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Comparison of Subpopulations of Tumor Cells with Altered Migratory Activity, Attachment Characteristics, Enzyme Levels and *In Vivo* Behavior*

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Abstract—*A murine fibrosarcoma cell line was established in culture and maintained on medium with* 10% *fetal calf serum. A subpopulation of cells that was adapted to grow in medium with* 10% *normal human serum was derived from this parent line. The two cell lines were compared with regard to their ability to migrate and to their ability to attach to plastic flasks. Cells from the parent line migrated actively and attached efficiently in medium with fetal calf serum. The migratory activity and attachment efficiency of these cells was greatly reduced, however, in medium with human serum. The human serum-adapted cells migrated and attached almost equally well in either serum. Activities of various enzymes of the two cell lines were compared. The human serum-adapted cells had lower levels of β-glucuronidase, acid-protease and glycosidase activities but elevated levels of neutral protease activity. The ability of these two groups of cells to form tumors in syngeneic mice was compared. The human serum-adapted cells induced tumors in a much lower percentage of the animals than did the parent fibrosarcoma cells. Furthermore, the human serum-adapted cells did not spontaneously metastasize while the parent cells did.*

INTRODUCTION

THE BIOLOGICAL properties of tumor cells that account for their ability to invade surrounding tissues and metastasize to different sites probably include cell movement, decreased adhesiveness and the production of lytic enzymes (for reviews see [1, 2]). It has been shown that tumor cells, in contrast to nonmalignant cells, contain elevated protease and glycosidase activities [3–6]. Differences between tumor cells and nontumor cells with regard to adhesiveness and migratory characteristics have also been shown [7–10]. The relationship between these properties and the growth characteristics and metastatic behavior of tumors is not completely understood and is undoubtedly very complex. One way to approach the problem would be, as Fidler has done, to select from the same parent population subpopulations of cells with different metastatic potential and then to compare the various subpopulations with regard to other characteristics [11–14]. Another approach would be to select subpopulations that have different *in vitro* characteristics and then to compare these cells with regard to their *in vivo* behavior. We describe in this report the selection from a mouse fibrosarcoma cell population of a subgroup of cells with altered *in vitro* migratory behavior, attachment capabilities and levels of protease and glycosidase enzyme activities. This subgroup is much less malignant than the parent line.

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MATERIALS AND METHODS

Parent cells

The parent cell line was established from a tumor induced in a C57 bl/6 mouse by 3-methylcholanthrene. The cells were cultured *in vitro* for approximately one year prior to establishment of the subpopulation. The parent cells were maintained in medium 199 (M199) supplemen-

ted with 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. The fetal calf serum was obtained from four lots purchased from Grand Island Biological Company (Grand Island Biological Company (Grand Island, New York). Cells were maintained at 37°C in 5% CO_2.

Establishment of the human serum-adapted cells

In a previous report [15] we described the migratory characteristics of the parent fibrosarcoma cells when suspended in medium with either fetal calf serum or normal human serum. The cells migrated less actively in human serum than in fetal calf serum. We subsequently found that when the cells were suspended as single cells in medium with either human serum or fetal calf serum, the number of cells that attached to plastic flasks was much lower in the presence of human serum [16]. The few cells that did attach in medium containing human serum into large colonies. When these cells were harvested and then suspended as single cells in human serum, most again did not attach. However, by continually selecting those cells which did attach after plating in this medium, we were able, over the course of 4–5 months, to establish a population of cells which would routinely attach to the flasks in medium with normal human serum and grow to monolayers. The cells selected in this way were then compared to the parent population with regard to migration characteristics and attachment capabilities in both fetal calf serum and normal human serum. These cells were also compared to the parent cells with regard to the levels of activity of protease and glycosidase enzymes.

