Inhibition of In Vitro Tumor Cell Invasion by *Arg-Gly-Asp*-containing Synthetic Peptides

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Abstract. The interaction of cells with extracellular matrix components such as fibronectin, vitronectin, and type I collagen has been shown to be mediated through a family of cell-surface receptors that specifically recognize an arginine-glycine-aspartic acid (RGD) amino acid sequence within each protein. Synthetic peptides containing the RGD sequence can inhibit these receptor-ligand interactions. Here, we use novel RGD-containing synthetic peptides with different inhibition properties to investigate the role of the various RGD receptors in tumor cell invasion. The RGD-containing peptides used include peptides that inhibit the attachment of cells to fibronectin and vitronectin, a peptide that inhibits attachment to fibronectin but not to vitronectin, a cyclic peptide with the opposite specificity, and a peptide, GRGDTP, that inhibits attachment to type I collagen in addition to inhibiting attachment to fibronectin and vitronectin. The penetration of two human melanoma cell lines and a glioblastoma cell line through the human amniotic basement membrane and its underlying stroma was inhibited by all of the RGD-containing peptides except for the one that inhibits only the vitronectin attachment. Various control peptides lacking RGD showed essentially no inhibition. This inhibitory effect on cell invasion was dose-dependent and nontoxic. A hexapeptide, GRGDTP, that inhibits the attachment of cells to type I collagen in addition to inhibiting fibronectin- and vitronectin-mediated attachment was more inhibitory than those RGD peptides that inhibit only fibronectin and vitronectin attachment. Analysis of the location of these cells that were prevented from invading indicated that they attached to the amniotic basement membrane but did not proceed further into the tissue. These results suggest that interactions between RGD-containing extracellular matrix adhesion proteins and cells are necessary for cell invasion through tissues and that fibronectin and type I collagen are important for this process.

THE metastatic process is comprised of a complex series of events the details of which are largely unknown. For a tumor cell to be metastatic, it must be capable of attaching to the extracellular matrices that separate tissues and must penetrate such matrices (reviews, Fidler et al., 1978; Liotta et al., 1983; Nicolson, 1984). Extracellular matrices are composed of macromolecules that include fibronectin, laminin, collagens, and proteoglycans. These molecules can promote cell adhesion and migration (reviews, Hynes and Yamada, 1982; Ruoslahti and Pierschbacher, 1987) and are believed to play a role in embryonic cell migrations (Bronner-Fraser, 1986; Duband and Thiery, 1982; Erickson et al., 1980; Goodman and Newgreen, 1985) and tumor cell invasion (Lacovara et al., 1984; Liotta et al., 1986; McCarthy and Furcht, 1984; Mignatti et al., 1986; Ruoslahti, 1984). The interaction of cells with the extracellular matrix is mediated by cell surface receptors.

Receptors for fibronectin, type I collagen, vitronectin, and laminin have been identified (reviews, Liotta et al., 1986; Ruoslahti and Pierschbacher, 1987). Of these proteins at least fibronectin, type I collagen, and vitronectin have the three amino-acid sequence, arginine-glycine-aspartic acid (RGD) as the core structure recognized by the receptors (Pierschbacher and Ruoslahti, 1984*a*,*b*; Ruoslahti and Pierschbacher, 1987). Synthetic peptides containing the RGD sequence are capable of competing with each of these adhesion proteins for its receptor thereby inhibiting cell attachment and migration of both normal and tumor cells on substrates coated with the adhesion proteins (Hayman et al., 1985*a*; Pierschbacher and Ruoslahti 1984*a*,*b*; Yamada and Kennedy, 1985). Thus, such peptides have become valuable tools for the analysis of cell adhesion phenomena.

In this report, we have used a series of novel synthetic RGD-containing peptides (Pierschbacher and Ruoslahti, 1987) to obtain information about tumor cell invasion through the human amniotic membrane in vitro. We find that the migration of several types of tumor cells through the amnion can be inhibited with RGD-containing peptides, thus suggesting an important role for the RGD receptors in cell migration through tissue matrices. We also find that peptides which are selective for the various receptors show different degrees of activity, allowing conclusions to be drawn about

the relative importance of the individual receptors in the invasive process.

