EFFECT OF SULFATED POLYSACCHARIDES ON BLOOD-BORNE PULMONARY METASTASIS IN RATS

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The inhibitory effect of sulfated polysaccharides on blood-borne metastasis was examined. As a model of blood-borne metastasis, the ascitic form of hepatoma AH-109A tumor was injected intravenously into Donryu strain rats. Examination of the pulmonary metastatic nodules developed 2 weeks later showed inhibitory effect of the five sulfated polysaccharides tested. Xylan sulfate was the most inhibitory, and exerted its inhibitory effect when the tumor cells were in the pulmonary capillary beds.

However, from the rapid disappearance of radioactivity from the lungs after injection of 125IUDR-labeled AH-109A cells, tumor cells seemed to be retained in the lungs for only a very short time. Measurement of the anticoagulative and fibrinolytic activities of three sulfated polysaccharides showed that the inhibitory effect of these compounds on blood-borne metastasis was proportional to their anticoagulative and fibrinolytic activities, xylan sulfate showing the highest activities.

These results suggest that sulfated polysaccharides may inhibit blood-borne pulmonary metastasis by inhibiting the lodging of tumor cells in the pulmonary capillary beds.

The most insidious feature of malignant tumors is metastasis. Hematogenous spread of tumor cells is thought to consist of four stages; (1) release from the primary tumor, (2) transport in the blood stream, (3) lodging in capillary beds far from the primary site, and (4) growth of these lodged cells.

The stage of lodging in capillaries of another organ may be the most important in metastasis formation and much attention has been paid to it. Its relation to coagulation has been reported in the past decade. Anticoagulants, such as heparin, dextran sulfate, and coumarins, as well as fibrinolytic agents, such as plasmin and antifibrinogen (Arvin) have been found to reduce the number of metastases, because they inhibit formation of microthrombi around the tumor cells attached to the capillary endothelium.

Conversely e-aminocaproic acid and trans-4-aminomethylcyclohexyl-1-carboxylic acid increased the number of metastases. However, results with these compounds were not consistent under different experimental conditions.

Therefore, we attempted to find a more reliable antimetastatic agent through studies on an experimental model of blood-borne pulmonary metastasis. The effect of five sulfated polysaccharides with different molecular weight and sulfur content on metastasis was examined and compared with their anticoagulative and fibrinolytic activities.

**Materials and Methods**

**Animals and Tumor** Eight- to 10-week-old female Donryu strain rats, weighing approximately 100 g, were purchased from the Nihon Rat Co. (Urawa). Rat ascites hepatoma AH-109A, which originated from a Donryu strain rat, was kindly supplied by Dr. M. Kato of the Institute of Development, Aging, and Cancer, Tohoku University School of Medicine.
Table I. Sulfated Polysaccharides Tested

<table>
<thead>
<tr>
<th>Sulfated polysaccharides</th>
<th>Molecular weight</th>
<th>Sulfur content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylan sulfate (XS)</td>
<td>$3 \times 10^8$</td>
<td>ca. 18</td>
</tr>
<tr>
<td>Dextran sulfate (DS)</td>
<td>$6 \times 10^8$</td>
<td>ca. 18</td>
</tr>
<tr>
<td>Chondroitin polysulfate</td>
<td>$6 \times 10^3$</td>
<td>ca. 15</td>
</tr>
<tr>
<td>Chondroitin sulfate (CSN)</td>
<td>$30 \times 10^3$</td>
<td>ca. 6</td>
</tr>
<tr>
<td>Glucose polysulfate (GPS)</td>
<td>360</td>
<td>ca. 18</td>
</tr>
</tbody>
</table>

supplied by Dr. H. Sato, Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University, Sendai. The tumor take rate in Donryu strain rats after intraperitoneal or intravenous inoculation of hepatoma AH-109A is approximately 100%.

Tumor Cell Suspension Five to 8 days after intraperitoneal inoculation of AH-109A cells into rats, the ascites fluid was harvested and tumor cells were separated from red blood cells by washing them several times with Hanks’ balanced salt solution. The viability of tumor cells was confirmed by the Trypan Blue dye-exclusion method. A suspension of about $5 \times 10^6$ cells in 0.2 ml of Hanks’ balanced salt solution was injected into rats via a tail vein.

Sulfated Polysaccharides The sulfated polysaccharides used were xylan sulfate, dextran sulfate, chondroitin polysulfate, chondroitin sulfate, and glucose polysulfate, obtained from Tokyo Research Institute, Seikagaku Kogyo Co. (Table I). Dose of 10 to 100 mg/kg of these sulfated polysaccharides in saline was injected into rats intraperitoneally. Control rats were injected with the same volume of saline by the same route.