Cell migration in agarose

The assessment of cell migration was done using a modification of methods described by Carpenter [17] and by Harrington and Stastny [18]. Cells were removed from monolayers, washed 4 times and centrifuged into a pellet. A typical cell pellet consisted of approximately 5×10^6 cells in a 50 μl vol. To this was added 150 μl of M199 containing 10% serum (either fetal calf serum or normal human serum), and 0.2% (w/v) Seaplaque agarose (Marine Colloids, Inc., Rockland, Maine). One to two microliter-sized drops of the cell suspension were delivered to the wells of a microtiter dish (Linboro Scientific Co., New Haven, Connecticut). The microtiter dish was then placed in a refrigerator for 10 min to allow the agarose to solidify. After cooling, the agarose droplets were covered with 200 μl of chilled M199 containing 10% serum of the same type used in the agarose droplets. The overlay medium was added gently so as not to disturb the agarose drops. Tests were performed in quadruplicate. The microtiter dishes were incubated in 5% CO_2 at 37°C. Migration of the cells was measured after 18 hr by phase-contrast microscopy using an inverted tissue culture microscope. The microscope contained a calibrated grid in the eyepiece. The width of one grid space represented 100 μm actual distance at a magnification of 100 ×. The distance from the edge of the agarose drop to the leading edge of migrating cells was determined on 4 sides of each droplet. Since tests were done in quadruplicate, 16 readings were obtained for each measurement. The migration of cells out of the agarose drop produced a uniform, expanding corona of cells around the agarose drop. While the majority of the cells migrated out of the agarose drop in the corona, a few cells migrated much farther out than the major population of cells. The cells that migrated beyond the corona were counted individually. A detailed description of the migratory characteristics of the parent cells has been recently published [15].

Cell migration in Boyden chambers

Modified Boyden chambers were also used to assess migration based on a method described by Romualdez and Ward for chemotaxis [19]. Cells were added to the top half of the chamber at a concentration of 5×10^5 cells per chamber in M199 with 10% serum M199 with serum was added to the bottom half of the chamber. The chambers were incubated for 4 hr at 37°C, after which time the membranes were fixed and cell migration assessed in the normal manner. The number of cells that migrated into the filters in 5 high power fields was counted. Selectron filters (Schleicher and Schuell, Kenne, New Hampshire) of 12 μm porosity were used. Tests were performed in triplicate.

Preparation of extracts and enzyme assays

Extracts of tumor cells were prepared as described previously [3] except that cold 0.5% Triton X-100 was used in place of 0.1% Triton X-100. The protein content of each extract was determined by the method of Lowry [20] and adjusted to a common value by dilution of the more concentrated extract with Triton X-100. These extracts were then assayed for the various enzyme activities. Normally 100 μl of each extract (containing 50–100 μg of protein) was used. β-Glucuronidase activity was measured using the substrate phenolphthalein-β-glucuronide [21]. Glycosidase activity was mea-

sured using the *p*-nitrophenyl derivatives of *N*-acetyl-β-D-glucosaminide, *N*-acetyl-β-D-galactosaminide, β-D-galactopyranoside, α-D-mannoside and β-D-fucoside [3] and protease activity was measured using *N*-acetyl-DL-phenylalanine-β-naphthylester, a substrate specific for chymotrypsin-like enzymes [22].

In vivo *behavior*

Syngeneic mice (4–8 weeks old, C57 bl/6 females) were injected in the right rear footpad with 1.5×10^5 viable tumor cells from each population. The diameters of the injected footpads were measured at 3–4 day intervals with a caliper. After 35 days the animals were killed by etherization and the lungs examined for metastatic nodules after India Ink inflation [23].

RESULTS

Comparison of the migratory responses of parent fibrosarcoma cells and the human serum-adapted subpopulation

Flasks of both cell lines were harvested and washed four times in serum-free M199. After the final wash the cells were resuspended in agarose-containing medium with either fetal calf serum or normal human serum and migration assays carried out as described in the Methods section. The results are shown in Table 1. The parent line of cells (maintained in fetal calf serum) migrated much better in fetal calf serum medium than in medium containing human serum. This was true regardless of whether the major populations of cells (which migrated in the corona) were compared or whether the cells that migrated beyond the corona were compared. These results are similar to what we have previously reported [15]. When the migratory patterns of the cells that had been adapted to human serum were examined, much less difference between their responses in the two sera were found. When the distance migrated by the cells in the corona was compared, fetal calf serum still supported migration better than human serum (Table 1). However, the difference was much less with this population of cells than with the parent cells. Furthermore, when the number of cells that migrated beyond the corona was compared, no discernible difference in cell migration patterns was evident.

We also studies migration of the two cell lines using the Boyden chamber assay. Both cell lines were harvested and washed four times in M199 without serum. Following the last wash, cells were divided into two groups and added to M199 containing either fetal calf serum or human serum. Migration assays were then carried out as described in the Methods section. Again, it was found (Table 1) that the parent line of cells migrated much better in fetal calf serum than in human serum, while the human serum-adapted cells migrated equally well in the presence of either serum.

