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Cooperative interactions between extracellular matrix, integrins and parathyroid hormone-related peptide regulate parietal endoderm differentiation in mouse embryos

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SUMMARY

The outgrowth of parietal endoderm (PE) cells from precursor endodermal cells is one of the first differentiation events that occur in mouse embryos. We have analyzed the molecular determinants of this process by placing isolated inner cell masses (ICMs) on defined extracellular matrix substrata in microdrop cultures. Differentiation and outgrowth of PE required a fibronectin substratum. Laminin supported the adhesion and outgrowth of visceral endoderm (VE) and actively suppressed the differentiation of PE in mixtures of fibronectin and laminin. Collagen type IV, gelatin, vitronectin or entactin supported little or no endodermal outgrowth. Trophectoderm (TE) cells have been implied to be important in PE induction in vivo. We found that recombination of ICMs in culture with TE cells, or with medium conditioned by TE cells, greatly increased the differentiation of PE. TE cells stimulated PE outgrowth on substrata other than fibronectin. One cytokine secreted by trophoblast and endodermal cells, parathyroid hormone-related peptide (PTHrP), was critical for outgrowth on any substratum. A function-perturbing antibody to PTHrP reduced the number of PE cells, whereas the addition of PTHrP increased that number. Furthermore, addition of PTHrP changed the substratum requirements for outgrowth, making laminin, vitronectin and low concentrations of fibronectin permissive for PE outgrowth. Immunostaining with anti-integrin antibodies showed that fully differentiated PE cells outgrowing on fibronectin expressed α_5, α_6 and $\alpha_V\beta_3$ integrins. However, analysis of outgrowths in the presence of function-perturbing antibodies to α_5, α_6 and $\alpha_V\beta_3$ integrins showed that these integrins directed PE outgrowth only on fibronectin, laminin and vitronectin substrata, respectively. We have shown that there is a cooperative interplay of extracellular matrix, integrins and PTHrP that modulates PE outgrowth.

Key words: blastocyst, extracellular matrix, fibronectin, laminin, inner cell mass, parietal endoderm, trophectoderm, trophoblast, vimentin

INTRODUCTION

The formation of parietal endoderm (PE) is an early differentiation step in the development of the mammalian blastocyst. PE appears in the mouse at 4.5 days post coitum as a population of cells that dissociate from the primitive endoderm and from one another, and then migrate onto the inner surface of the blastocoel (Enders et al., 1978). PE is formed in all mammals, including the baboon (Enders et al., 1990) and the pig (Richoux et al., 1989). Before PE development, the trophectoderm (TE) secretes a basement membrane that fosters adhesion and migration of the developing PE (Schlafke and Enders, 1963; Nadijcka and Hillman, 1974). The collaboration of TE in the induction of PE has been observed in cultures of microdissected fragments of 6.5-day embryos (Hogan and Tilly, 1981), where trophoblast giant cell precursors induced the transdifferentiation of visceral extraembryonic endoderm into PE. These functional data, together with the observation that PE evolves at the TE-visceral endoderm (VE) contact zone (Hogan and Newman, 1984), suggest that cell-cell and/or cell-matrix interactions are involved in the induction. Several molecules have been localized to the TE-generated basement membrane, including fibronectin (FN), laminin (LN), type IV collagen (COL IV) and heparan sulfate proteoglycans (Hierck et al., 1993; Salamat et al., 1993; Thorsteinsdottir, 1992; Carnegie, 1991; Leivo et al., 1980; Wartiovaara et al., 1979).

PE differentiation in vivo is defined by the expression of a set of molecular markers, including the extracellular matrix (ECM) components LN (Hogan et al., 1980) and secreted protein acidic and rich in cysteine (SPARC) (Mason et al., 1986) and the intermediate filament vimentin (Lane et al., 1983). Expression of LN, SPARC and vimentin distinguishes

PE cells from others, such as primitive endoderm, visceral embryonic or visceral extraembryonic endoderm cells, or TE. During and after their migration, PE cells synthesize large amounts of COL IV, LN and entactin, and incorporate them into Reichert's membrane (Mazariegos et al., 1987; Wu et al., 1983; Hogan et al., 1980, 1982; Semoff et al., 1982), which acts as a filtrative layer between the conceptus and the mother.

The dependence of PE differentiation on soluble factors has also been studied. Retinoic acid in combination with increased intracellular cAMP has been shown to induce PE differentiation in cultures of embryonal carcinoma (EC) cells (Strickland et al., 1980). Parathyroid hormone-related peptide (PTHrP) and its adenylate cyclase-coupled receptor have been found in early embryos, and PTHrP can replace dibutyryl cAMP in the induction of PE differentiation in EC cells (van de Stolpe et al., 1993; Chan et al., 1990).