Materials and Methods

Cell Lines

The cell lines used included two human melanoma cell lines designated A375P and A375M, the derivation and metastatic properties of which have been described (Kozlowski et al., 1984). They were obtained from I. J. Fidler, (M. D. Anderson Hospital and Tumor Institute, Houston, TX). A human glioblastoma cell line (*RuGli*), was obtained from Dr. S. L. Goodman (Max Planck Institute, Munich, Federal Republic of Germany) (Goodman and Newgreen, 1985). All of the cells were cultured in DME (Gibco, Chagrin Falls, OH) supplemented with 10% heat-inactivated fetal bovine serum (Tissue Culture Biologicals, Tulare, CA) and 0.1% gentamicin (Gibco).

Cells were removed from culture dishes using 2 mM EDTA in PBS devoid of Ca^{++} and Mg^{++} for all assays. Cell viability in all assays was determined using the Trypan Blue exclusion test.

Proteins and Synthetic Peptides

Fibronectin and vitronectin were purified according to published procedures (Hayman et al., 1985*b*; Ruoslahti et al., 1982). Laminin, and types I and IV collagen were purchased (Collaborative Research, Bedford, MA). These proteins were used to coat polystyrene dishes as previously described (Ruoslahti et al., 1982).

Peptides were synthesized using an Applied Biosystems (Model 430A; Foster City, CA) automated peptide synthesizer using the chemistry recommended by the manufacturer and purified by reverse phase HPLC on a Biogel TSK SP-5-PW cation exchange column (Bio-Rad Laboratories, Richmond, CA). A cyclic peptide and the peptides containing D-amino acids were the same as described elsewhere (Pierschbacher and Ruoslahti, 1987).

For the invasion assays, the peptides were dissolved in DME with 2% fetal bovine serum, 0.1% gentamicin, and 10 mM Hepes buffer. The pH was maintained at 7.2–7.4 by adding Hepes buffer or sodium bicarbonate when necessary. In cell attachment assays the peptides were dissolved in the same medium except that serum was omitted. Peptide/media preparations were made fresh for each experiment.

Cell Attachment to Defined Substrates

Cell attachment assays were done as described (Ruoslahti et al., 1982). In some experiments, the cells used for the attachment assays were radiolabeled with [^{14}C]thymidine (ICN Radiochemicals, Irvine, CA; see below) and the cell-associated radioactivity was used to quantitate the attached cells. The assays were performed in triplicate.

Cell Invasion Assays

Invasiveness of tumor cells was determined using the Membrane Invasion Culture System (MICS, Gehlsen et al., 1984; Gehlsen and Hendrix, 1986; Hendrix et al., 1985*b*) which is a modification of the amnion invasion assay described by Liotta et al. (1980). Briefly, cells (5×10^4 to 1×10^5) were radiolabeled with 0.25 μCi/ml ^{14}C-thymidine or 0.30 μCi/ml [^{125}I]deoxyuridine ([^{125}I]Udr), both from New England Nuclear (Boston, MA), for 48 h in medium containing 2% serum and then seeded into the upper compartment of the MICS chambers. After a preattachment incubation period at 37°C in a 7% CO_2-air atmosphere and at 24-h intervals thereafter, medium in both the upper and lower compartments of the chambes was replaced with fresh, peptide-containing medium. At specific time intervals after the addition of peptides, medium from the upper and lower chambers was removed, the membranes were washed with fresh medium, and the medium and wash fractions were centrifuged and the number of cells in the cell pellets was determined by counting the cell-associated radioactivity by liquid scintillation or in a gamma counter. The total number of cells that had passed through the membrane was derived by adding the lower chamber value to those from the previous samplings. In several experiments, unlabeled cells were used and the cells were counted in a hemocytometer. In addition, the cell-associated radioactivity in the amnion tissue was also determined. The amnion was treated in tissue solubilizer (Amersham Corp., Arlington Heights, IL) before counting radioactivity.

Microscopy

The localization of cells during invasion was determined using A375M cells labeled with the lipophilic dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylinadocarbocyanine perchlorate (DiI; Molecular Probes, Inc., Eugene, OR), as described (Honig and Howe, 1986). DiI was dissolved in 100% ethanol (2 mg/ml) and added to cells in culture medium at a final ethanol concentration of 3% for 12–24 h. Cells were then washed with PBS followed by fresh medium before seeding into MICS chambers. After a 72-h incubation period the membanes were washed thee times with PBS, fixed in 3.75% paraformaldehyde and frozen for cryostat sectioning. Membrane preparations were then viewed and photographed using a Nikon Diaphot-TMD inverted microscope equipped with fluorescence optics.

