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Fibronectin and integrins in invasion and metastasis

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Summary

The adhesive glycoprotein fibronectin and integrin receptors appear to play important roles in the progression of metastatic disease. Fibronectin is a multifunctional extracellular glycoprotein that has at least two independent cell adhesion regions with different receptor specificities. The cell adhesive region in the central portion of fibronectin is comprised of at least two minimal amino acid sequences – an Arg-Gly-Asp (RGD) sequence and a Pro-His-Ser-Arg-Asn (PHSRN) sequence – which function in synergy. Another cell adhesive region is located near the carboxy-terminus in the alternatively spliced IIICS module. The critical minimal sequences for this region are Leu-Asp-Val (LDV) and Arg-Glu-Asp-Val (REDV) which function in an additive rather than synergistic fashion. Integrins are heterodimeric, transmembrane cell adhesion receptors for fibronectin and other extracellular matrix molecules. Several different integrins bind to fibronectin. The $\alpha_5\beta_1$ fibronectin-specific integrin binds to the central RGD/PHSRN site. The $\alpha_4\beta_1$ integrin binds to the IIICS site. Fibronectin-integrin interactions are important in tumor cell migration, invasion, and metastasis. In addition to promoting cell adhesion to the extracellular matrix, these proteins may also function in chemotaxis and control of proliferation. Peptide and antibody inhibitors of fibronectin and integrin functions have been shown to be effective inhibitors of metastasis, and are potentially important reagents for the study and control of cancer.

Introduction

A major cause of morbidity and death due to cancer is the metastasis of cells from the primary tumor to distant sites where secondary tumors become established. As shown schematically in Fig. 1, metastasis is a multistep process [reviewed in 1–8]. These steps include detachment of cells from the tumor mass, degradation of basement membrane, migration to and invasion into the vascular or lymphatic systems, arrest at a distant site, adhesion to the vascular endothelium, degradation of basement membrane, extravasation, and migration to and proliferation at the secondary site. Many of these steps require cells adhesive interactions or loss of adhesion.

Tumor cell adhesion to components of the extracellular matrix and basement membranes is mediated by specific cell surface receptors that bind to extracellular adhesive proteins. The fibronectin-integrin system has provided a valuable model system for the study of molecular mechanisms of ligand-receptor interactions involved in cell adhesive steps in metastasis. This review is not intended to be comprehensive but, rather, focuses on topics of particular interest to the authors. Numerous outstanding reviews cited throughout this report provide more

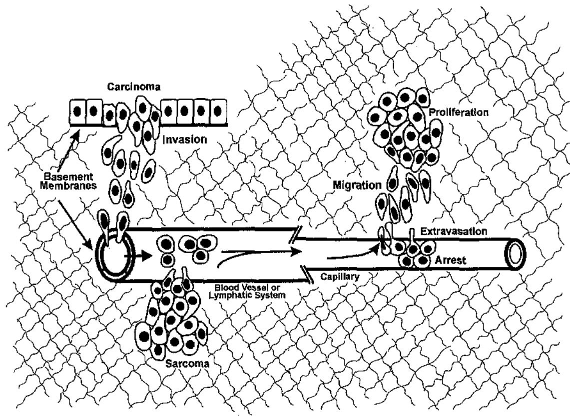

Fig. 1. Model of the major steps of metastasis. Tumor cells are shown invading the circulatory system from a carcinoma by degrading and migrating through a basement membrane or from a sarcoma. Cells separate from the primary tumor, enter the vasculature and eventually arrest in capillaries, where they penetrate and migrate through basement membrane and underlying connective tissue to the metastatic site, where colonization and proliferation occur.

comprehensive summaries on fibronectin and/or integrins.

Fibronectin

Fibronectin is a large adhesive glycoprotein found in extracellular matrices and body fluids [reviewed in references 9–11]. The primary structure of fibronectin is comprised of three different types of homologous repeating units or modules (Fig. 2). There are several alternatively-spliced forms of fibronectin that result from the deletion or insertion of complete type III modules. In addition, one particular region, designated IIICS, can be partially inserted or deleted in some isoforms by alternative splicing. The homologous modules comprising fibronectin are arranged into protease-resistant domains that are separated by more flexible, protease-susceptible regions. When cleaved from intact fibronectin by partial proteolysis, or expressed in bacterial or mammalian cells, and individually purified, these domains often retain the specific binding functions of intact fibronectin, such as those for heparin, fibrin, denatured collagen (gelatin), and cell surface receptors. Although fibronectin has many biological activities, its most important function for the purposes of this review are to promote cell adhesion and migration.

Fibronectin contains at least two distinct regions that can interact independently with distinct cell surface receptors. The first fibronectin cell-adhesive site to be identified was isolated in the form of protease-resistant fragments of 110–120 kDa, 75 kDa, and 37 kDa [12–15] derived from the central portion of the protein. Such fragments of fibronectin retained similar cell adhesive activities as those of intact fibronectin [13, 15, 16]. The cell adhesive activity attributed to these fragments was initially localized to the tenth type III module in the form of an 11.5 kDa pepsin fragment [17] and to an even smaller peptide with the sequence Gly-Arg-Gly-Asp-Ser (GRGDS) [18, 19]. Although the 11.5 kDa fragment and synthetic peptides containing the RGD sequence can inhibit fibronectin cell-adhesive functions *in vitro* and *in vivo* when added as soluble inhibitors [16, 18–21], they only poorly promote cell adhesion mediated by the major fibronectin receptor $\alpha_5\beta_1$ integrin and their affinities are too low to be estimated in direct binding studies

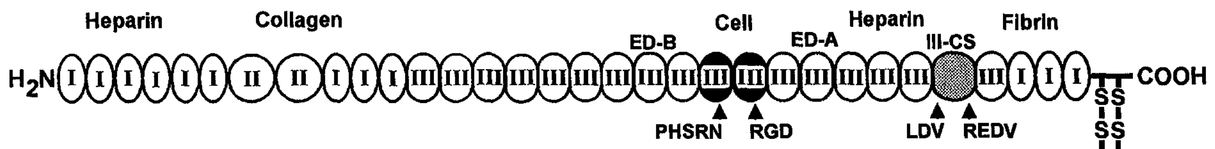

Fig. 2. Model of the structure of fibronectin. Fibronectin is composed of three types of internal repeating modules designated type I, type II, and type III. The ED-A, ED-B, and IIICS modules can be present or absent in some forms of fibronectin as a result of alternative splicing. There are interchain disulfide bonds at the carboxy-terminal end of fibronectin. The binding domains of fibronectin are indicated at the top. The central cell binding domain consists of the ninth and tenth type III modules containing the minimal PHSRN and RGD cell recognition sequences, respectively. The IIICS module contains a cell adhesive site that functions independently of the central cell binding region and contains the LDV and REDV minimal cell recognition sequences.

