Relationship of a $M$, 140 Fibronectin Receptor and Other Adhesion-related Glycoproteins to Tumor Cell-Cell Interaction

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ABSTRACT

Primary melanocytes attach poorly to collagen type IV and laminin, in contrast to their firm attachment to collagen type I/III and fibronectin [Gilchrist et al., In vitro (Rockville), 21: 114–120, 1985]. We have now found that metastatic B16 melanoma cells attach well to collagen type IV, laminin, vitronectin, and fibronectin but show a selective defect in attachment and cell aggregation on native collagen type I. Both flattened and aggregated melanoma cells revealed the presence of a $M$, 120,000 surface-iodinated species with affinity for a matrix containing the hexapeptide (glycylarginylglycylaspartylserylproline) which includes the fibronectin cell attachment sequence, but only flattened cells showed significant exposure of a $M$, 140,000 iodinated component with affinity for a large cell attachment-promoting fibronectin polypeptide. Decrease of the $M$, 140,000 fibronectin-binding external protein in the collagen-cultured melanoma cells was also associated with an inability to respond to the cell attachment activity of fibronectin, laminin, or vitronectin added to the collagen gels. Metabolic labeling with $[^{125}]$I-iodosulfate and electrophoretic analysis showed that lack of attachment and cell aggregation was associated with an increase in high molecular weight wheat germ agglutinin-binding glycoconjugates and an increase in $M$, 55,000 concanavalin A-binding glycoprotein species. Our data suggest that: (a) melanoma cell attachment requires the expression of the $M$, 140,000 fibronectin receptor which appears to be down regulated in cells exposed to poorly adhesive substrates; (b) expression of the $M$, 120,000 iodinated species with affinity for the fibronectin attachment sequence (arginylglycylaspartic acid) may be necessary but not sufficient for firm cell-substratum interactions; (c) increased tumor cell-cell interaction may involve a decreased attachment to substrate and the expression of different glycoproteins which may modulate cell-cell association.

INTRODUCTION

Within the several stages of metastasis, a decrease in substrate attachment to the primary site of growth is perhaps one cause of cell release from the primary tumor. Subsequently, tumor cell survival and invasiveness may be enhanced by increased intercellular adhesive properties of metastatic tumor cells. Then, metastatic cell aggregates presumably interact reversibly with extracellular matrix endothelial components for subsequent extravasation to reach the target organs where metastatic growth occurs (1, 2). Hence, it is important to gain further insight into the changes that occur in malignant cells when placed on extracellular matrix proteins that promote either attachment to substratum or intercellular adhesiveness. Cells are known to bind to extracellular matrices and to some of their protein constituents like fibronectin, laminin, vitronectin, and collagen (1, 3, 4). However, although metastatic tumor cells may not themselves produce significant amounts of adhesive proteins like fibronectin and vitronectin, nevertheless they may be exposed to these proteins in the blood at various times during their transit in the circulation to colonize select target tissues, characteristic of specific tumor types. One possible mechanism to explain the lack of indiscriminate attachment of metastatic cells during their transport in the body would be a decreased exposure or defective functionality of surface receptors for extracellular matrix proteins involved in cell substrate attachment and the concurrent expression of different glycoproteins characteristic of poorly attached cells.

While studying adhesion properties of a highly metastatic variant of B16 melanoma, we noticed that these cells attached rapidly to fibronectin, laminin, vitronectin, collagen type IV, and serum but failed to attach stably to native type I collagen gels, known to have a role in cell attachment and modulation of gene expression (5, 6). We now demonstrate that the cells that fail to attach to the native collagen gel even when supplemented with fibronectin, laminin, or vitronectin, exhibit an increased intercellular adhesion and a decrease in a $M$, 140,000 fibronectin-binding protein and in a $M$, 90,000 surface component. We also show that these properties are paralleled by an increased expression of Con A$^2$-binding glycoprotein species with a molecular weight of about 55,000 and by the greater detection of high molecular weight WGA-binding glycoconjugates.

