Hydrolytic Enzyme Activities, Migratory Activity, and in Vivo Growth and Metastatic Potential of Recent Tumor Isolates

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ABSTRACT

We isolated from an uncloned population of mouse fibrosarcoma cells a subpopulation of cells by repeated passage of the cells in medium with human serum in place of fetal calf serum. The human serum-adapted cells had an altered pattern of hydrolytic enzyme activities, migrated less actively than did the uncloned cells, and were much less malignant than were the parent cells. Both the uncloned cells and the human serum adapted cells were used to establish tumors in syngeneic mice. A number of isolates from primary tumors or metastatic tumors were obtained from these mice and grown in culture. The enzyme and migratory activities of the isolates were compared, and the degree of cancer was examined. It was found that, relative to their respective parent cells, the recent tumor isolates all showed increased chymotrypsin-like esterase activity, increased glycosidase activity, and increased β-glucuronidase activity. Of the three, glycosidase activity was increased the most. Migratory activity of the isolates obtained from the uncloned parent cells was also increased. Relative to the primary tumor isolates derived from the uncloned parent fibrosarcoma cells, the metastatic isolates had higher levels of enzyme activities and greater migratory activity. Although all recent isolates had certain common features (i.e., tendency toward higher levels of enzyme activities), the basic pattern of characteristics of each isolate reflected the characteristics of its respective parent. The isolates derived from the human serum-adapted cells, like the human serum-adapted cells, showed increased esterase activity but decreased glycosidase and glucuronidase activities and decreased migratory activity relative to the uncloned parent cells. Likewise, the degree of cancer of the isolates was still much lower than the degree of cancer of the uncloned parent cells or of the isolates derived from the uncloned parent cells. Thus, a single passage of the human serum-adapted cells through syngeneic mice did not result in a reversion of the phenotype to that of the uncloned cells.

INTRODUCTION

Tumor cells have been shown to differ from normal cells with regard to adhesive characteristics (5, 18) and migratory activity (1, 20, 27). The relationship between these characteristics is not fully understood, but the low adhesiveness of tumor cells may contribute to their abnormal migratory behavior and response to contact with other cells (24). Tumor cells have also been shown to possess elevated levels of protease and glycosidase activities relative to their normal counterparts (2, 8, 9, 19), and the high hydrolase levels may contribute to both decreased adhesiveness and abnormal migratory responses. These characteristics may, in addition, contribute to the malignant behavior of tumor cells in vivo (16, 25).

Following the work of Fidler (7) with the B16 melanoma, several studies have shown that cultures of uncloned tumor cells contain phenotypically distinguishable subpopulations (6, 10, 17). We have recently described the establishment, from an uncloned population of mouse fibrosarcoma cells, of a subpopulation of cells by selection of the cells in medium containing 10% human serum in place of fetal calf serum (22). As compared to the uncloned fibrosarcoma cells, the human serum-adapted cells had lower levels of glycosidase and glucuronidase activity and elevated levels of esterase activity when measured using a substrate specific for chymotrypsin-like enzymes. In addition, the human serum-adapted cells did not migrate as actively as did the uncloned cells in medium with fetal calf serum. When injected into syngeneic mice, the human serum-adapted cells were much less malignant than were the uncloned cells. While the uncloned cells produced tumors in 20 of 20 animals and spontaneously metastasized in 7 of these animals, the human serum-adapted cells produced tumors in only 6 of 30 animals with none demonstrating spontaneous metastases.

The current study was done as part of our efforts to determine the relationship between various tumor cell properties and the ability of these cells to demonstrate malignant potential in vivo. In this study, we compared the in vitro properties and in vivo behavior of tumor cell populations isolated in culture from either primary or metastatic tumors from animals given injections of the uncloned fibrosarcoma cells or from animals given injections of the human serum-adapted cells.

MATERIALS AND METHODS

Cells. The origin of the uncloned fibrosarcoma cells and the conditions under which these cells were grown has been described in previous reports (20, 21). Briefly, the cells were obtained from a tumor induced in a C57BL/6 mouse with 3-methylcholanthrene. The cells were grown at 37° in 5% CO2 with M199 supplemented with 10% fetal calf serum and antibiotics. Media, serum, and antibiotics were obtained from Grand Island Biological Co. (Grand Island, N. Y.). We have also described in a previous report the procedure used to isolate the human serum-adapted cells from the uncloned parent population (22). After 50 passages in medium containing 10% human serum (the serum was obtained from volunteers in the laboratory), the cells were switched over to M199 containing 10% fetal calf serum and maintained in this medium ever since. The conditions under which these cells were grown were

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2 The abbreviation used is: Medium 199, M199.
In addition to these 2 populations, we also established cultures from primary or metastatic tumors induced in C57BL/6 mice by either the uncloned fibrosarcoma cells or the human serum-adapted variant cells. The mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Fragments of tissue from primary tumors in the footpad or from visible metastatic lung tumors were aseptically removed, placed in sterile culture dishes containing a small amount of M199 with 10% fetal calf serum, and minced. Tumor cells grew out of the fragments within 1 to 2 weeks, and after 2 to 3 passages, the cells obtained from these fragments produced monolayers in culture.

