On the Role of Stickiness of Tumor Cells in the Formation of Metastases

Kiyohide Kojima and Isao Sakai
(Department of Pathology, Nagoya City University Medical School, Nagoya, Japan)

SUMMARY

A comparative study of stickiness of tumor cells to glass surfaces was undertaken with the use of three strains of rat ascites hepatoma, AH 13, AH 130, and AH 7974. Stickiness was measured by calculating the percentage of cells clinging to a glass surface of the calculating plate after it was washed with salt solution.

A significant difference was observed in the cell stickiness among these three strains. AH 13 showed the most active stickiness, AH 130 moderate, and AH 7974 the least active. These results indicate that cell stickiness is inversely correlated with the adhesiveness between cells in the above tumors.

When these three tumor strains were inoculated intravenously, the most active strain with respect to cell stickiness caused the largest number of metastatic nodules in the lung. The same result in frequency of lung metastasis was also obtained with intraperitoneal inoculation of each tumor. Such a correlation between metastatic frequency and cell stickiness suggests that the cell stickiness may be an important property of the cell surface responsible for metastatic spread of tumor cells via blood vessels.

In order to study the nature of stickiness, chemical treatments of tumor cells in vitro were undertaken with the use of Ehrlich ascites carcinoma, AH 13, and Yoshida sarcoma. It was found that the stickiness of tumor cells was decreased by treatment with protamine sulfate and trypsin, whereas stickiness was increased by treatment with Tween-80. Significant effects on stickiness could not be observed with polyvinyl sulfate, sodium deoxycholate, calcium chloride, and disodium versenate. Furthermore, in acid medium, cell stickiness was significantly decreased, while in alkaline medium it was increased. Based on these results, stickiness of tumor cells to glass was discussed.

Surface properties of tumor cells have been considered as an important factor responsible for malignant transformation (1). The observations (9, 15) concerning passages of circulating tumor cells through capillaries suggested that the properties of tumor cells or their surface may be responsible for the difference in response for metastatic spread.

Recently, Coman (6) reported that the "adhesiveness" of cells to one another and their "stickiness" to a foreign substrate are independent phenomena, and that tumor cells are extremely sticky but poorly adhesive, whereas normal cells are strongly adhesive but not very sticky. It may be reasonable to assume that there is a difference not only in adhesiveness but also in stickiness, depending upon tumor strain. To test this possibility, use of tumor strains of the same origin and of different biological characteristics as experimental material is desirable, since these properties might depend upon those of the normal ancestral cells.

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From this point of view, a comparative study on stickiness was performed with the use of three strains of the ascites hepatoma of the rat (8, 12, 13) which are different in their mutual adhesiveness. Furthermore, the biological significance of stickiness for metastatic spread of tumor cells was also investigated. These results are reported in this paper.

MATERIALS AND METHODS

Tumor materials.—Three strains of the ascites hepatoma, AH 13, AH 130, and AH 7974, established by Yoshida (12), were used for studies on stickiness and intravenous transplantation. In the ascites hepatoma, the tumor cells, being epithelial in nature, make cell associations, forming "islands," and individually isolated cells are also present. The number of these isolated cells differs depending upon the hepatoma strain; i.e., AH 13 consists of almost entirely single isolated tumor cells; AH 130, of abundant isolated cells and a few small "islands"; and AH 7974, of isolated cells and "islands."
For chemical treatments in vitro, three strains of ascites tumor of the free cell type, Ehrlich ascites carcinoma, AH 13, and Yoshida sarcoma, were used.

In the present studies, rats of the closed colony in Moriyama Psychiatric Hospital, weighing about 100 gm., and dd/N mice, offered from the animal supply center of Nagoya University, weighing about 20 gm., were used. They were highly susceptible to these tumors.

Observation technic on stickiness of tumor cells.—Tumor suspensions were prepared by adding 0.1 ml. of tumor ascites to 5 ml. of phosphate-buffered saline at pH 7.2.

