An in Vivo Study of the Role of the Tumor Cell Cytoskeleton in Tumor Cell-Platelet-Endothelial Cell Interactions


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ABSTRACT

We recently reported that disruption of tumor cell microfilaments or intermediate filaments resulted in an inhibition of the ability of tumor cells to induce the aggregation of homologous platelets in vitro (H. Chopra et al., Cancer Res., 48: 3787-3800, 1988). Previous investigators demonstrated that disruption of the tumor cell cytoskeleton decreases the ability of these cells to form lung colonies. We proposed that this latter effect is due, in part, to decreased interaction of tumor cells with platelets, following their arrest in the microvasculature. To test this hypothesis, B16 amelanotic melanoma cell microtubules, microfilaments, or vimentin intermediate filaments were disrupted with colchicine (50 μm), cytochalasin D (50 μm), or cycloheximide (50 μm), respectively, and then cells were tail vein injected into syngeneic mice. Both cytochalasin D- and cycloheximide-treated cells formed fewer lung colonies than did control cells. Colchicine, however, failed to inhibit lung colony formation. Neither colchicine nor cycloheximide treatment altered initial pulmonary arrest; however, fewer cycloheximide-treated cells remained in the lungs 8 h postinjection. Greater than 90% of control or colchicine-treated cells were found to be associated with activated platelets, and they also demonstrated typical cell membrane process formation 10 min and 8 h post-tumor cell injection. In contrast, less than 10% of cycloheximide-treated cells were in contact with activated platelets 10 min post-injection. However, by 8 h ~90% of cycloheximide-treated cells were in contact with activated platelets. This recovery coincided with the reformation of the B16 amelanotic melanoma vimentin intermediate filament network and the reacquisition of the ability to induce platelet aggregation in vitro. Neither colchicine nor cycloheximide treatment altered initial B16 amelanotic melanoma cell adhesion to murine microvessel-derived endothelial cells. This study provides in vitro evidence in support of our previous findings that disruption of certain cytoskeletal elements (i.e., vimentin, intermediate filaments) inhibits the tumor cell ability to activate platelets. This study also suggests that platelet activation may stabilize the initial tumor cell arrest in the microvasculature.

INTRODUCTION

Tumor cell arrest in the microvasculature is a critical step required for the formation of metastatic foci (1, 2). Following arrest, tumor cells must establish stable contacts with the endothelium, induce endothelial cell retraction, migrate, and attach to the subendothelial matrix. Finally, they must proteolytically digest the subendothelial matrix, extravasate, and proliferate (1-9). The successful completion of this series of events requires the coordinated action of phenotypic characteristics (i.e., adhesion receptors, proteinases, etc.) which determines the metastatic phenotype. One phenotypic characteristic which may regulate and coordinate this response is the organization of the various tumor cell cytoskeletal components (10). Hagmar and Ryd (11) provided the first direct evidence for a role of the tumor cell cytoskeleton in metastasis. They observed a marked alteration in the organ distribution of metastatic foci following microfilament disruption in TA3 tumor cells and concluded that this was due to alterations in tumor cell locomotion. Following this observation, Hart et al. (12) demonstrated that the disruption of B16F10 tumor cell microtubules or microfilaments prior to tail vein injection resulted in a decreased number of pulmonary tumor colonies, an alteration in the distribution pattern of metastasis, and a decreased adhesion to bovine endothelial cells. These effects were not the result of alterations in initial pulmonary arrest, cell volume, or growth rate. Since this pioneering work, several investigators have demonstrated the importance of the organization of actin filaments (13), intermediate filaments (14), and microtubules (15) in several aspects of metastasis (i.e., invasion, adhesion, lung colony formation, etc.). A variety of mechanisms have been proposed to explain the effects of cytoskeletal disruption on tumor cell metastasis (10). These include cell deformability, decreased homotypic and heterotypic aggregation, decreased motility, and decreased adhesion to endothelial cells.