MATERIALS AND METHODS

Cell Culture, Attachment Assays, and Affinity Chromatography of Iodinated Cells. In this study, we used B16 melanoma BL6 cells, selected for increased invasive ability to the lung and bladder of syngeneic animals (7). These cells were grown in vitro in Dulbecco’s medium supplemented with 1 g/liter glucose, 1× nonessential amino acids, and sodium pyruvate, including a 1× supplement of insulin, transferrin, and selenium (ITS Premix Lot 40350; Collaborative Research, Boston, MA), or with a 10% fetal calf serum supplement, whenever indicated. Subconfluent monolayer cultures were grown for 24 h on culture plates coated with fibronectin (5 μg/ml) in complete medium supplemented with 10 μCi/ml of $[^{125}]$I-methyl-L-phenylalanine. $[^{125}]$I-Labeled cells were then washed with PBS, detached with (10 μg/ml) 5× crystalline trypsin in PBS, and then harvested with a 2-fold excess of soybean trypsin inhibitor or detached with 1 mM EDTA in PBS, whenever indicated. Cells were then washed twice in PBS and seeded in unsupplemented Dulbecco’s medium for attachment to substrates coated for 16 h at 37°C with 10 μg/ml of plasma fibronectin (8), plasma vitronectin (9), EHS tumor-derived laminin (10), EHS tumor-derived collagen type IV (11), and rat tail collagen type I gels (6, 12). Surface iodination was carried out by harvesting cell monolayers on fibronectin or cell aggregates on collagen type I gels, with a rubber policeman in PBS containing 2 mM PMSF. Then a comparable number of cells was labeled by the Iodogen procedure (13).

Iodinated cells were ruptured by lysis in L-buffer and spun at 12,500 × g for 15 min at 4°C. The supernatant was dissociated for 3 min at 90°C in the presence of 2% SDS, 0.1 M β-mercaptoethanol, 0.1 M Tris-HCl (pH 6.8), 2 mM PMSF, and TLCK, followed by separation in 7.5% SDS-polyacrylamide gels (14). For affinity chromatography on fibronectin-Sepharose, iodinated cells lysed in an equal volume of L-buffer were slowly passed through a 2-ml matrix containing 3 mg/ml of the $M$, 120,000 chymotryptic cell-binding fragment of fibronectin coupled to CNBr-Sepharose (15). For analysis of binding to the fibronectin cell attachment peptide sequence, the same extracts in L-buffer were passed through a matrix prepared by coupling 100 mg of the...
(Gly–Arg–Gly–Asp–Ser–Pro) peptide to 2 ml of CNBr-activated Sepharose (4, 15). After extensive washing in L-buffer, specific elution was achieved from both columns by slowly running through, 1 volume of L-buffer supplemented with 1 mg/ml of the (Gly–Arg–Gly–Asp–Ser–Pro) peptide over a period of 1 h (4, 5). Electrophoretic analysis was carried out in 7.5% polyacrylamide gels (14) and detection of iodinated bands was obtained in vacuum-dried gels exposed to X-ray films at -70°C.

Glycoprotein Labeling and Detection of Con A-binding and WGA-binding Species. Cells were incubated on fibronectin coatings or collagen gels in Eagle's medium without glucose supplemented with 1 x sodium pyruvate, 10% fetal bovine serum, nonessential amino acids and 100 µCi [3H]glucosamine (NET-190; New England Nuclear, Boston, MA), for glycoprotein labeling (16). After 48 h of incubation, cells were harvested with a rubber policeman in PBS-containing 2 mM PMSF and centrifuged at 500 x g for 10 min. For affinity chromatography on Con A or WGA, we used 500 µl of Con A coupled to Sepharose 4B (Pharmacia) or an equal volume of WGA-Sepharose 6MB (Sigma) washed with PBS, which were incubated for 4 h with aliquots from an extract of [3H]glucosamine-labeled cells disrupted in 5 x urea-1% Triton X-100-0.1 M β-mercaptoethanol-1 mM TLCK-2 mM PMSF. After incubation, the mixture was spun in an Eppendorf microcentrifuge at 12,500 x g for 10 min to obtain the unbound fraction. Subsequently, the resin was washed with 15 volumes of PBS-1% Triton X-100, and bound glycoproteins were released from WGA-Sepharose glucosamine in electrophoresis dissociation buffer (2% SDS-0.1 M β-mercaptoethanol-0.1 M Tris-HCl (pH 6.8)-2 mM PMSF and TLCK) containing 0.1 M N-acetylglucosamine followed by removal of insoluble material. Elution of bound glycoproteins from Con A-Sepharose was achieved by treatment of the matrix with 100 µl of 0.1 M α-methyl mannioside in the same electrophoresis dissociation buffer. For electrophoretic analysis, samples were boiled in the eluting-dissociation buffer for 3 min at 90°C and separated in 7.5% SDS-polyacrylamide gels (14).