Results

Effects of RGD-containing Peptides on Melanoma Cell Invasion

We have used RGD-containing cell adhesion peptides to study the role of cell adhesion receptors in the penetration of tumor cells through amniotic membrane tissue. As previously noted (Hendrix et al., 1985*a*), the metastatic melanoma cell lines, A375M and A375P, were each able to traverse the human amniotic membrane in the MICS invasion assay with an increasing number of cells reaching the lower chamber with time. The RuGli glioblastoma cells also penetrated the membrane. 7–9% of the A375M cells, 3–5% of the A375P cells, and 7–12% of the RuGli cells seeded onto

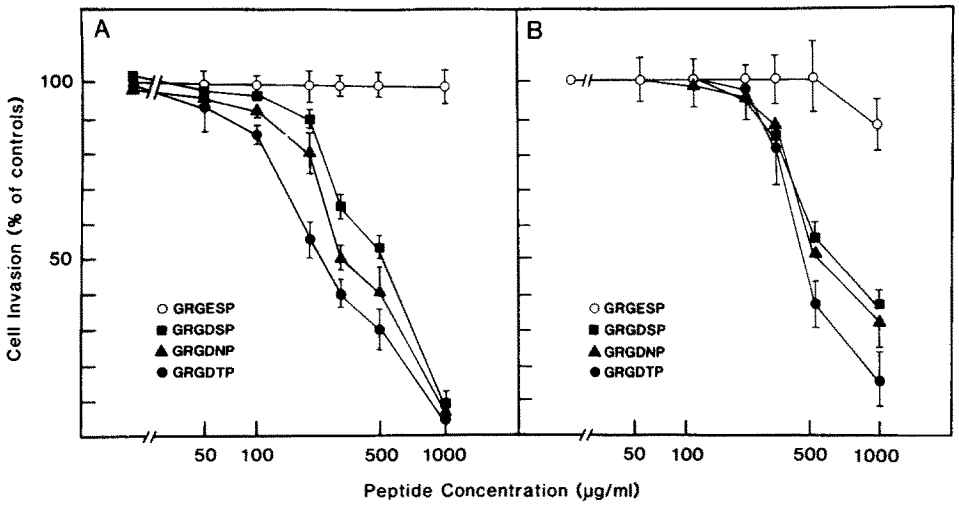

Figure 1. The effect of RGD-containing peptides on tumor cell invasion. A375M melanoma (*A*) and *RuGli* glioblastoma (*B*) cells were tested in MICS invasion chambers as described. The numbers of cells that had accumulated to the lower chamber compartment at 72 h were used to calculate invasion. The uninhibited control values were set at 100% with the data derived from nine observations for each point. The mean ±SD is shown as a percentage of tumor cell invasion relative to the controls.

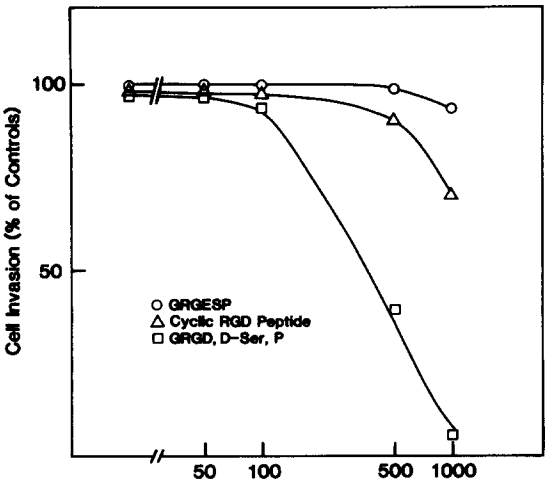

Figure 2. The effects of two selective synthetic RGD-containing peptides on A375M melanoma cell invasion. GRGESP (○); GRGD,D-Ser,P (□); or the cyclic RGD peptide (△) were added to MICS chambers and their effects on cell invasion was assayed as described in the text and the legend of Fig. 1.

