The Role of Extracellular Matrix in the Migration and Differentiation of Parietal Endoderm from Teratocarcinoma Embryoid Bodies

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Abstract. Embryoid bodies formed from teratocarcinoma stem cells differentiate an outer layer consisting of parietal and visceral endoderm or of visceral endoderm exclusively. We have previously shown that when these embryoid bodies are plated on collagen-coated substrates a parietal endoderm-like cell migrates onto the substrate, whereas all of the visceral endoderm remains associated with the stem cell mass, suggesting a role for substrate contact in parietal endoderm differentiation. We now identify fibronectin as the migration-promoting component in these cultures, and note that laminin and collagen type IV are 10-fold less effective at promoting both attachment and endoderm outgrowth. The RGDS tetrapeptide (arg-gly-asp-ser) from the cell attachment domain of fibronectin can specifically block attachment and outgrowth on both fibronectin- and laminin-coated substrates. In addition, the involvement of the 140-kD fibronectin receptor is demonstrated using an antibody directed against this molecule.

RECENT studies have provided much information concerning the molecular nature of the cell substrate interactions involved in promoting the migration of certain already existing cell types (19, 30). However, instances in which contact with a specific substrate may promote the differentiation of a new cell type, as well as its migration, have not been observed. One of the first instances of directed cell migration in the mouse embryo is the migration of parietal endoderm around the inner surface of the trophectoderm (9). This occurs subsequent to the differentiation of a layer of primitive endoderm on the side of the inner cell mass facing the blastocoele cavity. Although a role for the extracellular matrix in the migration and differentiation of parietal endoderm has been proposed (9), definitive studies have been limited by the fact that these events occur subsequent to implantation (26). For this reason, we have turned to the teratocarcinoma stem cell embryoid body system.

Teratocarcinoma stem cells provide an excellent model system for studying certain events of early mouse embryogenesis (14, 22). We have recently demonstrated that the outgrowth of parietal endoderm from teratocarcinoma-derived embryoid bodies provides an in vitro model system for studying the comparable event in the embryo (12). Initial studies demonstrated that considerable parietal endoderm outgrowth was observed when appropriate stage aggregates were plated upon certain substrates even if the aggregates contained only visceral endoderm before substrate contact, as is the case with retinoic acid-treated F9 aggregates (12, 14). Interaction with a migration-promoting substrate was sufficient to trigger parietal endoderm differentiation. We report here that the matrix component responsible for differentiation and outgrowth in these cultures is fibronectin. A comparative study indicates that although laminin and type IV collagen can also promote endoderm outgrowth, fibronectin is significantly more effective. In addition we demonstrate that the same hierarchy of matrix component effectiveness is observed for attachment of embryoid bodies. Consistent with these observations, the cell attachment tetrapeptide RGDS (arg-gly-asp-ser; 3, 24) can inhibit both attachment and outgrowth of endoderm on fibronectin- and laminin-coated substrates. Furthermore, antisera directed against the 140-kD fibronectin receptor (4, 5, 25) can block the outgrowth of parietal endoderm cells on fibronectin and laminin substrates, supporting the contention that laminin as well as fibronectin can interact with this receptor in an RGDS-inhibitable fashion (15). The in vivo significance of these results is discussed.

Materials and Methods

Cell Culture and Embryoid Body Differentiation and Outgrowth

PSA1 cells were cultured and embryoid bodies formed as previously described (21). Briefly, after 3 d of culture in the absence of feeder cells (day 3 + 0) the embryoid bodies were pipetted off of the dish and maintained in suspension for 4 d (day 3 + 4). The embryoid bodies were then plated on acid-soluble calf skin collagen- (Calbiochem-Behring Corp., La Jolla, CA) coated coverslips (0.1 mg/ml) as previously described (10), in the presence of normal calf serum or fibronectin-free calf serum. Fibronectin was removed from the serum by passage over a gelatin-agarose column (Sigma Chemical Co., St. Louis, MO), as described by Ruoslahti et al. (27). Fibronectin-coated coverslips were prepared by incubating the coverslips in 30 μg/ml (or the concentration described in the text) of bovine plasma fibronectin (Bethesda Research Laboratories, Gaithersberg, MD) or purified from bovine plasma by gelatin affinity chromatography, as described by Ruoslahti et al. (27), for 1 h. The coverslips were then washed three times

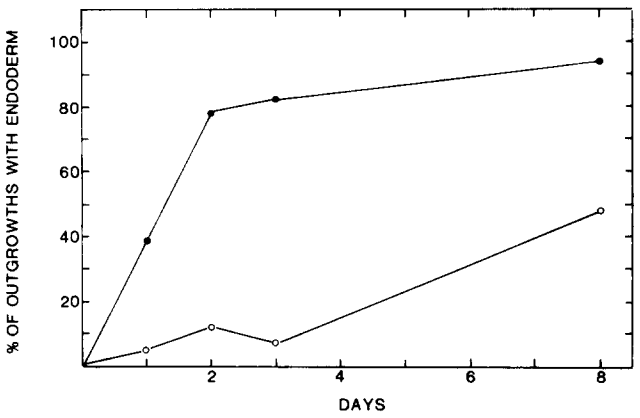

Figure 1. Endoderm outgrowth on collagen in the presence and absence of fibronectin. Day 3 + 4 embryoid bodies were plated on collagen type I-coated coverslips in the presence of fibronectin-containing (*solid circles*) or fibronectin-free serum (*open circles*), and the extent of endoderm outgrowth determined as described in Materials and Methods.

in PBS. Laminin was the generous gift of Dr. H. Kleinman (Laboratory of Developmental Biology, National Institutes of Health, National Institute of Dental Research), and collagen type IV was from Bethesda Research Laboratories. Laminin and collagen IV coverslips were prepared as described for fibronectin.