Stickiness was measured by settling the cell suspension into the calculating plate, which was made of a clean glass slide (75 X 26 mm.). At the center of this slide, a square, 2 X 2 mm., was cut in. Thin plastic tapes (about 50 µ in thickness), 4 mm. wide, were stuck to the two longer edges of the glass slide. A cover slip (50 X 22 mm.) was fixed to the plastic tapes with Vaseline, and then pressed with care to keep a constant space or slit of 0.10-0.13 mm. between the glass slide and the cover slip. A 5-min. period was allowed to elapse while the cells settled on the glass surface. A longer incubation did not give any change in the number of stuck cells. Then the cells within the square were counted. Subsequently the calculating plate was placed on a stand slanted at angle of 30° from the horizontal, and the tumor suspension in the plate was washed by allowing 1 ml. of buffered saline to flow through the thin slit from a side on which the tumor suspension had not been inserted. Drops of fluid on the latter side were drained off with filter paper. Thereafter, the cells that remained stuck to the glass surface were counted. Small "hepatoma islands" composed of two to four cells were counted as one, whereas the large "islands" were not counted at all, and the percentage of cells clinging to the glass was calculated.

Chemical treatments of tumor cells in vitro.—Ascites tumor (0.1 ml.) was added to 5 ml. of test medium adjusted to pH 7.2, and the suspension thus prepared was incubated at 37° C. After the incubation, the treated cells were gathered centrifugally and resuspended in 5 ml. of phosphate-buffered saline. Thereafter, the stickiness of the treated cells was compared with that of similarly treated controls.

The test media and incubation time were as follows. Protamine sulfate (L. Light & Co., Ltd.) and polyvinyl potassium sulfate (Wako Pure Chemical Industries, Ltd.) were used at concentrations of 0.2 per cent in 0.25 M sucrose solution adjusted to pH 7.2 with a small volume of 0.1 N HCl or NaOH. The tumor suspension was incubated in each of these media for 15 min. Tween-80 was used at a concentration of 0.5 per cent in phosphate-buffered saline, and the suspension was incubated for 45 min. Sodium deoxycholate was used at a concentration of 0.02 per cent in phosphate-buffered saline, and the suspension was incubated for 30 min. Trypsin (Difco Laboratories, Inc.) was used at a concentration of 0.5 per cent in phosphate-buffered saline. Disodium versenate was used at a concentration of 5 X 10⁻⁴ M in phosphate-buffered saline. The trypsin, CaCl₂, and versenate suspensions each were incubated for 1 hr. Control media consisted of the appropriate medium for each reagent used, but without the reagent.

As alkaline and acid media, borate buffer (0.05 M Na₂B₄O₇·10H₂O in 0.1 M NaOH added to 0.85 per cent NaCl), pH 9.5; carbonate-bicarbonate-CO₂ buffer (0.1 M Na₂CO₃ and 0.1 M NaHCO₃ added to 0.85 per cent NaCl), pH 9.5; citrate-HCl buffer (0.1 N HCl and 0.1 M sodium citrate added to 0.85 per cent NaCl), pH 4.6; and acetate buffer (0.2 M acetic acid and 0.2 M sodium acetate added to 0.85 per cent NaCl), pH 4.6, were used. Phosphate-buffered saline, pH 7.2, was used as a control. Ascites tumor (0.1 ml.) was incubated in 5 ml. of each of these buffers for 10 min. at 37° C. Then the stickiness of the treated cells was directly observed in these buffers without resuspension in phosphate-buffered saline.

RESULTS

Stickiness of the ascites hepatoma cells.—Tumor cells, 5 days after inoculation into the peritoneal cavity, were used for this study in each tumor strain. As shown in Table 1, AH 13, a free cell type of tumor, was most active in stickiness of the tumor cells to the glass surface (88.9 per cent), and AH 7974, containing abundant "hepatoma islands" and free cells, was least (57.6 per cent). AH 130, consisting of a few "islands" and abundant free cells, was intermediate (75.0 per cent) in stickiness between AH 13 and AH 7974. The differences