The interplay between adhesive and soluble signals from the extracellular milieu is critical in regulating cell behavior (reviewed by Damsky and Werb, 1992; Adams and Watt, 1993). The confluence of ECM molecules and peptide hormones at the junction of TE and primitive endoderm suggests that these stimuli collaborate to produce PE differentiation. By culturing immunosurgically isolated inner cell masses (ICMs) on defined substrata in serum-free medium, we investigated the molecular mechanisms regulating PE differentiation in mouse embryos and the integration of signals from ECM and growth factors in the differentiation pathway.

MATERIALS AND METHODS

Materials

Gonadotropin from pregnant mares' serum and mouse LN (prepared from mouse Engelbreh-Holm-Swarm tumors and containing entactin; referred to as LN/EN) were from Sigma Chemical Co. (St Louis, MO). Human chorionic gonadotropin was from Serono (Randolph, MA). Female mice of the CF1 or CD1 strain, 7-8 weeks old, and male mice of the CD1 strain were from either Harlan Sprague Dawley (Indianapolis, IN) or Charles River Laboratories (Wilmington, MA) and housed for one week before use. Human COL IV was from Calbiochem (La Jolla, CA). Human FN and human vitronectin (VN) were from Boehringer Mannheim (Indianapolis, IN). Mouse entactin-free LN and COL IV were from Collaborative Biomedical Products (Bedford, MA). Recombinant entactin was the generous gift of Dr Albert Chung, University of Pittsburgh, Pittsburgh, PA. PTHrP 1-34 (human) was from Peninsula Laboratories (Belmont, CA). Dulbecco's modified Eagle's medium (DME) was prepared at the Cell Culture Facility, University of California, San Francisco. Fetal bovine serum (FBS) was from Hyclone (Logan, UT).

Rabbit anti-mouse LN (affinity-purified IgG) was from Collaborative Research (Lexington, MA). Rabbit anti-mouse α-fetoprotein (AFP) (purified IgG) was from ICN Immunobiologicals (Lisle, IL). Goat anti-SPARC (unpurified antiserum) was a gift of Dr Helene Sage, University of Washington, Seattle, WA (Sage et al., 1989). Rabbit anti-human PTHrP (affinity-purified IgG) was from Oncogene Science (Uniondale, NY). Rat anti-mouse VLA-5 (α_5 integrin) (purified IgG) was from Pharmingen (San Diego, CA). Rabbit anti-mouse VLA-6 (α_6 integrin) (ion exchange-purified IgG from GoH3 culture supernatant) was from Serotec (Oxford, England); anti-α_6 GoH3 unpurified culture supernatant was a gift of Dr Arnoud Sonnenberg, The Netherlands Cancer Institute, Amsterdam. Rabbit anti-chicken $\alpha_{6A}\beta_1$ and rabbit anti-chicken $\alpha_{6B}\beta_1$ integrin cytoplasmic domains (purified IgG) were a gift of Dr Louis Reichardt, University of California, San Francisco. Rabbit anti-mouse $\alpha_V\beta_3$ antiserum was a gift of Dr James Gailit, State University of New York at Stony Brook and Dr Erkki Ruoslahti, La Jolla Cancer Research Foundation, La Jolla, CA; IgG was purified from this serum and from normal rabbit and normal rat serum by chromatography on protein A Sepharose from Pharmacia (Alameda, CA). Polyclonal rabbit anti-mouse FN (absorbed with mouse plasma proteins minus FN) was from Gibco/BRL, Gaithersburg, MD. Polyclonal rabbit anti-mouse placental lactogen (purified IgG) was a gift of Dr Frank Talamantes, University of California, Santa Cruz. Anti-mouse simple epithelial cytokeratins (Lane et al., 1983) were a gift of Dr E. B. Lane, Imperial Cancer Research Fund, London, England. Monoclonal anti-stage-specific embryonic antigen (SSEA)-1 (purified IgM) (Solter and Knowles, 1978) was a gift of Dr Davor Solter, Wistar Institute, Philadelphia, PA. Rat anti-mouse fetomodulin (Imada et al., 1987) was a gift of Dr Eileen Adamson, La Jolla Cancer Institute, La Jolla, CA. Goat anti-human tissue plasminogen activator (purified IgG) was from American Diagnostica, New York, NY. Polyclonal rabbit anti-mouse urokinase-type plasminogen activator (IgG enriched by ammonium sulfate precipitation) was a gift of Dr Liliana Ossowski, Rockefeller University, New York, NY (Ossowski, 1988). Rabbit antiserum to mouse erythrocytes and fluorescein-conjugated goat anti-rat IgG (absorbed with mouse serum) were from Organon Teknika (Durham, NC). Rat serum was from Harlan Bioproducts (Indianapolis, IN). Monoclonal mouse anti-pig vimentin antibody (purified IgM) and monoclonal rat anti-mouse E-cadherin (purified IgG) were from Sigma. Fluorescein-conjugated rabbit anti-mouse immunoglobulins and swine anti-rabbit IgGs were from Dakopatts (Santa Barbara, CA). *Hxt* was localized by in situ hybridization as described (Cross et al., 1995).