Immunofluorescence

Cells were fixed and prepared for indirect immunofluorescence as previously described (10). SSEA-1 monoclonal antibody was the generous gift of Dr. Davor Solter (Wistar Institute, Philadelphia, PA) and was used at a 1:50 dilution. Monospecific rabbit antiserum to mouse laminin (Bethesda Research Laboratories) was used at a 1:50 dilution and rabbit antiserum to human plasma fibronectin (Bethesda Research Laboratories), or goat antiserum to rat plasma fibronectin (Calbiochem-Behring Corp.), were used at a 1:100 dilution. Fluorescein-conjugated goat anti-mouse IgG, IgA, IgM mix and rhodamine-conjugated goat anti-rabbit IgG (all IgG fractions from Cappel Laboratories, West Chester, PA) were used at a 1:100 dilution. The specificity of these antibodies was determined previously by SDS-PAGE of immunoprecipitated material as well as by indirect immunofluorescence with a variety of cell types (12).

Endoderm Outgrowth and Embryoid Body Attachment Assays

The percent endoderm outgrowth was determined as previously described (12). Briefly, the percent of the total attached embryoid bodies containing endoderm within the outgrowth was determined based upon morphology and SSEA-1 staining (endoderm cells are SSSE-1 negative, whereas stem cells are SSEA-1 positive). 100 outgrowths were counted for each determination. For attachment assays embryoid bodies at the designated stage were labeled for 4 h in 3.8 μCi/ml [^{3}H]leucine (5 Ci/mmol; New England Nuclear, Boston, MA) in leucine-free medium. They were washed three times in PBS and then distributed equally into tissue culture 96-well microtest plates (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA). Wells were left untreated or coated with collagen type I, fibronectin, laminin, or collagen type IV, as described above. Embryoid bodies were allowed to settle overnight at 37°C. The wells were then washed three times with PBS and the attached material was harvested in 1% SDS, transferred into scintillation vials, treated with hydrofluor (National Diagnostics, Sommerville, NJ), and counted in a scintillation counter (Packard Instrument Co., Downer's Grove, IL). Each data point was done in triplicate. Data are expressed as percent attachment based on the amount of material added to the well at the beginning of the assay. Tetrapeptides were from Penninsula Laboratories, Belmont, CA. Partially purified IgG preparations of normal goat antiserum and antiserum directed against the 140-kD fibronectin receptor were generous gifts of Dr. P. Brown in Dr. R. Juliano's laboratory. See reference 5a for a recent characterization of this antiserum.

Results

The Role of Fibronectin in Endoderm Outgrowth

We have previously described the outgrowth of parietal endoderm-like cells when day 3 + 4 teratocarcinoma embryoid bodies are plated on substrates precoated with 0.1 mg/ml type I collagen (12). The identification of the cell type contained within the outgrowth was based upon the morphology of these cells as well as their staining pattern by indirect immunofluorescence for a number of markers. SSEA-1 antigen is present on stem cells and not their differentiated derivatives (29), laminin is made in large amounts by parietal endoderm (6, 16, 20), and alpha-fetoprotein is specific for only visceral endoderm cells in these cultures (1). Cells within these outgrowths were SSEA-1 negative, displayed extensive laminin-positive areas, and contained no immunologically identifiable alpha-fetoprotein, consistent with their identification as parietal endoderm (12). When fibronectin was removed from the medium of these cultures by gelatin-affinity chromatography of the calf serum, little endoderm outgrowth was observed after 3 d (10%) as compared with the outgrowth in fibronectin-containing cultures where 70–80% of the outgrowths contained endoderm by this time (Fig. 1). Fig. 2 *a* shows that endoderm outgrowth obtained in the presence of fibronectin consists primarily of large flattened cells that are SSEA-1 negative (Fig. 2, *a* and *b*). Any outgrowth observed (only 10% of the attached embryoid bodies) in the absence of fibronectin consisted essentially of cells that were SSEA-1 positive (Fig. 2, *c* and *d*). Fig. 3, *a* and *b* demonstrate that the outgrowth obtained in the presence of fibronectin is characterized by extensive laminin-positive areas organized into a complex matrix. These data suggest that in the presence of fibronectin parietal endoderm outgrowth occurs, whereas in its absence stem cells constitute any outgrowth observed. These results suggest that the initial cultures supported endoderm outgrowth due to the well-characterized binding of fibronectin to collagen (18). To substantiate this conclusion, outgrowths cultured on collagen substrates in fibronectin-containing medium were stained with anti-fibronectin antiserum. Uniform areas of fluorescence were observed in cell free regions, indicating the presence of substrate-bound fibronectin (Fig. 3, *c* and *d*). Regions onto which endoderm cells had migrated appeared clear of fluorescence, suggesting that these cells had digested the fibronectin (Fig. 3, *c* and *d*). Note the fluorescence in the upper left corner of Fig. 3 *d* due to embryoid body-associated fibronectin. The substrate-associated fibronectin observed in these cultures derived from the medium and was not a cell product since regions of uniform fluorescence could be visualized when collagen-coated substrates were incubated in fibronectin-containing medium in the absence of embryoid bodies (data not shown). It is also of interest that no extensive cell-associated deposits of fibronectin could be visualized within the endoderm outgrowth, suggesting the absence of fibronectin synthesis by these cells. These results suggest that fibronectin promotes the outgrowth of endoderm from embryoid bodies. To more directly test the role of fibronectin in endoderm outgrowth, embryoid bodies were plated on substrates coated with 30 μg/ml of bovine plasma fibronectin and cultured in fibronectin-free medium for 3 d. Under these conditions 70% of the outgrowths contained endoderm (Table I). As in previous ex-