the membranes traversed the amnion in 72 h. A marked decrease in the invasion was seen in the presence of RGD-containing peptides with all three cell lines. This inhibition of invasion was apparent at all time points analyzed, but it was greatest at 72 h (data not shown). For this reason, this time point was used in the subsequent experiments. Results from such an experiment in which several RGD-containing peptides and a control peptide were tested with the A375M and RuGli cells are shown in Fig. 1. The data show that each of the RGD-containing peptides inhibited invasion, whereas the control peptide in which the aspartic acid has been substituted with glutamic acid rendering it inactive in cell attachment assays (Pierschbacher and Ruoslahti, 1984*b*) was without effect. A hexapeptide with a D-alanine in the place of the second glycine (Pierschbacher and Ruoslahti, 1987) was also inactive (results not shown).

Peptide Specificity in the Inhibition of Invasion

The results shown in Fig. 1 suggested that RGD-containing adhesion proteins and their receptors play a role in tumor cell invasion. We next attempted to determine which adhesion protein(s) and receptor(s) might be important in this invasion using peptides that preferentially inhibit cell attachment to individual adhesion proteins. The effects of the various peptides that are shown in Fig. 1 are informative in this regard. The prototype, fibronectin-derived GRGDSP peptide inhibits the binding of the fibronectin and vitronectin receptors to their ligands (Pytela et al., 1985*a*,*b*). The GRGDNP peptide in which an asparagine follows the RGD sequence has a similar specificity but is about sixfold more active than the prototype peptide at inhibiting attachment of cells to fibronectin compared with vitronectin (Pierschbacher and Ruoslahti, 1987). The GRGDTP peptide which, unlike the other peptides, inhibits the attachment of cells to type I collagen, as well as to fibronectin and vitronectin (Dedhar et al., 1987), was consistently more active in the invasion assay than GRGDSP, suggesting that type I collagen may also play some role in the invasion process.

To distinguish between the involvement of the fibronectin and vitronectin receptors we used two peptides that inhibit only one but not the other of these receptors. A cyclic RGD peptide inhibits attachment predominantly to vitronectin and not to fibronectin, whereas a peptide in which a D-serine residue replaces the L-serine has the opposite specificity (Pierschbacher and Ruoslahti, 1987). As shown in Fig. 2, the cyclic peptide was an inefficient inhibitor of invasion, whereas the D-serine peptide was as active as the standard peptide GRGDSP.

Effects of Peptides on Tumor Cell Attachment

The effects of the peptides used in the experiments described above on cell attachment had been studied with cell lines different from the ones used here. Since a mouse melanoma cell line has been found to attach to fibronectin through a site different from the RGD site (Humphries et al., 1986*b*; McCarthy et al., 1986), it was necessary to determine the effects of the peptides on the attachment of the A375 and RuGli cells. Moreover, since the amniotic basement membrane contains laminin and type IV collagen (Liotta et al., 1983) it was also important to know whether the peptides would affect attachment of the tumor cells to these proteins. Earlier results have indicated that the RGD-containing peptides tested so far do not inhibit the attachment of various types of cells to laminin or type IV collagen (Aumailley and Timpl, 1986; Goodman et al., 1987; Graf et al., 1987), but a laminin receptor recognizing RGD has been described (Horwitz et al., 1985). The RGD peptides inhibited the attachment of the A375 and RuGli cells to fibronectin, vitronectin and type I collagen with the same specificities as has been previously found with other cells (Dedhar et al., 1987, Pierschbacher and Ruoslahti 1984*a*,*b*, 1987). Little or