Isolation of embryos

Female mice were induced to superovulate by injections in the afternoon with 10 units of gonadotropin from pregnant mares' serum and 46 hours later with 5 units of human chorionic gonadotropin. Blastocysts were harvested from the uterus at 3.5 days post coitum, washed through three drops of flushing medium II (Spindle, 1980), transferred to an organ culture dish and incubated for 24 hours at 37°C in an atmosphere of 5% CO_2. Blastocysts were cultured 24 hours before use in 0.5 ml T + 2XAA medium (Spindle, 1990), modified to contain insulin, Mito+ (Collaborative Research), sodium pyruvate and calcium lactate (Sutherland et al., 1993), and incubated at 37°C in an atmosphere of 5% CO_2.

Isolation of ICMs

ICMs were prepared by immunosurgery (Solter and Knowles, 1975). Expanded blastocysts were transferred to a 45 μl drop of rabbit anti-mouse erythrocyte antiserum diluted 1:4 with DME and incubated 10 minutes at 37°C. The blastocysts were then washed through four 50 μl drops of DME with 10% FBS, placed in a source of complement (a 45 μl drop of rat serum diluted 1:4 with DME), and incubated for 30 minutes at 37°C. The blastocysts were washed through seven drops of DME with 10% FBS. The dead TE and/or zonae pellucidae were removed from the ICMs by passage through smaller pipets. Finally, the ICMs were washed through three more drops of T + 2XAA medium before being plated. Mouse ICMs at this stage contain an average of 20 cells (range 11-33) (Hogan and Tilly, 1978). Our reported variation in total number of cells in the outgrowths may be a result of initial variations in ICM cell number.

Preparation of ECM substrata

FN, LN, COL IV, gelatin, entactin or VN in phosphate-buffered saline (PBS) was applied as 4 μl or 5 μl drops on Corning 60 mm tissue culture dishes. The dishes were placed in a humidified chamber at 37°C for 2 hours. Each spot was then washed twice with 5 μl of PBS, replaced with 5 μl of T + 2XAA medium and covered with mineral oil. The dishes were equilibrated for 1 hour before the introduction of 1 ICM per drop.

Experimental treatment of trophoblast/ICM co-cultures

For the production of pure trophoblast cells, blastocysts were X-irradiated with 10 Gy (Goldstein et al., 1975) to kill the ICMs. A day later, blastocysts with healthy trophoblast were placed in drops and allowed to attach and spread for 30 hours before addition of ICMs.

Morphologic analysis of ICM outgrowths

ICMs were photographed daily. Attached ICMs were characterized according to two criteria: (1) the total number of outgrowing cells was counted, regardless of cell type; (2) the number of PE cells was counted. For the purposes of morphologic analysis, PE cells are those cells that separate themselves from the patch of cells surrounding the ICM; cells that shared no more than 20% of their border with any other cells were scored as PE cells. Numbers of cells on days 1 and 2 were counted directly at the microscope; for larger outgrowths at day 3 and beyond, counts were determined by marking cells on a photograph of the outgrowth. PE cells were additionally scored as individual cells expressing the markers SPARC or LN.

Immunocytochemical analysis of ICM and blastocyst outgrowths

Outgrowths to be assayed by immunofluorescent localization of vimentin, LN, SPARC, AFP, α_5 integrin, α_6 integrin and $\alpha_V\beta_3$ integrin were fixed in 1:1 (v:v) methanol-acetone at ambient temperature for 10 minutes. Anti-vimentin antibody was diluted 1:40 in DME with 10% FBS for 1 hour, washed briefly in water, exposed for 1 hour to a 1:50 dilution of fluorescein-conjugated anti-mouse IgG, or mouse immunoglobulins in the case of vimentin, and washed again. The other primary antibodies were diluted 1:50 or 1:100 in PBS with 1% bovine serum albumin, which was used for the washing steps, followed by a 1 hour incubation in a 1:100 dilution of the appropriate fluorescein-conjugated anti-IgG. Anti-SPARC IgG was also localized with the use of biotin-conjugated anti-goat IgG purchased from Hyclone (Logan, UT) followed by Texas Red-streptavidin purchased from Amersham (Arlington Heights, IL). After at least four 10 minute washes in PBS/bovine serum albumin and a 1 hour wash in PBS with 0.1% Tween 20, the outgrowths were photographed on a Zeiss Photomicroscope III, Zeiss Axioskop or Biorad MRC 1000 confocal microscope. All immunolocalization experiments were performed at least twice.

