Correlation between Cell Deformability and Metastatic Potential in B16-F1 Melanoma Cell Variants

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ABSTRACT

Four B16 melanoma cell variants were investigated to determine if there exists a correlation between their deformability and their metastatic potential. Cell deformability was measured as the percentage of cells traversing 10-µm diameter Nuclepore filter membranes at constant pressure as a function of time. A method was devised to circumvent common problems encountered in cell filtration experiments, i.e., cell aggregation and adhesion to the filter and failure to recover the input. F1a cells with the lowest spontaneous metastatic rate required 44 s for 50% of the cell input to traverse the filter, whereas No. 4 cells, featuring the highest metastatic rate, needed 12 s despite the fact that the cells had identical dimensions. Other variants tested showed intermediate filterability which also correlated with their metastatic potential. Cells, when pretreated with cytochalasin B at a final concentration of 21 µM exhibited increased filterability (75% and 42% greater than control for F1a and No. 4 cells, respectively). Somewhat smaller increases were observed after colchicine treatment. The findings imply major involvement of the cytoskeleton in the filterability and thus deformability of these B16 variants. Such physicochemical factors may play an important role in the metastasis of this and possibly other tumor types.

INTRODUCTION

The mechanical properties of tumor cells can influence their ability to survive stresses occurring in the microcirculation, thus modifying their metastatic potential (1-3). Despite other more specific antitumor defense mechanisms, tumor cells surviving in the microvasculature must be resistant to the shear stresses arising in the vascular bed (4), the frictional forces arising between their peripheries and vessel walls (5) as well as have the ability to traverse capillaries which generally are rigid and smaller in diameter than tumor cells (1, 2, 5). Depending on the type of tumor, the deformability of tumor cells or lack of it may play a critical role in their ability to form neoplastic foci. In the work presented here, we have directly addressed the question of whether a correlation exists between the deformability characteristics of variants of B16 melanoma cells and their metastatic potential. The influence and the effect of the presence of cytoskeletal elements on deformability of these B16 variants were also examined.

Although a precise definition of cellular deformability does not exist, numerous methods are available for measuring properties of cells which are related to their deformability (1, 2, 6). Micropipet aspiration and filtration methods have been most widely used in this regard. The former involve, in their simplest application, measuring the negative pressure required for a cell or a portion thereof to enter a pipet the opening of which is smaller than the diameter of the cell. Alternatively, filtration methods generally rely on the time it takes for a given volume of a cell suspension to pass through micropore membranes at constant pressure or measuring the pressure rise on passing a suspension through the filter at constant flow. A limitation of the filtration method is that information regarding material properties of the cell, such as fluid mechanical parameters, cannot be deduced and it is possible to obtain only average information on a cell population, i.e., the presence or the effects of subpopulations are easily missed. The advantages of the micropore filtration method lies in the fact that it is simple, economical, and well suited to answering the questions addressed by this investigation. The methodology for measuring B16 tumor cell filterability was specifically devised and adapted with regard to differences in their adhesive and aggregative properties from other tumor cell types investigated thus far, e.g., ascites and leukemia cells (7). With this system we were able to show that the deformability characteristics of variants of the B16 melanoma system are related to their metastatic potential.

MATERIALS AND METHODS

Investigations were carried out on three variants of F1 B16 melanoma cells which were selected through culturing on Nuclepore filters, as described earlier (8). These variants were designated No. 1, No. 2, and No. 4 and a fourth nonselected variant of F1 was designated F1A.

The metastatic capacity of all variants was assayed in vivo by i.m. injection and removal of primary tumor and a s.c. injection assay with the primary tumor left intact (8, 9).

Cell Culture. Cells were cultured as described earlier (9). Briefly, the cells were grown in a humidified incubator in 100- x 20-mm Petri dishes in Eagle's minimum essential medium (GIBCO) supplemented with 10% heat-inactivated calf serum, 1% glutamine, vitamins, and antibiotics in 5% CO2. An initial cell concentration which allowed cells to approach subconfluence 2 days after seeding was used. Care was taken to control cell density strictly since it was found to influence the filterability. Cells were harvested by rinsing the monolayers with an EDTA solution (50 µM) and incubating them at 37°C for 5 min. After resuspension, the cells were transferred to new culture dishes at the desired concentrations.

