Biodistribution of Injected Tritiated Hyaluronic Acid in Mice: A Comparison Between Macromolecules and Hyaluronic Acid-derived Oligosaccharides

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Abstract. Background: Hyaluronan (HA) has been reported to bind specifically and with high affinity to various cell types and to directly modify cell behaviour. In a previous report we demonstrated that both high molecular weight molecules (HAH) and HA-derived oligosaccharides were efficient at triggering terminal differentiation of acute myeloid leukemia (AML) blasts, in vitro, through CD44 ligation. Materials and Methods: To explore the possibility of using HA for a differentiation therapy in AML, we investigated whether intravenous injection of tritiated HAH and/or HA-derived oligosaccharides (HA10-20) into mice accumulated in bone marrow, the main site of AML cell proliferation. Results: The present work showed that the level of HA in bone marrow: 1) was maximum 5 hours after injection of either HAH or HA10-20; 2) was about 40 times higher after HAH than after HA 10-20 injection. The amount of HA in bone marrow (5.8% ID/g) was two-fold higher than in serum, indicating that it was not due to circulating blood. Finally, using chromatographic analysis, we showed that about 34% of tritiated HA present in bone marrow 5 hours after HAH injection displayed a size higher or equal to HA 10. Conclusion: After a single injection of macromolecular hyaluronan in mouse bone marrow we obtained a concentration of oligosaccharides close to the one shown to trigger AML cell differentiation in vitro. A part of the oligosaccharides had a size higher than or equal to the minimal one required to interact with HA receptors.

Hyaluronan (hyaluronic acid, HA) is a large, ubiquitous glycosaminoglycan (GAG) found in extra-cellular matrix and especially abundant in loose connective tissues. It is synthesized in most tissues and partly degraded close to its origin; a high rate of catabolism also occurs in the lymph nodes. The remaining HA enters the blood stream from where it is removed by the endothelial cells of the liver sinuses and further degraded (reviewed in (1,2)).

Numerous works have reported that HA or HA-derived oligosaccharides can directly trigger different properties in cells, through their binding to specific cell surface receptors such as RHAMM and CD44. For example, HA has been shown to stimulate vascular cell proliferation (3-6) and, more recently, it has been evidenced that HA-derived oligosaccharides could inhibit tumor growth in mice (7,8) and tumor cell line proliferation when used as a carrier for drug delivery (9). Interestingly, Charrad et al. (10) recently demonstrated that both high molecular weight molecules (HAH) and HA-derived oligosaccharides were efficient at triggering terminal differentiation, through CD44 ligation, of acute myeloid leukemia (AML) blast in vitro, raising the possibility of using HA for a differentiation therapy in AML. Accordingly, it seemed worthwhile to investigate whether, following in vivo injection, HAH and/or HA-derived oligosaccharides (HA10-20) can accumulate in bone marrow, the main site of AML cell proliferation.

So far, it has been well documented that intravenously injected macromolecular HA is rapidly cleared from the blood stream, degraded primarily in the liver and, to a lesser extent, in the spleen, lymph nodes and bone marrow. The reticuloendothelial cells present in all these organs are thought to be responsible for this uptake and degradation (11-15). In contrast, neither the biodistribution nor the clearance pathways of HA-derived oligosaccharides have been so far analyzed in vivo. Considering that the size of an injected molecule might influence its biodistribution, we found it of interest to compare the biodistribution of HAH and HA-
derived oligomers in mice following intravenous injections. Using tritiated molecules, we here show evidence that injection of macro-molecular HA generates, in the bone marrow, a concentration of HA-derived oligosaccharides that is 40-fold higher than that following injection of HA-derived oligomers. Moreover, the size of these oligosaccharides is higher or equal to HA_{10}, indicating that they have the potential to bind HA receptors.