RESULTS

Different ECM ligands have distinct effects on PE differentiation

Our first interest was to determine the adhesive requirements for PE differentiation. FN supported rapid adhesion and outgrowth of cells (Fig. 1A). ICMs adhered within 1 day. Then, at day 1 or 2, cells migrated away from the ICM as individual cells. The first cells to move onto the substratum were often closely associated with one another (Fig. 1A). This behavior was also noted inside whole, expanded 4.5-day blastocysts (data not shown). Outgrowths spread with an average of 25±8% (mean ± s. d., n=9 experiments) of cells at the periphery separating to form cells that morphologically resembled PE. In other cultures, PE-like cells grew out directly from the adherent ICM.

We next established a panel of molecular markers to allow us to distinguish the cell types forming in early embryo outgrowths (Table 1). In addition to morphologic examination of whole blastocyst outgrowths, we used LN, SPARC and vimentin to identify PE cells, and AFP to identify VE cells in the ICM experiments described below (Fig. 2). A population of small cells in the cohesive sheets of the outgrowth stained positively for AFP (Fig. 2H); staining was homogeneous near the center of the outgrowth and more heterogeneous near the edges. The cells that had delaminated from the periphery of this sheet, or that migrated individually from the ICM, however, never stained for AFP. Instead, they stained with the PE markers (Fig. 2B,D,F) and expressed the flat, broad morphology typical of PE cells when they first grow onto TE in vivo (Enders et al., 1978). LN was expressed at low levels by VE and ICM; SPARC was expressed weakly by peripheral VE but not by ICM; vimentin was expressed by neither cell type (Table 1).

When ICMs were plated on LN/EN, they adhered, but the time course of cell outgrowth was delayed compared with that on FN (Figs 1B, 3). At 1 day there was little outgrowth. By day 3, the number of cells surrounding the ICM grew to 60% of the outgrowth observed on FN (Fig. 1B). However, LN/EN did not support PE cell migration or differentiation (Figs 1B, 3A), even though PE cells grown out on FN secreted abundant LN as a trail on the substratum (Fig. 2B).

