Ameboid Motility of the Ascites Hepatoma Cells and Its Significance for Their Invasiveness and Metastatic Spread

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The motility of tumor cells and the liberation of a spreading factor, such as hyaluronidase, have hitherto been regarded as responsible for the invasive growth of malignant tumors. However, since hyaluronidase does not exist in some kinds of malignant tumors (1, 12), it must be regarded as a nonessential factor in invasiveness (6, 7). Recently, Cliffton and Grossi (2) reported fibrinolytic activity of human tumors as revealed by the fibrin plate method, but the role of this enzyme in the invasive growth of tumor is not yet completely known. Consequently, motility of cells may be regarded as at least one of the essential factors responsible for the invasiveness of malignant tumors. That the invasive character of cancer might depend upon the ameboid movement of tumor cells has already been suggested by Waldeyer (15). Recently, many workers in tissue culture have demonstrated beyond all doubt that cancer cells possess ameboid motility (3, 9, 11, 13). Cowdry (8) suggested that ability to move probably depends on the stickiness of cell membranes, to some extent, and that decrease in stickiness of carcinomatous cells is a necessary prelude to their invasiveness. Coman and his co-workers (4-7, 14) made the most exhaustive study of invasiveness of cancer. They emphasized that decrease of mutual adhesiveness allows cancer cells to separate from one another and that such unattached cells are enabled to invade the interstitial spaces of the adjacent normal tissues by their ameboid motility. However, confirmative evidence that cancer owes its capacity to invade surrounding tissues to the motility of tumor cells has not as yet been completely presented. To confirm the role of motility in invasiveness, it seemed desirable to study the relationship between the migrating capacity of tumor cells and the intensity of invasive growth, with the use of several tumors of the same origin. Furthermore, it would also seem desirable to observe the motility of tumor cells with fresh material, without applying any medium, under the same conditions as exist within the body as far as possible, because in the tissue culture method it is doubtful whether the medium used is optimal for each strain of tumor and whether the order of motility in several strains of tumor obtained by tissue culture methods represents the order within the body. Using three strains of the ascites hepatoma (16), which are transplantable ascites carcinomas of the rat, we directly observed the motility of fresh tumor cells without applying any medium and studied the relationship between the motility and invasive growth of these tumors. Thus, confirmative evidence concerning the importance of the role of motility in invasiveness and metastasis is presented in this report.

MATERIALS AND METHODS

Tumor materials.—Three strains of the ascites hepatoma, AH 180, AH 608, and AH 7974, established by Yoshida (16), were used in this study. As reported by him, the ascites hepatoma is not a free-cell tumor in the strict sense of the word. The tumor cells, being epithelial in nature, make cell associations, or "islands," suspended in the ascitic fluid, and individually isolated cells are also present. The number of these isolated cells differs, depending upon the hepatoma strain. They are most abundant in strain AH 180. Isolated cells are also observed in strain AH 7974, but they are rarely seen in AH 608. Tumor "islands" continue to increase in number, with widespread invasion into the peritoneal tissues, resulting in death of the host animal in approximately 2 weeks or thereabouts after inoculation. In the present study, Wistar rats weighing about 100 gm. were used. They were highly susceptible to these tumors. Transplantation of the ascites hepatoma was performed intraperitoneally with the use of sterile glass pipettes by routine methods.

Observation technic on the motility of tumor cells.—A small amount of ascitic fluid containing "hepatoma islands" was withdrawn at intervals from the peritoneal cavity by means of a fine, sterile glass pipette (drawn from 6.5-mm., outside diameter, glass tubing). Without application of a medium, one drop of fresh, undiluted ascitic fluid was immediately dropped on the central part of a clean cover-slip, to take up a round space of about 5 mm. in diameter. The excess of ascitic fluid was removed with a glass pipette, leaving a thin flat covering of fluid. The cover-slip was then inverted over a depression slide having a depression 15 mm. in diameter and 0.5 mm. in depth, around the rim of which a sufficient amount of vaseline was applied. Such slides were then immersed in a water bath at 37°C and observed with a phase-contrast microscope.
had been previously smeared to act as a seal. A slight pressure was applied on the cover-slip to seal it completely with the depression slide. The hanging-drop preparation thus made was ready for observation under the microscope. The observation was carried out at a temperature of 37°C in a chamber equipped with an electric warmer. The procedure for making a hanging-drop preparation was quite simple, as shown above. Therefore, unless the materials dried out, the results obtained in many preparations of the same ascitic fluid were almost constant.