Preparation of Cells for the Filtration Assay. The medium was aspirated and the cells were washed with 10 ml of Ca2+-Mg2+-free PBS (CMF-PBS) and then incubated with 5 ml of CMF-PBS for 5-10 min. Cells harvested in this manner were then suspended and allowed to rest at room temperature for 30 min in 20 ml serum-free minimum essential medium which previously had been supplemented with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and filtered through 0.45-µm Millipore membranes. The viability of these cells was checked by the trypan blue dye exclusion test.

Drug Treatment of Cells before Filtration. Cells at a concentration of 2 x 104 cells/ml, in a total volume of 10 ml were incubated for 45 min with 100 µM colchicine (Sigma) end concentration; 21 µM cytochalasin B (CB) end concentration or 100 µM colchicine + 21 µM CB end concentration. The abbreviations used are: PBS, phosphate buffered saline; CB, cytochalasin B; CMF-PBS, calcium magnesium free phosphate buffered saline; T0, time in seconds at which 50% of the total cell input passed the filter.
concentrations, at 22°C or at 4°C without drugs. All cell suspensions, including controls, contained 20 µl of dimethyl sulfoxide, which was used as the solvent. After the incubation period, cell viability was checked by dye exclusion and the cell concentration was appropriately adjusted. Drug or cold-treated cells were filtered immediately after treatment to avoid cell regeneration. A 10-fold dilution of the incubated cell suspension which was normally performed in making concentration adjustments still maintained high enough drug concentrations to disrupt the cytoskeleton (10).

Mean cell size distributions of all investigated cell variants in pre- and postfiltrates and after drug or cold treatments were determined using a Coulter Counter Model ZBI with a C-1000 Channelizer interfaced with an Apple IIe microcomputer (11). Size measurements of nuclei were performed in the same manner following cell disruption with Zappoglobin (Coulter), a commercial cell lysing agent.

Selection of Filters. One lot (51 F2D9) of 1.3-mm diameter Nuclepore filters with 10-µm diameter pores was used. It was found that although the pore diameter was uniform there was considerable variability in pore density. Therefore, a device for preselection of filters was constructed according to a principle previously described (7). It consisted of an air pump, a water manometer, a pressure regulator, and an outlet equipped with a valve connected to the filter holder shown in Fig. 1A. Initially, the system was maintained under a constant air pressure of 30 cm H2O. With the filter in place, opening the valve resulted in a rapid decrease in pressure which was proportional to the total pore area. Only filters which gave a uniform decrease in pressure of 5-10 cm H2O were used in all experiments.

Cell Filtration. A 20-ml suspension with an appropriate cell concentration was prepared. The cells were counted and sized using the Coulter Counter. The preselected filters were placed in a custom-designed plexiglass filter holder (Fig. 1). The active filtration area of the filter was 19.6 mm2 which corresponds to 1.96 x 104 pores (calculated on the basis of data provided by Nuclepore). The chamber of the holder was filled with 1 ml of a cell suspension and connected with silicon tubing to a reservoir filled with filtration medium. The pressure was kept constant during filtration by dropwise addition of fresh medium to the reservoir. Fractions of filtrate were collected in siliconized tubes and postfiltrates and after drug or cold treatments were determined using a Coulter Counter Model ZBI with a C-1000 Channelizer interfaced with an Apple IIe microcomputer (11). Size measurements of nuclei were performed in the same manner following cell disruption with Zappoglobin (Coulter), a commercial cell lysing agent.

RESULTS

Metastatic Potential. The metastatic capacity of the cells was assayed using an i.m. and a s.c. method and was reported previously (8). Two assays were used due to the drawback that the i.m. assay, although less time consuming than the s.c. assay, involves surgical removal of the primary tumor with the inherent risk of accidental seeding of cells and other artifacts (12). Furthermore, the position of the primary tumor does not correspond to the origin of a melanoma, namely the skin.

The results, expressed as the percentage of animals with metastasis of FIA cells, were 28 ± 2 in the i.m. assay and 17 ± 16 in the s.c. assay. For No. 1 cells the values were 56 ± 11 and 50, for No. 2 cells 64 ± 1 and 88 ± 9, and for No. 4 cells 79 ± 9 and 94 ± 5 (8).

Cell and Nucleus Size. The mean cell diameter of all variants was the same and equalled 17.4 ± 0.21 µm. Size distribution curves of cells in pre- and postfiltrates were essentially identical (Fig. 2). Treatment with colchicine and CB and incubation at 4°C did not result in measurable changes in cell size distribution or mean cellular diameter (data not shown). The mean diameter of the cell nuclei was 9.8 ± 0.27 µm for all the cell variants and did not change as a result of filtration or drug treatment.