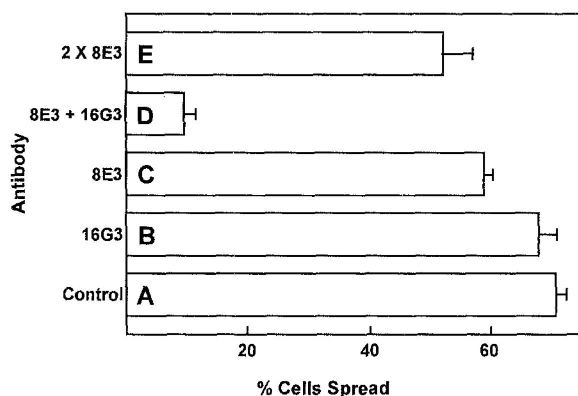

Fig. 3. Synergistic inhibition of cell adhesion by monoclonal antibodies that inhibit the synergy and RGD cell adhesion sites. Shown is the average percent BHK cells spread in the presence of no antibody (A), 1.6 μg/ml mAb 16G3 (B), 1.6 μg/ml mAB 8E3 (C), a mixture of 1.6 μg/ml mAb 16G3 plus 1.6 μg/ml mAB 8E3 (D), and 3.2 μg/ml mAb 8E3 (E) ± s.e.m. on substrates prepared with 2 μg/ml fibronectin. The experimental procedure is described in reference 15.

[22], suggesting that sequences outside of the tenth type III module are also important for maximal cell binding and adhesion.

This additional fibronectin sequence important for maximal cell adhesive activity was identified and characterized using a series of mutants of the central fibronectin cell adhesive region expressed in E. coli [23–25] and anti-fibronectin monoclonal antibodies [15]. Fragments containing the RGD sequence but truncated approximately 10 to 14 kDa to the amino-terminal side of the RGD sequence were < 4% as active as intact fibronectin in mediating cell adhesion, whereas fragments containing these amino terminal sequences retained > 97% of the activity of intact fibronectin [23]. The novel, amino-terminal (non-RGD) site appeared to act synergistically with the RGD site to promote cell adhesion, leading to its designation as a 'synergistic adhesive site' or 'synergy site'.

The biological function of the synergistic cell adhesive site was characterized by using a panel of anti-fibronectin monoclonal antibodies (mAbs), developed to bind a 37 kDa cell adhesive fibronectin fragment [15]. One of these antibodies, designated 8E3, bound to the ninth type III module at a site approximately 14 to 16 kDa to the amino-terminal side of the RGD sequence and close to the synergy site identified by mutational studies. Other mAbs that inhibited cell adhesion, such as 333 [16] and 16G3 [15], bound to the tenth type III module and inhibited the RGD site. Interestingly, there was also an antibody, designated 13G12, that bound to fibronectin between the RGD and synergy sites but did not inhibit cell adhesion. Antibodies that bound near the RGD and synergy sites could each individually inhibit cell spreading at high concentrations [15]. Furthermore, mAb 8E3 and mAb 16G3, at concentrations too low to inhibit spreading individually, can be highly inhibitory in combination, underscoring the synergistic nature of the two cell adhesive sites (Fig. 3). Antibody inhibition experiments have also shown that both the RGD and synergy sites function in cell migration and cytoskeleton assembly on fibronectin substrates, as well as in the assembly of a fibronectin extracellular matrix [15] indicating that both sites are required for a range of fibronectin activities.

A short polypeptide sequence corresponding to the synergy site has been identified using site-specific mutagenesis and 'homology scanning' of bacterially-expressed fragments consisting of the ninth and tenth type III modules of human fibronectin [26]. To avoid possible artifacts due to gross conformational alterations which can occur in deletion mutants, chimeric fibronectin fragments in which portions of the ninth type III module were replaced with homologous segments from the inactive eighth type III module were expressed and assayed for inhibitory activity. The major activity attributed to the synergistic site was localized to a Pro-His-Ser-Arg-Asn (PHSRN) peptide sequence located in the ninth type III module from amino acid residues 1376 through 1380, a portion of the fibronectin primary structure contained in the binding site for

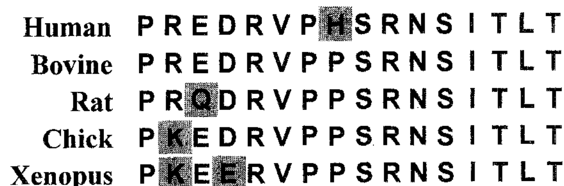

Fig. 4. Comparison of the sequence of human fibronectin in the vicinity of the PHSRN synergistic site with that of fibronectins from other species. Adapted from reference 26.

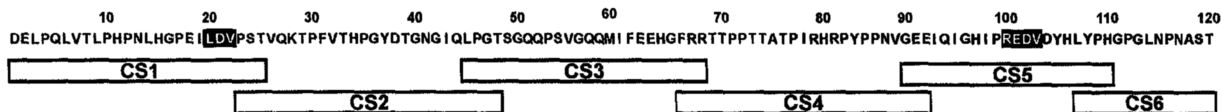

Fig. 5. Sequence of the IIICS module of human fibronectin showing the locations of the CS peptides. The sequences of the overlapping CS peptides are indicated by the white boxes. The LDV and REDV minimal adhesive sequences are in the black boxes. Adapted from reference 30.

mAb 8E3 [26]. This part of the fibronectin sequence is highly conserved among diverse species (Fig. 4), consistent with its identification as a functionally important site. The most crucial amino acid of the PHSRN sequence is the arginine. Even a conservative substitution of lysine for the arginine resulted in a loss of adhesive activity [26].

Soluble synthetic peptides containing the PHSRN sequence had no detectable activity as inhibitors of cell adhesion to fibronectin mediated by the $\alpha_5\beta_1$ integrin, nor could they promote cell adhesion when coupled to IgG carrier protein and adsorbed to plastic. Similarly, when mixed with soluble peptides containing the RGD sequence, no synergistic effect could be detected. However, when the PHSRN peptide was covalently coupled to a mutant fibronectin peptide consisting of the eighth and tenth type III modules, substantial activity could be restored [26]. Thus, the relative positioning or orientation of the PHSRN and RGD sites appears to be important for their functions.