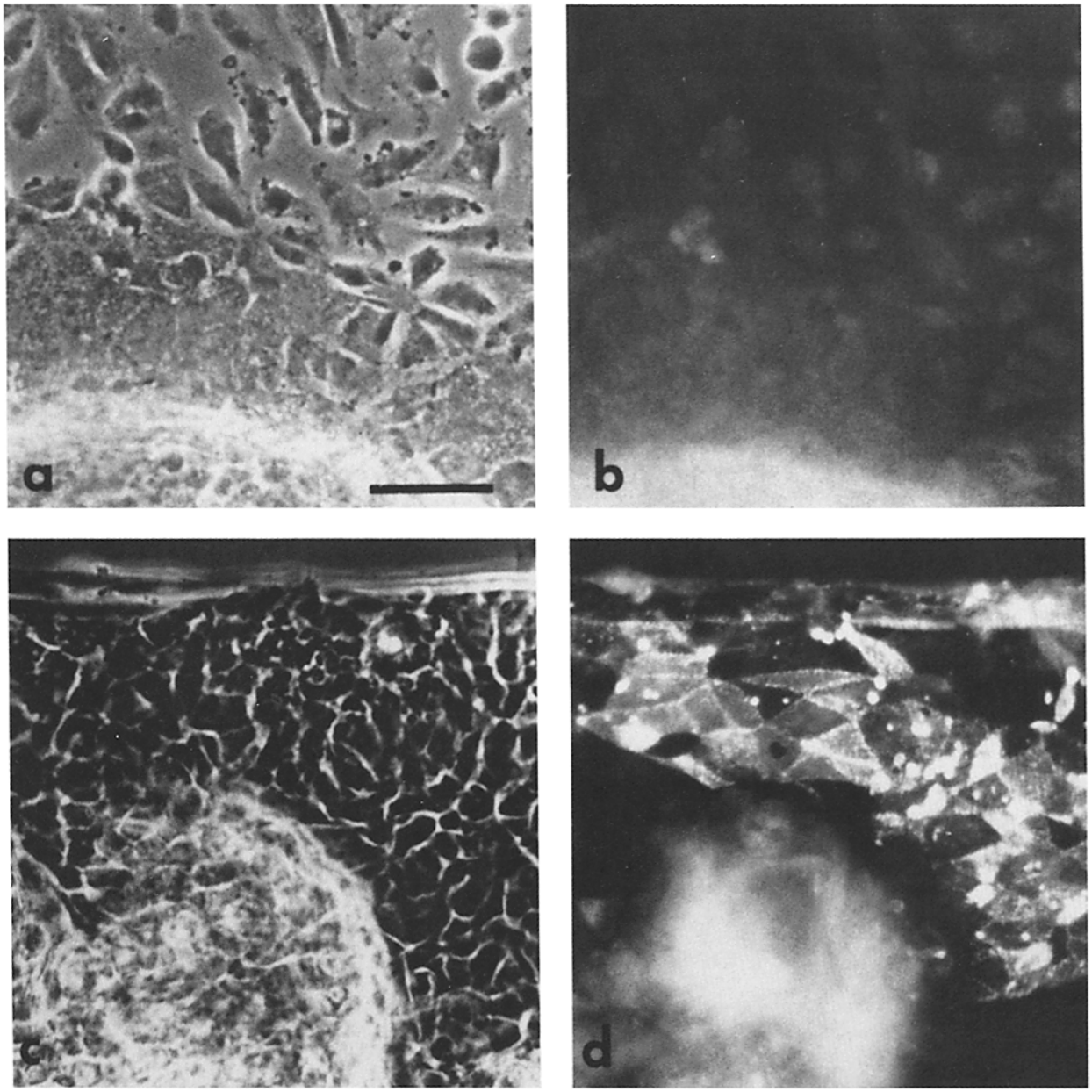

Figure 2. SSEA-1 staining of outgrowths in the presence and absence of fibronectin. Day 3 + 4 embryoid bodies were plated on collagen type I coverslips and stained by indirect immunofluorescence for SSEA-1 antigen after 3 d of culture in the presence (*a* and *b*) or absence (*c* and *d*) of fibronectin. *a* and *c* are phase-contrast images and *b* and *d* are fluorescence images. Bar, 50 μm.

periments, 78% of the outgrowths cultured on collagen in fibronectin-containing serum demonstrated endoderm outgrowth and only 8% displayed endoderm outgrowth when cultured in the absence of fibronectin (Table I). The combined presence of collagen and fibronectin did not increase endoderm outgrowth beyond the level observed as fibronectin alone (72%), indicating that fibronectin is responsible for the endoderm outgrowth observed under the initial conditions.