RESULTS

Motility of the ascites hepatoma cells.—In a hanging-drop preparation made with AH 130, the circumference of “hepatoma islands” was smooth, and they had a tortoise shell-like appearance (Fig. 1). Shortly after that, not only did individual, isolated tumor cells display ameboid motion, but “islands” were also found to move as a unit, the total mass changing in shape, with pseudopods thrust from its periphery (Figs. 2, 3). With the lapse of time, almost all tumor cells became actively ameboid, and individual tumor cells forming “islands” were also observed to detach from one another, showing ameboid movement (Fig. 4). The rate of locomotion was relatively slow. About 4 hours after the preparation was made, the structure of “islands” observed at the beginning could not be detected anywhere. Intermingled with isolated tumor cells and “islands,” a few polymorphonuclear leukocytes, lymphocytes, and macrophages were also seen in the ascitic fluid. These cells could easily be distinguished from the tumor cells, not only by their size and shape, but even more accurately by the speed and character of their movement.

According to this method, tumor cells usually displayed active ameboid motion for about 6 hours without showing any signs of degenerative cells. Occasionally, they displayed ameboid motion actively for over 10 hours. On the other hand, in strain AH 7974, tumor cells showed usually what less activity in the ameboid movement as compared with the activity seen in AH 130, i.e., most of the pseudopods of the cells were not typical but were tongue-like in appearance (Fig. 5). Also, alterations in shape and position of “islands” as a whole were not so remarkable as in AH 130. In strain AH 602, no separate tumor cells were seen. Almost all “islands,” as a rule, remained unchanged for about 5 hours (Fig. 6); thereafter, they began to degenerate, resulting in their separation into free round cells. In rare cases, the cells in the periphery of only a few “islands” became tongue-like in appearance, but no changes in the shape of “islands” themselves as a whole were observed. The results obtained in such observations of three strains of the ascites hepatoma were presented in Table 1. In about 70 per cent of all observations of AH 130, tumor cells showed excessively active ameboid movement. In AH 7974, active ameboid movement was observed in about 47 per cent of all observations. Furthermore, these movements were less active as compared with those of AH 130, as described above. On the other hand, in 73 per cent of all cases of AH 602, tumor cells did not show any movement and remained unchanged, and in the remaining cases a few tumor cells showed only slight motility. From these results, it may be evident that the order of decreasing motility in these three strains was AH 130, AH 7974, and AH 602, i.e., AH 130 was the most active and AH 602 the least active in motility.

Invasive growth and metastasis of the ascites hepatomas.—To study the invasiveness and metastasis production of these three strains of the ascites hepatoma, in total 59 Wistar rats were given intraperitoneal inoculations of 0.05 ml of undiluted tumor ascites of each strain in a state of “nearly pure culture.” In total, eighteen animals were sacrificed at successive intervals, and histological examinations were made of the internal surface of their abdominal wall, omentum, diaphragm, mesentery, and other abdominal organs. In the animals inoculated with AH 130 and AH 7974, respectively, a slight infiltration was noticed in the omentum on the 3rd day after inoculation (Fig. 7). The infiltration became more and more pronounced, and on the 5th day omentum, mesentery, and internal surface of the abdominal wall were completely invaded (Fig. 8). No significant difference in invasiveness has been observed so far between animals inoculated with AH 130 and those with AH 7974. In those cases in which AH 602 was used, no infiltration whatsoever could be detected in the peritoneal structure examined up to the 8th day. On the 8th day, tumor cells were found merely to adhere to the surface of the omentum (Fig. 9). On the 11th day, a slight infiltration could be seen in the omentum and mesentery (Fig. 10).

