Inhibition of Tumor-Cell Attachment to Extracellular Matrix as a Method for Preventing Tumor Recurrence in a Surgical Wound

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Studies with four different transplantable murine tumors demonstrated that surgical instruments contaminated by contact with a tumor mass could produce tumors in a surgical wound. Eighty-seven per cent of mice with wounds made by invisibly contaminated scissors developed tumors. Irrigation with water did not prevent tumor growth. Before spilled tumor cells can invade and grow into a recurrence in the wound site, they must first attach to underlying extracellular matrix. We have devised a simple *in vitro* assay to identify inhibitors of tumor-cell attachment to develop therapeutic compounds that can prevent tumor-cell reimplantation. Various test compounds, including proteases (trypsin and Dispase), known modulators of matrix metabolism (proline analogues, cycloheximide, heparin, cortisone, cortexolone, and heparin-steroid combinations), large molecular weight polymers (agarose, dextran, polyethylene oxide), and synthetic fibronectin peptides were tested for their ability to inhibit mouse melanoma (B16-F10) cell attachment to gelatinized dishes. Most of these compounds had little or no effect on tumor-cell adhesion when cells were plated in serum-containing medium. However we identified three compounds that inhibited tumor-cell attachment in a reversible fashion: (1) a specific inhibitor of collagen deposition (L-azetidine-2-carboxylic acid); (2) a bacterial neutral protease (Dispase); and (3) synthetic fibronectin peptides that contained the arginine-glycine-asparate (RGD) sequence that is responsible for cell binding. Dispase and the RGD-containing peptides also inhibited cell implantation and prevented tumor formation in a surgical wound. We propose that inhibitors of attachment might be used either alone or with other biologic modifiers to prohibit implantation of free tumor cells at the time of surgery and thus, to prevent local tumor recurrence.

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THERE ARE SEVERAL explanations as to why a tumor grows back after an apparently complete resection. Usually the recurrence is blamed on a nidus of disease that was not removed in the initial resection. However that explanation is not always correct. For example when carcinomatosis follows iatrogenic rupture of a cystic ovarian tumor, it is clear that local recurrence is due to implantation of cells spilled during the resection. We now present experimental evidence of another less-obvious way in which tumor cells can be spilled into the wound during a resection, that is, from surgical instruments that have been invisibly contaminated by touching the tumor mass.

Before free cells in a wound can invade and grow into a clinical recurrence, they must first attach to underlying tissue structures. Cell attachment is a dynamic process that involves specific adhesive interactions between the cells and extracellular matrix.[1,2] It may be possible to interfere with this process at the time of surgery and thereby lessen the likelihood of local recurrence. We have devised a simple *in vitro* assay for identifying molecules that block tumor-cell attachment to extracellular matrix and present several agents that inhibit the implantation of tumor cells transferred to a surgical wound by contaminated instruments.

Materials and Methods

Animals

Adult (8 to 12 weeks old) male C57 B1/6 mice were obtained from Charles River Labs (Wilmington, MA) and housed in a temperature-controlled, 12-hour light/dark cycle environment for at least seven days before performing any procedures. Procedures were performed aseptically under methoxyflurane anesthesia (Pitman-Moore Inc., Washington Crossing, NJ).

Tumors

Four different transplantable murine tumors were used: Lewis lung carcinoma, B16-F10 melanoma, Reticulum

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cell sarcoma, and MB-49 bladder carcinoma. These tumors originated in C57 B1/6 mice and were maintained in our laboratory by subcutaneous passage.

Cell Culture

The B16-F10 melanoma variant (provided by Dr. Bruce Zetter) was used for the attachment assays. These cells were grown in Dulbecco's Modified Eagles Medium (DMEM; GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; GIBCO), 2 mM glutamine, 100 U/mL penicillin and 100 U/mL streptomycin.

Contamination of Surgical Instruments by Tumor

The tips of a pair of iris scissors were contaminated by taking two snips of a tumor (approximately 500 mm^3) growing subcutaneously on the back of one animal. These scissors were then used to create a lateral subcutaneous pocket off of a dorsal midline wound in another animal (*i.e.*, nontumor bearing). The dorsal midline wound was closed with skin clips. After each contamination, and before use in the experimental animal, the scissors were inspected to assure that there was no visible tissue on them. Scissors were carefully wiped clean with an alcohol sponge between uses on different experimental animals. The number of cells on the scissor tips after a typical contamination was determined for each tumor type by rinsing the scissor blades with 15 mL of phosphate buffered saline without calcium or magnesium (PBS; GIBCO), centrifuging this suspension, resuspending the pellet in 1 mL DMEM, and then counting the cells with a hemacytometer or a Coulter counter.