Using W256 cells, previously, we demonstrated that disruption of microtubules, microfilaments, or vimentin intermediate filaments resulted in the inhibition of the ability of tumor cells to induce macroscopic platelet aggregation (16). However, at the ultrastructural level, there was evidence of platelet-tumor cell interactions in colchicine (i.e., microtubule disrupted)-treated cells, but not in those cells in which the microfilaments or vimentin intermediate filaments were disrupted. Subsequent studies, using a cell line (i.e., B16a) in which colchicine did not disrupt intermediate filaments, demonstrated that inhibition of macroscopic TCIPA by colchicine was due to a secondary effect of disrupting vimentin intermediate filaments (Ref. 16; Footnote 4). The observation that TCIPA is dependent upon cytoskeletal integrity suggests that the in vivo results of previous investigators using cytoskeletal disrupted tumor cells (11, 12, 14) may be due, in part, to an inhibition of tumor cell-platelet interaction following arrest, as platelets are proposed to stabilize the bond between the tumor cell and the vessel wall (17-19) in addition to facilitating endothelial cell retraction (20). Therefore, in this study, the effects of cytoskeletal disruption on lung colony formation by B16a cells were examined. In addition, morphometric and ultrastructural evidence for cytoskeletal dependent tumor cell-platelet interactions in vivo is presented.

The abbreviations used are: W256, Walker 256 carcinosarcoma cells; B16a, B16 amelanotic melanoma; PBS, fetal bovine serum; Glp/IIa, platelet glycoprotein IIb/IIIa complex; HBSS, Hanks' basic salt solution; IRGp/IIa, immunoreactive glycoprotein IIb/IIIa; MEM, minimal essential medium; PAb/IIa, polyclonal antibody raised against human platelet IIb/IIIa complex; PBS, phosphate-buffered saline; TCIPA, tumor cell-induced platelet aggregation; TXB2, hydrolysis product of thromboxane A2.

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2 To whom requests for reprints should be addressed.

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MATERIALS AND METHODS

Tumor Lines

The B16a tumor cell line was obtained from the Division of Cancer Treatment, NIH (Frederick, MD) and was passaged in syngeneic male C57BL/6/J mice (Jackson Laboratories, Bar Harbor, ME) as previously described (Ref. 21; Footnote 4).

Tumor Cell Culture

B16a tumors were removed aseptically. They were disaggregated and plated in tissue culture flasks containing MEM (Gibco, Grand Island, NY) supplemented with 5% FBS. Adherent tumor cells were grown to confluency and serially passaged as previously described (Ref. 21; Footnote 4). Low passage (i.e., second to fifth) cell cultures were used in this study. All cells were at 55% to 65% confluency when used in the experiments described below. Cells were harvested using EDTA (0.45 mmol), washed, and resuspended in the appropriate medium or buffer.

Cytoskeletal Inhibitors

Cytochalasin D (Sigma; St. Louis, MO), a microfilament inhibitor (22), was dissolved in dimethyl sulfoxide. Colchicine (Sigma), a microtubule inhibitor (23), was dissolved in glass-distilled water. Cycloheximide (Sigma), an inhibitor of the intermediate filament network (24), was dissolved in absolute ethanol. Treatment of tumor cells with colchicine (50 µmol, 15 min, 25°C) or cytochalasin D (50 µmol, 15 min, 25°C) led to the disruption of their microtubules and microfilaments, respectively. Treatment of tumor cells with cycloheximide (50 µmol, 8 h, 37°C) disrupted their vimentin-containing intermediate filaments. All treatments were conducted as described previously (Ref. 16; Footnote 4).

Platelet-rich Plasma and Aggregation Assays

Blood was drawn from the inferior vena cava of anesthetized mice into a solution of heparin sulfate (25 units/ml) and 4.8% dextrose in 0.9% saline solution (heparin:blood ratio = 1:9, v/v). Platelet-rich plasma and platelet-poor plasma were prepared as previously described (Ref. 15; Footnote 4). Aggregometry studies were performed with a Model DP-247E dual channel aggregometer (Seinco, Morrison, CO) as described previously (Ref. 25; Footnote 4). Tumor cells were pre-treated with various cytoskeletal disrupting agents or solvent controls, washed with MEM, assayed for their ability to aggregate platelets, and then used for the in vivo or in vitro assays described below.