Materials and Methods

Production of tritiated hyaluronan. Production of tritiated hyaluronan was performed according to a previously described procedure (16). Tritiated hyaluronan was produced by the CB193 cell line fed with tritiated acetate (4 MBq / ml of culture medium) in RPMI supplemented with 2 mM glutamine and 10% foetal calf serum for 60 h. The cell culture medium was processed as follows: 1) digestion by Streptomyces griseus protease (type XIV, 4.6 units / mg, 1 mg / ml) from Sigma (St Quentin Fallavier, France) in 0.1 M Tris supplemented with 0.1 M NaCl and 0.25 g / l sodium azide at pH 8, packed in a 100 x 1 cm column (Econocolumn, BioRad, Marnes la Coquette, France). The radioactive molecules of high molecular mass (over 10^6 Da) present in the exclusion volume of the chromatography column were checked for their hyaluronan composition by digestion with 200 TRU / ml Streptomyces hyaluronidase (a hyaluronan-specific hyaluronidase). Hyaluronidase digestion led to a complete degradation of the radioactive macromolecules in small fragments. Protease digestion had no effect on the radioactive molecules. The preparation was concentrated under pressure and chromatographed through Ultrogel A4 (Sepracor, Villeneuve la Garenne, France) in 0.1 M Tris supplemented with 0.1 M NaCl and 0.25 g / l sodium azide at pH 8, packed in a 100 x 1 cm column (Econocolumn, BioRad, Marnes la Coquette, France). The radioactivity of a 0.1 ml sample mixed with 2 ml of Liquid Scintillation cocktail was checked once a month by gel permeation chromatography. Stability of the preparation was checked for their hyaluronan composition by digestion with 200 TRU / ml Streptomyces hyaluronidase (a hyaluronan-specific hyaluronidase). Hyaluronidase digestion led to a complete degradation of the radioactive macromolecules in small fragments. Protease digestion had no effect on the radioactive molecules. The preparation was concentrated under pressure to 1 ml, desalted on Biogel P6-DG (BioRad) in RPMI with glutamine, sterilized by ultrafiltration through a 0.22 µm Millipore filter and stored at 4°C. This preparation was mixed with 2 ml of Liquid Scintillation cocktail. Fractions of each peak were pooled and radioactivity in a 50 µl sample mixed with 2 ml of Liquid Scintillation cocktail. Fractions of each peak were pooled and radioactivity was counted in a gamma scintillation counter after being mixed with 2 ml Liquid Scintillation cocktail. Nine results per point (except for the bone marrow the quantity of which allowed only 3 results per point) were expressed as a percentage of the injected dose per gram of tissue (%ID/g) and the s.e. was calculated.

Intending to inject the highest quantities of hyaluronan in mice, in one experiment increasing amounts of unlabelled HA (50, 100, 150 or 200 µg) were mixed with 15 µg of radio-labelled HA in 200 µl PBS. Each dose was administered intravenously to mice and, 5 h after the injection, the blood and the bone marrow were removed and processed as described for the other tissues. Radio-labelled HA used as a tracer represented a percentage of the whole injected HA. From the radioactivity found in bone marrow extracts, we calculated the corresponding whole HA amount accumulated in the tissue.

Oligosaccharide size in organ extracts. Tissue samples were solubilized (W/V = 1:5) overnight at 50°C and centrifuged for 20 min at 20,000 g. Two hundred microliters of the supernatant were chromatographed through Aca 202 gel (Sepracor) in PBS packed in a 50 cm / 0.5 cm column. The flow rate was 4 ml / h and 0.5 ml fractions were collected. Eluted peaks were detected by the measure of the radioactive in 200 µl of each fraction. The column was calibrated with purified HA_{10}, HA_0, HA_{10} and HA_{12}, giving elution volumes of 12 ml, 10 ml, 8 ml and 7 ml, respectively. The radioactivity counted in the fractions up to 8 ml was added up and expressed as a percentage of the whole radioactivity present in the fractions of the chromatography. This result was further expressed as a percentage of ID per gram of tissue for each sample and referred
to as the "active fraction"; i.e. the fraction containing oligosaccharides that were long enough to interact with the HA receptors of the cells. To ensure that the radioactivity found in organ extracts was associated with HA, a sample of organ extract in deionized water was mixed with Streptomyces hyaluronidase (20 TRU/μg HA) overnight at 37°C and chromatographed through AcA 202 gel. The radioactivity detected in the peaks was associated with small molecules showing a complete digestion of the radioactive molecules by hyaluronidase, demonstrating that they were composed of HA.

Results

The tritiated oligosaccharides were separated with regard to their size by chromatography through AcA 202 gel. Eluted oligosaccharides were detected by the measurement of radioactivity and glucuronic acid concentration. The chromatographic profiles obtained by the two different methods were superimposable, showing that the radioactivity found in the peaks was associated with the oligosaccharides. Peaks from HA10 to HA20 were pooled and represented the HA10-20 fraction injected into the mice.