Radioactive Labeling of Tumor Cells Four intraperitoneal injections (at 8-hr intervals) of 0.5 µCi/g body weight of 5-iodo[¹²⁵I]-2-deoxyuridine (¹²⁵IUDR) (Radiochemical Centre, England) were given to rats bearing AH-109A tumor. The rats were sacrificed 1 hr after the fourth injection of ¹²⁵IUDR and a tumor cell suspension was prepared by washing the cells with saline as described above. No labeled isotope could be detected in the supernatant of this suspension. Rats were given 0.1% KI in their drinking water for 4 days and then injected with 10³ labeled tumor cells in 0.2 ml of saline. They were killed at certain intervals after the injection and total radioactive activity in their lungs was counted in a well-type scintillation counter (Aloka DT-601).

Assay of Pulmonary Metastasis Rats were autopsied 14 to 16 days after tumor inoculation in each experimental group, and their organs were examined grossly and histologically. Pulmonary metastasis was assayed simultaneously, grossly by counting the number of metastatic nodules on the pulmonary surface.

Assay of Coagulative and Fibrinolytic Activities The coagulative activity of blood after intraperitoneal injection of 100 mg/kg of xylan sulfate, chondroitin polysulfate, or chondroitin sulfate into rats was measured by determining the whole blood clotting time, partial thromboplastin time, prothrombin time, and thrombin time. The whole blood clotting time was measured by the method of Lee and White, with the partial thromboplastin time by that of Langdell et al., with partial thromboplastin (Platein) from Warner-Lambert Co., U.S.A. The prothrombin time was measured by the one-stage method of Quick et al., and the partial thromboplastin time by that of Geigy Co., Switzerland. Bovine thrombin (Mochida Co., Tokyo) was used for measurement of thrombin time. Fibrinolytic activity was measured as the euglobulin clot-lysis time, and as the fibrinolytic area on a standard fibrin plate. Bovine plasminogen-free fibrinogen was obtained from Daiichi Chemical Co., Tokyo.

Lipoprotein Lipase Activity Lipoprotein activity was determined with Ediol as a substrate. The assay mixture contained 0.1 ml of 5% Ediol, 0.1 ml of human serum, 0.5 ml of 0.2M NH₄Cl-NH₄OH buffer (pH 8.5) containing 10% bovine serum, 0.2-0.5 ml of rat plasma, and distilled water to a final volume of 2.0 ml. After incubation at 37°C for 1 hr, free fatty acids were determined by the method of Dole, and the amount of free fatty acid released was calculated. One unit of the activity was defined as the amount of enzyme liberating 1 mol of free fatty acid per hour.

Results

Time Course of Change in Radioactivity in the Lung after Inoculation of ¹²⁵IUDR-labeled AH-109A Cells Viable ¹²⁵IUDR-labeled AH-109A cells were inoculated intravenously into rats and the time course of change in radioactivity in the lung was measured. As shown in Fig. 1, radioactivity in the lung decreased very rapidly in the first 4 hr and only 1% of the total radioactivity remained in the lung after 12 hr. These results suggest that injected tumor cells are retained in the lung for only a short time.
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Fig. 1. Change in radioactivity in the lung of rats after intravenous inoculation of \(^{125}\)IUDR-labeled AH-109A cells

Each rat was inoculated intravenously with \(10^7\) cells. Each point is expressed as the average of five rats.

Effect of Sulfated Polysaccharides on Pulmonary Metastases

Nodules on the surface of the lung were counted 14 to 16 days after intraperitoneal injections, first of the sulfated polysaccharides and then intravenous inoculation of tumor cells 1 hr later. Doses of 100 mg/kg of xylan sulfate and dextran sulfate strongly inhibited development of nodules in the lung (Table II). At the same dose chondroitin polysulfate was less inhibitory, and chondroitinsulfate and glucose polysulfate were not inhibitory. Inhibition by xylan sulfate and dextran sulfate was dose-dependent. As shown in Table III, a single injection of 10 mg/kg of xylan sulfate 1 hr before inoculation of tumor cells was slightly inhibitory (Expt. 1). Four injections of 10 mg/kg of xylan sulfate at 6-hr intervals before and after tumor inoculation were strongly inhibitory (Expt. 2). Injections of 50 mg/kg of xylan sulfate once daily from the 3rd to 7th day after tumor inoculation had no inhibitory effect (Expt. 3). Thus, the inhibition of pulmonary metastases by these sulfated polysaccharides depended on their dosage and time of injection.