The importance of the relative orientation or positioning of the PHSRN and RGD sites has also been directly demonstrated with a non-fused, bacterially-expressed 20 kDa polypeptide corresponding to the ninth and tenth type III modules of human fibronectin [27]. Although this fragment was highly active as a competitive inhibitor of cell adhesion to immobilized fibronectin, it had only minimal cell adhesive activity when adsorbed onto plastic even when the relative molar amount of fragment actually present on the substrate was taken into account. However, the adhesive function was restored to a level similar to that for the intact fibronectin if the 20 kDa fragment was first bound to the non-inhibitory anti-fibronectin antibody mAb 13G12 pre-adsorbed to the plastic substrate. These results suggest that the apparently inactive 13G12 antibody can function to 'present' the fibronectin cell adhesive sites to cells in order to preserve maximal biological activity, consistent with the hypothesis that the PHSRN and RGD sites must be oriented or spaced correctly in order to maximally promote cell adhesion.

A second important, independent cell-adhesive region of fibronectin is located in the alternatively-spliced IIICS (or V) module [28, 29]. The IIICS region is used for adhesion by neural crest cells, neural crest derivatives such as melanoma cells, and certain leukocytes. At least two non-adjacent peptide sequences within the IIICS module contain cell adhesive activity, one each located near the amino- and carboxy-termini. A 25 amino acid synthetic peptide derived from the amino-terminal sequence of the IIICS module (Fig. 5), designated CS1, was only 2- to 3-fold less active than intact fibronectin [31]. The minimal cell-adhesive peptide sequence from this Leu-Asp-Val (LDV) [32]. A 21 amino acid synthetic peptide derived from the sequence of the IIICS module near its carboxy-terminus, designated CS5 (Fig. 5), is much less active – approximately 1% that of the amino-terminal region – and has the minimal sequence Arg-Glu-Asp-Val (REDV) [30].

Unlike the central RGD and PHSRN cell adhesive sequences, the LDV and REDV sequences do not act in synergy but appear to have an additive effect on cell adhesion [30]. Also, simple mixtures of LDV-containing and REDV-containing peptides immobilized on IgG and adsorbed to plastic substrates can promote adhesion, indicating that their relative spacing and/or orientation is probably not of major importance for maximal activity.

Integrins

Cell adhesive interactions with extracellular matrix proteins such as fibronectin are mediated by specif-

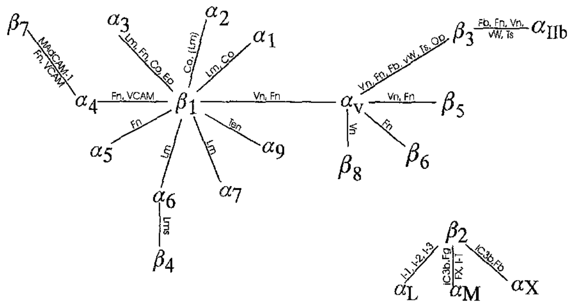

Fig. 6. The integrins. The known heterodimeric combinations of α and β subunits are shown by lines with the known ligands for each heterodimer indicated on the lines by the following abbreviations: Co, collagen; Fb, fibronogen; Fn, fibronectin; FX, factor X; Lm, laminin; Op, osteopontin; Ten, tenascin; Ts, thrombospondin; Vn, vitronectin; vW, von Willebrand factor.

ic cell surface receptors. The best characterized of these receptors are the integrins [reviewed in references 33–42]. Integrins are all non-covalent, heterodimeric complexes of an α subunit and a β subunit. So far, at least 15 different α subunits and eight different β integrin subunits forming at least 22 heterodimers have been identified (Fig. 6). The general structural features of all integrins appear to be similar. Both the α and β subunits are transmembrane glycoproteins with large globular amino-terminal extracellular domains that together make up an ellipsoidal head (Fig. 7). Each subunit provides a relatively thin leg that traverses the plasma membrane and ends in a relatively small cytoplasmic tail of less than 60 amino acids. The only known integrin which does not fit this general description is β_4 integrin, which has a cytoplasmic domain of close to 1000 amino acids [44–46].

The β_1 integrins that can bind fibronectin include $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$. Many integrins, including the $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha v\beta_5$, $\alpha_v\beta_6$, and the $\alpha_{IIb}\beta_3$, recognize the RGD site in adhesive proteins [47–53]. The fibronectin-specific integrin, which consists of an α_5 subunit and a β_1 subunit, is the major fibronectin receptor on most cells. This integrin mediates such cellular responses to fibronectin as adhesion, migration, assembly of a cytoskeleton and assembly of the fibronectin extracellular matrix [54]. The $\alpha_5\beta_1$ integrin interacts with the central cell adhesive region of fibronectin and, as shown in Fig. 8, requires both the RGD and synergy sites for maximal binding [27, 55]. The major platelet integrin $\alpha_{IIb}\beta_3$ also recognizes a similar synergy site in fibronectin for mediating platelet interactions with fibronectin [56].

The $\alpha_4\beta_1$ integrin is the receptor for the IIICS region [50, 57–59] of fibronectin. It binds to both the CS1 (LDV) and CS5 (REDV) sequences of the III CS region [57, 60], although it is currently unclear whether the CS1 and CS5 sequences bind to the same or distinct sites on $\alpha_4\beta_1$. Both the CS1 and CS5 peptides inhibit cell adhesion to substrates prepared with CS1-conjugates [60]. Likewise, both peptides inhibit cell adhesion to substrates prepared with CS5-conjugates (Fig. 9). These results are consistent with the CS1 and CS5 peptides bind-