The B16 melanoma was used to determine whether irrigation of the wound with distilled water could diminish the chances of tumor growth. A lateral subcutaneous pocket was made with scissor tips that had been contaminated with tumor cells as described above. The pocket was then filled with either saline (n = 8) or distilled water (n = 10) until the liquid ran out of the wound (approximately 2 mL). The remaining fluid was then expressed and the wound closed as before.

Assay of Tumor-Cell Attachment

Assays were done in gelatinized 96-well tissue culture plates (Costar, Cambridge, MA). The plates were gelatinized in the following manner: 100 μL of 1.5% gelatin (Difco, Detroit, MI) in PBS was added to each well and incubated at 37 C for at least 24 hours. The gelatin solution was aspirated and the wells washed twice with 200 μL of PBS before use.

A dose range of test substances was diluted to two times final concentration in culture medium (DMEM containing 10% FCS) and 100 μL were added to each well. Aliquots of the B16-F10 cell suspension (10^4 cells/100 μL culture medium) were then added to each well and plates were incubated at 37 C for 4 or 16 hours. At the end of the incubation period, the medium was aspirated, each well was washed twice with 200 μL of PBS, and the number of cells that remained attached was determined using an acid phosphatase colorometric assay as previously described.[3] Briefly, 100 μL of an acid phosphatase reagent (0.1 M sodium acetate, 0.01 M P-nitrophenyl phosphate, 0.1% Triton-X 100) were added to each well, the plates were incubated at 37 C for two hours, then 10 μL of 1 N NaOH were added to each well. Optical density of each well was read at 405 nm on a Biotek ELISA reader. Optical density has been shown to be directly related to cell number with this method. A linear correlation between optical density and cell number in our assay was confirmed by trypsinizing and counting the number of cells in parallel wells. Results are expressed as a percentage of the optical density in untreated control wells and represent the mean of at least three wells per point. Control values had a standard error of the mean of less than 10%.

Four classes of compounds were assayed for their ability to inhibit tumor-cell attachment in this system. The first group tested were potential or known modulators of extracellular matrix metabolism. These included heparin (Hepar Industries Inc., Franklin, OH), hydrocortisone-21-phosphate disodium salt (Sigma Chemical Co., St Louis, MO), 4-pregnene-17-alpha-21-Diol-3,20-Dione (cortexolone; Steraloids Inc., Wilton, NH), and combinations of heparin with hydrocortisone or cortexolone. An inhibitor of total protein synthesis, cycloheximide (Sigma), and a specific inhibitor of collagen deposition, the proline analogue L-azetidine-2-carboxylic acid (LACA; Calbiochem, La Jolla, CA), were also tested.

The second category of compounds tested were enzymes that are commonly used to disrupt cell adhesive contacts during passaging of cultured cells. We studied the general protease, trypsin, in a tissue culture formulation that included a calcium chelator (trypsin-EDTA; GIBCO). The bacterial neutral protease, Dispase (Collaborative Research, Bedford, MA) was also tested.

The third class of compounds included large molecular weight polymers, such as dextran (10,000 MW; Sigma), agarose (Sigma), and polyethylene oxide[4] (35,000 MW and 100,000 MW; provided by Cynthia Sung, Department of Chemical Engineering, MIT). These polymers were tested for their ability to physically interfere with cell attachment by altering medium viscosity.

The final group of test compounds included small synthetic peptides, such as glycine-arginine-glycine-asparate-serine-proline (GRGDSP; Peninsula Labs, Belmont, CA) and glycine-arginine-glycine-asparate-serine (GRGDS; Peptides International, Lexington, KY), which can spe-

cifically interfere with binding of fibronectin to cell surface receptors.[5] These peptides contain the RGD sequence that has previously been shown to be responsible for most of the cell binding activity of fibronectin. A related peptide that contains a single amino acid substitution, glycine-arginine-glycine-glutamate-serine-proline (GRGESP; Peninsula Labs), was also tested.