Experiments were performed to test if the effect of cycloheximide on TCIPA was reversible. Tumor cells were treated with cycloheximide as described above. At the end of 8 h the cells were washed (3 times) with sterile MEM containing 5% FBS and allowed to recover for 8 h at 37°C. The cells were then harvested with EDTA (0.45 mmol), washed, and tested for their ability to aggregate platelets as described above.

Experimental Metastasis Assay

Tumor cells were harvested and treated with colchicine as described above or treated with cycloheximide (50 µmol) for 8 h at 37°C and then...
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Fig. 3. Morphometric evaluation of the effects of disrupting the tumor cell microtubules (colchicine, CL, 50 μmol) or the vimentin intermediate filaments (cycloheximide, CX; 50 μmol) on tumor cell association with platelet aggregates (a, b) and tumor cell arrest and retention (c, d). CTL, control. Columns, mean; bars, SD.

Histology, Electron Microscopy, and Morphometry

For histological and morphometric evaluation, animals were sacrificed by cervical dislocation at 10 min and 8 h post-tail vein injection; their lungs were removed, processed, and analyzed as described below. Five animals were used per treatment group.

Specimen Preparation. The lungs were immediately removed and placed in fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2). Six to ten 1-mm cubes were dissected from the lower lobes of the lung of each animal and stored in the above fixative at 4°C. Six pieces of lung tissue per mouse (30 per experimental group) were routinely processed and embedded in Medcast resin (Pella, Inc., Redding, CA). Ninety to 120 sections 1 μm thick were cut per group, stained with a mixture of 1% methylene blue and 1% Azure II, and examined by light microscopy for B16a cell-platelet interaction. In addition, morphometry studies as described below were conducted at the light microscopic level to corroborate with ultrastructural evidence the extent of B16a cell interaction with platelets and endothelial cells. Three to 12 thin sections (60 to 70 nm) per experimental group were cut with a diamond knife from blocks of lung tissue. The thin sections were stained with aqueous uranyl acetate and lead citrate and examined with a Zeiss EM 109 electron microscope.

Morphometric Analysis. Thirty randomly selected 1-μm sections per
experimental group were used for further analysis. Analysis included the percentage of intravascular B16a cells associated with platelets and the number of B16a cells per unit area (unit area measured as mm² of lung section).

To examine the association with platelets, sections were scanned at ×400 magnification, and B16a cells with and without associated platelet aggregates were counted. The number of tumor cells observed varied from a minimum of 84 to a maximum of 466 per experimental group. Results are expressed as the mean ± SD.

To determine the number of arrested B16a cells/unit area, the area of each section was determined using a Bioquant image analysis system (R & M Biometrics Corporation, Nashville, TN). For each experimental group a minimum area of 186 mm² of lung parenchyma was evaluated. The number of B16a cells/mm² was calculated. Results are expressed as the mean ± SD.

Radioimmunoassay

The determination of serum TXA₂ (measured as the stable hydrolysis product, TXB₂) was used as another indicator of the extent of platelet activation in vivo post-B16a cell injection. B16a cells (3.7 x 10⁶), the same number as that used in the morphological studies described above, were injected into the tail vein of mice. Six animals were used per group. Animals were then anesthetized (ether inhalation), and blood samples were removed from the inferior vena cava for TXB₂ analysis by radioimmunoassay as previously described (25). The animals were bled at 10 min and 8 h post-tumor cell injection. Blood was drawn into syringes containing 25 units of heparin, indomethacin (cyclooxygenase inhibitor; final concentration, 25 μmol), and prostacyclin (final concentration, 100 nmol) to prevent platelet activation during sampling. TXB₂ levels were measured by radioimmunoassay. Briefly, following centrifugation (150 x g), supernatants were transferred to acetone (4°C) and extracted by octodecyl (C₁₈) reverse-phase chromatography prior to radioimmunoassay analysis as previously described (25). Prior to assay, samples were dissolved in 1 ml of phosphate buffered saline-bovine γ-globulin (Miles Laboratories, Inc.). Anti-TXB₂ antiserum was purchased from Seragen (Cambridge, MA) and [³H]TXB₂ was purchased from Du Pont-New England Nuclear (Boston, MA). Standard and experimental analyses were computed with a Hewlett Packard Model 41CV computer using a weighted data reduction program (25). Results are expressed as ng of TXB₂/ml of blood.