In addition to the various populations of tumor cells, we also prepared fibroblasts from the kidneys of normal C57BL/6 mice. These cells were used as primary cultures only.

**In Vitro Properties.** Migration was assessed using both the agarose drop assay and the Boyden chamber assay, as previously described (20). In the agarose drop assay, the tumor cells are pelleted into an agarose drop and placed at the center of microtiter wells. The agarose drops are overlaid with culture medium, and the cells migrate out of the drops. Migration is assessed by measuring the distance migrated by the cells in 18 hr.

In the Boyden chamber assay, a single-cell suspension of cells is made and 5 x 10^5 cells are added to the top half of the chamber, which is separated from the bottom half of the chamber by a 12-μm pore size cellulose acetate filter (Schleicher & Schuell, Inc., Keene, N. H.). Since both halves of the chamber are filled with M199 containing 10% fetal calf serum, there is no gradient present, and random migration rather than chemotaxis is measured. The chambers are incubated for 4 hr, after which time the filters are fixed and stained in the normal manner, and the number of cells which have migrated into the filters are counted.

The assay procedures used to measure the various enzyme activities have also been described (22). Cell extracts were prepared with 0.5% Triton X-100 detergent (Sigma Chemical Co., St. Louis, Mo.) (2). Protein concentrations were determined using the Lowry method (11). Chymotrypsin-like enzyme activity was determined using the ester substrate, N-acetyl-0-l-phenylalanine-β-naphthyl ester (4). β-Glucuronidase and glycosidase activities were assayed using the substrates phenol-β-glucuronide and p-nitrophenyl-N-acetyl-β-d-glucosaminide, as previously described (2, 12). All substrates were obtained from Sigma Chemical Co.

**In Vivo Properties.** Syngeneic 4- to 8-week-old female C57BL/6 mice (Jackson Laboratory) were given injections of 1 to 2 x 10^6 viable tumor cells in the right rear footpads. Animals were observed at 3- to 4-day intervals, and the number of animals with visible tumors were noted. The diameters of the footpads given injections were measured with a caliper to quantitate tumor growth. Experiments were terminated 30 days after injection. Animals were killed by etherization, and the lungs were examined for metastatic nodules after inflation with India ink (26). The nodules appeared as white, raised growths against the smooth, black background of the normal lung tissue. Nodule size ranged from <1 mm to 4 mm in diameter. Only those nodules which could be clearly identified as metastatic tumors were counted. By counting only the clearly positive lesions, it is possible that a few micrometastatic lesions went undetected. In several additional animals, the lungs were removed without inflation. The large macroscopic nodules were plainly visible on these lungs, and tumor cell cultures were established from these lesions. On the other hand, lungs which demonstrated no visible lesions did not give rise to tumor cell cultures when “blind” culture of the tissue was attempted (22).

**RESULTS**

**Isolation of Cells from Primary and Metastatic Tumors.** Tumor cell cultures were established from the primary tumors of 3 mice that had been given injections of the uncloned fibrosarcoma cells and from 2 mice that had been given injections of the human serum-adapted cells. Cultures were also established from the pulmonary metastatic tumors of 3 mice that had been given injections of the uncloned cells. However, animals given injections of the human serum-adapted cells developed no detectable metastatic lesions (22), and attempts to isolate tumor cells from the lungs of these always failed. In all cultures where tumor cells were isolated, only 1 or 2 colonies/flask developed. The tumor cells were readily distinguished from the contaminating normal epithelial cells, fibroblasts, and macrophages by (a) their morphology, (b) their tendency to grow without any orientation and to lie across one another, and (c) by the extremely high density to which the cells would grow within individual colonies. In addition to this, the fibrosarcoma cells were extremely trypsin sensitive relative to the various types of normal cells, and the tumor cells were easily separated from the normal cells by a very brief (30 sec to 1 min) exposure to trypsin. By the second subculture, there was no evidence of any cells other than the tumor cells present.

Morphologically, the isolated tumor cells resembled the parent cells used to initiate the tumors. Cells from tumors induced by the uncloned cells were small and flat and grew with no orientation. The progeny isolates from the human serum-adapted cells appeared to be more spindly; they tended to grow somewhat parallel to one another, although there still was much overlapping of cells and nuclei.