Mice received i.v. injections of either tritiated HA10 or tritiated HA10-20; they were sacrificed 5h or 24h after the injection and organ radioactivity was measured. Following an administration of tritiated HA10-20 (Figure 1A), the highest radioactivity levels were found in kidney, spleen and liver 5h after the injection (0.46±0.04 %ID/g, 0.38±0.06 %ID/g and 0.29±0.03 %ID/g, respectively) as well as 24h after the injection (0.26±0.01 %ID/g, 0.29±0.04 %ID/g and 0.18±0.01 %ID/g).
Between 5h and 24h, a significant decrease in radioactivity was seen in kidney, liver and spleen: 43 %, 38 % and 24 %, respectively. Only 0.15±0.03 % of the injected dose was found per g of bone marrow 5h after i.v. injection and 0.11±0.01 % ID/g at 24h. At both times, bone marrow activities were lower than in serum (0.22±0.02 % ID/g at 5h and 0.17±0.02% ID/g at 24h).

Radioactivity tissue uptake 5h or 24h after an i.v. administration of tritiated HA14 is shown in Figure 1B. The highest radioactivity level 8.6±0.4 %ID/g, was found in the
liver 5 h after the injection, decreasing to 4.4±0.4 % ID/g at 24 h. The amount of radioactivity in the bone marrow was 5.8±0.9 % of the injected dose per g, 5 h after the injection. This radioactivity was higher than that of each organ investigated (liver excepted) and represented twice that in serum. At 24 h the radioactivity decreased to the same value found in serum i.e. 2.9±0.4 % ID/g. To summarize, the highest levels of radioactivity accumulated in bone marrow were observed 5 h following injection of either tritiated HAH or HA 10-20. However, more importantly, the use of HAH led to a level of radioactivity 40-fold higher than that obtained following HA 10-20 injection.

The addition of increasing amounts of unlabelled HA to the tritiated HA had no effect upon the percentage of accumulation of radio-labelled-HA within the bone marrow (Table I) measured 5 h after the injection of HAH, under the conditions studied, but increased the total HA amount found in the bone marrow.

Five hours after tritiated HAH injection, the size of radioactive molecules in organ extracts was analyzed by chromatography through AcA 202 gel (Figure 2). The chromatogram of each extract (serum excepted) showed three distinct peaks. The first peak corresponded to the exclusion volume of the column (V0=6 ml) and represented molecules with a MM higher than 20,000 Da (i.e. higher than HA 100). This peak was predominant in the liver extract (56.7%), whereas in the other organs it corresponded to 1/3 of the total chromatography. The second peak had an average elution volume of 14 ml and represented molecules with MM in the range of 600 Da (the size of HA3) and the third peak contained small molecules whose size was that of the monosaccharidic constituent or that of tritiated acetyl groups. This last peak was more predominant in kidney and lymph node extracts and in serum. Comparable chromatograms were obtained with organ extracts made 5 h after a tritiated HA 10-20 administration. The macromolecular fraction at the V0 was indeed composed of HA since it was completely reduced to small molecules by Streptomyces hyaluronidase. From each chromatogram the percentage of molecules present in the fraction up to 8 ml was calculated and further expressed in % ID/g of tissue (Table II). This fraction was referred to as the "active fraction" and its HA size was higher or equal to HA 10. In the case of HA 10-20 injection, the highest percentage of "active fraction" was found in the kidney (0.12% ID/g) and was 0.05 %ID/g in the bone marrow. In the case of HAH injection, the highest percentage of "active fraction" was obtained in the liver (4.8% ID/g) whereas in the bone marrow it was 2.2 %ID/g, corresponding to 44-fold more than that obtained for this same tissue after an HA 10-20 injection.

**Discussion**

The present experiments indicate that, after a single administration of tritiated HA into the blood stream, the biodistribution of the tracer in the mice varies depending on the size of the administered hyaluronan. HA 10-20 oligomers were more rapidly eliminated from the blood than HAH, since 5 hours after the injections 10-fold more isotope was present in the blood of mice in the case of HAH administration. The analysis of the radioactivity extracted from the organs showed that the decrease of HA 10-20 concentration in the blood from 5 hours to 24 hours after the injection was due to a high uptake of the oligosaccharides by the kidney, where the activity was higher than in all other organs. The rapid rate of HA 10-20 elimination by the kidney probably prevents the oligomer diffusing into and accumulating in other organs. On the other hand, the HAH clearance was less rapid and extra-renal, the liver being the main organ responsible for the polysaccharide elimination. These observations are in agreement with those reported by other authors, indicating the preferential uptake of the largest polymers by the liver cells (21,22). Indeed, it is well established that the liver is

Table I. HA accumulation in mouse bone marrow extracts following administration of increasing amounts of unlabelled HA4H mixed with 15 µg (*) of radio-labelled HAH

<table>
<thead>
<tr>
<th>Injected HA4H (µg) added to 15 µg HA*</th>
<th>Calculated HA in bone marrow extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%ID/g</td>
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<tr>
<td>0</td>
<td>5.8</td>
</tr>
<tr>
<td>50</td>
<td>4.99</td>
</tr>
<tr>
<td>100</td>
<td>4.8</td>
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<tr>
<td>150</td>
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</tr>
<tr>
<td>200</td>
<td>5.2</td>
</tr>
</tbody>
</table>

HA bound to bone marrow was calculated from the radioactivity measured in bone marrow extracts.