Endothelial Cell Culture

C57BL/6J mice were utilized to obtain the pulmonary-derived endothelial cells used in these studies. Mice were decapitated, and lungs were aseptically removed and rapidly placed into cold Ca²⁺- and Mg²⁺-free HBSS. Following this initial wash, the pleural lining of the lung was fixed gently by applying 70% ethanol over the lung surface. This procedure eliminated mesothelial cell contamination in the developing culture preparation. After rinsing in HBSS, small tissue pinches, using fine forceps, were obtained close (1 to 2 mm) to the lung periphery to avoid obvious large vessels. Tissue explants were then treated with 0.1% collagenase (Type II; Worthington, Malvern, PA) in Ca²⁺- and Mg²⁺-free HBSS for 20 min at 37°C. The collagenase-treated tissue was carefully removed and plated as explants into 100-mm tissue culture dishes containing Dulbecco’s modified Eagle’s medium supplemented with 20% FBS. After 12 to 14 days in culture, the explants were removed, and areas containing endothelial cells, identifiable by their typical cobblestone growth pattern, were marked and allowed to grow an additional 7 days. Isolated colonies of endothelial cells, devoid of spindle cells, were selectively trypsinized using cloning penicylinders. Three separate endothelial cell clones were isolated, grown to confluency, and recloned. One surviving clone was established, routinely subcultured at 1:2 split ratios every 7 to 10 days, and designated CD3. Endothelial cell characteristics were verified by growth behavior pattern, the presence of Factor VIII, and prostanoid production according to published procedures (27, 28). These cells were used in adhesion assays as described below. Endothelial cells were grown in Dulbecco’s modified Eagle’s medium and 10% FBS in the presence of 10% CO₂ at 37°C in tissue culture flasks.

Adhesion

Endothelial cells (CD3) were plated on 24-well plates (Falcon; Becton Dickinson, Oxnard, CA) at a concentration of 250,000 cells/well and grown to confluency. Tumor cells were pretreated with the cytoskeletal inhibitors colchicine (50 μmol) or cycloheximide (50 μmol) as described above, washed, resuspended in platelet wash containing 2 mM CaCl₂ and 2 mM MgCl₂, and plated on wells containing CD3 cells. Tumor cells (5 x 10⁵) were plated on wells containing confluent CD3 cells and incubated for 10 min at 37°C. Nonadherent cells were removed by washing each well (twice) with platelet wash (phenol red-free MEM, pH 7.2; Gibco) containing 2 mM CaCl₂ and 2 mM MgCl₂. In addition, tumor cells were treated with cycloheximide as described above, washed with sterile MEM containing 5% fetal calf serum, allowed to recover for 8 h at 37°C, and then tested for their ability to adhere to CD3 endothelial cells.

Immunofluorescence

B16a cells (5 x 10⁴ cells/ml) were grown on coverslips placed in 6-well culture plates (Falcon) for 48 h at 37°C. These cells were incubated with cycloheximide (50 μmol) or ethanol (0.1%) for 8 h at 37°C, rinsed in PBS buffer (pH 7.2), and drained to remove excess buffer. To localize the vimentin-containing intermediate filaments, cells were permeabilized with acetone and processed for routine immunofluorescence as described previously.⁴ Antivimentin antibody (ICN, Lisle, IL) and fluorescein isothiocyanate-labeled rabbit anti-goat antibody (ICN) were used to localize the vimentin-containing intermediate filaments as described previously.⁴

Statistics

Raw data from tumor colony-forming assays were analyzed for significance using nonparametric statistical analysis (Kruskal-Wallis) and presented as the percentage of respective solvent control. Other data were analyzed by the analysis of variance test and the Scheffe test using the STATATA VIEW 512 + software (Brain Power, Inc., Calabasas, CA) on a Macintosh Plus computer (Apple Computer, Cupertino, CA). Differences were considered significant when \( P < 0.05 \).