Cell Filtration. The standard deviations of the number of cells filtered were less than 5% and that of the filtration times less than 7% of the mean when filter membranes were preselected as described above. Pilot studies with different cell-to-pore ratios, driving pressures, and temperatures were designed to establish optimal conditions of filtration. Based on the analysis of filtration times, filtration curve slopes, and percentages of cells filtered, the following optimal conditions were found: 20 cm H2O driving pressure; cell-to-pore ratio, 1:1; and temperature, 22°C.

The differences in filterability of cells of the four investigated melanoma variants manifested themselves in their filtration time and kinetics of filtration (Fig. 3 and Table 1). In all cases...
The initial slope of the cumulative percentage of filtered cells which exhibited the lowest metastatic potential, was about 1.7 equalled about 90%. The filtration time of FIA-variant cells, for FIA (A) and No. 2 (•). N = 6 for No. 1 (O) and No. 4 cells (D). The abscissa and with intersections on the curves. Bars, SD of the mean. N = 5 for FIA total filtration time, longer intervals were chosen as the number of cells filtered per unit time decreased. /'*,,values are indicated with arrowed symbols on the slope of FIA cells. Analysis of T50, i.e., the time necessary for 50% of the cells to pass through the filter shows even more pronounced differences in filtration behavior. The values are presented in Table 3.

Treating the cells with agents which disrupt the cytoskeletal elements of the cells (colchicine and CB or incubation at 4°C) reduced filtration times and increased the slopes of the curves, but the percentage of cells passing through the filter still remained about the same (Fig. 4, Tables 1 and 2). CB, which damages microfilaments, modifies the filterability to a greater extent than colchicine and incubation at low temperature, both of which destroy the microtubular network (Tables 1 and 2). Following CB treatment, the filtration time of the four variants was significantly reduced in comparison with the filtration time of untreated cells. The initial slope of FIA cells treated with CB becomes nearly equal to that of untreated No. 4 cells (Table 2). The T50 values of CB-treated cells calculated from the experimental data presented in Fig. 2 are not only shorter, but the range of the means is smaller in comparison to untreated cells (Table 3). As can be seen from Table 3, the difference between FIA and No. 4 is reduced to 4.3 s in comparison with 32.2 s for untreated cells.

Colchicine-treated cells express a pattern of changes which resembles that of cells which were incubated at 4°C. A decrease in filtration time and steeper initial slopes are observed for all four variants, but these changes are smaller when compared with those observed after CB treatment (Tables 1 and 2). The T50 values of colchicine treated cells are shorter than that of untreated cells, but longer and more dispersed when compared with the T50 values of CB-treated cells (Table 3). Incubating cells simultaneously with CB and colchicine resulted in changes similar to those caused by CB treatment alone, although a bit more pronounced (Tables 1 and 2).

**DISCUSSION**

It was the aim of this investigation to assess melanoma cell deformability and to determine whether this parameter is associated with metastatic potential. The relevance of cell filterability in vitro to the rheological or other types of behavior of cells in vivo is controversial and has been widely discussed. Certainly cell filterability cannot be regarded as a sufficient model for a number of aspects of cell behavior in the microvasculature (13, 14). For example, tumor cell filterability cannot be applied for selecting cell variants on the basis of their resistance to mechanical trauma, because the resistance to mechanical stress is not heritable (15). However, it was not the intention here to consider micropore filtration as a model of the microvasculature, but to study tumor cell deformability in relation to metastatic potential.

Considerable attention has been paid to eliminating, as far as possible, interference by cell properties other than deformability, such as their adhesive or aggregative behavior, which may become manifest, depending on the experimental conditions. In contrast to previous studies (2, 6, 7) filtration time is not defined here as the time necessary for passing a given volume of cell suspension through a filter, but as the time necessary for filtering all cells capable of passing through the filter under given conditions, i.e., driving pressure, pore size, and cell-to-pore ratio. It was found in pilot studies that attempts at correlating deformability of melanoma cells with filtration time as defined by the passage of a given volume of a cell suspension did not only yield unsatisfactory results, but made them difficult to interpret. This appeared to result from the increase in cell concentration in prefiltrates (higher cell-to-pore ratio) which led to cellular aggregation, a problem which could potentially be eliminated by lowering the cell concentration thereby decreasing the cell-to-pore ratio and making cell-cell interactions less likely to occur. However, when this is done, not all potentially filterable cells are in fact filtered since the driving fluid, i.e., the suspension medium passes ahead of the cells through unoccupied pores. The unlimited supply of suspending medium used here eliminates this possibility. It also ensures that the problem of counting cells in the final stages of filtration becomes the only limiting factor since the number of
In order to obtain values of cell passage rates at the beginning of filtration, the slope during the 0–4.6-s interval (corresponding to two fractions) was calculated from all experiments. Additional filtrations were run with untreated cells for this purpose and for the \( T_{50} \) calculations presented in Table 3.

