Evidence that an RGD-Dependent Receptor Mediates the Binding of Oligodendrocytes to a Novel Ligand in a Glial-derived Matrix

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Abstract. A simple adhesion assay was used to measure the interaction between rat oligodendrocytes and various substrata, including a matrix secreted by glial cells. Oligodendrocytes bound to surfaces coated with fibronectin, vitronectin and a protein component of the glial matrix. The binding of cells to all of these substrates was inhibited by a synthetic peptide (GRGDSP) modeled after the cell-binding domain of fibronectin. The component of the glial matrix responsible for the oligodendrocyte interaction is a protein which is either secreted by the glial cells or removed from serum by products of these cultures; serum alone does not promote adhesion to the same extent as the glial-derived matrix. The interaction of cells with this glial-derived matrix requires divalent cations and is not mediated by several known RGD-containing extracellular proteins, including fibronectin, vitronectin, thrombospondin, type I and type IV collagen, and tenascin.

The importance of cell surface interactions in tissue development is probably commensurate with the complexity of the organ, therefore, these interactions would seem to play a crucial role in developing brain. Of the many biosynthetic events which take place in brain development, one of the most dramatic is myelination of neuronal axons by oligodendrocytes. Since the brain contains many process-bearing cells, including some neurons, which are not myelinated, specific recognition and adhesion events between certain neurons and oligodendroglia are likely to be critical in determining the ability of oligodendrocytes to form a stable myelin sheath.

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Materials and Methods
Polystyrene 24-well plates, not tissue culture treated, were manufactured by Linbro and obtained from Flow Laboratories (McLean, VA). Calf serum
was obtained from HyClone (Logan, UT). The Labline Junior Orbital orbital shaker used in both cell culture and adhesion assays was purchased from American Scientific Products (McGaw Park, IL). [7SS]Translabel was purchased from ICN (Cleveland, OH). Bovine plasma was purchased from Irvine Scientific (Santa Ana, CA). Rat fibronectin was purchased from Coltech (La Jolla, CA). Human vitronectin and rabbit antibodies to human vitronectin were a gift of Dr. Erkki Ruoslahti of the La Jolla Cancer Research Foundation (La Jolla, CA). Antibodies to mouse fibronectin were the gift of Dr. Judy Berliner of the Department of Anatomy, UCLA. Antibodies to human thrombospondin were a gift of Dr. David Roberts of the National Institute of Health (Bethesda, MD). Antibodies to glial fibrillary acidic protein were the gift of Dr. L. Eng of the V.A. Medical Center (Palo Alto, CA). Antibodies to rat type IV collagen were a gift of Dr. John Fessler of the Department of Biology, UCLA. Antibodies to chick tenascin were a gift of Dr. Douglas Fambrough of the Department of Biology, Johns Hopkins University (Baltimore, MD). Preparation of antibodies to galactocerebroside have been described elsewhere (39). All other chemicals were from standard commercial suppliers.

Methods

Cell Culture. Purified oligodendrocytes were prepared from neonatal rat cerebral cortex by a modification of the method of McCarthy and de Vellis (25), as described (39). This method is based on the differential adhesion of oligodendrocytes and astrocytes in mixed glial cultures. Oligodendrocytes are released into the culture medium upon extended shaking, while astrocytes remain attached to the plastic surface.

Astrogial Matrix Preparation. Media was removed from confluent mixed glial cultures and 1 ml water per well or 10 ml per 75 cm² flask was added. After 2 h at room temperature the plates or flasks were swirled briefly and the lyed cell material removed. The surfaces were washed two times with PBS and then stored in PBS at 37°C until use. Surfaces treated in this way contained no microscopically detectable cell debris or membranous structures. The material remaining on the culture surface after water lysis and wash is referred to as the astrogial matrix (AGM)1. A typical AGM preparation contained 0.75–1.0 µg protein/cm² of culture surface. AGM was removed from the plastic surface for analysis by solubilization in 62.5 mM Tris, pH 6.8, containing 3% SDS, 10% glycerol, and 5% β-mercaptoethanol for 1 h at room temperature.

