In Vitro and *In Vivo* Adherence of Tumor Cell Variants Correlated With Tumor Formation

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Murine fibrosarcoma variants that differed greatly in tumorgenicity, *in vivo* growth rate, and rate of spontaneous metastasis formation were compared for their ability to induce tumors in the lungs of syngeneic mice after intravenous injection of a suspension of single cells. Significantly more tumors were observed in the lungs of mice that received 1×10^5 of the cells of high malignant potential than in the lungs of animals that received 1×10^5 cells of lower malignant potential (54 tumors per animal vs 3 tumors per animal). When the tumor cells were prelabeled in culture for 24 hours with ^{125}I-IUDR prior to intravenous injection, it was found that both the "high" and "low malignant" cells rapidly accumulated in the lungs (55–59% of the radioactivity was found in the lung tissue by 5 minutes after injection). However, by 4 hours only 4% of the low malignant cells (as indicated by the amount of radioactivity present) were still in the lungs, while a significantly higher percentage (13%) of the high malignant cells were still present in the lungs. The difference between the high and low malignant cells with regard to ability to remain sequestered in the lungs of syngeneic mice and to subsequently form tumors in the lungs of these animals correlated with the ability of the cells to form stable attachments to monolayers of endothelial cells in culture. While both the high and low malignant cells attached at the same rate to monolayers of bovine endothelial cells, once the cells were attached, the low malignant cells were released by trypsin treatment more easily than the high malignant cells. These observations suggest that the difference in malignant potential between the variants may be due, at least in part, to differences in ability to form stable attachments. (Am J Pathol 1980, 101:345–352)

WE HAVE ISOLATED from a murine fibrosarcoma tumor in culture several variants that differ greatly in their malignant potential.[1,2] The "high malignant" variant lines form tumors in 100% of syngeneic C57 bl/6 mice after injection of 1×10^5 cells. The tumors grow rapidly, and spontaneously metastasize to the lungs of 35–80% of the animals. A few animals also develop extrapulmonary metastases. The "low malignant" lines form tumors in 20–45% of the animals after injection of 1×10^5 cells. The growth of the tumors is slower and more variable and spontaneous metastases to the lungs is extremely rare.

In a recent study[3] we observed that the high malignant cells were much more resistant to protease-mediated release from plastic flasks than were the low malignant cells. No differences were seen between the vari-

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Supported in part by NIH Grant CA-26263 and by a grant from the Connecticut Research Foundation.

Accepted for publication May 27, 1980.

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0002-4440/80/1110-0345$01.00

ants when their rates of release from monolayers by EDTA treatment or by mechanical agitation were compared. There was no difference between the variants when the rates of attachment to plastic flasks were compared. It was the object of the present study to determine whether differences in the *in vitro* adhesive behavior of the high and low malignant lines would be reflected by a difference in adhesiveness *in vivo* and whether the difference in *in vivo* adhesiveness would correlate with a difference in tumorgenicity.

Materials and Methods

Cells

Two of the variant tumor lines previously characterized were used in the present study. The variants chosen were typical of the high and low malignant phenotypes previously described.[1,2] Both variants were isolated from the uncloned fibrosarcoma that had been induced in a C57 bl/6 mouse with 3-methylcholanthrene. The high malignant variant was isolated from a pulmonary metastatic tumor that developed in a syngeneic mouse after injection of the parent cells into the right rear paw of the mouse and subsequent development of a primary tumor. The cells isolated from the pulmonary tumor were established in culture and maintained on Medium 199 supplemented with 10% fetal calf serum and antibiotics (as previously described). All reagents were purchased from Grand Island Biological Company (Grand Island, New York). The low malignant variant was isolated *in vitro* by serial passage of the parent cells in Medium 199 to which 10% normal human serum had been added in place of the fetal calf serum. After several passages in this medium these cells were transferred back to Medium 199 containing 10% fetal calf serum rather than normal human serum. They have been maintained since on the same medium and under the exact same conditions as the high malignant cells. Our recent reports describe *in vitro* and *in vivo* biologic differences between these two variant lines.[1-3]

In Vitro Assays of Adhesiveness

The rates of cell attachment to bovine endothelial cells *in vitro* and the rates of cell detachment from bovine endothelial cells were measured as described previously.[3] The bovine endothelial cells were kindly provided by Dr. William Douglas (Tufts University Medical School, Boston, Mass). To measure attachment, the monolayers of endothelial cells in culture dishes 35 mm in diameter were washed in Medium 199 with 10% fetal calf serum. High and low malignant tumor cells harvested by brief trypsinization were washed in Medium 199, and 5×10^4 cells were added to replicate endothelial cell monolayers. At times between 5 minutes and 60 minutes later, the nonattached cells were removed by vigorous washing with a pipette. We counted the number of released cells were counted using a hemocytometer, and by subtracting this number from the total, we determined the number of attached cells.