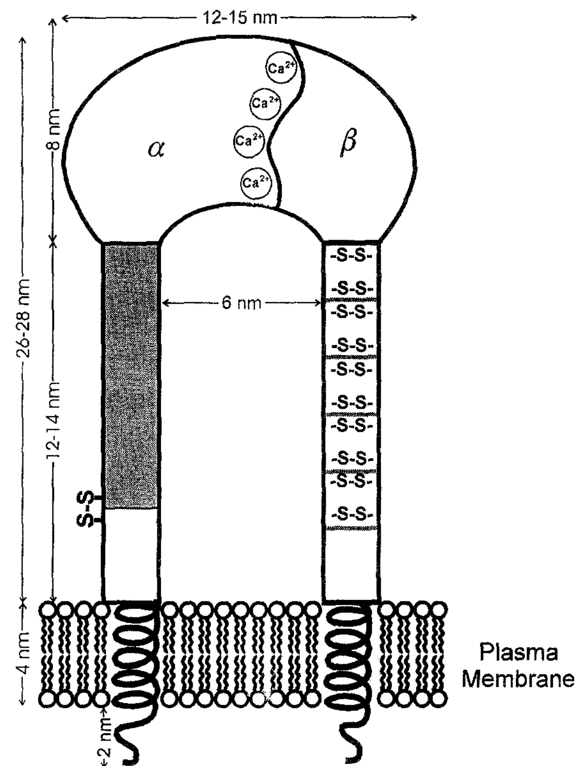

Fig. 7. The $\alpha_5\beta_1$ integrin. A structural model based on predicted primary structure and electron microscopy visualization of purified integrin is shown. The α_5 subunit consists of part of the globular extracellular domain, and one leg which contains predicted 12 beta strands (gray box). The β_1 subunit makes up the remainder of the globular extracellular domain. The β_1 subunit also contains five cysteine-rich repeats. Both the α_5 and β_1 subunits have transmembrane domains and small cytoplasmic domains. Adapted from reference 43.

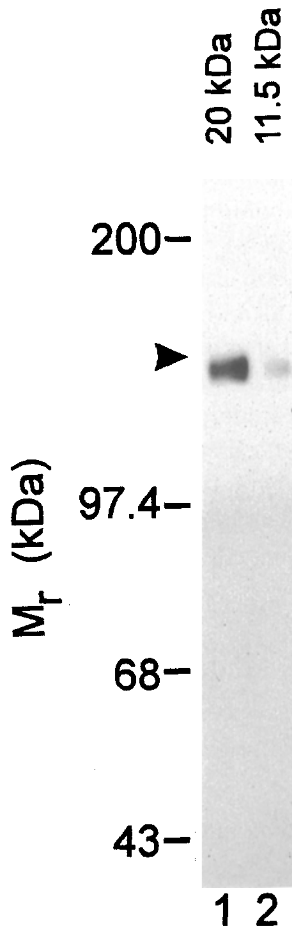

Fig. 8. The binding of $\alpha_5\beta_1$ integrin to fibronectin requires both the RGD and PHSRN sites. Detergent lysates of HT-1080 fibrosarcoma cells were applied to affinity columns prepared with either a 20 kDa recombinant fragment of human fibronectin consisting of both the ninth and tenth type III modules (lane 1), or an 11.5 kDa fragment of human fibronectin consisting of only the tenth type III module (lane 2). Bound material was eluted with EDTA, analyzed by SDS gel electrophoresis and western blotting using α_5-specific polyclonal antibodies. The 20 kDa fibronectin fragment contains both the PHSRN and RGD adhesive sequences, and binds the α_5 integrin well. The 11.5 kDa fragment contains the RGD sequence but lacks the PHSRN sequences and binds the α_5 integrin poorly. The positions of molecular weight markers are indicated on the left. The position of the α_5 integrin subunit is indicated by an arrowhead. Full experimental details and controls are provided in reference 27.

ing to either the same site or overlapping sites. Interestingly, activation of the $\alpha_4\beta_1$ integrin broadens its specificity, enabling cells that use this integrin to interact with proteins containing RGD and RGE sequences [61].

Early interest in the role of fibronectin-cell interactions in cancer resulted from the observation that the level of cell-surface fibronectin was greatly reduced in transformed cells [reviewed in 62–64], suggesting that some properties of cancer cells could be due to alterations in their fibronectin-binding properties. However, no clear pattern of changes in the expression of β_1 integrins upon oncogenic transformation has emerged. Normal and transformed cultured human cells have been found to contain similar total amounts of β_1 integrins [65–67], although the distribution can be different [67]. In normal human fibroblasts, there is a relatively large intracellular pool of integrins, partially due to the unusually long processing time required for maturation, and cell surface β_1 integrins are localized predominantly in distinct streaks that often coincide with focal adhesions. In transformed cells, the intracellular pool is much smaller due to a dramatically increased rate of processing, and the cell surface integrins are present predominantly in a diffuse pattern [67]. In contrast, rat cells transformed either by transfection with *ras* or with Rous sarcoma virus were found to have decreased expression of β_1 integrins [68]. However, Rous sarcoma virus-induced tumors *in vivo* have increased expression of β_1 integrins [69]. There may be a correlation between expression of β_3 integrins with tumor progression or metastatic potential [70, 71]. However, the functions of β_3 integrins may be very complex and include possible functions in angiogenesis and apoptosis [72].

Fibronectin and integrins in migration and invasion

Although it is clear that cells can migrate effectively on fibronectin substrates, and that integrins are involved in this migration, monoclonal antibodies that bind to the human β_1 integrin and especially those that bind to the human α_5 integrin do not uniformly inhibit migration on fibronectin by all cell types (Table 1). The reasons for these highly variable results are presently unclear, but probably have to do with the inherent complexity of the process of cell migration, which can involve multiple integrin receptors functioning in concert [24, 75], even on well-defined substrates.

Cell migration requires exquisite control of adhesion to the substrate. Clearly, a certain minimal level of adhesion is required to enable cells to adhere to the substrate with sufficient strength so that they can pull themselves along. Therefore, inhibitors that reduce cell adhesion to a level below the threshold required for traction will inhibit cell migration. On the other hand, interactions resulting in very strong substrate adhesion could also inhibit migration, presumably by immobilizing cells. These interactions could include, but are not necessarily limited to, binding to high affinity adhesive substrates and those that result in the clustering of adhesion receptors, which could subsequently promote higher affinity, multivalent adhesion-receptor binding.

These concepts have been broadly confirmed with experiments involving anti-integrin monoclonal antibodies. An antibody that generally inhibits all β_1 integrin-mediated cell adhesion was also found to inhibit cell migration [54, 73, 74], probably by inhibiting cell adhesion to a level below the threshold required for traction. In contrast, an antibody that selectively inhibits α_5-mediated adhesion was found to actually *increase* cell migration, possibly by inhibiting the formation of receptor clusters which could then result in less cell substrate adhesion [54]. Furthermore, cells can migrate on substrates prepared with low concentrations of high affinity anti-integrin antibodies as well as on moderate-affinity fibronectin substrates, but substrates prepared with high concentrations of high-affinity antibodies promote migration relatively poorly compared to substrates prepared with similar concentrations of moderate-affinity fibronectin [73, 76]. The inhibition of cell migration at both high and low levels of cell adhesion on the same substrate has been shown directly in an elegant study in which migration of human smooth muscle cells was quantitated under conditions where the strength of the substrate adhesive interactions was known [77]. These experiments clearly showed a biphasic dependence of migration on the adhesive strength of cells on two different substrates, fibronectin and collagen type IV, with maximal cell migration occurring only at intermediate adhesion levels.