Adhesion Assay. Oligodendrocytes released into the media upon shaking mixed glial cultures (39) were collected by centrifugation in a clinical centrifuge. The cell pellet was suspended by trituration in 10 ml of methionine-and glutamine-free Modified Eagle’s Medium supplemented with 1% calf serum and 100–200 µCi [35S]Translabel. The cells were labeled for 6–8 h at 37°C on an orbital shaker at 150 rpm. This gentle agitation prevented the oligodendrocytes from adhering to the surface of the flask and elaborating their processes

Results

Oligodendrocyte Adhesion to Various Substrates

A simple adhesion assay was used to test the ability of oligodendrocytes to adhere to various substrates. To determine the viability of adherent cells and to ensure that cells bound in the assay were indeed oligodendrocytes, adhesion was carried out on AGM for 30 min as described in Materials and Methods. Rather than extracting the adherent cells immediately for quantitation, though, culture media was added to the wells and the cells were grown for 2 d. As shown in Fig. 1, adherent cells display a morphology typical of cultured oligodendrocytes; the elaborate processes are particularly characteristic of this cell type. Immunofluorescence staining using anti–galactocerebroside, a specific marker for

1. Abbreviations used in this paper: AGM, astrogial matrix; CHAPS, 3-(3-cholamidopropyl)dimethyl-ammonio)-1-propanesulfonate.

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the oligodendrocyte, revealed positive staining in >80% of the cells. Only 7% of cells present after 2 d in culture were stained with anti-glial fibrillary acidic protein, a specific marker for astrocytes. Most of those cells which were scored as negative for galactocerebrosides nonetheless possessed a typical oligodendrocyte morphology and were weakly reactive with galactocerebroside antibody. We chose to immunolabel the cells as soon as it was technically possible (i.e., when the cells had become attached well enough to withstand the numerous wash steps required) so that the population of cells closely reflected that used in adhesion experiments and was minimally effected by cell proliferation. Since the synthetic rate of galactocerebrosides develops with time in culture (39), many oligodendrocytes are likely not detected by immunofluorescence after only 2 d in culture. The value of 80% is therefore a low estimate of the purity of oligodendrocytes used in these experiments. While fewer cells adhered to an untreated plastic surface, the percentage of galactocerebrosides-positive cells was similar to the percentage of galactocerebroside-positive cells that adhered to AGM (data not shown).

As shown in Fig. 2, the initial rate of oligodendrocyte adhesion varied considerably depending on the nature of the substrate. If the incubation time was increased to 90 min, all surfaces, including untreated plastic, were essentially equally effective in promoting the adhesion of oligodendrocytes (data not shown). 30 min was chosen as the incubation time for future assays, since the effects of different surface treatments on the initial rate of adhesion were readily apparent at this time. Among surfaces examined for the ability to promote oligodendrocyte adhesion, AGM, bovine plasma fibronectin, rat plasma fibronectin, and chondroitin sulfate all promoted the attachment of oligodendrocytes to a similar extent, with a 2.5–3-fold greater adhesion than seen for untreated plastic. Of all surface treatments examined, poly-L-lysine was most effective in the 30-min assay, increasing binding of cells nearly fivefold over the plastic level. Virtually 100% of the radioactivity associated with trichloroacetic acid precipitable material adheres to poly-L-lysine in a 15–30-min incubation. In a 90-min incubation essentially all of the radioactivity associated with TCA precipitable material adhered to all culture surfaces, indicating that given enough time all cells are competent to form stable adhesive interactions with a wide variety of substrates.