### Table 2: Initial slope of filtration curves of B16 variants

<table>
<thead>
<tr>
<th>Cell</th>
<th>None</th>
<th>4°C</th>
<th>Colchicine</th>
<th>CB</th>
<th>Colchicine + CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIA</td>
<td>3.69 ± 0.32(^a)</td>
<td>3.91 ± 0.19(^a)</td>
<td>4.24 ± 0.17</td>
<td>5.96 ± 0.24</td>
<td>6.76 ± 0.22</td>
</tr>
<tr>
<td>No. 1</td>
<td>4.41 ± 0.23</td>
<td>4.91 ± 0.10</td>
<td>5.39 ± 0.17</td>
<td>7.25 ± 0.22</td>
<td>8.41 ± 0.15</td>
</tr>
<tr>
<td>No. 2</td>
<td>5.62 ± 0.33</td>
<td>7.50 ± 0.18</td>
<td>7.55 ± 0.23</td>
<td>8.53 ± 0.12</td>
<td>9.78 ± 0.12</td>
</tr>
<tr>
<td>No. 4</td>
<td>6.10 ± 0.29</td>
<td>7.05 ± 0.20</td>
<td>7.68 ± 0.19</td>
<td>8.41 ± 0.29</td>
<td>9.76 ± 0.25</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean ± SD derived from the number of experiments shown in parentheses.

### Table 3: \( T_{50} \) values

<table>
<thead>
<tr>
<th>Cell</th>
<th>None</th>
<th>Colchicine</th>
<th>CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIA</td>
<td>43.7 ± 12.5(^a)</td>
<td>19.2 ± 1.0</td>
<td>11.0 ± 0.6</td>
</tr>
<tr>
<td>No. 1</td>
<td>17.3 ± 1.3</td>
<td>13.6 ± 0.4</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>No. 2</td>
<td>11.8 ± 0.6</td>
<td>8.4 ± 0.4</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>No. 4</td>
<td>11.5 ± 0.5</td>
<td>7.9 ± 0.2</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>No. 4</td>
<td>14.6(^b)</td>
<td>11.0</td>
<td>7.6</td>
</tr>
<tr>
<td>No. 4</td>
<td>11.5–43.7(^b)</td>
<td>(7.9–19.2)</td>
<td>(6.7–11.0)</td>
</tr>
</tbody>
</table>

\(^{a}\) Mean ± SD derived from \( T_{50} \) values calculated from the individual experiments presented in Table 2. All drug treatments led to significant decreases in \( T_{50} \) vs. untreated cells at \( P < 0.001 \). Untreated cells differed from each other at \( P < 0.001 \) except for No. 2 and No. 4 cells which did not differ significantly.

\(^{b}\) Median values of the means in each category with the range in parentheses.

The results show that the ability of the variants of B16 melanoma cells to pass through pores having a diameter equal to about 57% of the cell correlates with their metastatic potential \((P = 0.97\) and 0.98 for the percentage metastasis versus final filtration time using the i.m. and s.c. assays, respectively, \( P < 0.05 \) in both cases). Filtration curves of all the variant cell types exhibit steep slopes in the beginning and then decrease so that 50% of cell input passes through the filter after 10–50 s, i.e., over 10 times shorter than the total filtration time. The changes in slope with time suggests that the cell variants tested may be comprised of several subpopulations differing in their filterability. It appears that the cells which undergo filtration within \( T_{50} \) are more deformable. However, the proportion of these cells are different in the various cell types. For example, \( T_{50} \) for No. 4 is 11.5 s at which point only 34% FIA cells have passed through the filter. With time the conditions of filtration undergo changes. Microscopic examination of filters fixed with 4% formaldehyde in PBS at a time corresponding to \( T_{50} \) shows that about 20% of the pores are occupied by cells. This transiently decreases the chances of succeeding cells which reach the filter to pass through the pores and results in some delay of filtration of potentially equally deformable cells. At the filtration end point only a few percent of the pores remain obstructed by cells.