The Role of Laminin and Collagen Type IV in Endoderm Outgrowth

The ability of fibronectin to promote the outgrowth of endoderm did not preclude a similar activity for other extracellu-

Table I. Endoderm Outgrowth from Day 3 + 4 Embryoid Bodies 3 d after Attachment

Conditions		
Substrate	Medium (±fibronectin)	Percent endoderm-containing outgrowth
Collagen*	+	78
Collagen	−	8
Fibronectin‡	−	70
Fibronectin/collagen	−	72

* Substrate coated with 0.1 mg/ml collagen as described in Materials and Methods.
‡ Substrate coated with 30 μg/ml fibronectin as described in Materials and Methods.

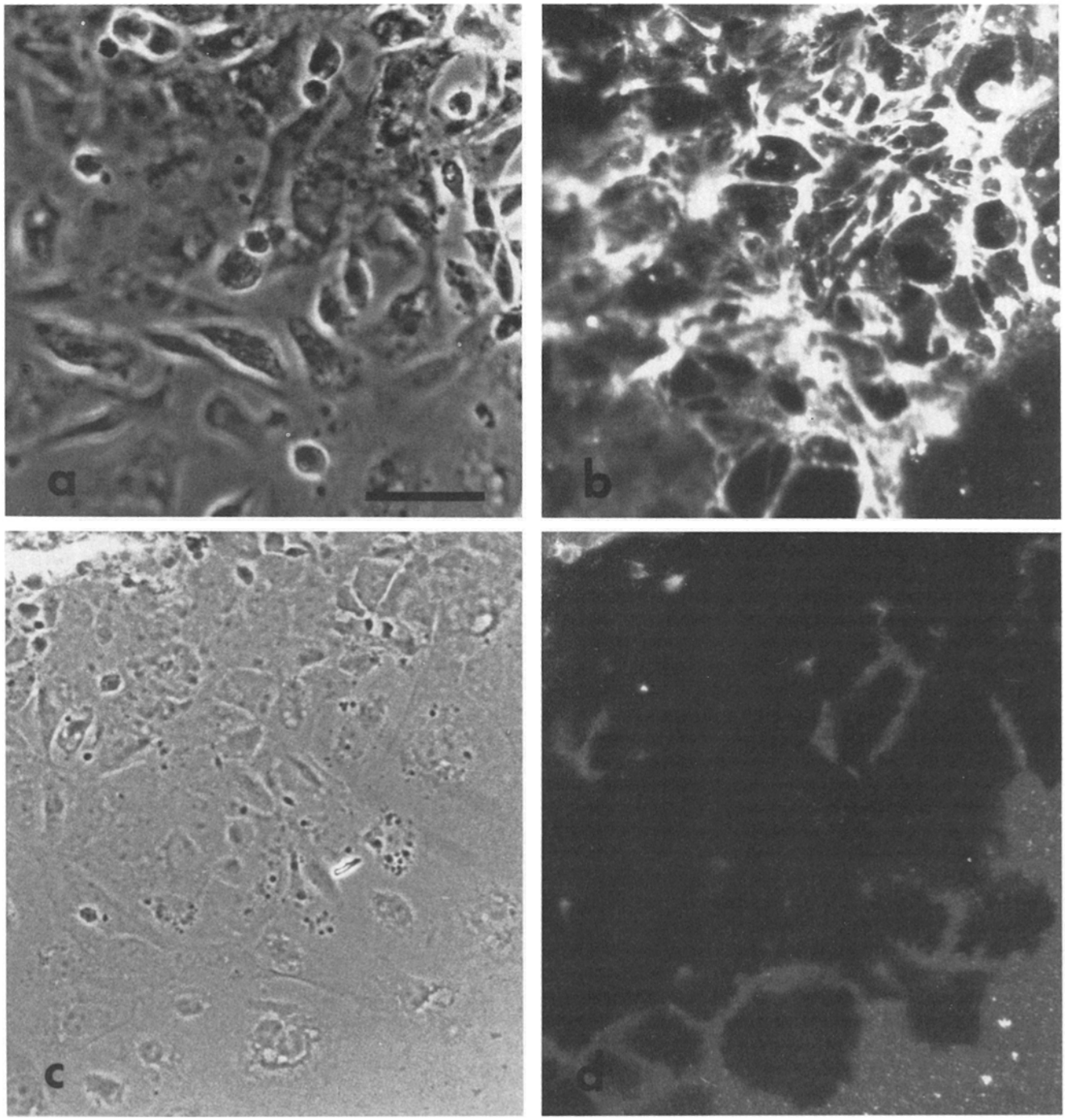

Figure 3. Laminin and fibronectin staining of outgrowths in the presence of fibronectin. Day 3 + 4 embryoid bodies were plated on collagen type I, cultured for 3 d in fibronectin-containing medium, and stained by indirect immunofluorescence for laminin (*a* and *b*) or fibronectin (*c* and *d*). *a* and *c* are phase-contrast images and *b* and *d* are fluorescence images. Bar, 50 μm.