RESULTS

Effect of Cytoskeletal Disruption on Lung-colonizing Ability. B16a cells were treated with colchicine, cytochalasin D, cycloheximide, or the appropriate solvents, and they were tested for their ability to aggregate homologous platelets and tail vein injected into C57BL/6J mice as described in “Materials and Methods.” Treatment of B16a cells with these compounds disrupted microtubules, microfilaments, and vimentin intermediate filaments.⁴ Under the conditions used, cycloheximide treatment did not alter the indirect immunofluorescent staining...
Fig. 5. Ten min postinjection, a control B16a cell is arrested in the pulmonary microvasculature with its plasma membrane intimately juxtaposed to an endothelial cell plasma membrane (arrow). In a, a localized aggregate of activated platelets can be observed in contact with the B16a plasma membrane (*). In b, as first reported by Menter et al. (43), characteristic tumor cell processes interdigitate with the platelet aggregate (*). Similarly, in c, 10 min postinjection, colchicine-treated B16a cells were observed in intimate contact with endothelial cells (arrow) and associated with focal aggregates of activated platelets (*). In d, colchicine-treated B16a cells demonstrated evidence of process formation in areas of contact with activated platelets (arrow). a, \( \times 5,500 \); b, \( \times 6,000 \); c, \( \times 6,500 \); d, \( \times 9,193 \).
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Fig. 6. Ten min postinjection cycloheximide-treated B16a cells were observed in the microvasculature in contact with endothelial cell plasma membrane (arrow). Although unactivated platelets are visible immediately adjacent to the B16a cell plasma membrane (*), no platelet aggregates were observed, and the B16a cell plasma membrane is smooth without process formation (a). A higher magnification of the above micrograph shows the area of tumor cell-platelet contact. The pattern for either microtubules or microfilaments (data not shown). As reported earlier, colchicine treatment did not alter the ability of B16a cells to induce platelet aggregation, whereas treatment with cytochalasin D or cycloheximide consistently inhibited their ability to aggregate homologous platelets (data not shown). Enumeration of lung colonies 21 days later revealed significantly decreased lung colony formation by cytochalasin D or cycloheximide-treated B16a cells (Fig. 1). In contrast, colchicine-treated cells did not form a decreased number of lung colonies but rather, a slight increase was observed in some experiments (Fig. 1).

Morphology and Morphometric Analysis. In vivo, tumor cell-platelet-endothelial cell interactions by solvent control and colchicine- and cycloheximide-treated cells were evaluated directly by light and electron microscopy at 10 min and 8 h post-tumor cell injection. Selection of these time intervals was based on the morphological studies of Crissman et al. (3, 4). Using elutriated B16a cells, significant tumor cell-platelet interaction was observed 10 min following tail vein injection and was maximal at 8 h post-tumor cell injection (3). Morphometric analysis and quantitation of tumor cell-platelet interaction was performed by light microscopic examination of 1-μm sections and confirmed by ultrastructural examination as described in “Materials and Methods.” B16a cells arrested in the microvasculature were identified by their large size and abundant cytoplasm (Fig. 2) as described by Crissman et al. (3, 4). Fig. 2 compares an ethanol-treated control cell with a cell treated with cycloheximide. At 10 min postinjection, morphological examination revealed large platelet aggregates associated with intravascular tumor cells in all control groups (Fig. 2a) and the colchicine-treated group 8 h postinjection (data not shown). However, the cycloheximide-treated group showed numerous intravascular tumor cells within the pulmonary vessels, surrounded by occasional erythrocytes, but without evidence of associated platelet aggregates (Fig. 2b). Light microscopic examination of lungs in all control and experimental groups at 8 h postinjection demonstrated numerous platelet aggregates in direct association with tumor cells. Morphologically, no qualitative or quantitative differences were observed between individual groups (data not shown). Quantitative analysis revealed that 90 to 95% of control cells were associated with platelet aggregates in both the 10-min and 8-h groups (Fig. 3, a and b). Similarly, about 95% of colchicine-treated cells were associated with platelet aggregates at 10 min and 8 h postinjection (Fig. 3a). In contrast, we observed a significant (P < 0.0001) decrease in the number of cycloheximide-treated cells associated with platelet aggregates at 10 min postinjection (Fig. 3a). However, at the 8-h interval, there were no significant differences with respect to the platelet association with cycloheximide-, colchicine-, or solvent-treated B16a cells (Fig. 3, a and b), suggesting that the effect of cycloheximide on the integrity of B16a vimentin intermediate filaments and tumor cell-platelet aggregating ability is reversible (see below).