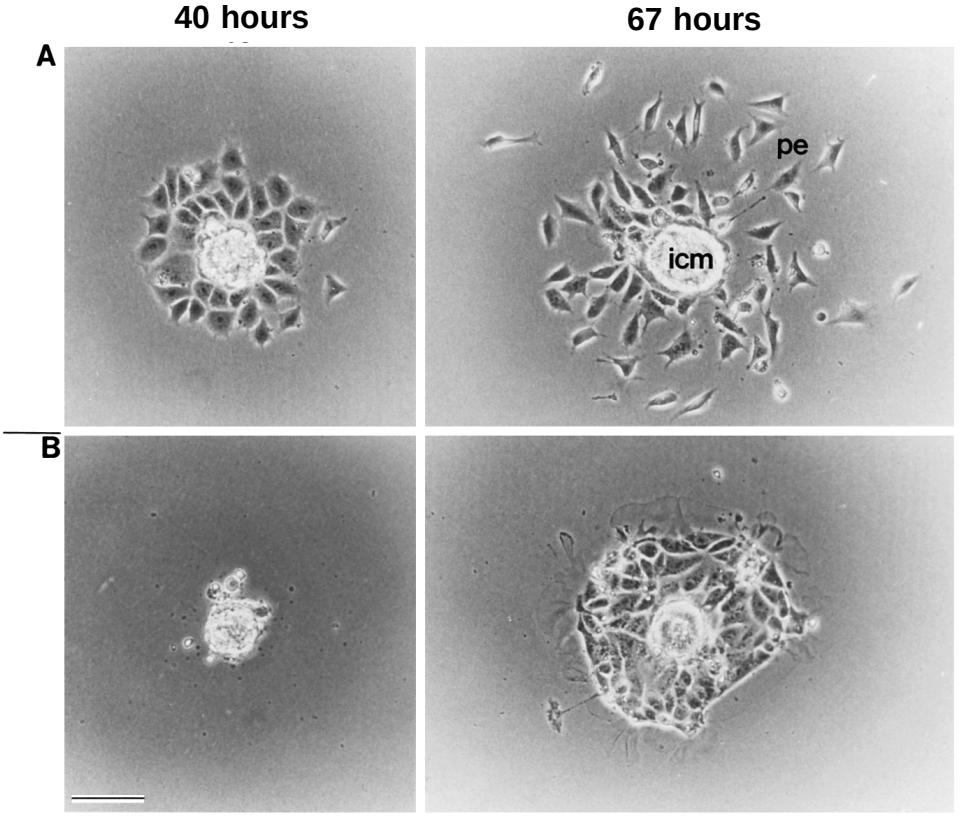

Fig. 1. ICM outgrowths cultured on (A) 20 μg/ml FN and (B) 20 μg/ml LN/EN at 40 and 67 hours after plating. pe, parietal endoderm; icm, inner cell mass. Bar, 20 μm.

LN has been shown to be both an adhesive and an anti-adhesive protein (Calof and Lander, 1991). To determine whether the lack of effect of LN/EN on PE outgrowth could be attributed to diminished adhesion of the ICMs, we plated ICMs on FN mixed with various amounts of LN/EN. As the concentration of LN/EN increased, the number of PE cells, as well as the total number of cells, decreased (Fig. 4A,B). These data suggest that LN/EN was not only nonpermissive, but was inhibitory to PE outgrowth. To control for a possible alteration in the concentration of bound FN produced by prior mixing with LN, we added LN after precoating the dishes with FN. At all concentrations tested, LN was highly inhibitory to PE outgrowth (Fig. 4C). However, once differentiated, PE cells adhered to LN or LN/EN (data not shown). Taken together,