Comparison of the attachment capabilities of the parent fibrosarcoma cells and the human serum-adapted line

Cells from both groups were harvested and washed four times in M199 without serum. A suspension of single cells was prepared from each and cells were added to 25 cm^2 flasks containing 4 ml of M199 with either 10% fetal calf serum or normal human serum. After 18 hr the number of cells that had attached and regained their typical spindle shape was counted. The results are shown in Table 2. Both populations attached with a high efficiency (>95%) in the presence of fetal calf serum, but only the human serum-adapted cells attached in medium containing

Table 1. *Comparison of the migratory activity of the parent fibrosarcoma cells and the normal human serum-adapted fibrosarcoma cells*

Cell type	Seru,	Agarose assay		Boyden chamber assay
		Distance migrated by cells in corona (μm ± S.E.M.)*	Number of cells beyond corona (± S.E.M.)†	Number of cells per high power field (± S.E.M.)‡
Fibrosarcoma (parent line)	Fetal calf serum	240 ± 10	12 ± 5	21 ± 2
	Normal human serum	100 ± 10	1 ± 1	5 ± 3
Fibrosarcoma (human serum-adapted)	Fetal calf serum	200 ± 10	9 ± 2	16 ± 1
	Normal human serum	170 ± 10	7 ± 1	17 ± 2

*The distance migrated by cells in the corona was determined from a total of four readings on each of four agarose drop cultures.
†The number of cells beyond the corona was determined by counting the number of cells beyond the edge of the corona in each of four agarose drop cultures.
‡The number of cells per high power field was determined from three fields in each of three membranes.

normal human serum. Although the data was routinely tabulated after 18 hr, flasks were examined at 30 min, 1, 2, 4, 8 and 18 hr to see if the parent cells might attach early in the human serum medium and then detach. No evidence to support this possibility was found. In contrast, the human serum-adapted cells began to attach quickly (within 30 min to 1 hr) and some cells began to regain their typical shape by 1–2 hr.

In order to test the stability of the migratory characteristics and attachment characteristics of the human serum-adapted cells, flasks of these cells were cultured for five passages in medium with fetal calf serum and then examined for their ability to migrate in medium with human serum and to attach in this medium. Both characteristics showed no evidence of reversion. These cells attached as efficiently and migrated as efficiently as the human serum-adapted cells that had not been subcultured in fetal calf serum.

Comparison of enzyme activities in extracts of the parent fibrosarcoma cells and the human serum-adapted subpopulation

Monolayers of both cell types were washed 4 times in phosphate-buffered saline (without pH indicator) and then treated with Triton X-100 to prepare extracts. After adjusting the protein concentration so that both extracts contained the same amount of protein, the following enzyme activities were measured: glycosidase activity against a variety of substrates; glucuronidase activity and protease activity at pH 3.7 and 7.4. Results are shown in Table 3. Activities of the enzymes at acid pH were all reduced in the human serum-adapted cells. Glycosidase activity was reduced by 28–42%. Glucuronidase activity was reduced by 21% and protease activity at pH 3.7 was reduced by 26%. In contrast, neutral-protease activity was elevated in the human serum-adapted cells.

In vivo *growth and metastatic potential of the parent fibrosarcoma cells and the human serum-adapted cells*

Both the parent cells and the human serum-adapted cells were harvested from culture by trypsinization, washed three times in serum-free M199 and resuspended at a concentration of 1.5×10^5 viable cells in 20 μl of M199. Twenty C57 bl/6 mice were injected with 1.5×10^5 viable cells from each group in the right rear footpads. A third group of animals was injected with 1.5×10^5 viable human serum-adapted cells that had been cultured in medium with 10% fetal calf serum for five passages before injection. Animals were observed every 3–4 days for signs of tumor growth. In the group of animals injected with parent cells, 8 of the 20 had grossly-visible tumors by day 10 and 20 of 20 animals had visible tumors by day 20. In the group of animals injected with the human serum-adapted cells no animals had visible tumor by day 10, only two had visible tumor by day 20, and only four had tumor by day 35 when the animals were killed. Likewise, of the 10 animals injected with the human serum-adapted cells that were cultured for five passages in fetal calf serum, only one had a grossly-visible tumor by day 10. No additional animals showed signs of tumor by day 20, and by day 35 the number of animals with gross tumor had risen to only two. These data are shown in Table 4.