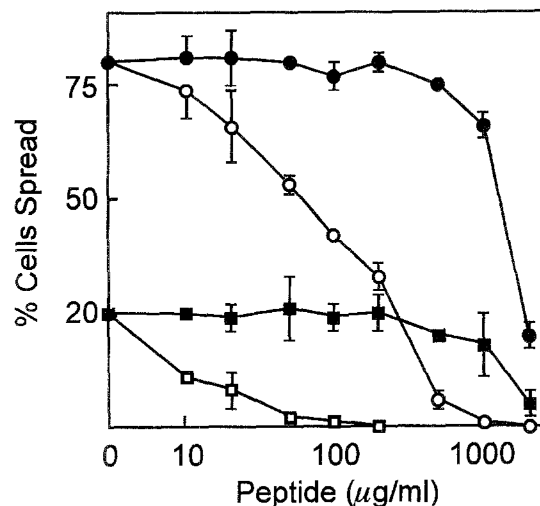

Fig. 9. Inhibition of cell spreading on CS1 and CS5 substrates. Inhibition of A375-SM human melanoma cell spreading on substrates prepared with 125 μg/ml CS1-IgG (squares) or 150 mg/ml CS5-IgG (circles) in the presence of soluble CS1 peptide (open symbols) or soluble CS5 peptide (closed symbols). Data taken from reference 60.

The process of invasion involves the adhesion of tumor cells to basement membrane, partial proteolytic digestion of basement membrane proteins followed by cell migration through the membrane. One of the most useful assays for modeling this very complex process *in vitro* involves migration of tu-

Table 1. Antibody inhibitors of human cell migration on fibronectin

Cells	Inhibition by Anti-β_1 Antibody[1]	Inhibition by Anti-α_5 Antibody[1]	Reference
WI38 fibroblasts (normal)	+	–	54
HT-1080 fibrosarcoma	+	+	73
5637 bladder carcinoma	++	–	73
VA13	+	+	73
MDA-231 breast carcinoma	+	+	74

[1] – denotes no inhibition, + denotes partial inhibition, ++ denotes complete inhibition.

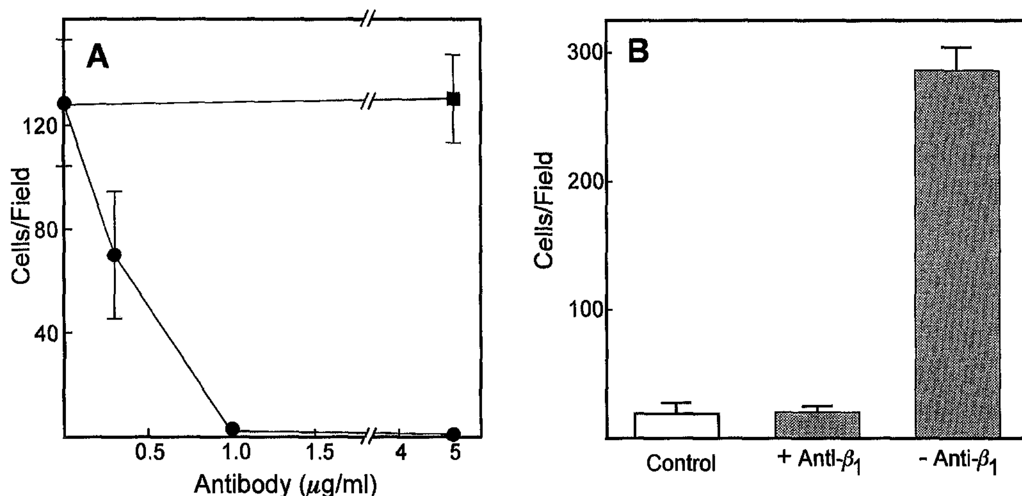

Fig. 10. Inhibition of fibrosarcoma cell invasion by an anti-integrin monoclonal antibody. A. HT-1080 fibrosarcoma cell invasion across a reconstituted Matrigel artificial basement membrane in the presence of the indicated concentrations of an anti-β_1 monoclonal antibody 13 (●) or an anti-α_5 monoclonal antibody 16 (■) was quantitated by counting cells that had crossed the membrane. In this experiment, antibodies were mixed with the cells prior to their addition to the Matrigel basement membrane and were present throughout the assay. Each point is the average of five determinations ± s.e.m. B. Inhibition of HT-1080 fibrosarcoma cell invasion in a modified invasion assay. Cells were allowed to attach to the reconstituted Matrigel artificial basement membrane for 1 hour in the absence of chemoattractant and antibodies. Invasion was subsequently allowed to proceed in the presence (+Anti-β_1) and absence (−Anti-β_1) of 5 μg/ml anti- integrin β_1 monoclonal antibody 13 for 4.5 hours. The extent of HT-1080 cell invasion at the time of addition of anti-β_1 monoclonal antibody is indicated by the open control bar. All bars represent the average of 5 determinations ± s.e.m. Adapted from data published in reference 73.

mor cells through a reconstituted Matrigel basement membrane coated on a filter in response to a chemoattractant in the form of fibroblast-conditioned medium [78, 79]. An anti-β_1 integrin monoclonal antibody was a very effective inhibitor of invasion of both HT-1080 fibrosarcoma cells (Fig. 10A) and MDA-MB-231 breast carcinoma cells [73, 74]. The processes of adhesion to, and migration through, the membrane were tested separately in a modification of the invasion assay in which cells were allowed to attach to the membrane prior to the addition of inhibiting antibodies. As shown in Fig. 10B, the anti-β_1 antibody completely inhibited invasion through the Matrigel membrane even when added after the cells were allowed to attach, indicating that the antibody was acting to inhibit a later step in the invasion process, such as migration through the Matrigel.