lar matrix components. Both laminin and collagen type IV are synthesized in large amounts by parietal endoderm cells and are major components of Reicherts' membrane. We directly tested their ability to support endoderm outgrowth under fibronectin-free conditions. Fig. 4 shows that although both laminin and collagen type IV can promote endoderm outgrowth, they are 10-fold less effective, requiring 40 μg/ml to promote 25% endoderm outgrowth, whereas fibronectin demonstrates this level of activity at only 4 μg/ml. The addition of collagen type IV or laminin to fibronectin-coated substrates did not increase the effectiveness of fibronectin at promoting outgrowth (data not shown). This suggests no cooperative enhancement of outgrowth on mixed substrates.

Attachment of Embryoid Bodies to Matrix-coated Substrates

Initial observations suggested that in the absence of fibronectin there was substantially less substrate attachment of embryoid bodies. We therefore examined directly the attach-

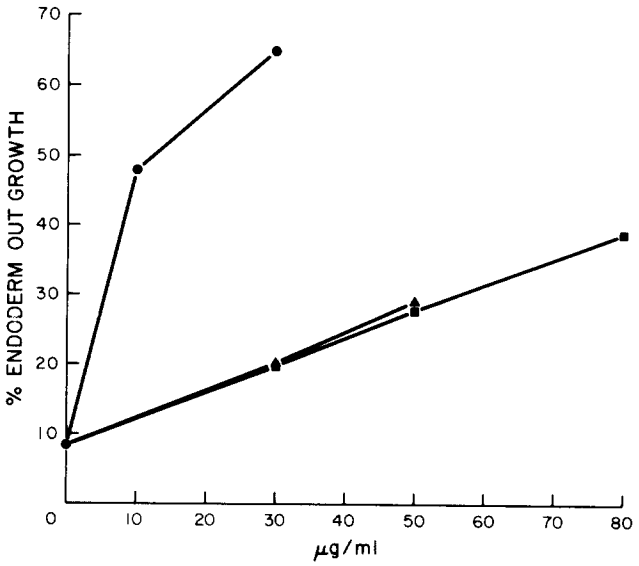

Figure 4. Extent of endoderm outgrowth on fibronectin, laminin, and surfaces coated with fibronectin (*solid circles*); laminin (*solid squares*); or collagen type IV (*solid triangles*); and the extent of endoderm outgrowth observed after 3 d of culture.

ment of day 3 + 0 and day 3 + 4 embryoid bodies to a variety of substrates. These stages were chosen in order to provide a comparison between attachment of stem cell aggregates and aggregates surrounded by endoderm; day 3 + 0 aggregates are essentially endoderm-free whereas >90% of the 3 + 4 aggregates contain endoderm. Embryoid bodies were labeled with [^{3}H]leucine for 4 h, and allowed to attach overnight onto untreated tissue culture plastic or collagen-coated

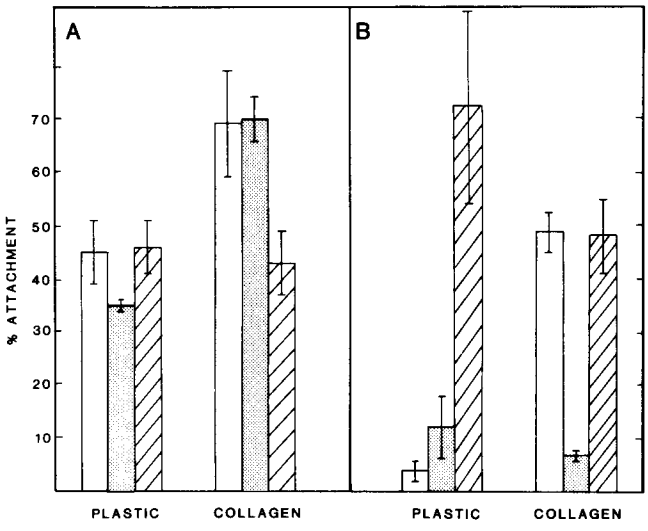

Figure 5. Attachment of embryoid bodies to collagen type I and fibronectin substrates. Day 3 + 0 (*A*) and day 3 + 4 (*B*) embryoid bodies were labeled with [^{3}H]leucine and allowed to attach to tissue culture plastic, or collagen-coated tissue culture plastic in the presence of fibronectin-containing serum (*open bars*), in the absence of fibronectin-containing serum (*stippled bars*), or in the absence of fibronectin-containing serum with fibronectin-coated substrate (*striped bars*). Percentage of attachment was determined 16 h after plating as described in Materials and Methods.