**An RGD-containing Peptide Inhibits Oligodendrocyte Adhesion to Fibronectin, Vitronectin, and AGM**

Fibronectin and vitronectin are among the best characterized
Figure 2. Time course of oligodendrocyte adhesion. 35S-labeled oligodendrocytes were incubated on surfaces treated with poly-L-lysine (●), bovine fibronectin (▲), AGM (○), prepared as described in Materials and Methods, or on untreated plastic (○). After the indicated times the plates were shaken, the media aspirated, and the adherent cells extracted and counted as described in Materials and Methods. Each point represents the average of three wells and variation between wells was less than 10%.

adhesion proteins. Different cell surface receptors for each of these proteins have been purified and, in each case, shown to recognize a defined sequence of the fibronectin or vitronectin primary structure (1, 35, 36, 45). To examine the specificity of the oligodendrocyte's interaction with fibronectin and vitronectin we used, as an inhibitor of adhesion, a synthetic hexapeptide (GRGDSP) derived from the known sequence of the fibronectin cell-binding domain. At concentrations of 0.1 mg/ml, the peptide decreased binding of cells to fibronectin by 79% and to vitronectin by 92% (Fig. 3). Increasing the concentration of peptide above this level did not increase the specific inhibition. Interestingly, the peptide also inhibited oligodendrocyte binding to AGM, decreasing adhesion by 60% at 0.1 mg/ml (Fig. 3). The RGD-containing hexapeptide had no effect on cell binding to plastic, poly-L-lysine or chondroitin sulfate (data not shown).

The specificity of this inhibition was examined by screening a group of peptides, both related and unrelated to GRGDSP. As shown in Table I, these peptides had no effect on adhesion of the oligodendrocyte to either AGM or to an untreated plastic surface. One of these peptides differed from the active peptide by the single substitution of alanine for glycine within the RGD sequence. Another inactive peptide, a tetramer with the sequence SDGR, represents the inverse sequence of the active region within the GRGDSP peptide, which has been shown to interfere with cell spreading of both baby hamster kidney cells and chick embryo fibroblasts on fibronectin, vitronectin, collagen, and laminin coated surfaces (52). The peptide GRGES has been shown to inhibit the spreading of certain cell types on fibronectin coated surfaces (15). This peptide, however, did not inhibit the adhesion of oligodendrocytes to either fibronectin or AGM (data not shown).

RGD-dependent Component of AGM Is Produced by Mixed Glial Cells and Is Not Derived from Serum

Since AGM is prepared from cells that have been grown in media containing calf serum, the RGD-dependent binding of oligodendrocytes to AGM might be explained by the presence of serum-derived proteins in the AGM which can bind to cells in a RGD-inhibitable manner. Fibronectin and vitronectin are the most abundant and the best characterized proteins of this family. The binding of cells to both fibronectin and vitronectin is known to require divalent cations. As shown in Fig. 4, binding of oligodendrocytes to either fibronectin, vitronectin, or AGM, is diminished to the level of untreated plastic by addition of 2 mM EDTA, consistent with the notion that cells are binding to each of these proteins by a related mechanism.

To further assess the role that serum-derived factors may play in the adhesive properties of AGM, tissue culture wells

Table 1. Inhibition of Oligodendrocyte Adhesion with Synthetic Peptides

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AGM (%)</th>
<th>Plastic (%)</th>
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<tbody>
<tr>
<td>GRGDSP</td>
<td>53</td>
<td>100</td>
</tr>
<tr>
<td>GRADSP</td>
<td>106</td>
<td>101</td>
</tr>
<tr>
<td>SDGR</td>
<td>101</td>
<td>104</td>
</tr>
<tr>
<td>VYPNGA</td>
<td>103</td>
<td>113</td>
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The specificity of oligodendrocyte adhesion by GRGDSP was examined by testing several other peptides for inhibitory activity. All peptides were used at a final concentration of 0.1 mg/ml. The percent cell binding to the plastic surface was determined by calculating the 35S radioactivity retained in wells containing peptide relative to wells which received no peptide. The percent cell binding to AGM was calculated by first subtracting the background level of 35S radioactivity bound to untreated plastic from the values determined for binding to AGM.