Analysis of B16a cell distribution at 10 min (measured as cells/mm²) revealed no difference among solvent controls and colchicine- or cycloheximide-treated cells (Fig. 3, c and d), indicating a similar pattern of initial arrest in the microvasculature. Eight h following the arrest, the number of cells/mm² in both control groups decreased from the number observed at 10 min. However, there was no difference between control groups at 8 h (Fig. 3, c and d). In contrast, at 8 h the number of platelets are clearly not activated, as indicated by their round profiles and intact secretory granules. (*) a, × 9,300; b, × 21,818.

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Fig. 7. Eight h postinjection, control B16a cells (arrow in a) were observed within the microvasculature in contact with platelet aggregates (*). At this time interval, both cycloheximide (b) and colchicine (arrow in c) treated B16a cells were observed within the microvasculature associated with aggregates of activated platelets (*). A colchicine-treated B16a cell, 8 h postinjection, is in direct contact with the subendothelial matrix (arrows) following endothelial cell retraction (d). Similarly, control and cycloheximide treated B16a cells were also observed in contact with the subendothelial matrix 8 h postinjection (not shown). a. × 8,374; b. × 5,962; c. 7,040; d. 23,225.
of cells/mm² in the cycloheximide-treated group was significantly ($P < 0.02$) lower than the number of cells/mm² in the colchicine-treated group (Fig. 3, c and d).

In addition to direct morphological examination, circulating TXB₂ levels were used as another indicator of the extent of in vivo platelet activation. Thromboxane B₂ is the hydrolysis product of the short-lived ($t_{1/2} = 30$ s) thromboxane A₂, the major cyclooxygenase metabolite of arachidonic acid produced by agonist-stimulated platelets (29), including tumor cell agonists (30). Serum thromboxane B₂ levels in animals given injections of cycloheximide-treated cells were significantly lower than those of animals given injections of solvent-treated B16a cells at 10 min (Fig. 4). However, no difference was observed at 8 h (Fig. 4), consistent with the morphological data presented above (Fig. 3b).

Ultrastructural Observations. Ultrastructural examination of lung tissue removed 10 min post injection revealed control cells arrested in the microvasculature with their plasma membranes intimately juxtaposed to endothelial cell plasma membranes (Fig. 5a). A focal aggregate of activated platelets was in contact with a limited portion of the B16a cell plasma membrane, the latter of which formed processes projecting into the platelet aggregate (Fig. 5b). Similarly, colchicine-treated B16a cells were also juxtaposed to the endothelial cell plasma membrane and demonstrated focal platelet aggregates (Fig. 5c) and cell membrane process formation (Fig. 5d).

Ultrastructurally, contact between the plasma membrane of cycloheximide-treated B16a cells and the plasma membrane of endothelial cells was similar to controls (Fig. 6a). However, platelet activation was absent, even with platelets which appeared to be in contact with the tumor cell plasma membrane (Fig. 6b). Endothelial cell retraction was evident 8 h post injection of control and colchicine- or cycloheximide-treated B16a cells; this time interval also demonstrated B16a cell contact with the subendothelial cell matrix (Fig. 7a). Focal aggregates of activated, degranulated platelets were observed in contact with cells from all three groups (i.e., control and colchicine or cycloheximide treated) at 8 h post injection as were tumor cell processes (Fig. 7a).

Reversibility of the Cycloheximide Effect. Ben-Ze’ev et al. (14) demonstrated that the effect of cycloheximide on disruption of B16-F1 melanoma cell vimentin intermediate filaments was reversible. In vivo B16a cells regain their ability to activate platelets 8 h following arrest (this study). We have demon-
cells were removed. This time interval was chosen to coincide with the time interval used in the in vivo studies. As previously demonstrated, B16a cells exhibit a distinct intermediate filament network as visualized by indirect immunofluorescence. Cycloheximide treatment disrupted B16a intermediate filaments which partially reformed 8 h following removal of cycloheximide.