To determine whether inhibition of cell attachment was reversible, B16-F10 cells were incubated for 16 hours in the presence of LACA (100 μg/mL), Dispase (5 units/mL), GRGDSP (125 μg/mL), or GRGDS (125 μg/mL). Unattached cells were then collected, washed twice with serum-containing medium, and replated on gelatinized 96-well plates in new culture medium. Control cells were handled in a similar manner except that because they attached to the plates after 16 hours of incubation, they were collected by trypsinization. After incubation for four hours (LACA and Dispase) or 16 hours (GRGDSP and GRGDS), the medium was aspirated, wells were washed twice with 200 μL of PBS, and remaining attached cells were trypsinized and counted on a Coulter counter.

Inhibition of Tumor-Cell Implantation In Vivo

Two inhibitors of tumor-cell attachment *in vitro,* Dispase and GRGDS, were tested for their ability to block tumor-cell implantation in a surgical wound. Wounds were made on the backs of C57 B1/6 mice with scissors invisibly contaminated by B16-F10 melanoma, as described above. Wounds were then vigorously irrigated with 3 mL of Ringers lactate, either alone or supplemented with Dispase (5 units/mL) or GRGDS (1 mg/mL). Excess fluid was expressed and the wound closed with skin clips. On day 21, 30, and 45 after surgery, the number of animals that developed tumors was determined and the maximal length and width of each tumor was measured using calipers. Tumor volumes were calculated using the following formula:

$$\text{length} \times (\text{width})^2 \times 0.52 = \text{tumor volume } (\text{mm}^3)$$

Results

Contamination of Surgical Instruments by Tumor

In total 87% of the mice developed new tumors when scissors that had been previously contaminated with tumor cells from another animal were used to make the wound. However different tumor types varied in their ability to reimplant by this method (Table 1). Interestingly 100% of mice developed tumors when wounds were contaminated by instruments that were contaminated with B16 melanoma cells, whether the wounds were irrigated with saline, distilled water, or nothing at all.

TABLE 1. *Tumor-Cell Implantation in a Surgical Wound*

Tumor Type	n	Number of Tumor Cells on Scissors (±SD)	Animals with Tumors (%)
Lewis lung	23	4050 ± 495	87
B16 melanoma	28	2750 ± 2050	100
Reticulum-cell sarcoma	10	9700 ± 4667	90
MB-49 bladder	10	1013 ± 265	50

Inhibition of Tumor-Cell Attachment In Vitro

A variety of tested substances had little or no effect on tumor-cell attachment to gelatinized plates in the presence of serum-containing medium. Some substances, however, did inhibit this process. Combinations of heparin (100 μg/mL) and cortexolone (10 to 200 μg/mL) or hydrocortisone (12.5 to 200 μg/mL), which have previously been shown to modulate matrix metabolism in embryonic tissues *in vivo*,[6,7] produced less than 20% inhibition of tumor-cell attachment at either 4 or 16 hours of incubation (Fig. 1). Heparin (12.5 to 200 μg/mL), hydrocortisone (12.5 to 200 μg/mL), and cortexolone (10 to 200 μg/mL) had no effect when administered alone. High-dose cycloheximide (100 μg/mL) was only minimally effective after four hours of incubation, although lower doses completely inhibited attachment after 16 hours (half maximal inhibition at 0.5 μg/mL). LACA also inhibited tumor-cell attachment at 16 hours when administered at doses greater than 25 μg/mL (Fig. 1) and these inhibitory effects were found to be reversible. Cells that were prevented from attaching by exposure to LACA were able to replate in fresh medium when transferred to new dishes (reattachment was more than 50% of control attachment).

Trypsin, a general protease, had no inhibitory effect on attachment after 4 or 16 hours. In contrast Dispase completely prevented cell attachment after four hours (Fig. 2). The inhibitory effect of Dispase was maintained after 16 hours of incubation (half maximal inhibition 0.8 caseinolytic units/mL) and was reversible. Almost all of the cells that were prevented from attaching to the substrate replated in fresh medium (reattachment was 83% of control).

Agarose partially blocked attachment at 4 and 16 hours when used at high concentrations; an effect that coincided with increased viscosity of the solution (Fig. 3). In contrast neither polyethylene oxide (35,000 MW at 0.5 to 100 mg/mL or 100,000 MW at 0.25 to 50 mg/mL) nor Dextran (0.0025 to 25 mg/mL) produced a viscous solution or prevented cell attachment.