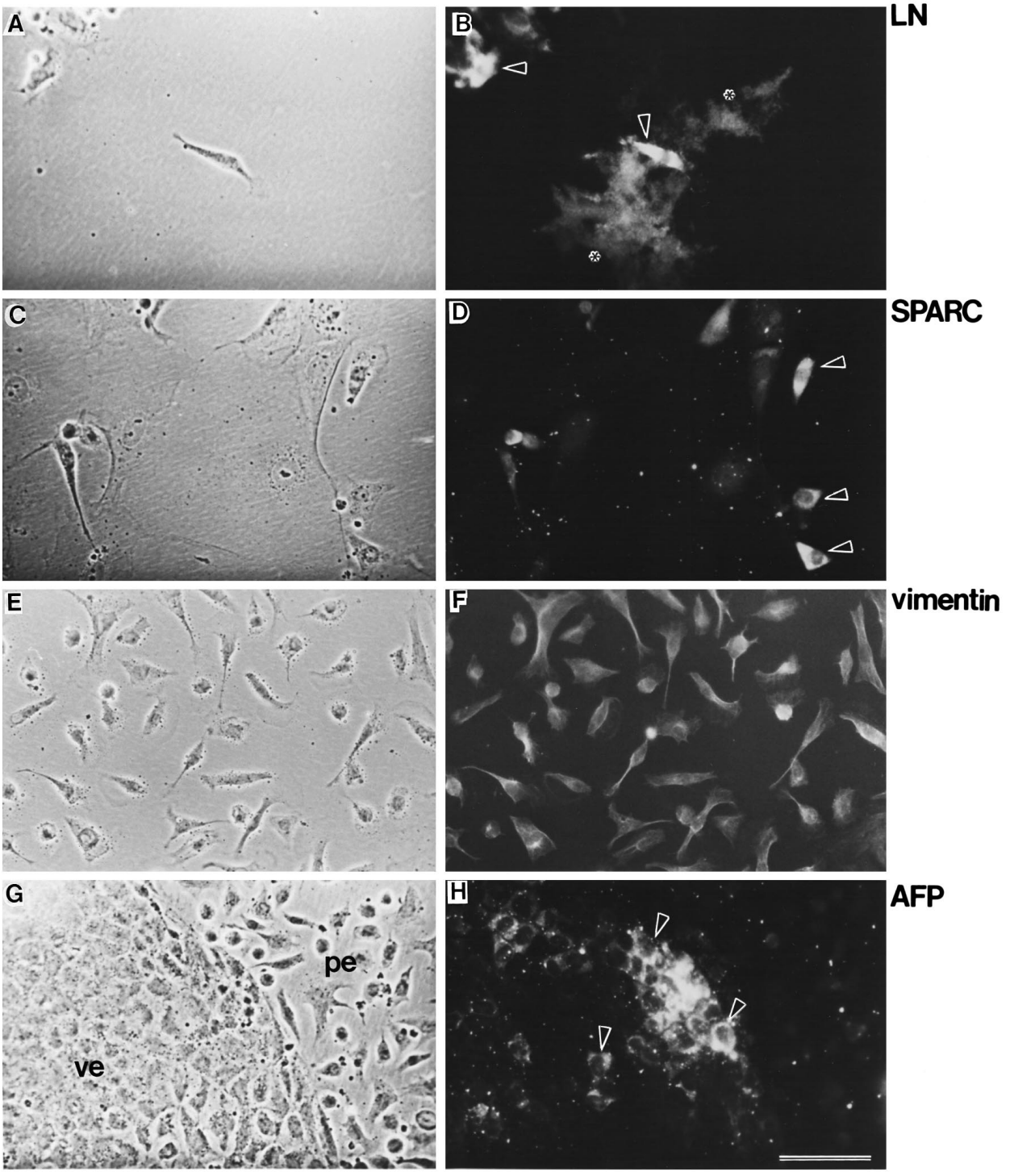

Fig. 2. Immunofluorescent localization of endodermal markers in ICM outgrowths cultured on FN (50 μg/ml) for 3 days. (A,B) LN; (C,D) SPARC; (E,F) vimentin; (G,H) AFP. (A,C,E,G) Phase micrographs; (B,D,F,H) fluorescence micrographs. pe, parietal endoderm; ve, visceral endoderm; asterisks, extracellular LN trail; arrowheads, positive cells. Bar, 20 μm.

Table 1. Summary of marker expression in blastocyst outgrowths

Marker	ICM/primitive Cuboidal cells; little obvious polarization or morphologic differentiation	PE Migratory, loosely associated cells; expanded endoplasmic reticulum	TE Very large, flat cells with giant nuclei	VE Cohesive sheets of polygonal cells	References
Secreted proteins					
Laminin (B2)	+/–	++	–	+/–	Hogan et al., 1980
Type IV collagen	–	+	–	–	Grover et al., 1987
SPARC	–	++	+/–	+/–	Mason et al., 1986
AFP	–	–	–	+	Dziadek and Adamson, 1978
Placental lactogen	–	–	+	–	Colosi et al., 1987
Fibronectin	+	+/–	+	+	Wartiovaara et al., 1979
Intermediate filaments					
Simple epithelial cytokeratins					
K8 + 18 (endo A + B)	–	+	+	+	Jackson et al., 1981
K19 (endo C)	–	+	+	–	Boller and Kemler, 1983
Vimentin	–	+	–	–	Lane et al., 1983
Cell surface markers					
SSEA-1	+	–	–	–	Solter and Knowles, 1978
Fetomodulin	+	–	+/–	–	Imada et al., 1987
E-cadherin	+	–	+/–	–	Damsky et al., 1983
PA isozyme					
tPA	–	+	–	+/–	Marotti et al., 1982
uPA	–	–	+	+/–	Sabbag et al., 1989
Other					
Hxt	–	–	+	–	Cross et al., 1995

Immunocytochemical analyses were performed on blastocyst outgrowths of 7-9 days cultured in 10% serum. –, no staining; +/–, trace staining; +, positive staining; ++, very strong staining. *Hxt* localization was by in situ hybridization. tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

these data suggest that the ICM outgrowth model is suitable for analyzing factors required for differentiation, and not just for adhesion or migration of fully differentiated PE.

ICMs plated on COL IV attached transiently, then detached and formed bilayered structures; outgrowth on COL IV was rare (Fig. 3A,B). Entactin, VN, LN and gelatin were not permissive substrata for ICM attachment (Table 2).

Trophectoderm promotes PE differentiation in ICM outgrowths

PE differentiates in vivo where primitive endoderm or VE contacts TE, whereas VE differentiates from primitive endoderm in contact with egg cylinder ectoderm. We combined ICM with TE to identify signals secreted by TE that might influence differentiation of endoderm from ICM. X-irradiated blastocysts developed well-spread TE cells. ICMs plated in TE cultures attached in a variety of ways: directly to TE cells, onto the FN substratum, or on both FN and TE at the edge of TE cells. ICMs that adhered directly on top of TE cells either showed no outgrowth (although the ICM grew larger) or showed delayed migration of PE during 3 days in culture. This behavior is similar to that observed in whole blastocyst cultures; presumably, the apical surface of a cultured TE cell is not a suitable substratum for PE migration. ICMs in direct contact with the FN substratum attached and grew out as described previously for FN substrata, except that PE cell formation was stimulated (Fig. 5A). This increase was accompanied by a decrease in VE cell number, resulting in approximately the same total cell number (Fig. 5B). This observation suggests that TE synthesizes a factor or factors that promote PE differentiation.