<table>
<thead>
<tr>
<th>Tumor strain</th>
<th>Character of tumor cells</th>
<th>Per cent of cells sticking to glass</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH 13</td>
<td>Almost all cells free</td>
<td>88.9 ± 2.2*</td>
</tr>
<tr>
<td>AH 130</td>
<td>A few &quot;islands&quot; and abundant free cells</td>
<td>75.0 ± 2.9</td>
</tr>
<tr>
<td>AH 7974</td>
<td>&quot;Islands&quot; and free cells</td>
<td>57.6 ± 4.7</td>
</tr>
</tbody>
</table>

B. Analysis of Variance

<table>
<thead>
<tr>
<th></th>
<th>s.s.</th>
<th>d.f.</th>
<th>m.s.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among groups</td>
<td>2490.73</td>
<td>2</td>
<td>1230.37</td>
</tr>
<tr>
<td>Errors</td>
<td>137.50</td>
<td>12</td>
<td>11.45</td>
</tr>
<tr>
<td>Total</td>
<td>2598.23</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

F₀ = 107.46 > F₁² = 6.93 (α = 0.01)

* Mean ± standard deviation. Five rats and 5-day-old tumor cells were used with each tumor strain. To avoid technical errors, three specimens were calculated from each rat. The total number of cell units observed was 8877 in AH 13, 8277 in AH 130, and 5163 in AH 7974. The difference among the strains is statistically significant at the 1 per cent level. Abbreviations: s.s. = sum of squares; d.f. = degree of freedom; m.s. = mean squares; F₀ = ratio of two mean squares.
A. Lung Metastases of Ascites Hepatoma Cells Injected Intravenously

Ten rats were used with each strain.

<table>
<thead>
<tr>
<th>Tumor strain</th>
<th>No. of rats with metastatic nodules in the lung (metastatic frequency)</th>
<th>Average no. of metastatic nodules per rat (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH 13</td>
<td>10 (100%)</td>
<td>30.4 (7-54)</td>
</tr>
<tr>
<td>AH 130</td>
<td>8</td>
<td>9.3 (0-30)</td>
</tr>
<tr>
<td>AH 7974</td>
<td>2 (20%)</td>
<td>0.4 (0-2)</td>
</tr>
</tbody>
</table>

B. Injected Cell Units and Their Population

Fifteen days after the intravenous transplantation of tumor cells, the rats were killed and autopsied.

<table>
<thead>
<tr>
<th>Tumor strain</th>
<th>No. of injected cell units</th>
<th>Population of cell units (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free cells</td>
<td>Small &quot;islands&quot; consisted of 2-4 cells</td>
</tr>
<tr>
<td>AH 13</td>
<td>1.4 X 10^4</td>
<td>99.6</td>
</tr>
<tr>
<td>AH 130</td>
<td>1.1 X 10^4</td>
<td>77.6</td>
</tr>
<tr>
<td>AH 7974</td>
<td>1.1 X 10^4</td>
<td>69.3</td>
</tr>
</tbody>
</table>

TABLE 3

Metastatic Frequency of Ascites Hepatoma Cells Inoculated Intraperitoneally in the Lung

Twenty rats were used with each strain. Rats were inoculated intraperitoneally with 0.1 ml. of undiluted tumor ascites in a state of "nearly pure culture" of each strain.

<table>
<thead>
<tr>
<th>Tumor strain</th>
<th>Mean survival time (days)</th>
<th>Metastases in lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats</td>
<td>Per cent</td>
</tr>
<tr>
<td>AH 13</td>
<td>7.0</td>
<td>16</td>
</tr>
<tr>
<td>AH 130</td>
<td>11.3</td>
<td>9</td>
</tr>
<tr>
<td>AH 7974</td>
<td>19.3</td>
<td>4</td>
</tr>
</tbody>
</table>

TABLE 4

Effects of Chemical Treatments in Vitro on Stickiness of "Free Cell Type" Tumor Cells