The synthetic peptide GRGDSP, which contains the RGD cell-specific binding sequence of fibronectin, inhibited tumor-cell attachment at concentrations greater than

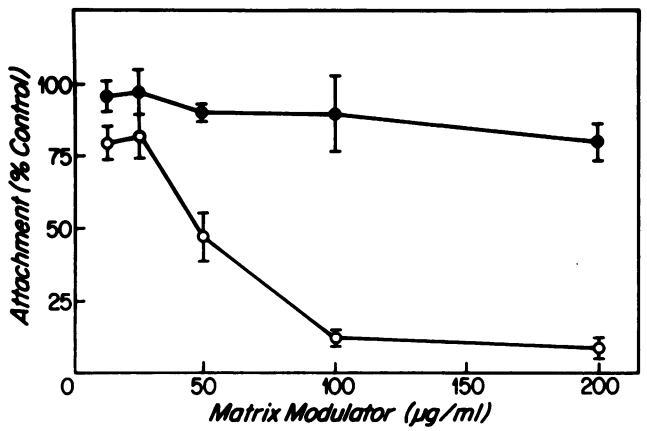

FIG. 1. Effects of modulators of extracellular matrix metabolism on tumor-cell attachment. L-azetidine 2-carboxylic acid (LACA) (O–O), a specific inhibitor of collagen deposition, completely inhibited B16-F10 melanoma-cell attachment to gelatin. Varying amounts of hydrocortisone (·–·), administered in combination with heparin (100 μg/mL), were only minimally effective (16-hour incubation; mean data is presented ± SEM).

10 ug/mL (Fig. 4). A concentration of 1 mg/mL completely inhibited attachment at four hours. The related peptide, GRGESP, also inhibited attachment but was significantly less effective than GRGDSP (Fig. 4). Cells prevented from attaching for 16 hours with GRGDSP could replate in fresh medium (reattachment was 80% of the control cells). Similar reversible inhibition of tumor-cell attachment was obtained with GRGDS (not shown).

Inhibition of Tumor-Cell Implantation In Vivo

Irrigation of a wound contaminated by B16-F10 melanoma cells with Ringers lactate containing either Dispase or RGD-containing peptide markedly delayed the appearance of tumor growing in the contaminated wound (Fig. 5). All of the animals in the group irrigated with

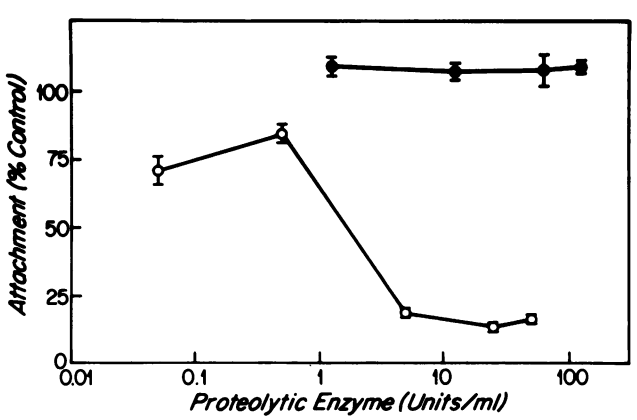

FIG. 2. Prevention of tumor-cell attachment using proteolytic enzymes. Dispase (O–O) (caseinolytic units) inhibited B16-F10 melanoma-cell attachment to gelatin-coated dishes, while Trypsin (·–·) (BAEE units) did not (4-hour incubation; mean data is presented ± SEM).

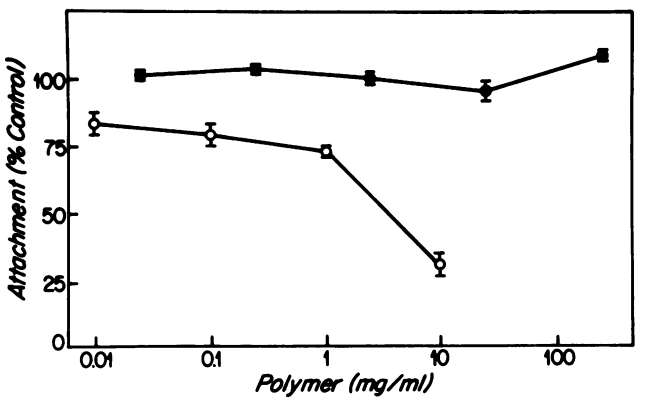

FIG. 3. Inhibition of tumor-cell attachment by altering medium viscosity. Agarose (O–O) partially blocked B16-F10 melanoma-cell attachment to the collagen matrix at concentrations that were highly viscous. Dextran (·–·) had no effect (4-hour incubation; mean data is presented ± SEM).