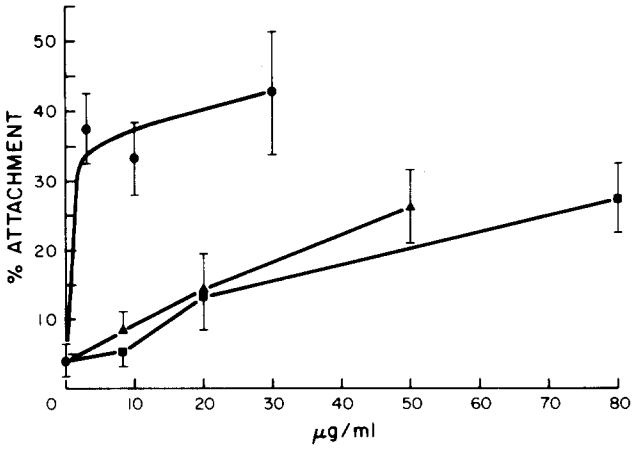

Figure 6. Attachment to fibronectin, laminin, or collagen type IV. Day 3 + 4 embryoid bodies were labeled with [^{3}H]leucine and allowed to attach to surfaces coated with fibronectin (*solid circles*), laminin (*solid squares*), or collagen type IV (*solid triangles*) for 16 h. The percentage of attachment was determined as described in Materials and Methods.

surfaces with no exogenous fibronectin source, with fibronectin present in the medium, or with fibronectin present as a substrate coating (30 μg/ml). Day 3 + 0 embryoid bodies attached reasonably well to plastic, regardless of the presence or absence of fibronectin, although collagen stimulated attachment under certain conditions (Fig. 5 *A*). Thus, the attachment of aggregates that contained little or no endoderm was fibronectin independent. In contrast, attachment of day 3 + 4 embryoid bodies was fibronectin dependent. On plastic, appreciable attachment occured only when the surface was fibronectin coated. On collagen, there was little attachment unless fibronectin was present either in the medium or on the substrate (Fig. 5 *B*). These results suggest a developmentally regulated change in the attachment requirements of teratocarcinoma cells. The differentiated endoderm cells acquire a fibronectin dependence not observed with the undifferentiated stem cells.

We have also investigated the attachment of day 3 + 4 embryoid bodies to laminin and type IV collagen. These results are shown in Fig. 6. As observed for endoderm outgrowth, fibronectin is substantially more active (>10-fold) in promoting attachment than either of the other matrix proteins.

Because our observations indicated that fibronectin promotes attachment as well as outgrowth, it was necessary to separate these two effects. It is conceivable, for example, that fibronectin selects for the attachment of endoderm-containing embryoid bodies and that subsequent outgrowth is perhaps matrix independent. To discriminate between these alternatives we determined whether or not embryoid bodies attached in the absence of fibronectin contained an endoderm outer layer. 50% of the aggregates attached 16 h after plating contained endoderm, although only 10% of them supported subsequent endoderm outgrowth. This indicates that although a significant number of the attached aggregates contained endoderm, most did not generate endoderm outgrowth in the absence of fibronectin. This observation, as well as some additional experiments described below, suggest that fibronectin is involved in specifically promoting outgrowth as well as attachment.

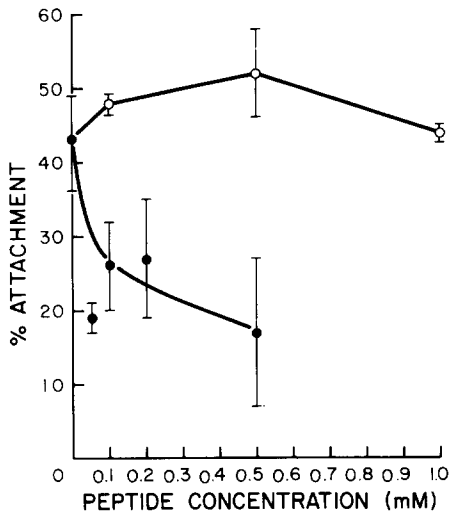

Figure 7. Inhibition of attachment by RGDS tetrapeptide. Day 3 + 4 embryoid bodies were labeled with [^{3}H]leucine and allowed to attach to surfaces coated with 10 μg/ml fibronectin for 16 h in the presence of RGDS (arg-gly-asp-ser) (*solid circles*); or VGSE (val-gly-ser-glu) (*open circles*). The percentage of attachment was determined as described in Materials and Methods.

Effect of the Fibronectin Cell-attachment Tetrapeptide and Antibody to the Fibronectin Receptor on Embryoid Body Attachment and Endoderm Outgrowth

A tetrapeptide sequence, RGDS (arg-gly-asp-ser), that can inhibit the attachment of cells to fibronectin as well as other matrix proteins, has recently been identified (3, 15, 24). This sequence is present in a number of molecules that mediate adherence and is contained within the cell attachment domain of fibronectin. We compared the effect of RGDS and a control tetrapeptide, VGSE (val-gly-ser-glu), on the attachment of day 3 + 4 embryoid bodies. RGDS inhibits attachment by 50% at 0.5 mM, whereas the control tetrapeptide shows no effect at 1.0 mM (Fig. 7). This level of inhibition is comparable to that observed for normal rat kidney cell attachment to fibronectin (50% at 1 mM; reference 24). When a similar experiment was performed using laminin-coated substrates (20 μg/ml) there was a slight but reproducible inhibition of attachment by RGDS relative to the control tetrapeptide, suggesting that this specific sequence can interfere with laminin-based interactions as well (data not shown).