Immediate following treatment, cycloheximide-treated cells failed to induce platelet aggregation. However, following an 8-h recovery from cycloheximide, B16a cells aggregated homologous platelets to approximately 70% of that observed with untreated B16a cells.

**Effect of Cycloheximide on Tumor Cell Attachment to Endothelial Cells.** Morphometric data indicated that 10 min following the intravascular arrest, an equivalent number of tumor cells/mm² were present in all groups in the lung microvasculature. Since lung colony formation is decreased following disruption of intermediate filaments, but not following disruption of microtubules, this suggested that initial tumor cell arrest and attachment to endothelial cells may be independent of intermediate filaments. To test the hypothesis we used an in vivo adhesion assay with lung microvessel endothelial cells derived from the C57BL/6J mouse and cycloheximided-treated B16a cells immediately following treatment and following an 8-h recovery period (see “Material and Methods”). The adhesion assays were conducted for 10 min before nonadherent cells were removed. This time interval was chosen to coincide with the time used in the in vivo studies to evaluate the initial B16a cell arrest in the microvasculature. After a 10-min adhesion period, no difference in the number of adherent cells was found between untreated and cycloheximide-treated B16a cells (Fig. 10). However, if the cells were allowed to adhere for 45 min before being washed and counted, a significant decrease in the number of adherent cells was observed in the cycloheximide-treated group, when compared with the untreated controls (data not shown). Colchicine treatment did not alter tumor cell adhesion in either a 10-min or 45-min adhesion assay (data not shown). When B16a cells were allowed to recover (8 h) from cycloheximide treatment, there was no difference in the number of adherent cells when compared with controls following a 10-min (Fig. 10) or 45-min adhesion assay (data not shown).

**DISCUSSION**

The tumor cell cytoskeleton is proposed to play a role in metastasis by mediating cell migration (11), adhesion (12, 31), invasion (15), and cell shape change (31). Recently, Chopra et al. (Ref. 16; Footnote 4) presented in vitro evidence demonstrating that disruption of microfilaments or intermediate filaments inhibits macroscopic TCIPA, and platelet adhesion to the plasma membrane of W256 cells and B16a cells. This adhesion response appears to be mediated by a tumor cell integrin receptor (i.e., IRGpIIb/IIa) (Ref. 6; Footnote 5) that is immunologically related to the platelet glycoprotein IIb/IIa (i.e., αIIbβ3). Pretreatment of tumor cells with monoclonal or polyclonal antibodies which react against IRGpIIb/IIa inhibits macroscopic TCIPA and platelet adhesion to the plasma membrane (Refs. 16 and 32; Footnotes 4 and 5), suggesting that this receptor mediates tumor cell-platelet interaction. Light fixation of W256 cells with paraformaldehyde inhibits plasma membrane receptor mobility (including IRGpIIb/IIa) without altering membrane fluidity (16). This treatment also results in an inhibition of TCIPA and adhesion of platelet aggregates to the tumor cell plasma membrane (16). Similarly, disruption of microfilaments or intermediate filaments also inhibits IRGpIIb/IIa receptor mobility, concomitant with an inhibition of TCIPA (16). Taken collectively, the above results suggest that the loss of platelet-aggregating ability following disruption of certain cytoskeletal elements is due in part to a loss of IRGpIIb/IIa mobility and/or the loss of the ability of the receptor to bind adhesion protein ligands.