An anti-α_5 monoclonal antibody had no significant effect on invasion by the HT-1080 cells [73] and only a small (but significant) effect on invasion by MDA-MB-231 breast carcinoma cells [74] in the presence of chemoattractant. In the absence of chemoattractant the MDA-MB-231 could still invade the Matrigel basement membrane, but then the anti-α_5 antibody had no significant inhibitory effect. Thus, the α_5 integrin may be functioning as a chemoattractant receptor for the MDA-MB-321 breast carcinoma cells, rather than as an adhesion receptor during invasion, suggesting that a possible role for the α_5 integrin is to promote migration of these cells towards fibronectin in the target organ.

RGD-containing synthetic peptides had little effect on invasion of the HT-1080 cells in the Matrigel assay [73]. This result is consistent with the composition of Matrigel, which is composed largely of laminin, collagens, and proteoglycans [78] with relatively little fibronectin or vitronectin. Although both laminins and collagens have been found to contain RGD sequences [80, 81], these potential adhesion sites are reported to be cryptic, requiring proteolysis for their exposure. RGD-containing peptides that specifically inhibit cell adhesion to fibronectin were found, however, to inhibit invasion

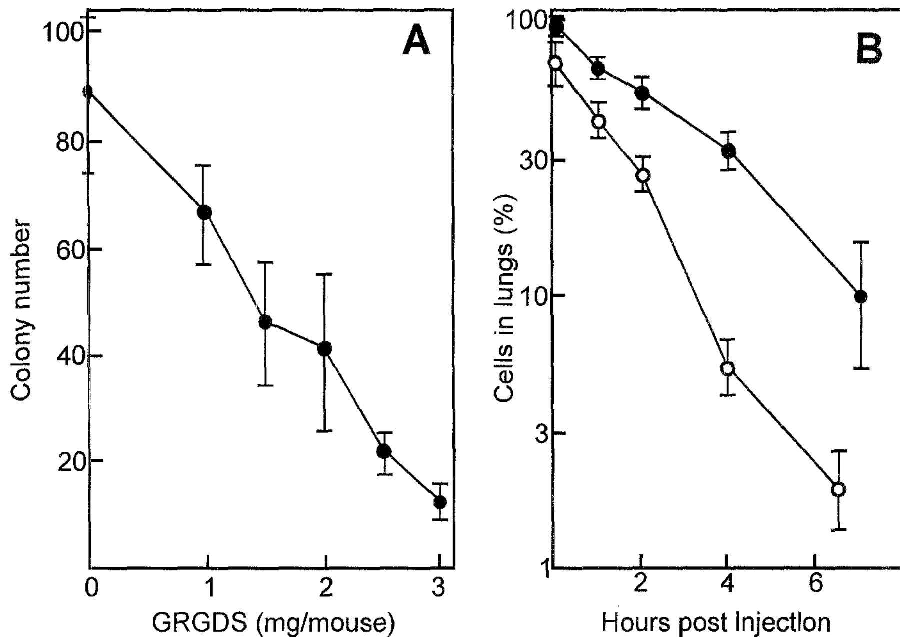

Fig. 11. Effect of GRGDS peptide on experimental metastasis of B16 murine melanoma cells. A. Inhibition of lung colonization of B16-F10 cells co-injected with the indicated doses per mouse of GRGDS synthetic peptide. Each point represents the average of eight mice ± s.e.m. B. Pulmonary retention of ^{125}I-labeled B16-F10 cells injected into mice alone (●) or in the presence of 3 mg GRGDS peptide (○). Each point represents the average of 4 determinations ± s.d. Data and experimental details originally published in reference 84.

through amnion basement membrane [82], although, similar peptides specific for vitronectin were not inhibitory. As was the case with the Matrigel invasion assay, the synthetic peptides had no effect on cell adhesion to the amnion basement membrane. The apparent discrepancy between the Matrigel and amnion assays can be explained, at least in part, by the difference in composition between Matrigel and amnion basement membrane. The latter contains substantial amounts of fibronectin [83], which might be important in mediating penetration of the invading cells into the basement membrane [82].

Fibronectin and integrins in metastasis

Several different assay systems have been used to analyze directly the role of fibronectin and integrins in *in vivo* metastasis, but they all fall into two broad classes. The 'experimental' metastasis models involve the intravenous injection of tumor cell suspensions into mice, and subsequent quantitation of either the number of metastatic colonies or the sizes of colonies usually in the lungs or liver. Experimental metastasis models omit the earlier steps of the metastatic process involving detachment from the tumor mass and invasion into the vasculature and replicate only the final steps of hematogenous metastasis. 'Spontaneous' metastasis models, in which tumors or cells are implanted into mice and subsequent metastases to distant organs are quantitated either by counting colonies or measuring colony size, are more complicated but are required to analyze of the earlier steps of the metastatic cascade.

Synthetic peptides containing the Gly-Arg-Gly-Asp-Ser (GRGDS) sequence derived from fibronectin were assayed by examining their effects on lung colonization of B16-F10 murine melanoma cells in syngeneic C57BL/6 mice using an experimental metastasis model [84]. GRGDS peptide, which had a circulatory half life of approximately 8 minutes [85], specifically inhibited lung colonization in a concentration-dependent manner (Fig. 11A). The major effect of the GRGDS peptide appeared to be to inhibit arrest of melanoma cells in the lungs (Fig. 11B) without affecting the size of either melanoma cell clusters in suspension or lung

colonies. The efficacy of peptide treatment was unaltered in animals with impaired platelet function and in animals lacking natural killer cells [85]. Taken together, these results suggests that the GRGDS peptide inhibits metastasis by disrupting an early adhesive process [84]. As shown in Fig. 12, a single administration of the GRGDS peptide also had a dramatic effect of increasing the survival of mice when co-injected with melanoma cells [85].

Anti-β_1 integrin monoclonal and polyclonal antibodies have been found to inhibit experimental metastasis in several different systems, apparently through diverse mechanisms. In addition, several integrin α subunits have been implicated in the metastatic process. Both anti-β_1 and anti-α_5 monoclonal antibodies were found to inhibit experimental metastasis resulting from intravenous injection of MDA-MB-231 breast carcinoma cells into athymic nude mice. This result suggests that tumor cell-fibronectin interactions are important in metastasis [74]. Fab fragments or pre-treating tumor cells with antibodies were as effective as co-injection of intact antibodies with tumor cells, apparently ruling out effects due to free, unbound antibodies, antibody-induced crosslinking, and antibody Fc regions. Although it was not possible to draw unequivocal conclusions about exactly which steps in the metastatic cascade were being inhibited by the antibodies, possibilities include initial adhesion during tumor cell arrest, and cell migration [74].