To determine if the tetrapeptide could block outgrowth as well as attachment, day 3 + 4 embryoid bodies were allowed to attach to fibronectin- (10 μg/ml) coated substrates overnight, and RGDS or VGSE was then added and the percent endoderm outgrowth determined after 24 h. Although inhibition of endoderm outgrowth was consistently observed with RGDS and not with VGSE, the extent was minimal and variable (10–30% at 0.5 mM). Since the amount of tetrapeptide available was limited, we used antibody directed against the 140-kD fibronectin receptor to substantiate the involvement of fibronectin–cell interactions in endoderm outgrowth.

Recent evidence indicates that the cell attachment site of fibronectin binds to a 140-kD cell surface fibronectin receptor that has been identified and characterized by a number of different laboratories (4, 5, 25). An antiserum (partially purified IgG preparation characterized in reference 5a) to the 140-kD receptor was added to cultures of day 3 + 4 embryoid bodies which had attached overnight to substrates coated with 10 μg/ml fibronectin. The cultures were observed 24 h later. Fig. 8 *a* and *b* show that this antiserum (500 μg/ml) could block endoderm outgrowth, whereas equivalent concentrations of normal goat serum (partially purified IgG preparation) had no effect. In addition, an antiserum against cell CAM 120/80 (7) had no effect on outgrowth (data not shown). This effect was not due to selective toxicity, since the treated embryoid bodies remained viable, based on trypan blue exclusion, 24 h after antibody addition. Numerous rounded cells appear in Fig. 8, *a* and *c* due to the fact that any cells that become dissociated from the aggregate are prevented from substrate attachment by the presence of the antiserum. When similar experiments were performed using laminin-coated substrates (20 and 80 μg/ml), the results shown in Fig. 8, *c* and *d* were obtained. Outgrowth was blocked by the anti-140 kD antibody and not by normal goat serum. These data suggest that the outgrowth of endoderm on either fibronectin- or laminin-coated substrates involves the 140-kD fibronectin receptor. They also support the contention that fibronectin supports outgrowth as well as attachment.

Discussion

A clear picture of the molecular interactions involved in morphogenesis is now emerging from the combination of recent information obtained from in vitro studies identifying the molecules responsible for cell adhesion and directed cell migration and studies that focus on the in vivo localization of these molecules during embryogenesis (8, 13, 30). The identification of molecules that promote cell migration during mammalian embryogenesis is difficult since these events take place subsequent to implantation (26). Teratocarcinoma stem cells have proven invaluable in identifying the molecules that mediate intercellular adhesion (7, 11, 17, 33). We demonstrate here that teratocarcinoma embryoid bodies can be used to study the substrate specificity of endoderm outgrowth. We have previously shown that when embryoid bodies are attached to collagen-coated substrates, parietal endoderm cells migrate out onto the substrate even under conditions in which no parietal endoderm was initially present. We show here that the outgrowth in these cultures was due to the binding of serum fibronectin to the collagen substrate. Endoderm outgrowth is observed on fibronectin-coated surfaces in fibronectin-free medium. Indirect immunofluorescence staining with anti-fibronectin antiserum suggested that the parietal endoderm cells themselves may not be capable of synthesizing and/or accumulating fibronectin when associated with a fibronectin substrate. Although this observation is difficult to follow up in these cultures because of the presence of the embryoid bodies that are actively synthesizing and secreting fibronectin, it has been proposed that PYS cells, a parietal endoderm cell line, do not actively synthesize fibronectin (32). This point is of interest in view of the well-documented observation that neural crest cells adhere and migrate on fibronectin surfaces in vitro and do not synthesize the protein (23).

In contrast, parietal endoderm cells synthesize large amounts of laminin (6) and type IV collagen (2), although