<table>
<thead>
<tr>
<th>Chemical treatment</th>
<th>Per cent of remaining cells*</th>
<th>Ehrlich ascites carcinoma</th>
<th>AH 13</th>
<th>Yoshida sarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± S.D.†</td>
<td>P difference</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td></td>
<td>49.6 ± 3.1</td>
<td>P &lt; 0.01</td>
<td>67.1 ± 11.2</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>100.0 ± 4.5</td>
<td></td>
<td>100.0 ± 3.1</td>
</tr>
<tr>
<td>Tween-80 Control</td>
<td></td>
<td>107.8 ± 2.5</td>
<td>0.01 &lt; P &lt; 0.05</td>
<td>104.6 ± 1.1</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>100.0 ± 2.4</td>
<td></td>
<td>100.0 ± 2.3</td>
</tr>
<tr>
<td>Trypsin Control</td>
<td></td>
<td>13.9 ± 3.9</td>
<td>P &lt; 0.01</td>
<td>27.6 ± 8.4</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>100.0 ± 2.1</td>
<td></td>
<td>100.0 ± 3.4</td>
</tr>
</tbody>
</table>

* This value is converted into per cent against the control.
† S.D. = standard deviation. For this observation three animals were used in each treatment and three specimens were calculated from each animal. The pH of the test medium was 7.2 in all cases.

among the three strains are statistically significant at the 1 per cent level.

Pulmonary metastasis of the ascites hepatoma.—The 5-day-old tumor cells were drained from the peritoneal cavity by the routine method and suspended in phosphate-buffered saline added to 1.0 per cent methylocellulose. To remove the large "hepatoma islands," they were centrifuged at 500 r.p.m. for 5 min. at 0° C. The supernatant was centrifuged three times in the same manner. All three types of hepatoma were treated alike. The cell suspension thus prepared contained free tumor cells and smaller "hepatoma islands." Both a free tumor cell and a "hepatoma island" were similarly counted as one cell unit. Then 10^4 cell units were injected into the tail vein of rats. Metastatic nodules in the lung were counted in each rat 15 days after injection. The inoculated cell units, their population, and the number of metastatic nodules per rat are summarized in Table 2. AH 13 was most active in producing lung metastasis, as revealed in terms of the average number of metastatic nodules per rat (30.4), AH 7974 was least active (0.4), and AH 130 was intermediate (9.3).

A total of 60 rats were given intraperitoneal inoculations of 0.1 ml. of undiluted ascites tumor of each strain in a state of "nearly pure culture." The rats were kept until the tumor caused death and then were autopsied. The metastatic frequency of each strain of ascites hepatoma in the lung is shown in Table 3. In animals inoculated with AH 13, lung metastases were observed in sixteen out of twenty animals (80 per cent), and, in those with AH 130 and AH 7974, nine out of twenty (45 per cent) and four out of twenty (20 per cent), respectively.

Effects of chemical reagents on stickiness of tumor cells in vitro.—Ehrlich ascites carcinoma cells 6 days after inoculation were mainly used for this observation. As shown in Table 4, the stickiness of the tumor cells was reduced to 49.6 per cent by treatment with protamine sulfate, a positively charged polymer, for 15 min., and also to 13.9 per cent by treatment with trypsin for 1 hr., compared with the stickiness of the respective controls. However,
STICKINESS (%)

- PROTAMINE SULFATE
  - 49.6%
- POLYVINYL SULFATE
  - 102.8%
- TWEEN 80
  - 107.8%
- DESOXYCHOLATE
  - 104.7%
- TRYP SIN
  - 13.9%
- CALCIUM CHLORIDE
  - 98.9%
- VERSENATE
  - 100.2%

**Chart 1.**—Stickiness of Ehrlich ascites carcinoma cells after various chemical treatments in *vitro*. Stickiness is indicated by the percentage of cells that remained stuck to the glass surface. This value is converted into per cent against each control. Dashed line indicates the control level (100 per cent). For this observation three animals were used in each treatment, and three specimens were observed from each animal.

The cells treated with Tween-80 for 45 min. were more sticky than the control; i.e., their stickiness increased to 107.8 per cent. The same results were also obtained in 4-day-old cells of AH 13 and Yoshida sarcoma. The difference between the reagent and the corresponding control is statistically significant at the 1 per cent level in the case of protamine sulfate and trypsin, and at the 5 per cent level in the case of Tween-80.