Because TE in culture was not always in direct contact with the ICM, we asked whether secreted factors contained in conditioned medium from TE cells could modify the phenotype of an ICM outgrowth. Medium conditioned by TE cells for 24 hours stimulated differentiation of outgrowths to PE by twofold (Fig. 5C,D), while having no effect on total cell number.

PTHrP stimulates PE outgrowth in cultures of ICMs on FN

TE produces many growth factors (Rappolee and Werb, 1994), including PTHrP. Endodermal cells also synthesize PTHrP, but less than TE (van de Stolpe et al., 1993). We first assessed the contribution of endogenous endodermal PTHrP to PE differentiation from ICM. In ICM cultures on FN, antibody to PTHrP depressed both the number and the proportion of PE cells in the population, while having no effect on the total cell number (Fig. 6A,B). Could this hormone mimic the effect of TE on PE differentiation? Addition of PTHrP to cultures of ICMs on FN increased the number of PE cells (Fig. 6C).

Table 2. Summary of ability of various ECM molecules to support outgrowth from ICMs by three days in culture

Substratum	Coating (μg/ml)	Total cells	PE cells	Other cells
Fibronectin	20-100	>50	5-25	25-50
Laminin/entactin	20-200	25-50	<5	25-50
Laminin	50-100	0	0	0
Type IV collagen	20-50	0	0	0
Entactin	50	0	0	0
Vitronectin	50-100	0	0	0
Gelatin	300	0	0	0

Data are expressed as range of cells in outgrowths in 3-5 experiments.

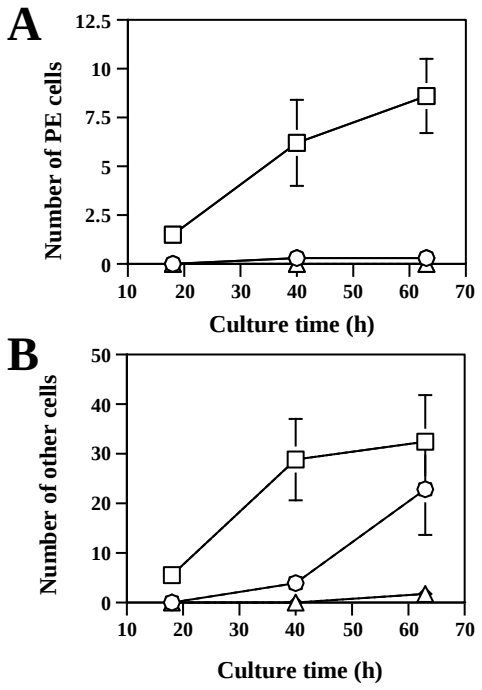

Fig. 3. Characteristics of ICM outgrowths on various substrata. ICMs were plated singly in 5 μl drops of medium on spots prepared with 20 μg/ml coating concentrations of FN (□), LN (○) or COL IV (△). Outgrowths were scored for (A) PE cells and (B) other cells. Note the absence of PE cells on the LN/EN substratum (n=10 ICMs, P<0.001, Student's t-test) and the overall poor suitability of COL IV as substratum. The experiment was performed 3 times; data shown are from one representative experiment.

LN, VN and low concentrations of FN become permissive substrata for PE differentiation and migration in the presence of PTHrP

It is clear that PE can develop from blastocyst outgrowths on ECM substrata other than FN, e. g., smooth muscle cell ECM (Behrendtsen et al., 1992; Glass et al., 1983), and LN (Sutherland et al., 1993). Could TE cells stimulate PE outgrowth on ECM substrata other than FN? TE cells from a single blastocyst stimulated PE outgrowth from a single ICM on COL IV at 6 days (control, 1.1±0.8; TE cell co-culture, 36.2±7.4; mean ± s.e.m., n=20 ICMs, P<0.001, Student's t-test). One observation prompted us to determine whether PTHrP had any effect on ICMs cultured on other ECM substrata: At suboptimal FN substratum concentrations, outgrowth from ICMs was low; the addition of PTHrP significantly increased the outgrowth of PE cells by day 6 (Table 3). These data suggested that PTHrP makes an ordinarily nonpermissive ECM capable of sustaining PE differentiation. Accordingly, we next plated ICMs on LN in the presence or absence of PTHrP. After 5 days of culture with PTHrP, PE cells began to appear around the ICMs and continued to grow through day 6 (Table 3). These cells were identified as PE because 67% of them expressed the PE marker SPARC; these cells did not, however, express immunohistochemically detectable LN (data not shown), unlike cells migrating on FN. Similarly, after 4 days of ICM culture on VN, morphologically identifiable PE cells began to differentiate only in the presence of PTHrP (Table 3); 99% of these cells expressed the PE marker LN and deposited it in a trail (data not shown).

