P, Forsberg N, Graf W (1995) Targeting of hyaluronan to tumours via binding to ICAM-1. In *"3rd International Workshop on Hyaluronan in Drug Delivery*." Royal Soc Med Press, Round Table Series 40 pp 1–9.
11 Samuelsson C. Gustafson S (1997) *Glycoconjugate J* **15**: 169–75.
12 Steele G Jr, Sjögren HO (1974) *Cancer Res* **34**: 1801–7.
13 Lebel L, Smith L, Risberg B, Laurent TC, Gerdin B (1989) *Am J Physiol* **256**: 1524–31.
14 Gustafson S, Björkman T, Westlin J-E (1994) *Glycoconjugate J* **11**: 608–13.
15 Gustafson S (1997) *Glycobiology* **7**: 1209–14.
16 Graf W, Sundin A, Glimelius B, Ahlström H, Carlsson J (1992) *Eur J Surg Oncol* **18**: 608–14.
17 Ullberg S, Larsson B, Tjälve H (1982) Autoradiography. In *Biologic Applications of Radiotracers* (Glenn HJ ed) pp 55–108. Boca Raton, Fl.; CRC Press, Inc.
18 Dencker L, Larsson B, d'Argy R, Ullberg S, Tjälve H (1991) *Pure Appl Chem* **63**: 1271–7.
19 Gustafson S, Björkman T (1997) *J Pharm Pharmacol* **49**: 29.

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