Protease-mediated cell detachment from monolayers of bovine endothelial cells was also measured. In these experiments about 5×10^4 high or low malignant tumor cells in Medium 199 with 10% fetal calf serum were added to monolayers of endothelial cells in 35-mm dishes and incubated for 1 day. The cells were then washed three times in Ca^{++} and Mg^{++}-free Hanks' balanced salt solution, and to each dish was added 3 ml of a protease solution (0.1% Difco trypsin in Ca^{++} and Mg^{++}-free Hanks' balanced salt solution, Difco Laboratories, Detroit, Mich.). The dishes were placed on an orbital shaker and rotated at 60 revolutions per minute at room temperature. Samples were taken at periods between 1 and 10 minutes and the number of released cells counted with a hemo-

cytometer. Although the endothelial cells were much more resistant to the protease solution than the tumor cells, in all experiments we also determined the number of these cells released by the protease solution. Pure cultures of endothelial cells (containing the same number of cells that were used in the detachment assays) were harvested and counted after 45 minutes' exposure to the protease, as were the remaining cells in the dishes actually used in the assays. By obtaining the difference in the number of cells released from the matched dishes, we could determine the number of cells that were released from the assay dishes during the initial 10 minutes of exposure to the protease solution, which released nearly all of the tumor cells. It was found that less than 10% of the endothelial cells were released during the initial exposure to the protease solution in the presence of the tumor cells. This finding was very similar to what was found when pure cultures of endothelial cells were treated with the protease solution for a period of 10 minutes.

In Vivo Tumor Cell Distribution

To quantitate the arrest of tumor cells in the lungs and the subsequent release of the cells, we used a procedure very similar to that used by Fidler [4] with the B16 melanoma cells. The cells were subcultured one day before use. The freshly subcultured cells were incubated in growth medium to which 10 μCi of ^{125}I-IUDR had been added. After incubation for 24 hours in this medium, the cells were harvested by brief trypsinization, washed three times to remove free radioactivity, and suspended in serum-free Medium 199. The clumps of cells were allowed to settle for about 10 minutes, after which the supernatant was gently decanted. The decanted supernatant was found to contain mostly single cells and a few aggregates of two or three cells. The concentration of cells was determined and adjusted to 1×10^5 cells per 200 μl. Typically it was found that the cells took up the radioactivity at between 0.1 and 1.0 counts per minute per cell. The low malignant cells typically contained about 10–20% more radioactivity per cell than the high malignant cells. Syngeneic mice were injected with 1×10^5 cells (200 μl) by the intravenous route. At 5 minutes and 4 hours after injection, blood samples were taken from each mouse via retroorbital bleeding, and the animals were killed by cervical dislocation. The lungs, liver, and the spleen were removed from each animal and placed in tubes containing 95% ethyl alcohol. The organs were left in the alcohol for 4 days so that any non-cell-associated radioactivity would be washed out. The alcohol was replaced with fresh alcohol daily. At the end of 4 days each sample was counted with a γ-scintillation counter (Searle, Model 1195). The amount of radioactivity was assumed to be related directly to cell numbers. Samples were not taken at periods longer than 4 hours, because as the cells begin to divide, the radioactivity may not be directly related to cell numbers. We have in the past shown that although both the high and low malignant cells have similar *in vitro* growth rates, the low malignant cells show a slight lag phase before cell division begins, while the high malignant cells do not. Furthermore, the *in vivo* growth rates are not the same.[2]

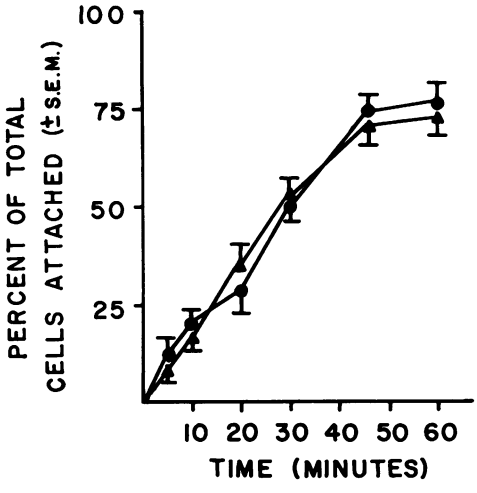
Tumor Formation in the Lungs

High and low malignant cells were harvested by brief trypsinization, washed three times, and suspended in serum-free Medium 199. The concentration of cells was adjusted to 1×10^5 cells per 200 μl and syngeneic mice were injected with 200 microliters by the intravenous route. Eighteen days later the animals were killed by etherization and the lungs examined for tumors after inflation with India ink.[5]