Ringers lactate alone died within 6 weeks of the initial procedure. In contrast 25% of the group with GRGDS-irrigated wounds and 50% of the animals with wounds irrigated with Dispase were still alive at 6 weeks. Furthermore the average volume of tumors that formed was significantly smaller in the drug-treated animals than in controls (Fig. 5). Most importantly three mice (25% of animals) from the Dispase-treated group never developed tumors. All of the surgical wounds produced in these experiments healed without breakdown or infection; all deaths were due to tumor growth.

Discussion

These data suggest that implantation of free tumor cells in a surgical wound may be an underappreciated cause of local tumor recurrence. In surgical practice it is easy to imagine that scissor tips cut across microscopic exten-

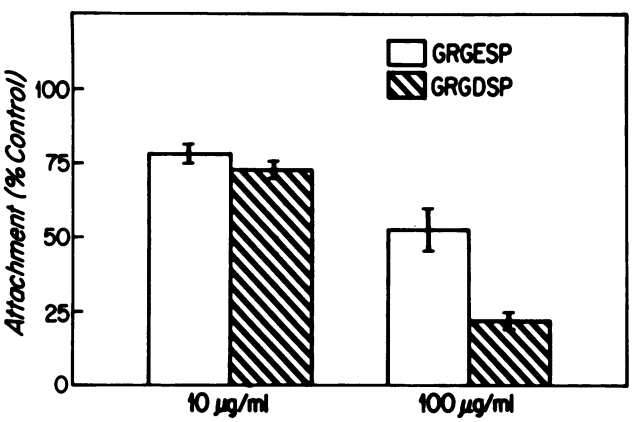

FIG. 4. Inhibition of B16-F10 melanoma-cell attachment using fibronectin-related peptides. On gelatinized dishes, both GRGDSP and GRGESP inhibited cell attachment, although the RGD-containing peptide was more effective (4-hour incubation; mean data is presented ± SEM).

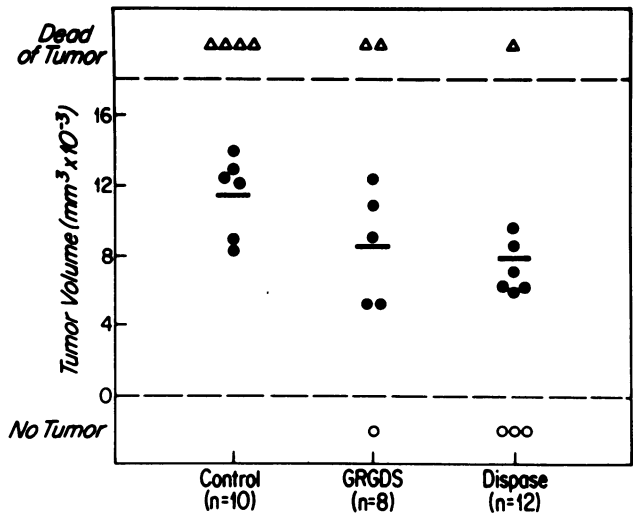

FIG. 5. Inhibition of B16-F10 melanoma implantation in a surgical wound with compounds that block tumor-cell attachment *in vitro*. Wounds were contaminated with B16-F10 melanoma cells as described in the text. Irrigation of the wound with GRGDS (1 mg/mL) or Dispase (2 units/mL) delayed the appearance of tumors compared to control (irrigation with Ringers lactate). Scatter plot shows animals that did not develop tumors (○), animals that died (△), and the volume of the tumors that were present in living animals (·) 30 days after contamination. Horizontal bar represents the mean tumor volume in living animals with tumors.

sions of tumor tissue during dissection around a mass that is being excised with close margins. In the present study contaminated scissors that were visibly clean consistently produced tumors at new sites. A major advantage of using an animal model system is that we could use scissors that had been contaminated by an exposed tumor in another animal. Therefore there was no possibility that subsquent tumors developed from a nidus of disease that had been missed during a resection, or from a systemic metastasis to the wound.[8]