Figure 3. Inhibition of oligodendrocyte adhesion by GRGDSP. Cells were incubated on bovine fibronectin (B-FN), vitronectin (VT), AGM, or untreated plastic. Hatched bars indicate the addition of 0.1 mg/ml of GRGDSP in the incubation media. Values given are the average of 3 wells and the error bars indicate SD.
Figure 4. Effect of EDTA on oligodendrocyte adhesion. Cells were incubated on bovine fibronectin (FN), vitronectin (VT), AGM (AGM), or untreated plastic. Hatched bars indicate the addition of 2 mM EDTA to the incubation media. Each bar represents the average of three wells and the error bars indicate the SD.

without cells were incubated with media containing 10% serum at 37°C. The wells were maintained in serum-containing media for 7 d and the media was replenished on the same schedule as parallel plates containing mixed glial cells. All wells were then treated with water lysis and subsequent washes by the same procedure used to prepare AGM from mixed cells. These surfaces were then used to compare RGD-dependent adhesion. As shown in Fig. 5, the mock-treated surface exhibited only 16% of the RGD-dependent adhesion of AGM.

Thus it appears that serum-derived factors are insufficient to account for the behavior of AGM. It is conceivable, however, that serum proteins such as fibronectin and/or vitronectin could combine with constituents of the AGM to show adhesion beyond that seen for either protein alone. However, if this were the case, antibodies which specifically inhibit adhesion to purified serum proteins might be expected to inhibit adhesion to the AGM as well. As shown in Fig. 6, pretreatment of wells with antibodies to fibronectin inhibited adhesion of cells to fibronectin-coated plastic, but were ineffective against either vitronectin, or AGM. Similarly, antibodies to vitronectin inhibited adhesion to vitronectin-coated surfaces but were ineffective against either fibronectin or AGM.

The antibodies against mouse fibronectin are equally effective in inhibiting adhesion to both rat and bovine fibronectin excluding the possibility that fibronectin secreted by the glial cells is the active adhesion component of the AGM.

In a separate experiment, surfaces pretreated with antibody to either fibronectin, vitronectin or both were tested for RGD-inhibitable adhesion. As shown in Fig. 7, fibronectin antibodies inhibit the binding of oligodendrocytes to fibronectin-coated surfaces to the background value of untreated plastic and addition of RGD-containing peptide caused no further inhibition. The same is true of vitronectin-coated surfaces; antibodies to vitronectin decrease binding to back-
Treatment:

- anti-FN
- anti-VT
- non-immune
- GRGDSP

Figure 7. GRGDSP inhibition of oligodendrocyte adhesion to surfaces pretreated with antibodies to fibronectin and vitronectin. Fibronectin-(FN) coated surfaces were preincubated with non-immune serum, or anti-fibronectin (anti-FN); vitronectin-(VT) coated surfaces were preincubated with nonimmune serum, or anti-vitronectin (anti-VT); AGM was preincubated with nonimmune serum, or with both anti-FN and anti-VT. Oligodendrocyte adhesion was measured in the presence and absence of 0.1 mg/ml GRGDSP. Each bar represents the average of 3 wells and the error bars indicate SD.

Ground levels and no RGD-inhibitable adhesion is evident on antibody treated surfaces. When AGM is treated with antibodies to both vitronectin and fibronectin, cell binding is diminished by 18%. However, 44% of the cells which bind to AGM treated with both antibodies are still inhibited by 0.1 mg/ml of RGD-containing hexapeptide, indicating that the RGD-inhibitable behavior of AGM is imparted largely by a component(s) other than fibronectin or vitronectin.

To estimate the amount of fibronectin or vitronectin that would be needed to account for the properties of AGM, we carried out titration curves, applying variable amounts of purified proteins to the plastic wells and measuring adhesion of oligodendrocytes to these surfaces. Maximal adhesion occurred at >1.0 μg/well fibronectin and at >0.5 μg/well vitronectin. The material extracted from AGM was directly analyzed on SDS-PAGE for the presence of these serum proteins. Neither protein is detected by western blotting (Fig. 8). In other experiments using more concentrated AGM we were able to detect 20 ng/well of fibronectin. This amount is less than 5% of what would be required to explain the RGD-inhibitable properties of AGM.