Table II. Effect of Sulfated Polysaccharides on Blood-borne Pulmonary Metastases

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Saline (control)</th>
<th>Number of metastatic nodules on the surface of the lung</th>
<th>XS</th>
<th>DS</th>
<th>CPS</th>
<th>CSN</th>
<th>GPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(15/15)</td>
<td>53.2±6.8</td>
<td>10.5±2.4*</td>
<td>14.1±4.0*</td>
<td>15.0±3.1*</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(15/15)</td>
<td>53.2±6.8</td>
<td>4.3±1.4*</td>
<td>2.3±1.3*</td>
<td>9.4±2.3*</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10/10)</td>
<td>62.3±6.3</td>
<td>0.7±0.4*</td>
<td>1.4±0.6*</td>
<td>1.9±0.5*</td>
<td>50.6±6.2**</td>
<td>65.1±9.0**</td>
<td></td>
</tr>
</tbody>
</table>

Each rat was inoculated intravenously with \(5 \times 10^6\) cells of AH-109A. A dose of 25, 50, or 100 mg/kg of sulfated polysaccharide was injected intraperitoneally 1 hr before tumor inoculation. Values are mean ± SE. The incidences of pulmonary metastases are shown in parentheses (number of rats with pulmonary metastases/number of rats tested). Significance of differences of values from that of the control by Student's \(t\)-test: *\(P<0.001\), ** not significant. NT = not tested.
Table III. Time Dependency of Inhibition of Pulmonary Metastases by Sulfated Polysaccharides

<table>
<thead>
<tr>
<th>Treatment schedule</th>
<th>Number of metastatic nodules on the surface of the lung (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>Expt. 1</td>
<td>39.0±5.2</td>
</tr>
<tr>
<td>(12/12)</td>
<td>(9/10)</td>
</tr>
<tr>
<td>Expt. 2</td>
<td>99.0±21.0</td>
</tr>
<tr>
<td>(12/12)</td>
<td>(11/12)</td>
</tr>
<tr>
<td>Expt. 3</td>
<td>17.3±2.8</td>
</tr>
<tr>
<td>(15/15)</td>
<td>(12/12)</td>
</tr>
</tbody>
</table>

Rats were inoculated intravenously with 5 x 10⁵, 7 x 10⁵, or 3 x 10⁶ cells of AH-109A in Expt. 1, 2, and 3, respectively. Values are mean±SE. The incidences of pulmonary metastases are shown in parentheses (number of rats with pulmonary metastases/number of rats tested). Significance of differences of values from that of the control by Student's t-test: * P<0.001, ** P<0.005, *** P<0.01, * not significant. NT = not tested.

Fig. 2. Change in coagulant activity of blood after intraperitoneal injection of sulfated polysaccharides into rats

Single dose of 100 mg/kg of xylan sulfate (XS), chondroitin polysulfate (CPS), or chondroitin sulfate (CSN) was injected into rats. Each point is expressed as the average of five rats.
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Fig. 3. Change in coagulative activity of blood after intraperitoneal injection of xylan sulfate

Each point is expressed as the average of five rats.
- 100 mg/kg ■ 50 mg/kg ▲ 25 mg/kg

Fig. 4. Change in fibrinolytic activity of blood after intraperitoneal injection of sulfated polysaccharides into rats

Single dose of 100 mg/kg of xylan sulfate (XS), chondroitin polysulfate (CPS), or chondroitin sulfate (CSN) was injected into rats. Each point is expressed as the average of five rats.
time, prothrombin time, and thrombin time, after injection of these compounds. As shown in Fig. 2, xylan sulfate had the greatest anticoagulative activity; it caused marked prolongation of the whole blood clotting time, partial thromboplastin time, and thrombin time, and some prolongation of the prothrombin time. Its anticoagulative activity was maximum 1 hr after its injection and gradually decreased during the next 5 hr, but some activity was still observed 6 hr after its injection. The anticoagulative activity of chondroitin polysulfate was less than that of xylan sulfate and of shorter duration, and this compound had no detectable effect on the prothrombin or thrombin time. Chondroitin sulfate had no anticoagulative activity. The dose-dependency of the anticoagulative effect of xylan sulfate is shown in Fig. 3.

The time course of change in fibrinolytic activity in the blood of rats after injection of these compounds was measured as the euglobulin clot-lysis time and as fibrinolysis on a standard fibrin plate. As shown in Fig. 4, xylan sulfate had the strongest fibrinolytic activity of the three compounds.

These results indicate that the inhibitory effect of sulfated polysaccharides on metastasis is well correlated with their anticoagulative and fibrinolytic activities. Their intrinsic anticoagulative activities may be especially important in inhibition of blood-borne metastases.

Liberation of Lipoprotein Lipase by Sulfated Polysaccharides It has been reported that xylan sulfate, chondroitin polysulfate, and chondroitin sulfate liberate lipoprotein lipase when injected intravenously into rats. This was measured as liberation of free fatty acids from Ediol by plasma of rats obtained at intervals after intraperitoneal injection of the three compounds. Fig. 5 shows that xylan sulfate caused greatest liberation of lipoprotein lipase and that the lipoprotein lipase activity was maximum 1 hr after injection of xylan sulfate or chondroitin polysulfate.