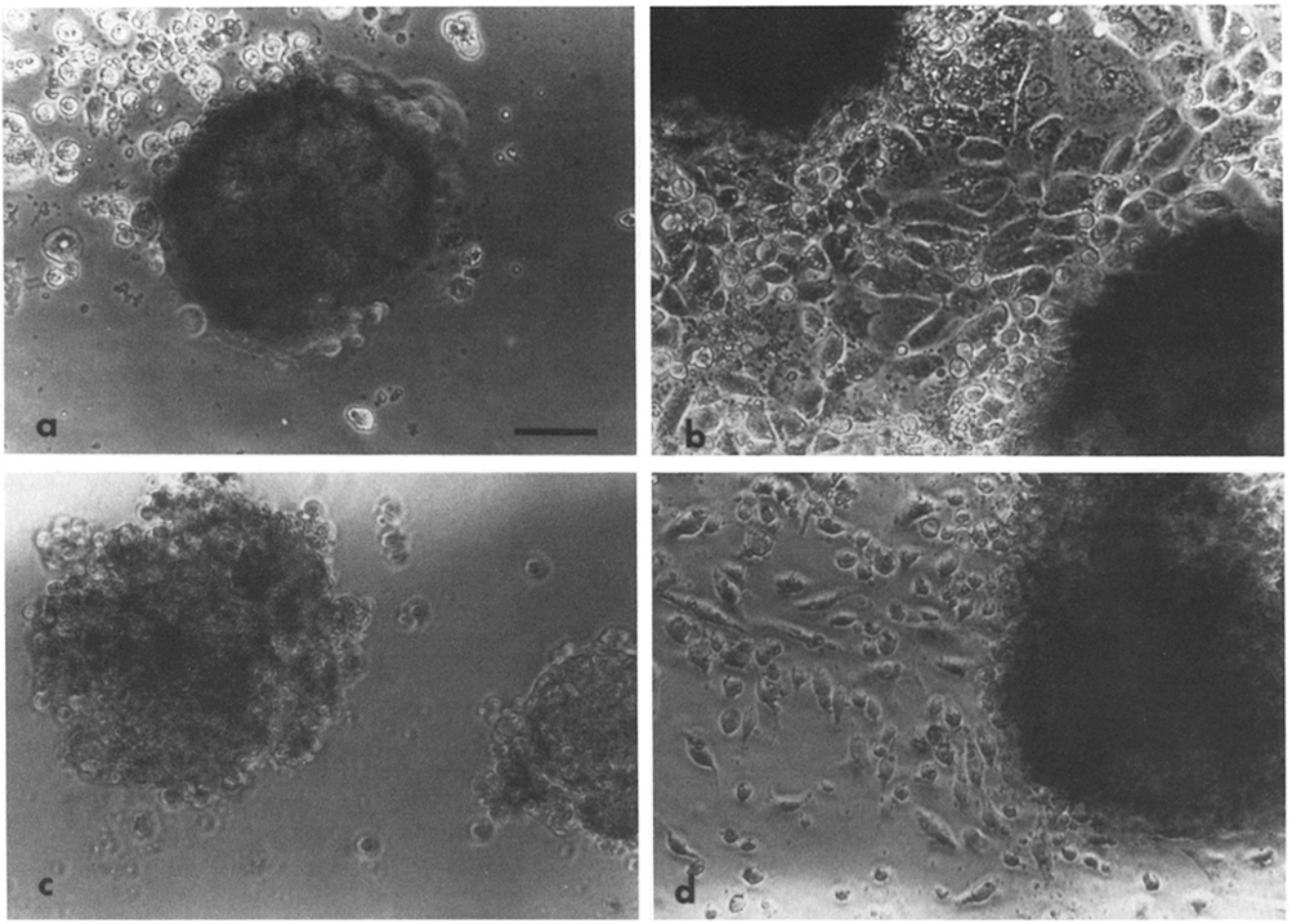

Figure 8. The effect of antibody to the 140-kD fibronectin receptor and normal goat serum on endoderm outgrowth. Day 3 + 4 embryoid bodies were allowed to attach to surfaces coated with 10 μg/ml fibronectin (*a* and *b*), or 20 μg/ml laminin (*c* and *d*) for 16 h. 500 μg/ml of partially purified IgG directed against the 140-kD fibronectin receptor (*a* and *c*) or 500 μg/ml of partially purified normal goat IgG (*b* and *d*) were added and the cultures observed after 24 h. Bar, 50 μm.

both of these matrix glycoproteins are each 10-fold less effective than fibronectin at promoting endoderm outgrowth. The attachment of endoderm-containing embryoid bodies is fibronectin dependent, and, again, both laminin and collagen type IV are 10-fold less effective. The observation that fibronectin-free cultures contain a substantial number of attached embryoid bodies that contain an outer layer of endoderm and yet are incapable of generating endoderm-specific outgrowth is consistent with the conclusion that fibronectin is important for both outgrowth and attachment. The similar dose–response curve obtained with the three matrix glycoproteins for both attachment and outgrowth suggests that the same cell surface receptor(s) may mediate both of these processes. In this regard it is of interest that the RGDS sequence can inhibit attachment to both fibronectin and laminin substrates and that antibody against the 140-kD fibronectin receptor is effective at blocking endoderm outgrowth on either fibronectin or laminin substrates, suggesting that the 140-kD receptor may be involved in interactions with a variety of matrix molecules. These observations are particularly interesting in light of those made by Horwitz et al. suggesting that laminin could interact with this fibronectin receptor in an RGDS-inhibitable fashion (15). It is also clear from recent studies that the RGD sequence is present in many proteins that are associated with cell attachment events, and that numerous receptors capable of recognizing this sequence may be present at the cell surface (28).

A role for cell–cell and cell–matrix interactions in directing the differentiation of parietal endoderm from primitive endoderm cells has been proposed (9). Our previous findings suggested that interaction with a migration-promoting substrate may initiate the differentiation as well as the migration of parietal endoderm (12). The data presented here indicate that fibronectin can provide such a substrate in vitro and that interaction with the 140-kD fibronectin receptor is also involved. In support of the relevance of these findings to the in vivo process, fibronectin has been localized to the inner surface of the trophectoderm at 4 d in the mouse embryo, just before the migration of parietal endoderm cells (31). The use of teratocarcinoma embryoid bodies promises to facilitate a molecular analysis of the events controlling migration and differentiation during early mammalian embryogenesis.

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