The influence of varying filtration conditions (filter pore size, temperature, pressure, cell adhesion) on the results has also been discussed earlier (6, 7). Our observations made in preliminary experiments were consistent with these observations. Therefore it needs only to be mentioned that no appreciable differences were found in the percentage of cells filtered or in the shapes of filtration curves at 37 and 22°C (data not shown). On the other hand, filtration at 4°C was characterized by a decrease in the percentage of filtered cells compared to filtration carried out at 22°C. This decrease occurred not only when cells were exposed to 4°C immediately before filtration, but also with cells incubated at 4°C for 45 min. Therefore, the increase in suspending medium viscosity was not compensated by slightly greater cell deformability resulting from disruption of the microtubular system by low temperature.

The differences in cellular rheological properties of the tumor cell variants reflect the sum of the differences in the physical-chemical properties of the cytoskeleton, the nucleus, and the plasma membrane. Cell nuclei are regarded as being less deformable than the surrounding cytoplasm (6, 15). The ability of melanoma cells to migrate through pores having a diameter five times smaller than their nucleus shows that significant deformation can take place under certain conditions. Filtration of Ehrlich Ascites cells and L1210 cells through pores having a diameter two times smaller than their nuclei results in cell damage revealing itself through inhibition of DNA synthesis and only later through changes in the integrity of the plasma membrane (14, 15). This suggests that the nucleus is more susceptible to mechanical stress than had previously been thought. Considering the low viscosity of the cytoplasm and the pore size chosen here, which roughly equals the diameter of the nucleus, it seems unlikely that differences in nucleus pliability would be the determinant responsible for the observed differences in deformability.

The aim of treating cells with CB or colchicine or incubating them at low temperature was to examine the effect that microtubules and microfilaments have on cell deformability. Treatment of cells with CB disrupts microfilament function (16, 17) whereas incubation at 4°C or treatment with colchicine results...
in microtubule dissociation (18, 19). The results strongly suggest that the cytoskeleton dominantly influences cellular deformability in the melanoma system. Damage to cytoskeletal elements increases the deformability of all cell variants in comparison to untreated cells. Disruption of microfilaments by CB is known to reduce heterotypical adhesion, homotypical comparison to untreated cells. Disruption of microfilaments by CB that the cytoskeleton dominates influences cellular deformability, and agglutinability by wheat germ agglutinin in the B16F10 melanoma system, in vitro (6). In the system described here, CB treatment not only increases cell filterability, but also decreases the differences in deformability of the melanoma cells in comparison with untreated cells. The differences in \( T_{50} \) between F1A and No. 4 cells decrease from 32.2 to 4.3 s while the slope of CB-treated F1A cells become equal to that of untreated No. 4 cells. In agreement with the literature, changes caused by CB are not followed by a change in cell volume (10). Incubation of cells with colchicine does not change differences between variants to the same degree as treatment with CB. This observation points to the fact that it is the CB-sensitive microfilaments of the B16 melanoma variants, rather than the microtubular structures, which are overriding responsible for their different deformability characteristics.

It remains to be seen why the small differences in the kinetics of filtration remain after treatment with CB. Previous investigations have shown that the degree of actin organization of 15 clones of melanoma K1775 cells was inversely correlated with their metastatic potential (20, 21). A similar relationship has also been shown for lectin-resistant mutants of the B16 melanoma (22). Therefore, it is possible that the amounts of the different distribution of the microfilament degradation products in cells with low metastatic potential results in greater rigidity of these cells in comparison to cells which have a poorly organized cytoskeleton. An influence of the different surface properties or architecture on intercellular interactions and on the interactions of the cells with the filter membrane or pore channels cannot presently be ruled out either. Furthermore, differences may exist in the rate at which the tumor cells deform under the given experimental conditions, thus affecting their filtration kinetics. This possibility is underscored by results reported by Weiss and Clement (23) who showed RPMI No. 41 cells to have bulk dilatant properties. The expression of these rheological properties depended on the magnitude as well as a threshold value of the applied shear stress. Other cell types have also been shown to have thixotropic properties (24).

In conclusion, the results of the present study suggest an important role of the cytoskeleton, especially the microfilament system, in the deformability of B16 tumor cells. The experimental design allowed us to circumvent two problems commonly encountered in cell filtration experiments, namely cell-cell adhesion and failure to detect all filterable cells. This approach may prove to be a valuable tool for investigating the role of tumor cell deformability in metastasis or survival in the circulation.

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