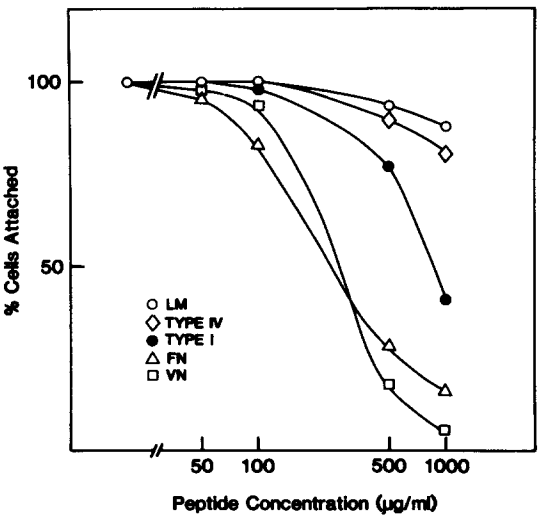

Figure 3. The effects of GRGDTP on A375M cell attachment to various extracellular matrix proteins. A375M cells were seeded on either fibronectin (*FN*), vitronectin (*VN*), laminin (*LM*), collagen type IV, or collagen type I in the presence of the GRGDTP peptide. About 90% of the cells attached to the wells coated with fibronectin and vitronectin in the absence of the peptide, while ~80% attached to laminin, 70% to type IV collagen and 46% to type I collagen. These values were designated at 100%. Inhibition of cell attachment is shown as a percentage of cells attached relative to controls. All assays were done in triplicate as described in the text.

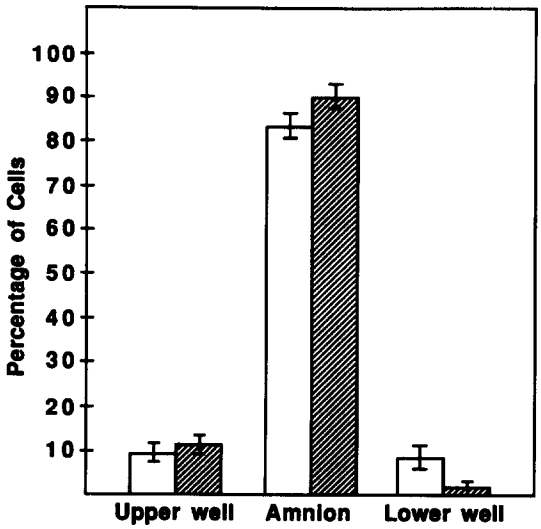

Figure 4. Distribution of tumor cells within the invasion system. A375M cells were allowed to attach to the amniotic membrane in MICS chambers for 4 h before the addition of either 1 mg/ml of GRGESP (□) or GRGDTP (▨). The invasion was allowed to proceed for 72 h and the number of cells located in either the upper well, within or adherent to the basement membrane portion of the amniotic membrane or in the lower wells was determined. The bar diagram represents the number of cell in each compartment as a percentage of the original number of cells seeded, ±SD.

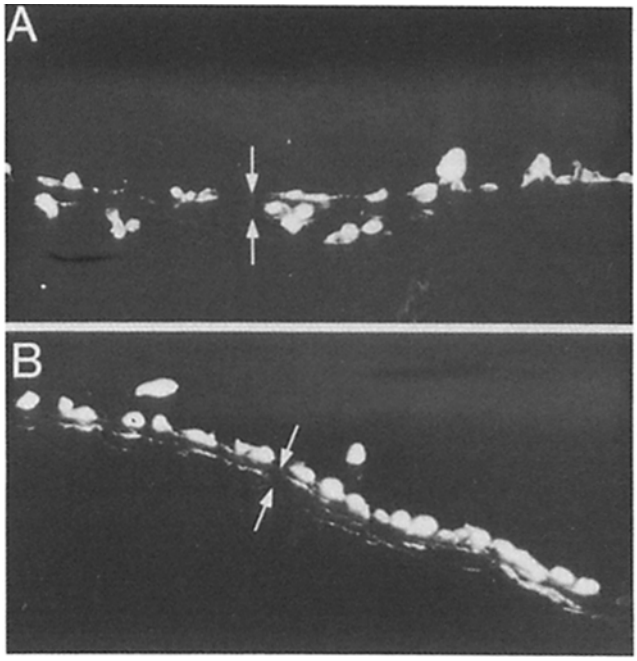

Figure 5. Visualization of invading tumor cells within the amniotic membrane. The A375M cells were labeled with the fluorescent lipophilic dye, DiI, and allowed to invade the amniotic membrane for 72 h in the presence of either GRGESP (*A*) or GRGDTP (*B*), as described for the previous invasion assays. The visualization of cell location during the invasion period was then determined by fluorescent microscopic examination of the sectioned amniotic membrane. Basement membrane surface (*upper arrow*) and the lower stromal surface (*lower arrow*) of the amniotic membrane are shown.

no inhibition of attachment to type IV collagen or laminin was seen with any of the peptides. The results for the A375M melanoma cells and the GRGDTP peptide were representative and are shown in Fig. 3.