Although the human serum-adapted cells and their isolates appeared to be larger than the uncloned cells, there was, in fact, no difference in protein concentrations. When extracts were made from a given number of cells from each population, protein concentrations were always very close to one another.

The growth rates of the various tumor isolates and parents were all similar (22 to 25 hr). Typical growth curves of one isolate from each group is shown in Chart 1. The progeny of the human serum-adapted cells reached saturation density at a lower level than did the progeny of the uncloned parent cells. Although growth curves are not shown for either parent population, their growth rates and saturation densities were similar to those of their progeny isolates.

**Enzyme Activities.** Extracts were prepared from each group of cells and assays were carried out as described in “Materials and Methods.” Tumor cells in the third through sixth subcultures were used. Normal mouse fibroblasts were used only in primary culture. When the tumor isolates were compared to one another and to their respective parent cells, some interesting observations were made (Table 1). First, all 3 activities measured were elevated in the recent tumor isolates. Chymotrypsin-like esterase and β-glucuronidase activities were slightly elevated, while glycosidase activity was elevated to a
greater degree. Second, activities of the metastatic tumor isolates were higher than were the corresponding activities in isolates from primary tumors, and third, the pattern of activities of the tumor isolates clearly reflected the pattern of activities in the respective parents. The human serum-adapted cells as well as their progeny tumor cells had elevated levels of chymotrypsin-like esterase activity and reduced levels of glycosidase and β-glucuronidase activities relative to the uncloned fibrosarcoma cells.

In comparison, the normal mouse fibroblasts produced (relative to the parent tumor cells) elevated levels of all 3 enzyme activities (Table 1). Chymotrypsin-like esterase activity was only slightly elevated, while both the glycosidase and β-glucuronidase activities were elevated to a greater degree.

In Vitro Migration. Migration studies were carried out with cells from each tumor cell population using both the agarose-drop and the Boyden chamber assays. It can be seen in Table 2 that the primary and metastatic tumor isolates derived from the uncloned parent fibrosarcoma cells migrated slightly more actively than did the parent cells. On the other hand, the primary tumor isolates (progeny of the human serum-adapted cells) did not show increased migratory activity relative to the parent cells. It can also be seen in this table that with regard to migratory activity, the tumor isolates reflected their respective parents. Relative to the uncloned cells and the isolates derived from these cells, the human serum-adapted cells and their progeny cells migrated much less actively.

In Vivo Growth and Metastasis. Two tumor isolates from each group were compared with regard to ability to induce tumors in syngeneic mice and to metastasize. Mice were given injections of 1.5 x 10⁶ cells from each isolate in the right rear footpad. Both of the parent cell types were also injected into mice. Tumor cell growth was observed and quantitated as described in "Materials and Methods." After 30 days, the animals were killed, and their lungs were examined for metastatic tumors. The results are shown in Table 3 and Chart 2. It can be seen in Table 3 that all animals given injections of the uncloned fibrosarcoma cells or progeny cells from this population developed tumors at the site of injection. A much lower percentage of animals that were given injections of the human serum-adapted cells or progeny from this group developed tumors. Not only did tumors develop in fewer animals given injections of these cells, but also the growth rates of the tumors induced by these cells were slower. This is shown in Chart 2. The growth rates of both the parent cell types are not shown in this chart, but they were very similar to the growth rates of their respective progeny isolates.

The metastatic potential of all groups of cells is also shown in Table 3. It can be seen that only one of 46 animals given injections of either the human serum-adapted cells or progeny from this group developed detectable metastatic lesions [the one positive animal had a single, small (<1 mm) tumor nodule]. On the other hand, the uncloned fibrosarcoma cells and the isolates from tumors initiated by these cells metastasized to the lungs in between 35 and 75% of the animals. The highest rate (75%) was obtained in animals given injections of one of the isolates from a pulmonary metastatic tumor. It can also be seen in Table 3 that the average number of lesions obtained per mouse was higher in the mice that were given injections of isolates from pulmonary tumors than in animals given injections of the parent cells or cells isolated from primary tumors.