RESULTS

Selective Lack of Cell Attachment to Native Collagen Gels Promotes Cell Rounding and Aggregation. Since metastatic tumor cells presumably interact with different extracellular matrix proteins during their spread to secondary sites of growth, we first carried out a comparative analysis of B16 melanoma cell attachment to coatings with various extracellular matrix proteins, using [3H]thymidine labeled cultures. Most cells were found to attach rapidly within 60 min of seeding on nonadhesive surfaces coated with collagen type IV, laminin, vitronectin, or fibronectin.

However, parallel assays consistently failed to show comparable melanoma cell attachment to collagen type I films or native collagen gels (Table 1), substrates which showed the ability to promote the rapid attachment of other nonmetastatic cells (not shown) (5, 6, 12, 17). Since a possible explanation for the low attachment of these melanoma cells to collagen could have been an inability to resynthesize collagen receptors damaged by trypsin subculturing of the cells, we did identical experiments using cells subcultured with ethyleneglycol bis(β-aminoethyl ether)-N, N', N''-tetraacetic acid in the absence of proteases or allowing the cells to interact longer with collagen. However, although some degree of flattening was observed on collagen gels after 1–2 days of culture, this association was very unstable and flattened cells were dislodged from the collagen substrate by streams of growth medium (not shown). In contrast with the firm cell attachment to the other extracellular matrix proteins tested (Table 1). Also, experiments in which the native collagen gels were supplemented with fibronectin, laminin, vitronectin, or serum proteins failed to reveal increased cell adhesiveness to collagen. This may indicate a low interaction of these proteins with native collagen and/or that their conformation when bound to three-dimensional collagen differs from that shown by the same proteins when bound to plastic substrates or insoluble extracellular matrices (18).

Table 1 Effect of extracellular matrix proteins on B16 melanoma cell attachment

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% of attached cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen gels (rat tail, type I)</td>
<td>12.5</td>
</tr>
<tr>
<td>Collagen films (rat tail, type I)</td>
<td>17.2</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>58.4</td>
</tr>
<tr>
<td>Laminin</td>
<td>73.0</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>79.2</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>83.7</td>
</tr>
<tr>
<td>Collagen gels + fibronectin</td>
<td>18.9</td>
</tr>
<tr>
<td>Collagen gels + laminin</td>
<td>15.5</td>
</tr>
<tr>
<td>Collagen gels + serum</td>
<td>21.7</td>
</tr>
</tbody>
</table>

Fig. 1. Differential effect of collagen and fibronectin substrates on cell-cell adhesion. B16 melanoma cells were seeded on surfaces coated with the substrates indicated below for culture in medium supplemented with insulin, transferrin, and selenium (ITS Premix Lot 40350) for examination under phase contrast microscopy (A and B).

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Also, cultures on collagen gels revealed a marked alteration in cell shape manifested by significant rounding and aggregation, in contrast with the flattening of the same cells on fibronectin (Fig. 1), in which the morphology was similar to that observed on laminin, vitronectin, or collagen type IV (not shown).