Two other studies have examined the effects of anti-integrin antibodies on metastasis. One study used rabbit polyclonal anti-β_1 integrin antibodies injected into mice bearing SP1 murine mammary carcinoma transplants and treated with anti-CD4 antibodies to suppress an immune response to the rabbit IgG [86]. In this spontaneous metastasis system, treatment with polyclonal anti-β_1 antibodies reduced the size of the metastatic colonies but had little effect on the number of colonies. These results may be indicative of the multiple roles of cell adhesion in metastasis. In the earlier steps of the metastatic cascade, inhibiting tumor cell adhesion may make it easier for cells to leave the tumor mass and might actually increase the rate of metastasis. This part of the metastatic cascade is missing from studies using the experimental metastasis model. Only in the later steps of metastasis should inhibiting adhesion also be expected to inhibit colony formation. Thus in models of spontaneous metastasis, it is possible that there may be no *net* change in the number of metastatic colonies following treatment with cell adhesion inhibitors. The decrease in colony growth seen in these experiments is consistent with the anti-adhesive properties of the anti-β_1 antibody, which may be inhibiting tumor cell interactions with the surrounding extracellular matrix, thus depriving the cells of adhesion-dependent growth signals.

Several integrin α subunits in addition to α_5 have also been examined in metastasis assays. A rat antibody that binds to the mouse α_6-containing laminin receptor integrins, designated EA-1, inhibited experimental metastasis of B16/129 murine melanoma cells to lung [87]. Direct examination of tissue showed that the EA-1 antibody inhibited adhesion of melanoma cells to the vascular endothelium in the lungs within five minutes of injection. The α_6-containing integrins are laminin-specific receptors [88], and laminin could be involved in multiple steps in the metastatic pathway including cell adhesion to the luminal endothelium of capillaries, adhesion to the subendothelium during extravasation, and invasion and migration to the eventual metastatic site. Surprisingly, the EA-1 antibody did not inhibit melanoma cell adhesion to laminin fragments in *in vitro* assays. Furthermore, the EA-1 antibody was found to bind to the luminal surface of vascular endothelium in host animals [87]. Thus, it was hypothesized that the EA-1 anti-α_6 antibody was inhibiting integrin binding to a possibly novel ligand that has yet to be identified.

The role of the α_2 integrin subunit was examined directly by transfecting human rhabdomyosarcoma cells, which do not normally express this integrin with an α_2 cDNA [89]. Expression of the α_2 integrin polypeptide resulted in increased adhesion to both laminin and collagen, and also in increased formation of metastatic colonies in both experimental and spontaneous metastatic model systems. The growth rate and tumorigenicity of the cells were unaffected. These results suggest that up-regulation of the α_2 integrin observed on these tumor cells could directly result in increased metastatic potential.

Over-expression of the α_4 integrin has been in-

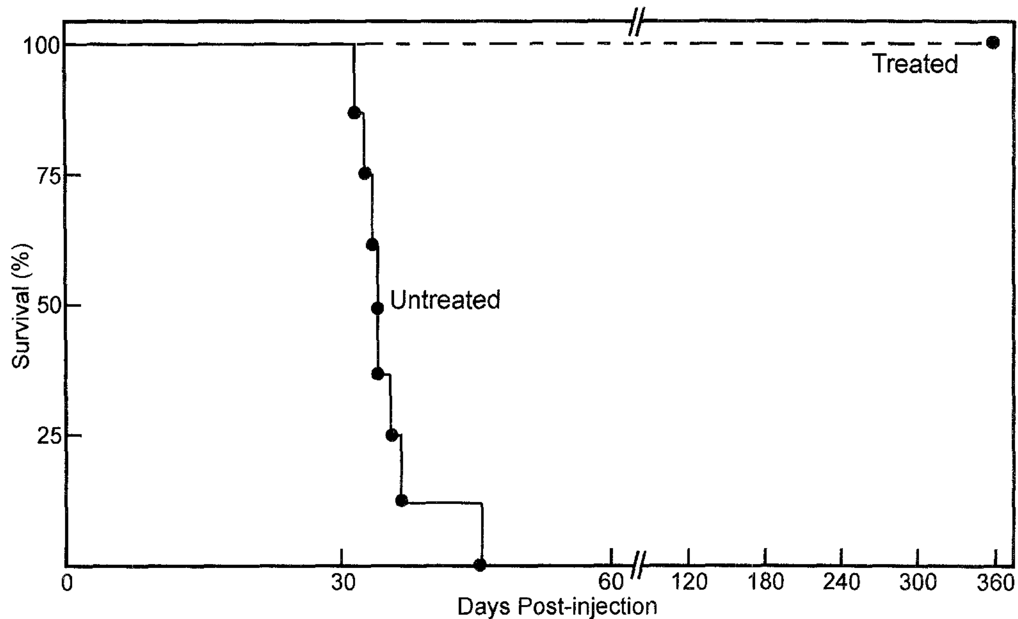

Fig. 12. Effect of GRGDS peptide on survival of C57BL/6 mice injected with B16-F10 murine melanoma cells. Mice were co-injected intravenously with 3×10^4 B16-F10 cells mixed with 3 mg GRGDS peptide. Survival of mice was initially monitored daily. Survival of untreated mice is indicated by the solid line, and survival of treated mice is indicated by the dashed line. Adapted from data and experimental details originally published in reference 85 and unpublished data (M. Humphries, K. Matsumoto, K.M. Yamada, and K. Olden).

versely correlated with invasive potential of B16 mouse melanoma cells [90]. Three different B16 melanoma cells variants were compared: poorly metastatic B16-F1; B16-F10, which produce few metastatic colonies after subcutaneous injection but numerous colonies after intravenous injection; and B16a, which are highly metastatic after either subcutaneous or intravenous injection. The B16-F1 and B16-F10 cells expressed high levels of cell surface α_4 integrin, whereas the B16a variants expressed little α_4. When the α_4 integrin was over-expressed by transfecting B16a melanoma cells with α_4 cDNA, both invasion through a reconstituted Matrigel basement membrane and the incidence of spontaneous metastasis were inhibited by 85% and 90%, respectively, compared to non-transfected controls [90]. In contrast, number of colonies formed by the B16a cells over-expressing the α_4 polypeptide in experimental metastasis assays was unchanged. When examined *in vitro*, the α_4 integrins appeared to function in cell-cell adhesion, which could be abrogated with anti-α_4 monoclonal antibodies. These results are consistent with α_4 playing a role during the early steps in the metastatic cascade, possibly to prevent release of cells from the primary tumor mass prior to invasion through the extracellular matrix [90].