### Table 1

Relative enzyme activities in cell extracts prepared from the various populations of fibrosarcoma cells

<table>
<thead>
<tr>
<th>Enzyme activity (%)</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chymotrypsin-like esterase activity</td>
</tr>
<tr>
<td></td>
<td>Glycosidase activity</td>
</tr>
<tr>
<td></td>
<td>β-Glucuronidase activity</td>
</tr>
<tr>
<td>Uncloned fibrosarcoma</td>
<td>100 ± 5²</td>
</tr>
<tr>
<td>Primary tumor isolates (3)</td>
<td>109 ± 5</td>
</tr>
<tr>
<td>Lung metastasis isolates (3)</td>
<td>124 ± 9</td>
</tr>
<tr>
<td>Human serum-adapted fibrosarcoma</td>
<td>169 ± 17</td>
</tr>
<tr>
<td>Primary tumor isolates (2)</td>
<td>177 ± 10</td>
</tr>
<tr>
<td>Normal mouse fibroblasts</td>
<td>136 ± 15</td>
</tr>
</tbody>
</table>

Table 2

Migratory activity of the various populations of fibrosarcoma cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Distance migrated by cells (μm)²</th>
<th>No. of cells/high-power field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncloned fibrosarcoma</td>
<td>260 ± 10³</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>Primary tumor isolates (3)</td>
<td>280 ± 20</td>
<td>24 ± 8</td>
</tr>
<tr>
<td>Lung metastasis isolates (3)</td>
<td>330 ± 20</td>
<td>27 ± 7</td>
</tr>
<tr>
<td>Human serum-adapted fibrosarcoma</td>
<td>220 ± 10</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>Primary tumor isolates (2)</td>
<td>190 ± 10</td>
<td>10 ± 4</td>
</tr>
</tbody>
</table>

² The distance migrated by cells in the agarose assay was determined from a total of 4 readings on each of 4 agarose drop cultures. The number of cells migrated in the Boyden chamber assay was determined from a total of 3 fields in each of 3 membranes.
³ Mean ± S.E.
⁴ Numbers in parentheses, number of isolates tested.

* Prepared from mouse kidney.

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Table 3
In vivo growth and metastatic potential of the various populations of fibrosarcoma cells

Animals were given injections of $1.5 \times 10^8$ viable cells in the footpad. Animals were checked for tumor growth at 3- to 4-day intervals, and the number of visible tumors was determined. Animals were killed at Day 30 by etherization, and the lungs were examined for metastatic nodules after inflation with India ink.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of tumor 'takes'</th>
<th>No. of animals injected</th>
<th>Av. no. of metastatic nodules/animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncloned fibrosarcoma</td>
<td>30/30</td>
<td>11/30</td>
<td>2 ± 1*</td>
</tr>
<tr>
<td>Primary tumor isolate 1</td>
<td>10/10</td>
<td>4/10</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Primary tumor isolate 2</td>
<td>8/8</td>
<td>5/8</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Lung metastatic isolate 1</td>
<td>20/20</td>
<td>9/20</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Lung metastatic isolate 2</td>
<td>20/20</td>
<td>15/20</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Human serum-adapted fibrosarcoma</td>
<td>5/20</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>Primary tumor isolate 1</td>
<td>9/20</td>
<td>1/20</td>
<td>1</td>
</tr>
<tr>
<td>Primary tumor isolate 2</td>
<td>2/6</td>
<td>0/6</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

DISCUSSION

We have recently described the establishment, from an uncloned culture of tumor cells, of a subpopulation by repeated passage of the cells in medium which contained human serum in place of fetal calf serum. The cells selected in this manner demonstrated reduced migratory activity in medium with fetal calf serum, as compared to the parent cells. These cells had lower levels of acid hydrolase activities but elevated levels of neutral esterase activity against a substrate specific for chymotrypsin-like enzymes. These cells were also much less malignant than was the uncloned population when injected into syngeneic mice. They induced tumors in a much lower percentage of the animals; the tumors which did develop grew much more slowly and failed to metastasize (22).

The present study was undertaken with the goal of identifying which of the in vitro characteristics we have examined contribute to the cancer in these tumor cells. We sought to do this by establishing a number of tumor cell cultures from primary or metastatic tumors of mice given injections of either the uncloned or the human serum-adapted cells and then comparing these populations to one another and to each parent line. Eight such cultures were established. Three were obtained from primary tumors induced by the uncloned parent cells, 3 were obtained from lung metastatic tumors induced by the same cells, and 2 were obtained from primary tumors induced by the human serum-adapted cells. Since none of the animals given injections of the human serum-adapted cells developed observable pulmonary metastases, no metastatic cells were isolated. (Unsuccessful attempts were made to isolate cells from possible micrometastatic lesions by blind culture of lung tissue from animals given injections.) The 8 isolates were examined for enzyme activities and for migratory activity in vitro, and of the isolates (2 from each group) were compared with regard to in vivo behavior.