**Relationship of M, 140,000 Fibronectin-binding Proteins and 90,000−100,000 Surface Components to Cell Adhesion.** Electrophoretic patterns of octylglucoside-soluble fractions revealed increased labeling in the M, 140,000 and 90,000 region in iodinated cells seeded on fibronectin, in contrast with greater iodination in the M, 94,000 region and in several lower molecular weight components in poorly adherent cell aggregates cultured on collagen gels (Fig. 2). Subsequently, aliquots from these extracts were used for sequential chromatography through plain Sepharose, a Sepharose matrix containing a large cell attachment-promoting fibronectin polypeptide (15), and another Sepharose matrix containing a hexapeptide (Gly-Arg-Gly-Asp-Ser-Pro) with the cell attachment sequence of fibronectin (4). After extensive washing, no iodinated species were released from plain Sepharose or from the other columns treated with octylglucoside containing 1 mg/ml of a control hexapeptide (Gly-Arg-Gly-Glu-Ser-Pro), which does not promote cell attachment or elution from fibronectin matrices (4, 15). Elution with the attachment-promoting peptide (Gly-Arg-Gly-Asp-Ser-Pro) released from flattened cells approximately comparable levels of a M, 140,000 species from the column containing the large attachment-promoting fibronectin fragment and a M, 120,000 species from the hexapeptide column (Fig. 3, a and b). In contrast, a similar elution of iodinated extracts from collagen-grown cell aggregates revealed predominantly the M, 120,000 species from the hexapeptide column and much lower levels of the M, 140,000 species eluting from the column containing the large cell attachment-promoting fibronectin fragment (Fig. 3, a' and b'). A similar result to that presented in Fig. 3, a' and b', was obtained from cell aggregates grown on collagen gels when these cells were seeded in medium containing insulin, transferrin, and selenium and supplemented with 5 μg/ml of fibronectin (not shown). This suggests that the presence of fibronectin and the cell surface exposure of the M, 120,000 component that binds to the Gly-Arg-Gly-Asp-Ser-Pro attachment-promoting peptide may be necessary but not sufficient to promote firm cell attachment since a complex involving both the M, 140,000 and the M, 120,000 species may be required for cell-substratum adhesion (19). Also, results qualitatively similar to those shown in Fig. 3, a' and b', were observed when using cell aggregates obtained after seeding these cells on bacterial dishes precoated with 100 μg/ml of bovine serum albumin to prevent cell attachment (not shown). This suggests that the findings presented in Fig. 3 may relate to the lack of adhesion to substratum and increased cell aggregation.

**Glycosylation Changes Associated with Modulation of Cell Adhesion.** Since the iodination data suggested a decrease in M, 140,000 and 90,000 surface proteins in cell aggregates, we also examined by metabolic labeling with [3H]glucosamine whether specific glycoproteins were increased in these collagen-cultured cells. A comparison of Con A-binding glycoproteins from fibronectin-grown monolayers grown on tissue culture plastic and on April 10, 2017. © 1987 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from
ADHESION-RELATED GLYCOPROTEINS AND TUMOR CELL INTERACTIONS

collagen-cultured cell aggregates also showed a decrease in the M, 140,000 and 90,000 region in cell aggregates (Fig. 4). In contrast, cell aggregation correlated with an increase in Con A-binding glycoproteins within the M, 55,000 range. These results are compatible with the iodination data in Fig. 2, indicating the preferential detection of surface components of lower molecular weight in collagen-cultured cells. However, no comparable increase in fast-migrating surface components was found when examining the WGA-binding glycoproteins from cell aggregates. Instead, this revealed an increase in a M, 160,000 glycoprotein and in a broad migrating glycoconjugate in collagen-cultured aggregates which were not comparably detected in cell monolayers (Fig. 5). This argues against a nonspecific proteolysis of glycoproteins in poorly adherent cell aggregates, suggesting that the mechanisms of cell adhesion involve a complex rearrangement of several cell surface molecules.