The lack of inhibition of the laminin and type IV collagen induced cell attachment by the RGD-containing peptides suggested that the attachment of the tumor cells to the amniotic basement membrane should not be affected by the RGD-containing peptides. When this was tested, partial inhibition of cell attachment was seen when the tumor cells were seeded on the amniotic basement membrane in the presence of the RGD-containing peptides (result not shown). However, if the cells were first allowed to attach to the membrane during a preincubation period, as was the case with the invasion assays, the number of unattached cells floating in the upper chamber after subsequent incubation with the peptides for 1–72 h was not significantly different from that in control incubations. The results for the 4-h preincubation are shown in Fig. 4. The 10% of nonadherent cells with or without the peptides may have been detached due to degradation of the amniotic membrane by the tumor cells, cell death, or cell proliferation. Unlike the results on the amniotic membrane, cells plated similarly on fibronectin or type I collagen substrate could be completely detached with the peptides (result not shown, see Hayman et al., 1985*a*). These results indicate that the effect of the RGD peptides on the tumor cell invasion is not likely to be due to inhibition of the initial cell attachment to the amnion, but rather appears to result from inhibition of subsequent invasion steps involving fibronectin, and collagen or other as yet unidentified molecules. A further control experiment showed that the soluble fibronectin added to the assay in the fetal bovine serum which was part of the culture medium is not a factor in the assay. The involvement of soluble fibronectin was excluded by seeding the A375M cells into the invasion chambers in the presence of 50 μg/ml of fibronectin. This addition, which represents a 10-fold increase in fibronectin concentration above what is present in 10% fetal bovine serum (Hayman et al., 1985*b*), had no effect on the invasion or on the ability of the peptides to inhibit it (result not shown).

Visualization of Cell Location during Invasion

To visualize the invading cells in the amniotic membrane, fluorescently labeled cells were seeded onto amniotic membranes in MICs chambers. The labeled cells invaded as efficiently as unlabeled cells and the GRGDTP peptide inhibited this invasion. Cross sections of the amniotic membranes were examined by fluorescent light microscopy to locate the cells. In experiments performed with a control peptide (GRGESP), cells at various stages of migration throughout the amnion were observed (Fig. 5 *A*), whereas examination of over 100 sections from experiments with the GRGDTP peptide revealed no cells within the amnion stroma. In this case the cells were located at the basement membrane surface or directly beneath the basement membrane (Fig. 5 *B*).

Discussion

In this paper, we report that RGD-containing synthetic peptides can inhibit in vitro tumor cell invasion through a human

amniotic membrane, and that this inhibition is concentration-dependent, nontoxic, and correlates with the ability of the peptides to interact with matrix adhesion receptors. The specificity of the effect was shown by the fact that control peptides had little or no effect on invasion.

For the tumor cells to invade through the amniotic membrane in our system, they must go through a series of steps one or several of which may be inhibited by the peptides. First, the cells have to attach to the basement membrane which in our experiments was denuded of cells and oriented toward the seeded tumor cells. The basement membrane attachment is likely to be an important step in this in vitro invasion system, as well as in vivo (Liotta et al., 1983, 1986). The RGD-containing peptides showed only minimal inhibition of the attachment of the tumor cells to the basement membrane and had no detaching effect after the cells had already attached to it. Therefore, because the invasion experiments reported here were performed by adding the peptides after a 2–4-h preattachment period, we can conclude that the mode of action of the peptides was not inhibition of the initial cell attachment. This is in agreement with the fact that the peptides did not substantially inhibit the attachment of the tumor cells to laminin or type IV collagen which are the two main adhesive proteins in basement membranes. In addition, the localization of fluorescently labeled tumor cells showed that the cells in the presence of the RGD-containing peptides remain attached at the surface of the amniotic membrane or penetrate only slightly into the basement membrane.

The localization of the cells that were prevented by the RGD-containing peptides from invading suggests that the invasion step inhibited is the penetration of the cells into and through the stromal portion of the amniotic membrane which is about four-fifths of the thickness of the membrane. In agreement with this is that stromal extracellular matrix contains the adhesive proteins fibronectin and type I collagen (Liotta et al., 1983), cell attachment to which is inhibited by the RGD-containing peptides we have used. Since only those peptides among a group of closely related peptides that can inhibit cell attachment also inhibit invasion, the cell surface receptors recognizing RGD sequences in adhesive proteins are likely to be involved in the invasion process.