When the 8 recent tumor isolates were compared to the parent cells with regard to enzyme activities, some interesting trends were observed. The recent tumor isolates all showed increased enzyme activities relative to the parent cells. Of the 3 enzymes measured, glycosidase activity showed the greatest increase. Although enzyme activities tended to increase, the basic pattern of activities in the recent isolates reflected the pattern of activity seen in the corresponding parent cells, i.e., isolates obtained from tumors induced by the human serum-adapted cells showed elevated neutral esterase activity but lower levels of glycosidase and β-glucuronidase activity relative to the uncloned parent fibrosarcoma cells. It appears from this that although all 8 recent tumor isolates showed certain common tendencies (e.g., toward elevated enzyme activities), there was no reversion of the phenotypic characteristics of the human serum-adapted cells as a result of one passage in vivo.

Similar results were obtained when migratory activity was compared. The migratory activity of the recent tumor isolates reflected the activity of the respective parent cells. Thus, the isolates obtained from tumors induced by the human serum-adapted cells, like the human serum-adapted cells, did not migrate as actively as did the uncloned parent cells or the progeny of the uncloned fibrosarcoma cells. When compared to the uncloned cells, both the primary and metastatic tumor isolates (progeny of the uncloned parent cells) actually migrated more actively than did the parent cells. The cells from the metastatic tumors were the most active. On the other hand, the isolates obtained from tumors induced by the human serum-adapted cells did not show increased migratory activity. In fact, these isolates showed slightly decreased migratory activity relative to the parent cells.

The relationship between the various enzyme activities and cell migratory activity is difficult to determine. It may be expected that the cells with the elevated chymotrypsin-like activity would migrate most actively, since it has been reported in the past that serum proteases such as plasminogen and prothrombin promote migration (3, 14, 15). On the other hand, the elevated levels of chymotrypsin-like activity could inhibit migration by interfering with the cell-substrate attachment process, which is necessary for optimal migration (24). We have found that the cells with high neutral esterase activity are much more protease sensitive than are the cells with lower activity. When harvesting cells from monolayers by trypsin treatment, the parent cells require 2 to 3 min of exposure to the enzyme, while the cells with elevated esterase activity are released after

* J. Varani, W. Orr, and P. A. Ward, unpublished observation.
The phenotype of the uncloned fibrosarcoma cells to induce tumors reflected the cells that induced the tumors to begin fibrosarcoma line (23) isolated in vitro) showed decreased reduced by the human serum-adapted cells, and an additional additional to the uncloned parent population.

In addition to comparing the in vitro properties of the various isolates, we also looked at the in vivo growth and metastatic potential of 2 isolates from each group and compared these to both parent lines. The uncloned parent cells produced tumors in 100% of the animals given injections of 1.5 x 10^5 cells (Ref. 22; Table 3), and the progeny isolates of the uncloned cells also produced tumors in 100% of the animals given injections. There was very little difference in the in vivo growth rates of tumors induced by the various lines. Some of the recent isolates did show a higher metastasis rate. Both of the isolates obtained from metastatic lung tumors showed a higher average number of metastatic tumors per animal than did either the parent cells or the isolates obtained from primary tumors, although the increase was slight and may only reflect normal biological variability.

When the cells isolated from tumors induced by the human serum-adapted cells were examined for in vivo growth, it was found that these cells produced tumors at a somewhat higher rate than did the parent cells (35 and 45%; versus 25%), but still at rates far below that of the uncloned parent cells and their progeny tumor isolates (100%). In addition, the growth rates of the isolates from tumors induced by the human serum-adapted cells were similar to the growth rate of the human serum-adapted cells and much lower than the growth rates of the other isolates or other parent line. Finally, the metastatic potential of the human serum-adapted cells and the isolates derived from these cells was very low. The low rate of metastases may reflect the relatively slow growth rate of these cells, or it may be a specific consequence of the altered enzyme pattern or altered migratory activity. It may also be a function of one or more entirely different properties. For example, the presence of a large primary tumor has been reported to suppress the development of secondary lesions (13). Perhaps the human serum-adapted cells are able to prevent the growth of metastatic lesions more effectively than do the uncloned cells. Additional studies will have to be done before this question can be answered.

In summary, it was found that tumor cells taken from recent tumor isolates showed certain differences from the parent cells, including elevated enzyme activities, increased migratory activity, and increased growth and/or metastatic potential in vivo. On the other hand, it was found that cells recently isolated from tumors reflected the cells that induced the tumors to begin with. The cells obtained from tumors induced by the human serum-adapted cells remained phenotypically similar to their parent cells. It was not necessary for these cells to revert to the phenotype of the uncloned fibrosarcoma cells to induce tumors (albeit, at a lower rate) in syngeneic mice.

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