DISCUSSION

Normal melanocytes were reported to attach well to collagen-type I/III compared to their poor adhesion to type IV collagen and laminin (20). In contrast, metastatic B16 melanoma cells have now been shown to attach much better to laminin and collagen type IV than to collagen type I. Our findings were in agreement with prior reports indicating that collagen type I is less adhesive for metastatic cells than for poorly metastatic cells (17). The lack of attachment to collagen type I persisted when this substrate was supplemented with either fibronectin or laminin. The latter was of particular interest since soluble laminin is assumed to be quite effective in promoting the adhesion of cells to surfaces that otherwise would be nonadhesive (21). An interpretation of the lack of cell attachment to collagen gels can be a deficiency in functional receptors for this substrate as evidenced in cell-binding assays in serum-free medium. However, the poor cell attachment to this substrate even in medium supplemented with soluble fibronectin or laminin may be due to the inability of fibronectin or laminin to form with native collagen type I, an insoluble matrix required for optimal B16 melanoma cell attachment, or to an effect of the native collagen type I substrate on the conformation of receptors for extracellular matrix proteins. The poorly anchored cells obtained by culture on collagen gels revealed a decrease in a M, 140,000 surface component with affinity for the large cell attachment-promoting fibronectin fragment. This agrees with our recent glycoprotein-labeling studies which described a M, 140,000 component preferentially in B16 melanoma variants with decreased metastatic ability and greater cell-substratum interaction (16). Our correlation of a decrease in a M, 140,000 putative fibronectin receptor with defective attachment to collagen gels and lack of response to soluble fibronectin on the same substrate is in essential agreement with findings in erythroid cells in which fibronectin associated with the interstitial matrix of the bone marrow promotes attachment of these cells whereas plasma fibronectin is unable to do so (22). This situation may be analogous to that of metastatic cells in the circu-
lation which are exposed to plasma fibronectin through their migration in the blood but colonize only at specific sites, where presumably they interact with exposed extracellular matrix proteins from denuded endothelium (1, 2). A possible explanation for the decreased detection of the MR 140,000 putative fibronectin receptor in poorly attached tumor cell aggregates may be the finding that in the early vertebrate embryo, poorly attached migrating cells reveal their fibronectin receptor in a much more diffuse and mobile manner on the cell surface. In contrast, anchored cells show their fibronectin receptors concentrated in clusters close to areas where a fibronectin-containing matrix is available (23). Hence, it may well be that the fibronectin receptor molecules are involved not only in attachment to fibronectin but also in the regulation of cell migration, cell-cell interaction, and tumor invasiveness. An intriguing observation was that the cell aggregates grown on collagen gels exhibited a MR 120,000 surface protein with affinity for the Arg-Gly-Asp sequence derived from fibronectin. This suggests that the presence of soluble fibronectin and the exposure of cell surface proteins with affinity for the Arg-Gly-Asp sequence may be helpful but not sufficient to promote cell attachment. It may be that a cooperative effect of a complex between different surface proteins (19) with affinity for the Arg-Gly-Asp sequence is required for greater attachment, as now found in B16 melanoma cells seeded on a fibronectin matrix, and in properly flattened MG-63 cells (15). Another alternative explanation may be that in addition to the ability to recognize the Arg-Gly-Asp sequence, the functionality of the membrane receptor involved in substrate attachment is influenced by conformation or charge of other glycoproteins.

Some possible candidates that may relate to cell-cell adhesion at the expense of cell-substratum adhesion are the wheat germ-binding MR 160,000 glycoprotein and large glycosylated components that bind to wheat germ-Sepharose, as well as the MR 55,000 Con A-binding glycoprotein species, increased in cell aggregates. These findings imply the participation of glycoconjugates bearing N-acetylgalactosamine and sialic acid residues recognized by WGA and the involvement of glycoproteins with d-mannose and d-glucose residues (24) in cell-cell interaction, suggesting also the use of lectin affinity chromatography in the isolation of glycoproteins involved in cell-cell adhesion. The combined use of fibronectin affinity chromatography and lectin affinity chromatography has now permitted us to postulate cell-cell adhesion as a complex phenomenon involving not only the modulation of receptors for extracellular matrix proteins but also an altered expression of several other glycoconjugates.

In summary, we have now shown a novel correlation of greater cell-cell interaction with an increased detection of specific lectin-binding glycoproteins and decreased expression of a MR 140,000 fibronectin-binding surface protein in poorly adherent melanoma cells. Our findings suggest that optimal tumor intercellular aggregation occurring in nonadhesive substrates may involve an altered expression of receptor for extracellular matrix proteins and the involvement of different cell adhesion molecules other than these receptors in cell-cell interactions.

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