Fig. 5. Change in lipoprotein lipase activity of plasma after injection of sulfated polysaccharides

Single dose of 50 mg/kg of xylan sulfate (XS), chondroitin polysulfate (CPS), or chondroitin sulfate (CSN) was injected intraperitoneally into rats. Each point is expressed as the average of five rats.

DISCUSSION

Wood first demonstrated the intravascular events associated with tumor metastasis formation in a rabbit ear chamber by the intravital microcinematography. He showed that circulating tumor cells adhered to the endothelium of the capillaries, and that a coagulum consisting of fibrin and platelets quickly formed around the cells. Changes then occurred in the capillary wall adjacent to this thrombus, and the tumor cells penetrated into the perivascular tissues and grew forming a metastatic focus. Similar events were observed by Sato by cinemicrography of the mesenteric arterioles of rats after intravenous injection of the ascitic form of hepatoma cells. He emphasized that changes of microcirculatory dynamics occurred after intravasation of tumor cells, and that subsequently the tumor cells became attached to the injured endothelium forming an extra-
vascular tumor growth. Tanaka et al.\textsuperscript{22} in electron microscopic studies observed aggregation of platelets and fibrin formation around tumor cell emboli in the lung of rats inoculated intravenously with AH-130. These morphological observations show that intravascular coagulation occurs at an early stage of tumor lodgement in capillary beds. There have been many studies on the inhibition or enhancement of metastasis by agents which affect coagulation and fibrinolytic systems. Fisher and Fisher,\textsuperscript{10,11} Agostino,\textsuperscript{1,2} and Clifton\textsuperscript{7,8} reported that anticoagulants, such as heparin, coumarins, or plasmin, inhibited blood-borne metastasis, while antiplasmins, such as ε-aminocaproic acid and \textit{trans}-4-aminomethylcyclohexyl-1-carboxylic acid enhanced metastasis.

Heparin is an acid mucopolysaccharide containing O- and N-sulfate groups and it is a polyanionic substance which has a strong anticoagulative activity because it binds with proteins. It inhibits blood clotting at three stages of the coagulation mechanism; it inhibits thromboplastin generation and thrombin formation, and has an antithrombin activity. Xylan sulfate, dextran sulfate, chondroitin polysulfate, chondroitin sulfate, and glucose polysulfate are acid polysaccharides, which are the so-called heparinoid and have O-sulfate groups. The difference in chemical properties of these sulfated polysaccharides is due to differences in their molecular weight, sulfur content, and polyanionic properties. The biological activities of these compounds have not been fully clarified but they undoubtedly have anticoagulative and fibrinolytic activities.

As described in this paper, we examined effect of these sulfated polysaccharides on blood-borne pulmonary metastasis, and its results showed that xylan sulfate had the strongest inhibitory effect. Dextran sulfate had almost as much inhibitory effect as xylan sulfate, and chondroitin polysulfate had less. Chondroitin sulfate and glucose polysulfate were not inhibitory. The biological activities of xylan sulfate were dose-dependent. Our most important finding was that inhibitory effect of these compounds on pulmonary metastasis correlated well with their anticoagulative and fibrinolytic activities. Another significant finding was that xylan sulfate showed maximum anticoagulative and fibrinolytic activities 1 hr after its injection and it also had a strong inhibitory effect on metastasis when injected just before or soon after tumor cell inoculation. Injection of labeled tumor cells showed that these cells remained in the lung for only a very short time. Thus, it seems likely that xylan sulfate inhibits blood-borne metastasis by interfering with the intravascular coagulation system in the pulmonary arteries at the early stage of tumor lodgement.

However, sulfated polysaccharides may have other biological activities besides anticoagulative and fibrinolytic activities, and their inhibitory effect on metastasis may be partly due to these other activities. In this work we showed that xylan sulfate caused liberation of lipoprotein lipase. Suemasu and Ishikawa\textsuperscript{21} pointed out that the antimetastatic effect of dextran sulfate might be partially due to a change in the charge on the surface of a tumor cell. Hagmar\textsuperscript{13} reported that the ionic properties of the surface of tumor cells affect tumor metastasis. Studies on other effects of xylan sulfate besides anticoagulation and fibrinolysis are now in progress in our laboratory.

Our studies raised the question of the final distribution of injected tumor cells in various organs after they had passed through the lung. In preliminary experiments rats treated with xylan sulfate survived longer than control animals after injection of tumor cells, although both groups finally died of tumor. Moreover, the number of metastatic foci in the lung was less in xylan sulfate-treated animals than in controls. These findings seem to be due to reduction in the number of viable tumor cells by xylan sulfate in the circulation after their passage through the
pulmonary capillary beds. The adjuvant chemotherapeutic efficacy of xylan sulfate will be reported in the near future.

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