The remaining 41 animals were kept until the tumor caused death and then autopsied. Median survival time of the rats inoculated with AH 130 was 10 days, and those with AH 7974 and AH 602, 13 and 15 days, respectively (Table 2). A large number of tissue samples from the abdominal cavities, lungs, and lymph glands were examined histologically. Metastatic frequency of each strain of the ascites hepatoma was shown in Table 2. In animals inoculated with AH 130, conspicuous infiltration of the tumor was observed macroscopically in the omentum, mesentery, internal surface of the abdominal wall, retroperitoneal fatty tissue, and diaphragm. The infiltration was also seen on

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TABLE 1

**MOTILITY OF TUMOR CELLS IN STRAINS AH 130, AH 7974, AND AH 602**

Five animals were used per group. Three preparations of each ascitic fluid were used, so that the total number of preparations tested each way was 15.

<table>
<thead>
<tr>
<th>Date after inoculation</th>
<th>Motility*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>AH 130</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<tr>
<td>total:</td>
<td>15</td>
</tr>
<tr>
<td>Per cent:</td>
<td>1.3</td>
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* All results were obtained by observations carried out 4 hours after the preparations were made. +++ = marked, a major portion of “islands” or individually isolated tumor cells showed ameboid motility; ++ = moderate, between +++ and +; + = slight, only a few tumor cells showed ameboid motility; = ameboid motility was not observed at all.

TABLE 2

**METASTATIC FREQUENCY OF THE ASCITES HEPATOMA**

Rats were inoculated intraperitoneally with 0.05 ml. of undiluted tumor ascites in a state of “nearly pure culture” of each strain.

<table>
<thead>
<tr>
<th>Strain of Tumor</th>
<th>Median Survival Time (days)</th>
<th>Median Metastases in Paratracheal Lymph Glands</th>
<th>Median Metastases in Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH 130</td>
<td>14</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>AH 7974</td>
<td>15</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>AH 602</td>
<td>14</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

* All results were obtained by observations carried out 4 hours after the preparations were made. +++ = marked, a major portion of “islands” or individually isolated tumor cells showed ameboid motility; ++ = moderate, between +++ and +; + = slight, only a few tumor cells showed ameboid motility; = ameboid motility was not observed at all.
the surface of the liver and spleen. The pancreas was completely invaded by the tumor in almost all cases, and no normal tissues of the pancreas remained. Furthermore, metastases in the paratracheal lymph glands were observed in twelve out of fourteen animals (85 per cent) (Fig. 11), and the lung metastases were confirmed histologically in 42 per cent of the animals (Fig. 12). In animals inoculated with AH 7974, infiltration of tumor was seen in the same tissues as in AH 130, but generally to a somewhat slighter degree than in AH 130. Nine out of thirteen animals (69 per cent) showed metastases in the paratracheal lymph glands, and two of thirteen animals, 15 per cent, showed lung metastases. Of the three strains, tumor infiltration was the least when AH 602 had been used, i.e., only slight infiltration was found in the omentum, the pancreas frequently being intact. Infiltration of the internal surface of the abdominal wall and diaphragm usually could not be detected macroscopically. Metastases in the paratracheal lymph glands were seen in 28 per cent of all cases. Lung metastases were never encountered.

From these results, it was evident that invasive growth of AH 130 was the first to begin after the inoculation and that it was the most vigorous of the three strains. Contrary to the AH 130, invasive growth of AH 602 started the latest and was the least; that in AH 7974 was intermediate. The frequency of metastasis also was highest in the animals inoculated with AH 130 and lowest in those inoculated with AH 602. The metastatic frequency of AH 7974 was between the highest and the lowest, as was true for invasive growth. Metastasis via lymph vessels was always observed more frequently, without regard to tumor strain, than that via the blood-stream.