The degree to which scissors became contaminated as a result of only two snips into tumor tissue was variable in our system and was related to the type of tumor (Table 1). Results also varied slightly with the method of cell counting; counts with a hemacytometer tended to be more accurate because multicellular clumps could be more easily distinguished. Nevertheless our results clearly demonstrate that an extremely low number of cells (a few thousand) could cause tumors in a high percentage of cases. This finding reflects the virulence of transplantable tumor models. Other investigators using a transplantable mast-cell tumor also have shown that a suture pulled through a slurry of cells reliably produces a tumor when it is then passed through the leg of another mouse.[9] In patients the presence of tumor cells in the wound does not necessarily mean that there will be a local recurrence.[10] This suggests that the implantation of these cells is a relatively inefficient process, similar to hematogenous metastasis.[11] However tumor cells probably implant more efficiently in a wound than they do on surfaces covered with intact epithelium or endothelium.[12] There are also clinical reports of tumors growing from cells innoculated by contaminated surgical instruments.[13] Thus our results justify traditional concerns about violating a tumor during surgery and suggest that instruments used during dissection around a tumor should not be used afterwards.

What else should be done? Adjuvant radiation therapy and systemic chemotherapy are often used, but toxicity makes it unwise to use these modalities without specific indications. Surgeons often fill the operative field with distilled water in an effort to lyse free tumor cells. Such treatment did not prevent tumors from developing in our experimental system. Past experiments have shown that flooding the wound with cytotoxic agents such as nitrogen mustard and formaldehyde can prevent tumor growth in the wound.[14] However this approach has not found much clinical favor because of concerns about wound healing and because it seems more sensible to administer cytotoxic adjuvant therapy systemically.

We propose that inhibition of cell attachment may be an effective way to prevent local recurrence due to implantation of tumor cells. Cell attachment to underlying extracellular matrix is an active process and the initial step that a free tumor cell must reach to grow into a vascularized tumor mass.[15] Unattached cells may well be more vulnerable to host immune responses and cells unable to attach would certainly be more easily displaced from a wound or serous cavity by irrigation. Finally cell geometry itself can exert a cytotoxic effect, especially with more highly differentiated tumor cells because these cells rapidly lose viability when maintained in a round, unattached state.[16,17]

To find agents that might accomplish this objective, we used a simple *in vitro* assay. Cell adhesion was promoted by the presence of adsorbed gelatin (*i.e.*, denatured collagen) as well as serum attachment factors (*e.g.*, fibronectin and vitronectin). Our assay is an initial approximation of the wound environment in as much as wounds contain serum as well as fibrillar and denatured (air dried, cauterized) collagens. However because it does not completely mimic the *in vivo* process, agents that inhibit cell attachment in this assay require *in vivo* testing to determine their real effectiveness. Nevertheless we expect that most compounds that cannot prevent tumor-cell attachment in this system will also be ineffective *in vivo*, and therefore consider the assay to be a useful initial screen.

Agents that modulate matrix metabolism take advantage of the dynamic nature of the attachment process because cell-matrix attachments are constantly being made and broken. Agents that tip the balance toward degradation should inhibit maintenance of cell attachment. A general inhibitor of protein synthesis, cycloheximide, only minimally inhibited initial attachment but did result in

significant detachment at later times. Cycloheximide previously has been shown to inhibit migration and invasion;[18] however these effects may in part be due to associated cytotoxicity.

Proline analogues, such as LACA, have been previously shown to inhibit attachment of both normal and tumor cells by blocking collagen deposition.[19,20] Interestingly LACA prevented tumor-cell attachment after 16 hours in the present study, even though we used collagen- coated plates. A possible explanation for this finding is that B16-F10 melanoma cells require basement membrane collagens or native interstital collagens, which they would have to synthesize in our system to remain attached.

Combinations of heparin and steroid that were previously shown to inhibit collagen accumulation *in vivo* were minimally effective in preventing B16-F10 melanoma-cell attachment. Heparin and steroid combinations cause regression of growing capillaries *in vivo*, an effect mediated by dissolution of capillary basement membrane and subsequent detachment of associated endothelial cells.[6,7] The matrix- modulating effects of these combinations may be specific for endothelial cells or for vascular mesenchyme. Additional disadvantages of these types of matrix modulators are the time it takes to achieve an effect and the possibility that such agents would inhibit wound healing *in vivo*.