The GRGDSP peptide from the fibronectin cell-attachment site inhibits the attachment of cells to fibronectin and vitronectin but not to collagen (Dedhar et al., 1987; Hayman et al., 1985*a*). That this peptide inhibits invasion suggested a role for the fibronectin and/or vitronectin receptors. It also suggested that collagen receptors would not play a substantial role in the invasion. However, an indication of some involvement of type I collagen receptors was obtained with the peptide, GRGDTP, which inhibits not only the attachment of cells to fibronectin and vitronectin, but also to type I collagen (Dedhar et al., 1987). This peptide was consistently the most active inhibitor among the peptides tested in the invasion assay. Thus, collagen receptors may also play a role in cell invasion. Two of the RGD peptides we have developed recently (Pierschbacher and Ruoslahti, 1987) allowed us to evaluate the relative contribution of the fibronectin and vitronectin receptors to the invasion process.

Introduction of a D-serine into the serine position of the standard GRGDSP peptide yields a peptide that inhibits the function of the fibronectin receptor but not of the vitronectin receptor. That this peptide had an essentially unaltered activity relative to the standard GRGDSP peptide in the invasion assay suggests that, of the two receptors, the vitronectin receptor does not play a substantial role in the invasion process. The low activity of the cyclic RGD-containing peptide which inhibits the function of the vitronectin receptor but not the fibronectin receptor, supports this conclusion. Perhaps the amniotic membrane contains an insufficient amount of vitronectin, or other ligands that the vitronectin receptor could recognize, for this receptor to play a role in the invasion. On the other hand, it is possible that other, as yet unknown RGD-directed receptors might also be important in addition to the fibronectin and collagen receptors.

The results with the cyclic peptide also allowed another important conclusion. Even though the tumor cells have the vitronectin receptor which the cyclic peptide binds to, this peptide did not inhibit invasion. This shows that mere occupancy of an adhesion receptor at the cell surface does not cause inhibition of invasion. The inhibition of invasion by the peptides that can interfere with the likely cell attachment phenomena in the amniotic stroma strongly suggests that attachment of cells to the stromal matrix is needed for invasion and tends to exclude alternative possibilities. For instance, the ability of tumor cells to degrade extracellular matrices is important in invasion (Liotta et al., 1986; Mignatti et al., 1986) and cell surface proteins are closely associated with adhesion sites (Chen et al., 1987, Pöllänen et al., 1987). Therefore, the peptides could conceivably inhibit invasion by inhibiting proteolysis. However, the peptide specificity favors the explanation that the peptides deny the cells the traction they would need to derive from matrix adhesion to move through tissue.

Humphries et al. (1986*a*) have recently found that RGD-containing peptides when injected together with melanoma cells into mice can inhibit the colonization of the lungs by the tumor cells. Although there is evidence that the entry of blood borne cells into tissues depends on RGD interactions (Savagner et al., 1986), we think it unlikely that the inhibitory effect on invasion we have demonstrated would be the basis of the effect on experimental metastasis found by Humphries et al., 1986*a*. The reason for this is that the peptides have an in vivo half-life of only a few minutes (Pierschbacher, M. D., and P. M. Cardarelli, unpublished results), whereas invasion into tissues is likely to take much longer. Perhaps the peptides in the experimental metastasis model inhibit the initial stages of cell attachment to vascular endothelial cells or to the underlying subendothelial matrix, which may contain more fibronectin and be somewhat different in structure and composition than the amniotic basement membrane.

It is commonly held that extracellular matrices are important for tumor cell attachment in their new, metastatic location, but in other ways these matrices have been viewed as barriers to tumor cell invasion (Liotta, 1986; Ruoslahti, 1984). The barrier concept may be correct in the special case of basement membranes, but an important suggestion from our present work is that at least the connective tissue matrix may be a facilitator of tumor invasion, not a barrier. The RGD-containing peptides may become useful in counteracting the invasion of tumor cells through such tissues, leading to possible future therapeutic roles for these peptides.

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