Table II. Comparison of "active fractions" (HA molecules with a size higher than or equal to HA 4H) in organ extracts 5h after injection of either HAH or HA 10-20. Results are expressed as percentage of injected dose per gram of tissue (%ID/g tissue).

<table>
<thead>
<tr>
<th></th>
<th>HAH</th>
<th>HA 10-20</th>
<th>HAH/HA 10-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4.8</td>
<td>0.09</td>
<td>53</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.8</td>
<td>0.09</td>
<td>20</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.6</td>
<td>0.12</td>
<td>5</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>2.2</td>
<td>0.05</td>
<td>44</td>
</tr>
<tr>
<td>Serum</td>
<td>0.79</td>
<td>0.05</td>
<td>16</td>
</tr>
<tr>
<td>Lymph node</td>
<td>0.3</td>
<td>0.05</td>
<td>6</td>
</tr>
</tbody>
</table>
the major site of circulating HA uptake and degradation. The hyaluronan fragments are further released into the blood and then eliminated by the kidney. The results of organ extract chromatograms obtained 5 hours after HA$_H$ injection are in agreement with that observation. In the liver, the majority of the radioactivity (56.7%) was associated with HA molecules whose MM was > HA$_{10}$, whereas in the kidney and the serum the majority of radioactive molecules corresponded to monosaccharidic constituents or to tritiated acetyl groups, assuming that deacetylation had occurred. The chromatogram of each organ extract exhibited three distinct peaks corresponding to macromolecular species and small molecules. The nature of the macromolecular fraction in V$_0$ was confirmed to be HA by the complete digestion of the molecules by Streptomyces hyaluronidase. In organs, the shift of the macromolecular HA towards the low molecular species without intermediate oligosaccharide breakdown indicates that, when the HA degradation process starts, it runs until completion. Hyaluronan degradation to monosaccharidic constituents may occur by the concerted action of three enzymes: a hyaluronidase that generates non-reducing termini and two exoglycosidases that remove sugars sequentially from the non-reducing termini (23).

A significant accumulation of hyaluronan i.e. 5.8% of the injected dose per gram of tissue, was found in the bone marrow 5 hours after a 15Ìg administration of tritiated HA$_H$ and represented twice that obtained in the serum in the same experiment, showing that the tritiated HA detected in the bone marrow was not due to contamination by serum but really represented specific uptake of HA by the bone marrow. It was calculated from the chromatogram that about 34% of these molecules were "active" molecules i.e. molecules whose size is higher than or equal to HA$_{10}$, able to interact with HA receptors on cells. Actually, several authors have shown that the smallest HA fragment that binds to HA receptors on the surface of various cells such as SV-3T3 transformed fibroblasts (24), liver endothelial cells (21) or keratinocytes (25) is the decasaccharide HA$_{10}$. Administration of increasing amounts of HA$_H$ led to an accumulation of the polysaccharide within the bone marrow, measured 5 hours after the injection, indicating that there was no HA$_H$ saturation of the tissue under the conditions studied. HA$_H$ accumulation reached a mean of 5% of injected dose per gram of bone marrow whatever the injected dose. This percentage became slightly lower if only "active" molecules were considered. In a previous work (10) it was shown that the blockage of human acute myeloid leukemia differentiation was reversed after addition of 10 Ìg/ml HA$_H$ in cell culture. In that experiment, we obtained a hyaluronan concentration in the bone marrow close to the effective concentration tested in culture, showing that HA can efficiently reach the main site of AML proliferation. Moreover, since the amount found in bone marrow was enhanced by increasing the injected dose of HA, these encouraging results could probably be improved by using longer periods of treatment such as a continuous infusion of hyaluronan instead of a single administration, the concentration of which is limited by the viscosity of the reagent.

In conclusion, in spite of the quick degradation and elimination of most injected tritiated HA from the blood stream, we observed a significant accumulation of hyaluronan in mouse bone marrow. This result was obtained when macromolecular hyaluronan was injected and when, after partial degradation, oligosaccharides with a size able to interact with cell receptors were produced. The oligosaccharide concentration obtained in the bone marrow was close to the one shown to trigger AML cell differentiation in vitro and it could probably be enhanced by a continuous infusion of hyaluronan.

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References


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