No significant effects on stickiness were observed in polyvinyl sulfate (a negatively charged polymer), deoxycholate, CaCl$_2$, and versenate compared with the corresponding controls (Chart 1).

The effects of the pH of the medium on the stickiness of the 6-day-old Ehrlich ascites carcinoma cells were examined. As shown in Table 5, the stickiness of these tumor cells was decreased remarkably in acid media, but increased in alkaline media, compared with that in the phosphate-buffered saline (pH 7.2) controls; in citrate buffer (pH 4.6) the stickiness was 61.3 per cent, in acetate buffer (pH 4.6) 48.7 per cent, in borate buffer (pH 9.5) 114.4 per cent, and in carbonate buffer (pH 9.5), 110.6 per cent. The difference between each acid or alkaline medium and the control is statistically significant at the 1 per cent level in the cases of citrate buffer, acetate buffer, and borate buffer, and at the 5 per cent level in the case of carbonate buffer.

**DISCUSSION**

Yoshida (12, 13) reported that each hepatoma is individual with regard to transplantability, number of chromosomes, and resistance of the cells to drugs. In the present study, a clear difference was observed in the stickiness of tumor cells to glass among the three strains of rat hepatoma. As described above, it is evident that there was good correlation between metastatic frequency in the lung and stickiness of tumor cells. These facts suggest that stickiness of tumor cells is at least one of...
the important factors responsible for the metastatic spread of tumor cells via blood vessels. These results seem to support Coman's opinion (6) that stickiness would favor the chance that tumor cells will lodge at distant sites in initiating metastases when transported through vascular channels. Furthermore, it is evident that there is a parallel correlation between the number of isolated free tumor cells and their stickiness in ascites hepatoma; i.e., a tumor strain containing abundant free tumor cells was more sticky than those containing a small number of free tumor cells. Hirono (8) has suggested from results on the motility of tumor cells that a large number of free tumor cells in the ascites hepatoma was due to a decrease in their mutual adhesiveness. On the basis of this fact, it may be assumed that increased stickiness of tumor cells to a foreign substrate is parallel to a decrease in their mutual adhesiveness. Reduced adhesiveness of tumor cells would facilitate separation and allow the motile tumor cells to invade adjacent tissues and vessels (14). In this connection, the parallelism between reduced adhesiveness and increased stickiness is very interesting with regard to metastatic spread via blood vessels.

Several workers (5, 7) have reported that lipase, phosphatase, elastase, neuraminidase, and trypsin show a marked effect on stickiness of tumor cells. Little is known about the physical aspects of cell stickiness, however.

Since most of tumor cells stuck to the glass surface within a short time and the time of contact had no detectable effect on their stickiness, stickiness may be due to simple contact, reflecting the physicochemical affinity between the surface of the cells and of the substrate. As shown in the present study, the treatment with protamine sulfate, a positively charged polymer, reduced the stickiness of tumor cells to glass, whereas no detectable changes in stickiness were observed after treatment with polyvinyl sulfate, a negatively charged polymer. That positively charged polymers bind to cell surfaces (2, 10) and that they act to decrease the negative charge in tumor cells (4) were reported by several workers. Recently, Terayama (10) has reported that clupeine became bound to the surface of ascites hepatoma cells and that the clupeine-binding capacity depended upon the strain of hepatoma. From these facts, it may be inferred that the negative charge of the cell surface is an important factor responsible for stickiness of tumor cells. Decrease of stickiness in acid medium and its increase in alkaline medium seem to support this assumption. Yamada (11) has reported that the protamine-binding capacity of hepatoma cells was increased by treatment with Tween-80 and decreased by treatment with trypsin. Thus, an increase in cell stickiness by treatment with Tween-80 and its decrease by treatment with trypsin seem also to support our assumption on the nature of stickiness.

The greater negative surface charge of tumor cells compared with that of the normal ancestral cells has been shown by many workers (1, 3), and it has been discussed in connection with reduced adhesiveness in tumor cells (1, 11). In this connection, it may be reasonable that a parallelism exists between reduced adhesiveness of tumor cells and their increased stickiness.

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