Evidence in the literature suggests that platelets are important during the arrest and attachment phase of the metastatic cascade. The studies by Crissman and coworkers (3, 4, 19) demonstrated that: (a) initial tumor cell arrest involves the juxtaposition of the tumor cell-endothelial cell plasma membranes; (b) platelet activation and their association with intravascular tumor cells probably occur postarrest; (c) platelet activation proceeds over several hours postarrest and coincides with the initiation of endothelial cell retraction; and (d) 24 h postarrest, many tumor cells remain intravascular and adherent to the subendothelial matrix with an absence of associated platelet thrombi. In general, this sequence of events is reported (Refs. 16 and 32; Footnotes 4 and 5), suggesting that this receptor mediates tumor cell-platelet interaction. Light fixation of W256 cells with paraformaldehyde inhibits plasma membrane receptor mobility (including IRGpIIb/IIa) without altering membrane fluidity (16).
adhesion to endothelium (25, 35, 36) and induce reversible endothelial cell retraction (20), thereby temporarily exposing the subendothelial matrix to tumor cell adhesion and proteolysis (20). Therefore it seems reasonable to propose that the loss of platelet-aggregating ability is, in part, responsible for decreased lung colonization following disruption of certain cytoskeletal elements.

Colchicine disruption of microtubules does not inhibit tumor cell-platelet interaction in vitro or in vivo (this study) and does not reduce lung colony formation (this study). However, disruption of microfilaments or intermediate filaments inhibits lung colony formation as well as tumor cell-platelet interaction in vitro and in vivo (this study). These results are in partial agreement with those of Hart et al. (12) in that we observed a decrease in lung colonization following disruption of microfilaments. However, we could not reproduce the previously reported decrease in lung colonization and adhesion to endothelial cells following microtubule disruption with colchicine (12). As mentioned previously, colchicine treatment can, in some cell lines, result in a secondary disruption of intermediate filaments (38, 39), which may account for the discrepancy between our results and those of Hart et al. (12). Our results are in agreement with those of Ben-Ze'ev and Raz (14) who observed that disruption of vimentin intermediate filaments reversibly reduced lung colonization. However, since cycloheximide, a protein synthesis inhibitor, was used to disrupt intermediate filaments, we cannot totally exclude other mechanisms.

When [125I]iododeoxyuridine-radiolabeled B16 cells were treated with colchicine, cytochalasin B, or a combination of both and tail vein injected, it was observed that initial pulmonary retention, measured at 2 min postinjection, was unchanged from that of controls (12). However, at 1 h postinjection, fewer colchicine- or cytochalasin B-treated cells remained in the lung (12), suggesting that cytoskeletal disruption does not affect initial arrest in the microvasculature and possibly does not affect initial adhesion to endothelium. The results of the present study support that hypothesis. First, we did not observe a difference in the number of B16 cells/mm² of lung parenchyma among the control and treated groups 10 min following injection, whereas a significant decrease was observed in the cycloheximide-treated group as compared with the colchicine-treated group at 8 h postinjection. Second, neither colchicine nor cycloheximide affected initial (i.e., 10 min) tumor cell adhesion to pulmonary microvessel-derived endothelial cells in vivo (this study; Footnote 6). However, in a longer (i.e., 45 min) adhesion assay there were fewer cycloheximide-treated cells adherent to endothelial cells, while the colchicine-treated group remained unchanged from controls. The cycloheximide results suggest that different receptor mechanisms may be responsible for initial and late stage tumor cell adhesion to endothelium and that the former is independent of cytoskeletal control. We reported previously that (a) tumor cell adhesion to endothelium can be stimulated by a number of factors, i.e., platelets (35), eicosanoids (37), thrombin (40), etc.; (b) this stimulated adhesion is mediated in part by the integrin receptor IRGpIIb/IIIa (37) and cytoskeletal involvement. (Ref. 41; Footnote 7). This study now demonstrates that the initial basal adhesion to endothelial cells is independent of IRGpIIb/IIIa (37) and cytoskeletal involvement.

The role of the tumor cell cytoskeleton in metastasis is not yet completely understood. Previous studies suggest that the tumor cell cytoskeleton may play a role in metastasis by modulating tumor cell motility and interaction with endothelial cells (10). While support for those hypotheses exists, the present study suggests that the tumor cell cytoskeleton may modulate yet another event (i.e., TCIPA), which may play an important role in tumor cell arrest and adhesion to the vessel wall during metastasis. TCIPA is a cytoskeleton-dependent, receptor-mediated phenomenon (Refs. 16 and 42; Footnote 4). However, the exact biochemical mechanisms involved in this process are as yet unknown and remain to be elucidated.

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