The results summarized above suggest that the component of AGM responsible for its RGD-inhibitable behavior is not derived from serum. In particular, they show that the active component is neither fibronectin nor vitronectin. Three other proteins which bind to cells via RGD-dependent mechanisms, thrombospondin (22), Type I collagen (8) and tenascin (7, 9), were also considered as the AGM active component. Antibodies to human thrombospondin and chick tenascin were ineffective in inhibiting the adhesion of oligodendrocytes to AGM and neither tenascin nor thrombospondin were detected in AGM extracts by western blot (data not shown). Plastic surfaces coated with either type IV or type I collagen did not promote adhesion above the level for untreated plastic. Antibodies to type IV collagen, which contains an RGD-sequence (43), did not inhibit the binding of cells to AGM (data not shown).

The chemical nature of the active factor(s) in AGM was probed by digestion of AGM using protease and glycosidases. As shown in Fig. 9, digestion of AGM with pronase B reduces the binding of cells to the level of untreated plastic. The pronase digested AGM shows essentially no RGD inhibition. In contrast, digestion of AGM with heparinase and chondroitinase ABC (Miles, Lisle, IL) had little or no effect on cell adhesion (data not shown).

Discussion

The use of cells in primary culture to study the differentiated...
properties which typify those cells in vivo, often requires the use of a specific substratum (11, 13, 38, 41). Schwann cells, the counterpart of the oligodendrocyte in the peripheral nervous system, when kept in culture under appropriate conditions, produce an extracellular matrix which is similar to the matrix formed in peripheral nerve endoneurium. In these cultures, basement membrane components have been shown to induce normal Schwann cell differentiation (6, 26). Our interest in the substratum produced by mixed glial cultures stems from the observations that oligodendrocytes plated on AGM, rather than plastic or poly-L-lysine, tend to more rapidly extend processes and develop morphologically. In addition, cells plated on AGM show a twofold higher rate of incorporation of [3H]thymidine into trichloroacetic acid precipitable material than cells grown either on plastic or poly-L-lysine (Bullock, P. N., and L. H. Rome, unpublished observations). As a first step in quantifying the role that the AGM plays in development of oligodendrocytes in culture, we have begun to characterize the initial adhesive interaction between oligodendrocytes and components of this surface.

The results presented here show that oligodendrocytes, like many cell types, bind to fibronectin and vitronectin in an RGD-dependent manner. More interestingly, oligodendrocytes bind to a protein component present in an extracellular matrix produced by cultures of mixed glial cells. This interaction is also inhibited by an RGD-containing hexapeptide. The AGM interaction is not inhibited by antibodies to either vitronectin or fibronectin even though the same antibody treatment prevented oligodendrocyte adhesion to a surface coated with the purified proteins. Furthermore, the AGM component does not appear to be derived from serum.

Cultures of astrocytes from neonatal rat cerebral cortex have been reported to synthesize fibronectin (34). These cultures synthesize and secrete fibronectin in amounts comparable to fibroblasts, but the form of the protein produced does not assemble into a matrix. Fibronectin has also been immunolocalized to the surface of astrocyte cultures which were derived from various brain regions. However, astrocytes from neonatal cerebral cortex, as used in our study to form the AGM, were negative when stained for cell surface fibronectin (23). Since our fibronectin antibodies recognize rat as well as bovine fibronectin, it is unlikely that fibronectin secreted by the mixed glial cells, or derived from serum, is the active component of the AGM.

It was possible that the RGD-dependent behavior of AGM could be imparted by fibronectin derived from either the serum or the mixed glial cells which, though interactions with other products of the glial cultures, were not recognized by the antibodies we used. For example, the binding of proteoglycans to a site distinct from the cell-binding site has been shown to inhibit the cell-binding characteristics of fibronectin and of type I collagen (41). Presumably the proteoglycan exerts this effect indirectly by obscuring the cell-binding domain. A similar interaction with proteoglycans secreted by the glial cells might result in the 'masking' of fibronectin antigenic sites from antibodies. However, since we could not detect an appreciable level of either fibronectin or vitronectin in AGM extracts by western blot, it is unlikely that these proteins are components of AGM.

Thrombospondin is another serum protein which binds to cells in an RGD-dependent manner (22). That this protein might be a component of the AGM is suggested by the report that cultures of human astrocytes synthesize thrombospondin (2). However, antibodies to human thrombospondin had no effect on the ability of oligodendrocytes to adhere to the AGM. Additionally, we were unable to demonstrate its presence in extracts of AGM by western blot.