Not only was the number of tumor "takes" much lower in the animals injected with the human serum-adapted cells, but the growth rate of the tumors in these animals was also much slower than the tumors in the animals that were injected with the parent cells. The tumors produced by the parent cells grew to 4–6 mm in diameter within 20–30 days. The tumors produced by the human serum-adapted cells grew only 1–2 mm in diameter in the same period. Thus there was a striking

Table 2. Comparison of attachment capability of the parent fibrosarcoma cells and the normal human serum-adapted fibrosarcoma cells

Cell type	Serum	Number of attached cells per mm^2
Fibrosarcoma (parent line)	Fetal calf serum	106 ± 11
	Normal human serum	< 1
Fibrosarcoma (human serum-adapted)	Fetal calf serum	114 ± 30
	Normal human serum	110 ± 19

Each experiment was done three times. Each value is the average number of attached cells per mm^2 determined from 8–1 mm^2 readings in each of two flasks from one such experiment.

Table 3. Relative enzyme activities in cell extracts prepared from the parent fibrosarcoma cells and the normal human serum-adapted fibrosarcoma cells

Test	Change in o.d. per hour per 100 μg of extract protein (±S.E.M.)	
	Parent fibrosarcoma cells	Human serum-adapted fibrosarcoma cells
Glycosidase activity using		
p-Nitrophenyl-*N*-acetyl-β-D-glucosaminide	2.146±0.016	1.236±0.006
p-Nitrophenyl-*N*-acetyl-β-D-galactasaminide	0.238±0.003	0.185±0.002
p-Nitrophenyl-β-D-galacto-pyranoside	0.307±0.003	0.235±0.016
p-Nitrophenyl-α-D-mannoside	0.189±0.006	0.122±0.003
p-Nitrophenyl-β-D-fucoside	0.281±0.003	0.200±0.003
β-glucuronidase activity	0.141±0.001	0.111±0.001
Protease activity at		
pH 3.7	0.156±0.002	0.116±0.012
pH 7.4	0.462±0.004	0.600±0.006

Each assay was run three times. Each o.d. value is the average of triplicate samples from one such experiment.

Table 4. Comparison of the in vivo *behavior of the parent fibrosarcoma cells and the normal human serum-adapted fibrosarcoma cells*

Cell type	Number of tumor "takes" / Number of animals injected	Number of animals with metastatic lesions in lungs
Fibrosarcoma (parent line)	20/20	7*
Fibrosarcoma (human serum-adapted)	4/20	0
Fibrosarcoma† (human serum-adapted)	2/10	0

*Refers to the number of animals with lesions odentified after India Ink inflation. A total of 15 lesions were observed in the 7 animals.

†These human serum-adapted cells were cultured in medium with fetal calf serum for five passages prior to injection into animals.

difference between the parent cells and the human serum-adapted cells regarding ability to produce tumors in syngeneic mice and the growth rates of these tumors.

Thirty-five days after the injection of tumor cells into the footpads, the animals were sacrificed and examined for metastatic nodules in the lungs as described in the Methods section. Of the 20 animals injected with the parent cells, 7 showed large, visible metastatic nodules, In addition to the 20 animals examined after India Ink injection, two additional animals that had been injected 35 days previously with the parent cells were killed and their lungs removed aseptically without inflation with India Ink. Large metastatic nodules were readily visible on the surface of the lungs from each animal. These nodules were sterilly excised and placed in culture. Clones of cells that were morphologically very similar to the parent cells grew out from these nodules. By the second *in vitro* passage these cells appeared homogeneous. When injected back into syngeneic mice, they induced the development of tumors. In contrast to these findings, none of the 30 animals injected with the human serum-adapted cells showed any evidence of metastatic lesions when their lungs were examined following India Ink inflation. Furthermore, attempts to culture tumor cells from the lungs of these animals always failed. These data are shown in Table 4.

DISCUSSION

The ability of tumor cells to metastasize to sites distant from the primary tumor implies a separation of cells (either individually or in

groups) from the primary mass. Three of the biological properties of tumor cells that are thought to be involved in the separation are low adhesiveness, active cell motility and production of lytic enzymes [1, 2]. The relationship of each of these properties to the metastatic process has not, however, been clearly defined, nor has the relationship of these properties, one to the others, been determined. In this study we compared the migratory characteristics and the ability of two fibrosarcoma cell lines to attach to plastic flasks. One of the fibrosarcoma lines was a subpopulation of the other. The parent cell line had been maintained in culture (containing 10% fetal calf serum) for approximately one year prior to the start of this study. The subpopulation of cells was derived from the parent line by culturing the cells over a period of 4–5 months in M199 using 10% normal human serum in place of the fetal calf serum. In a previous study [15] we found that the parent fibrosarcoma cells did not migrate as efficiently in medium with normal human serum as in medium with fetal calf serum. It was our intention to select a human serum-adapted population in the hope that the migratory activity of these cells would be enhanced.