The second type of agent tested enzymatically disrupt cell-matrix attachments. Trypsin and Dispase are both proteolytic enzymes used in tissue culture to detach cells from culture substrata. The inability of trypsin to inhibit attachment in this assay was not surprising because there are high concentrations of trypsin inhibitors in normal serum. Dispase is a neutral protease made by *Bacillus polymyxa*, which is not toxic. It is used for a wide variety of applications, including lifting epithelial cells from culture dishes as a sheet rather than as a dispersed cell suspension.[21] These findings suggest that proteins that mediate cell attachment to extracellular matrix may be preferentially degraded by Dispase. Our results clearly demonstrate that Dispase can also inhibit tumor-cell attachment to gelatinized dishes, even in the presence of serum. More importantly we have shown that Dispase effectively inhibits tumor-cell implantation *in vivo*. The usefulness of Dispase *in vivo* could be limited by its ability to detach normal cells from their basement membrane. However it is important to emphasize that concentrations of 2 to 5 units/mL injected into a wound or intraperitoneally were well tolerated by C57 B1/6 mice.

Increased viscosity also appeared to prevent tumor-cell attachment in our *in vitro* assay. Agarose caused partial inhibition at concentrations that were still liquid, but most of its inhibitory effect appeared at higher viscosities that probably inhibit cell attachment as a result of physical entrapment. Such an effect could be worthwhile *in vivo* as a means to prevent tumor cells from being nourished or vascularized. However healing through a solid slab of agarose gel may be difficult and infection a problem.

Specific interference with binding sites important for cell attachment is an alternative approach. Peptides containing the RGD sequence from the cell adhesion site on fibronectin effectively inhibited tumor-cell attachment *in vitro* in a reversible fashion. Relatively high concentrations of RGD containing peptide were required in this assay most likely because of the presence of high concentrations of fibronectin in serum as well as the use of collagen rather than fibronectin as a substrate. The control peptide sequence GRGESP also inhibited B16-F10 melanoma cell attachment but less effectively than GRGDSP. These results are consistent with the finding that both of these synthetic peptides inhibit B16-F10 melanoma-cell spreading on fibronectin.[22] Importantly solutions containing RGD peptides also inhibited tumor-cell implantation *in vivo*, although not as successfully as Dispase. Due to its lack of toxicity, much higher concentrations of RGD peptides may be used in future studies. Others have shown that RGD peptides can decrease experimental hematogenous metastases, presumably by preventing blood-borne cells from attaching to pulmonary capillary extracellular matrix.[23]

Further understanding of the specific mechanisms and molecules involved in tumor-cell attachment to extracellular matrix presents new therapeutic opportunities. For example peptide fragments of laminin, alone[24] and in combination with heparin-binding fragments of fibronectin,[25] have been shown to inhibit experimental metastases. Such combinations may be more immediately used to prevent local tumor recurrences because this is the one clinical situation in which therapeutic intervention can coincide with the initial release of tumor cells. In our study two compounds, Dispase and GRGDS, which reversibly inhibited tumor-cell attachment *in vitro*, also decreased the chance that a tumor would develop in a wound contaminated by tumor cells on surgical instruments. When tumors did develop after wound irrigation with either compound, their appearance was delayed and their growth rate was suppressed. The use of combined modalities, including immunologic adjuvants such as muramyl dipeptide,[26] in conjunction with agents that block attachment is another as-yet-unexplored therapeutic possibility. Nevertheless it is important to emphasize that 25% of mice contaminated with tumor cells and treated with Dispase alone are alive, healthy, and tumor free to this date (more than 5 months after surgery).

Our data suggest that tumor cells seeded by surgical instruments can be a cause of local recurrence, and more importantly, one which is preventable at the time of surgery. Our first goal was to find a nontoxic compound that could prevent spilled tumor cells from implanting in a

wound or serous cavity. A variety of compounds, categorized by their presumed mode of action, have been identified here. Although these specific agents may not ultimately prove valuable in clinical practice, we believe that this approach can be used to identify other effective agents. Thus we propose that inhibition of tumor-cell implantation may greatly benefit the subset of patients with localized neoplastic disease undergoing a local resection.

Acknowledgments

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