Results

In Vitro Adhesiveness of the High and Low Malignant Variants

The rate of attachment of the high and low malignant variants to monolayers of bovine endothelial cells was determined as described in the Ma-



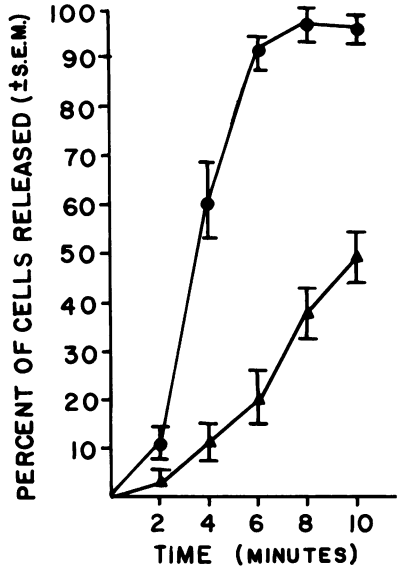
TEXT-FIGURE 1—Attachment of high and low malignant tumor cells to bovine endothelial cells. See Materials and Methods for details (high malignant cells, ▲; low malignant cells, ●).

terials and Methods section. The results are shown in Text-figure 1. It can be seen that the rate of cell attachment under the conditions employed was similar for both the high and low malignant cells. Both groups began to attach as early as 5 minutes after addition to the endothelial cells, and by 60 minutes most of the cells of each group were attached. It could be seen that by 30–45 minutes after the cells were added, some of the cells were already beginning to regain their spindle-shaped appearance. These results agree very closely with what we have reported previously.[3]

Cell detachment mediated by proteolytic enzymes was next examined. Assays were carried out as described in the Materials and Methods section. It can be seen (Text-figure 2) that the low malignant tumor cells were released from the surface of the endothelial cells by the protease solution more rapidly than were the high malignant cells. Again, these results agree closely with what we have reported previously, using plastic flasks as the substrate.[3]

Tumor Cell Distribution *in Vivo*

High and low malignant tumor cells were labeled with ^{125}I-IUDR as described in Materials and Methods, and 1×10^5 cells were injected into syngeneic mice by the intravenous route. Blood samples as well as samples from lung, liver, spleen, and kidneys were removed from the animals at 5 minutes and 4 hours, and the radioactivity was counted. The results are shown in Table 1. On the basis of the distribution of the radioactivity, by 5 minutes after injection 55% of the high malignant cells and 59% of the low malignant cells had accumulated in the lungs. Of the remaining radioactivity, some was found in the blood and other organs surveyed, but most



TEXT-FIGURE 2—Detachment of high and low malignant tumor cells from bovine endothelial cells mediated by proteolytic enzymes. See Materials and Methods section for details (high malignant cells, ▲; low malignant cells, ●).

was unaccounted for. By 4 hours, most of the radioactivity that had accumulated in the lungs of animals given injections of either cell type was released. However, a significantly greater amount of radioactivity remained in the lungs of animals given injections of the high malignant cells than in the lungs of animals given injections of the low malignant cells (13% vs 4%; $P < .01$). As radioactivity was lost from the lungs, a small increase was observed in the blood and in the other organs surveys. Most, however, remained unaccounted for and probably reflected broken-down cells.

Tumor Formation in the Lungs

High and low malignant cells were harvested, and a single cell suspension of each was prepared. Syngeneic mice were given injections of 1×10^5 cells from either group by the tail vein and left for 18 days. The animals were then killed, and their lungs were examined for tumors after inflation with India ink. Animals given injections of cells from the high malignant group had significantly more tumors in the lungs than did animals given injections of the low malignant cells (Table 2). In addition, a small number of animals were injected by the tail vein route with 1×10^6 cells from either group. All of the animals given injections of the high malignant cells died before day 18. Examination of the lungs of these animals at the time of death revealed massive colonization by tumors, with almost complete destruction of the normal lung architecture. Animals given injections of

Table 1—Accumulation of High and Low Malignant Tumor Cells in the Lungs After Intravenous Injection*

Time after injection	Number of cells in the lung (± SEM)	
	High malignant cells	Low malignant cells
5 minutes	$5.5 \pm 0.2 \times 10^4$	$5.9 \pm 0.8 \times 10^4$
4 hours	$1.3 \pm 0.2 \times 10^4$	$4.0 \pm 1.0 \times 10^3$

* Animals were given injections at Day 0 of 1.0×10^5 viable cells from a single cell suspension in 200 μl of serum-free Medium 199. The high malignant cells contained about 5.1×10^4 counts per minute of ^{125}I per 1.0×10^5 cells; the low malignant cells contained 5.5×10^4 counts per minute per 1.0×10^5 cells. Each group contained between 5 and 8 animals. The experiment was run three times; the data from one such experiment is shown here. See Materials and Methods for details.