Conclusions and future prospects

Although much progress has been made, several important issues remain unresolved. The roles of fibronectin and integrins in cancer biology and the possibility of developing novel therapeutics based on the biological activities of these proteins should remain important research topics for the foreseeable future. A current area of great interest is the role of integrins in signal transduction [reviewed in references 38, 41, 91–97]. Integrin-mediated cell adhesion to extracellular matrix proteins can trigger signal transduction in several forms including up-regulation of gene expression [98, 99], changes in intracellular pH and calcium ion concentration [100–103], and increased tyrosine phosphorylytion of the focal adhesion kinase, also known as $pp125^{FAK}$ and FAK [104–107]. An example of the latter is shown in Fig. 12. Signal transduction requires

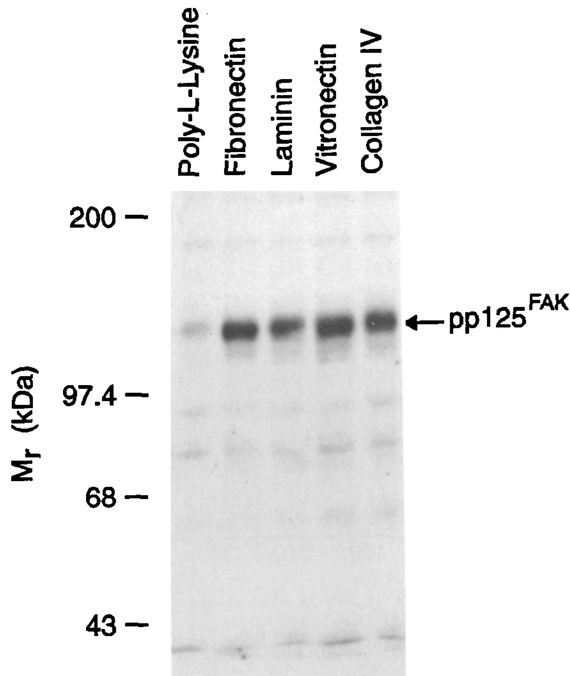

Fig. 13. Adhesion-induced tyrosine phosphorylation of focal adhesion kinase. Adhesion of NIH-3T3 murine cells to fibronectin, laminin, vitronectin, and collagen type IV all stimulate tyrosine phosphorylation of focal adhesion kinase ($pp125^{FAK}$ arrow). Non-physiological adhesion to poly-L-lysine does not stimulate tyrosine phosphorylation. Tyrosine phosphorylation was assayed using Western blotting of whole cell lysates as described in reference 104.

the cytoplasmic domains of β_1, β_3, or β_5 integrin but not the extracellular domains, and the ability of integrin β subunits can be regulated by alternative splicing [108]. Integrin-mediated cell adhesion results in activation and nuclear translocation of mitogen-activated protein (MAP) kinases in murine 3T3 cells and human skin fibroblasts [109–111] and the binding of the GRB2 adaptor protein to FAK [111].

Interestingly, the binding of GRB2 to FAK occurs independently of cell adhesion in v-src-transformed 3T3 cells [111]. These results along with the demonstration that a polyclonal anti-integrin can inhibit tumorigenicity of SP1 murine mammary carcinoma cells [86] are suggestive of an important role for adhesion-dependent signal transduction in stimulating tumor growth, implying a possible focus for attack by signal transduction-disrupting drugs.

Different studies have identified several possible biological roles for integrins and fibronectin in metastasis including cell-adhesion, migration, maintaining integrity of the tumor mass, stimulating proliferation, and inducing apoptosis. The mechanisms responsible for the inhibitory activities of the different antibodies need to be defined more precisely in order to determine whether these apparently diverse activities are truly distinct or are simply different manifestations of similar molecular interactions. This will require detailed mapping of antibody binding sites coupled with a more detailed knowledge of the functions of the different portions of the integrin primary structure. Regardless of the mechanisms involved, several anti-integrin antibodies have already been identified as effectively inhibiting cancer cell dissemination and/or proliferation in mouse model systems. Before such work can be extended to human studies, these antibodies must be 'humanized', either in the form of chimeric IgGs or as fragments containing only integrin binding sites.

RGD peptides and RGD mimetics are also very promising possibilities. RGD peptides not only inhibit metastatic colony formation but even a single administration of the peptide can dramatically increase animal survival. However, the use of such agents is not without drawbacks. One potential problem is the relatively high concentration of peptide required for activity. Possible solutions include the use of cyclic peptides and 'designer' peptides that have increased affinity for cell surface integrins [112] or the use of repeating polymers containing RGD units [113–115]. Polymers containing RGD sequences have the dual advantages of augmenting the affinity of small peptide sequences through multivalent interactions and possibly of solving the problem of the relatively short circulatory half-life of GRGDS peptides. An alternative approach would be to develop novel, small, synthetic integrin antagonists. Such compounds that inhibit RGD-dependent and LDV-dependent cell adhesion have already been synthesized and tested in mice [116, 117]. Work on second generation compounds that mimic multiple adhesive sites providing the potential for inhibitory activities greater on a molar basis than

that of intact fibronectin is undoubtedly already in progress.

In addition to interactions with fibronectin via integrins, metastasizing cells also depend on interactions with several other extracellular matrix molecules. For example, tumor cell interactions with laminins, collagens, and hyaluronan can contribute to the metastatic process [e.g. see reviews in reference 6]. It appears likely that blocking any one of several critical cell-matrix interactions can interrupt the multi-step metastatic cascade. This multiplicity of sensitive steps would appear to provide a source of considerable optimism for the potential therapeutic value of targeting malignant cell-matrix interactions. If several sensitive steps exist, it would appear more practical to consider tailoring therapeutic approaches to an individual tumor and to apply this strategy as an adjunct to standard cytotoxic therapy. In such a multi-step biological system, there would be less chance for cells to develop resistance to inhibitors of several distinct steps. Moreover, there could be less danger of inhibiting crucial normal processes such as renewal of epithelia and wound repair if it is possible to rotate targets of inhibition. Interactions such as those of integrins with fibronectin can therefore be viewed as one of several sensitive links in tumor progression, and the eventual rational targeting of such steps may provide a novel approach to the control of metastasis.

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