DISCUSSION

All tumor strains used in the present study are rat hepatomas, i.e., they are cancers having the same origin but developed in different animals. A clear difference was observed in the motility of tumor cells among these three strains. While it might be questionable whether the medium used was equally suitable for all three strains at the time the comparative observations were made by the tissue culture method, such a question may not be pertinent in the present study, because fresh materials were used without applying any medium. Consequently, such a difference as observed in the present study could be regarded as that in the in vivo tumor cells per se. Based on his study on chromosome number and drug resistance of hepatoma cells, Yoshida (16) reported that every hepatoma has its own individual biological properties. It is also very interesting that the motilities of tumor cells differed depending upon the hepatoma strain. Furthermore, from results described above, it may be evident that there was good correlation between the intensity of invasive growth or metastatic frequency and the motility of tumor cells. These facts prove that motility of tumor cells is at least one of the important factors responsible for the invasive growth and metastatic spread of the tumor. Furthermore, these facts facilitate Coman’s opinion (7) that such factors as multiplication rate, liberation of lytic substance, and loss of growth restraints can no longer be regarded as essential factors in invasiveness, since these qualities are either shared by noninvasive tumors or do not exist in malignant tumors. That the motility depends on the decrease of mutual adhesiveness was reported by previous investigators (4, 6, 8, 14). Based on this fact, it may be assumed that the large number of isolated tumor cells in AH 130 was due to decrease in adhesiveness. It may also be understandable that, on the contrary, AH 602, which has low motility, did not contain isolated tumor cells. Median survival time of the rats inoculated with AH 130 was 10 days, and of those with AH 7974 and AH 602, 13 and 15 days, respectively. From these results, it can be said that the more active the motility of tumor cells, the higher the incidence of invasive growth and the shorter the survival time of host animals.
FIGS. 7-10.—The greater omentum of rats sacrificed at successive intervals after intraperitoneal inoculation of the ascites hepatoma. X550.

Fig. 7.—Three days after inoculation with the AH 130. Slight infiltration of tumor cells can already be observed at the nodes on the surface of the great omentum. X550.

Fig. 8.—Five days after inoculation with the AH 130. Great omentum has been completely invaded by tumor cells. X550.

Fig. 9.—Eight days after inoculation with the AH 602. Tumor cells are found merely to adhere to the surface of the omentum. X550.

Fig. 10.—Eleven days after inoculation with the AH 602. The slight infiltration of tumor cells is observed at the nodes. X550.

Fig. 11.—Metastasis in the paratracheal lymph gland, 7 days after inoculation with the AH 130. X475.

Fig. 12.—Lung metastasis, 9 days after inoculation with the AH 130. X475.
These facts seem to support the conclusion of Goldie and his associates (10) that a short life span of animals given intraperitoneal inoculations of sarcoma and carcinoma was correlated with the massive infiltration of peritoneal tissue and of abdominal organs by tumor cells.

SUMMARY

1. With the use of fresh, undiluted ascitic fluid of three strains of the ascites hepatoma, AH 130, AH 602, and AH 7974, comparative observations on the motility of living tumor cells were performed. Furthermore, the relationship between the motility of tumor cells and the invasive growth or metastatic spread was studied.

2. Distinct differences were observed in the cell motility among these three strains. AH 130 showed the most active motility, AH 7974 a moderate, and AH 602 the least.

3. The tumor strain containing the greatest number of separated free-tumor cells as well as “hepatoma islands” showed more active motility.

4. When the three tumor strains were each inoculated into the peritoneal cavity of rats, the more active tumor strain with regard to cell motility invaded the surrounding tissues sooner after inoculation than the less active; also, the median survival time of host animals inoculated with the more active tumor strain was shorter than that of the others.

5. In the autopsy findings on rats which were allowed to die spontaneously of tumor, good correlation was observed between the motility of tumor cells and the intensity of invasive growth or metastatic frequency.

6. Furthermore, metastasis via lymph vessels was always observed more frequently than that via the bloodstream, without regard to tumor strain, when the ascites hepatomas were inoculated into the peritoneal cavity of rats.

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