1×10^6 low malignant cells also showed correspondingly more tumors when killed and examined on Day 18.

Discussion

In previous reports we have described the isolation and characterization of fibrosarcoma variants that differ greatly in their malignant potential.[1-3] Variants defined as "high malignant" form tumors in 100% of syngeneic mice after an injection of 1×10^5 cells. The induced tumors grow rapidly and spontaneously metastasize to the lungs. The "low malignant" variants form tumors in only 20–45% of the animals after injection of 1×10^5 cells. The tumors grow more slowly, and spontaneous metastasis occurs only very rarely, if at all. Several biologic properties distinguish the high and low malignant variants, including differences in levels of certain enzyme activities, differences in migratory activity, and differences in adhesiveness.[1-3] The goal of this study was the determination of whether differences in adhesiveness contributed to the differences in malignant potential exhibited by these populations. We sought first to establish a correlation between high adhesiveness *in vitro* and ability to adhere to and remain trapped in the lungs of syngeneic mice after the intravenous injection of the cells and second to establish a correlation between ability to remain in the lungs and ability to form tumors in the lungs. Both correlations were established. First, the tumor cells that were more adherent *in vitro* (as evidenced by resistance to detachment from monolayers mediated by proteolytic enzymes) was more adherent *in vivo* (as evidenced by a greater proportion of the cells remaining bound in the lungs after intravenous injection of labeled cells). And second, the more adherent cells subsequently formed a significantly greater number of pulmonary tumors than the less adherent cells after intravenous injection of the cells. These

Table 2—Development of Tumors in the Lungs of Mice After Intravenous Injection of High or Low Malignant Cells*

Tumor cell type	Average number of tumors per animal (± SEM)
High malignant cells	54 ± 16
Low malignant cells	3 ± 1

* Animals were given injections on Day 0 with 1×10^5 viable cells from a single cell suspension in 200 μl of serum-free Medium 199. On Day 18 the animals were killed by etherization and the lungs examined for tumors after inflation with India ink. Each group contained 10 animals. See Materials and Methods for details.

results, along with the results obtained by several other investigators, all implicate adhesiveness as being critically important in the process of tumor establishment.[6-10] It is also clear, however, from the differences reported in these various studies, that the role of adhesiveness in tumor establishment is complex. Fidler[6] and Nicholson and Winklehoke[8,9] observed, for example, that the high metastatic variants of the B16 melanoma attached to a greater extent to lymphocytes, lung tissue cells, and monolayers of 3T3 on SV40-transformed 3T3 cells than did the low metastatic variants. On the other hand, Briles and Kornfeld,[10] working with EDTA-sensitive and EDTA-resistant tumor cell variants, found no differences between them with regard to rates of attachment. Likewise, in this study and a previous study[3] we observed no difference between the high and low malignant variants of the murine fibrosarcoma in the rate of attachment of the cells to plastic flasks as to monolayers of endothelial cells but did find significant differences in rate of cell detachment from both substrates mediated by proteolytic enzymes. It was interesting that the difference between the cell lines was specific for susceptibility to detachment mediated by proteases. Both cell lines were equally resistant to release mediated by EDTA, and there was no difference between the lines with regard to release by mechanical force.[3]

These results correlating high adhesiveness (low sensitivity to trypsin-mediated release) with high malignant potential would seem at first to be in contrast to the early findings by Comon and others, who observed that tumor cells were more easily separated from tumor tissue than were normal cells from the corresponding normal tissue.[11-13] The studies by Coman have been duplicated by several other investigators,[14-16] including us (unpublished observation), with cultured cells, and their generality is not questioned. We believe that the observations by Coman and others regarding the ability of tumor cells to be easily separated from tumor tissue is extremely important in influencing the ability of tumor cells to invade surrounding tissues and ultimately to spread beyond the primary tumors.

The correlation between high adhesiveness and high malignant potential that we make here in no way contradicts these earlier observations; what we are looking at is a different phenomenon. What we are looking at is the ability of isolated, individual tumor cells (already separated from other cells) to establish themselves and grow into macroscopic tumors after injection into a suitable host. Our findings and those reported by others [6-10] clearly indicate that cells that demonstrate a higher degree of adhesiveness are able to establish more tumors than cells with lower adhesiveness.

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Acknowledgment

We would like to thank Mr. Chuck Ford and Ms. Patricia Perone for technical support.