When the migration characteristics of the two lines were compared, it was found that the human serum-adapted cells did show enhanced migratory activity in medium with normal human serum. While the parent line cells migrated optimally in medium with fetal calf serum but migrated less efficiently in medium with human serum (by any of the three criteria used), the human serum-adapted cells migrated almost equally well in either serum. A similar relationship was observed when the ability of the cells to attach to plastic flasks was compared in fetal calf serum and human serum. The parent cells attached efficiently in medium with fetal calf serum, but did not attach in medium with human serum. The human serum-adapted cells attached efficiently in either serum. It is not known what relationship these two properties (migration and attachment) have to one another, but it is not unlikely that they are related. It is necessary for a motile cell to be able to adhere to a substrate in order to "pull" itself along [1]. It may be that the inability of the parent cells to migrate optimally in human serum medium is reflective of the inability of the cells to attach properly under these conditions. It is interesting that although the parent cells did not attach in normal human serum when plated as single cells, they did attach (and regain their typical spindle shape) when plated in human serum-containing medium in agarose drops. It may be that subtle differences in the attachment properties of the cells in the two sera account for the differences in the migratory activity.

The differences in migration and attachment characteristics correlated with differences in the levels of activity of certain hydrolytic enzymes. The human serum-adapted cells had lower levels of glycosidase activity (measured against 5 different substrates), lower levels of β-glucuronidase activity and lower levels of acid-protease activity. There is a considerable amount of data which suggest that high lysozomal enzyme activities in tumor cells play a role in the detachment of tumor cells from both solid tumors and from artificial substrates [24–27]. It is interesting that the ability of the human serum-adapted cells to attach in human serum is correlated with these cells having lowered acid hydrolase activities. The relationship between the altered enzyme levels and the migratory activity of the cells is not clear. It has been reported that neutral proteases such as plasmin [28, 29] and thrombin [30] are necessary for cell migration *in vitro*. We found elevated levels of neutral protease activity in the adapted cells. The increased activity could contribute to the enhanced migration of these cells in human serum. The decreased acid hydrolase activity could, on the other hand, contribute to the increased motility by facilitating the attachment of cells to the surface of the flask or membrane. It is likely that the regulation of cell migration is complex and that multiple factors are involved.

Regardless of the mechanisms involved it is clear from these findings that the human serum-adapted cells showed a lower degree of malignancy than the parent cells when injected into syngeneic mice. Progressively growing tumors developed in 20 of 20 animals injected with the parent cells and in only 6 of 30 animals injected with the human serum-adapted cells. In addition, the tumors induced by the parent cells grew faster than the human serum-adapted tumors and spontaneously metastasized to the lungs in 35% of the animals. No evidence of metastases was found in the lungs of animals injected with the human serum-adapted cells.

Several interesting questions remain to be answered. We want to know, first of all, which of the *in vitro* differences that we have identified in the human serum-adapted cells

are causally related to the decreased malignancy of these cells. We are in the process of characterizing several additional variant populations in order to help us answer this question.

We would also like to know the difference between fetal calf serum and normal human serum which accounts for the disparity in their ability to support migration and attachment. We have found that human serum has less protease inhibitor activity than fetal calf serum when measured against trypsin, plasmin and the fibrosarcoma cell-associated protease [16, 31]. The low level of inhibitor activity in human serum could affect cell attachment either by itself or in combination with concomitant activation of the serum plasminogen. it is known that human serum plasminogen is more easily activated than the plasminogen in fetal calf serum [32] and we have shown (unpublished observation) that the addition of excess trypsin, plasmin or plasminogen does inhibit cell attachment. If the low level of protease inhibitor activity in human serum does contribute to its inability to support attachment, this may explain why the cells developed lower levels of activity of various hydrolytic enzymes during the process of adaptation to human serum. We did not, however, see a corresponding drop in neutral protease activity. More work will have to be done before the role of protease inhibitors can be adequately assessed.

Another question we would like to answer concerns the adaptation process itself. Does adaptation involve selection of cells with unique characteristics or does the process involve the activation of traits present in all the cells of the original population? So far we have shown that culturing the human serum-adapted cells in fetal calf serum for five pasages does not induce a reversion of the characteristics associated with these cells. Perhaps several passages will be necessary for reversions to be manifested. We would ultimately like to select from the human serum-adapted cells a population that has lost the ability to grow in human serum (perhaps by continued passage in medium with fetal calf serum). If this can be accomplished, we will then determine if the other characteristics associated with the human serum-adapted cells are also lost. It may, however, be difficult to select the cells in this manner, since the human serum-adapted cells already grow well in fetal calf serum.

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