The extracellular protein tenascin (7, 9), previously known as chick myotendinous antigen, is apparently identical to several independently isolated extracellular proteins, including cytotactin. Cell binding to this protein is inhibited by RGD-containing peptides. Cytotactin has been shown to be produced by glial cells and has been implicated in neuron-glia interactions (12). While we did see material which reacted with tenascin antibodies in extracts of mixed glial cultures, antibodies to tenascin were ineffective in inhibiting adhesion to AGM and the protein was not detected in AGM by western blot.

The chemical basis of the oligodendrocyte/neuron interaction has been the subject of much speculation. Several molecules with cell adhesive properties have been implicated as mediators of this interaction. The neural cell adhesion molecule, NCAM, is widely distributed among neuronal types and is a component of oligodendrocytes both in vivo (24) and in vitro (29). In contrast to the mechanism of fibronectin binding to its receptor, NCAM mediated cell–cell interactions are homophilic and Ca2+ independent. The myelin associated glycoprotein, MAG, has been suggested to play a role in the interaction on the basis of its periaxonal localization within myelin and on its adhesive properties seen with in vitro assays (33). MAG, NCAM, and cytotactin are members of a group of adhesion proteins which share a common carbohydrate epitope which is itself implicated in the adhesive properties of these proteins (20). Interestingly, cytotactin has been shown bind to cells in an RGD-dependent manner and, while MAG has not been shown to have such binding properties, it has been reported to have an RGD region in a proposed extracellular domain. The precise role that these proteins play in oligodendrocyte function remains to be determined but the potential overlap between these two classes of adhesive interactions is intriguing.

Oligodendrocytes were able to adhere in an RGD-dependent manner to at least three different ligands; fibronectin, vitronectin, and a component in AGM. Several cell surface receptors which recognize RGD sequences have been isolated (1, 8, 10, 14, 17, 30, 35–37). Fibroblasts bind to both vitronectin and fibronectin via distinct receptor complexes, which show exclusive binding specificities. There is precedent, however, for a single receptor complex binding to several different proteins in an RGD-dependent manner. The glycoprotein Ib/IIa complex of platelets binds to at least four different proteins in an RGD-dependent manner (9, 37). Integrin, previously called CSAT antigen, binds to fibronectin, vitronectin and to laminin (14). The binding of this receptor complex to all three of these proteins is inhibited by RGD-containing peptides. Our preliminary attempts to purify an oligodendrocyte cell surface protein by chromatography of detergent extracts of these cells on immobilized GRGDSP hexapeptide have so far been unsuccessful. However, affinity chromatography using an immobilized peptide has been generally less effective than chromatography with the intact protein or proteolytic fragments of the parent pro-
tein. For example, while both the vitronectin and fibronectin receptors from osteosarcoma cells can be affinity purified using the parent protein as affinity support, only the vitronectin receptor can be isolated using a peptide affinity column (35, 36).

The adhesive component of the AGM can be solubilized using detergents and chaotropic agents. We have not yet been able to reconstitute the adhesive activity of these extracts. A major obstacle to these studies has been the insolubility of the AGM under physiological conditions. For example, a buffer containing 4 M urea and 1% CHAPS completely removes the activity from the culture surface. Dialysis of this extract against PBS at room temperature results in the irreversible precipitation of most of the protein, and what remains in solution is inactive in promoting adhesion.

Recent work on the biology of the oligodendrocyte has suggested that nonspecific adhesion may play a role in the phenotypic development of these cells in culture (48, 53). Using a system in which mature oligodendrocytes can be maintained either floating or attached to a poly-1-lysine surface, it has been demonstrated that attached cells show increased levels of synthesis of the major myelin proteins (proteolipid protein and myelin basic protein), as well as increased phosphorylation of myelin basic protein. With our observation of a specific and easily inhabitable adhesive interaction as a starting point, we have begun to examine the contribution that this RGD-dependent interaction makes to development of the myelin-like properties of neonatal rat oligodendrocytes in culture (5).

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