For LN and VN substrata, PTHrP was about as effective as TE. Exogenous PTHrP also stimulated outgrowth of PE cells on COL IV at 6 days (Table 3); however, TE was more effective (see above), suggesting that PTHrP is not the only soluble factor from TE that is involved in PE migration and differentiation. PE outgrowth on LN/EN was stimulated at 3 days in the presence of TE cells (control TE cell co-culture, 4.2±1.2; TE cells and PTHrP, 15.3±2.8; mean ± s.e.m., n=20 ICMs, P<0.001, Student's t-test). These data indicate that LN, LN/EN, VN, COL IV and low concentrations of FN can all mediate good PE outgrowth when PTHrP is added to the cultures, but that other TE factors may be involved.

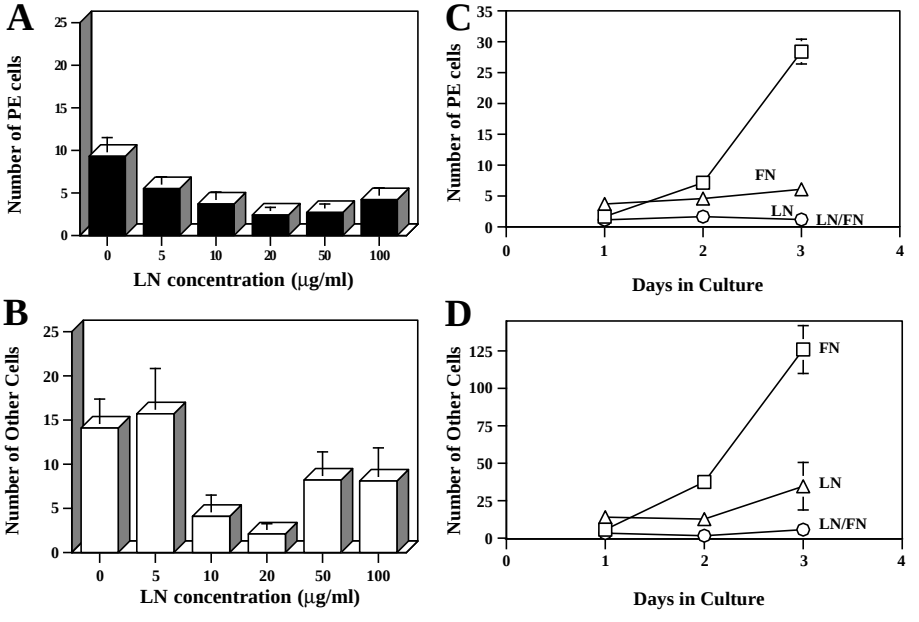

Fig. 4. LN inhibits PE outgrowth. (A,B) ICMs were plated singly on substrata prepared by coating dishes with 20 μg/ml FN alone or mixed with 5, 10, 20, 50 or 100 μg/ml LN/EN. Outgrowths were scored at 2 days for (A) PE cells and (B) other cells. (C,D) ICMs were plated singly on substrata prepared by coating dishes with 100 μg/ml FN or 100 μg/ml LN for 2 hours, or with 100 μg/ml FN for 2 hours followed by 100 μg/ml LN for 2 hours on top (LN/FN). Outgrowths were scored from 1 through 3 days for (C) PE cells and (D) other cells. The experiment was performed twice; data shown are from one representative experiment. LN in the mixed substrata (A) significantly decreased the number of PE cells in the outgrowth (20 μg/ml FN alone, 9.3±2.2 cells; mixture of 20 μg/ml FN and 20 μg/ml LN, 2.4±0.9 cells; mean ± s.e.m., n=10 ICMs, P=0.008, Student's t-test). In C, LN/FN also significantly inhibited PE outgrowth at 3 days (FN alone, 28.4±2.0; LN/FN, 1.2±0.3; mean ± s.e.m., n=20 ICMs, P<0.001, Student's t-test).

PE cells express α_5, α_{6A}, α_{6B} and $\alpha_V\beta_3$ integrins on various substrata

We performed immunocytochemistry to characterize expression of the α_5, α_{6A}, α_{6B} and $\alpha_V\beta_3$ integrins in mature ICM outgrowths on FN, LN and VN. These integrins were all expressed by PE cells growing out on FN. The α_5 subunit was expressed in adhesive sites at the leading edges of migrating cells (Fig. 7A), whereas the α_6 integrins were found in a punctate pattern throughout the PE cells, at the leading edges of the more flattened cells, or in spots along filopodia (Fig. 7B,C). $\alpha_V\beta_3$ integrin was more diffusely localized on PE cells, but was sometimes expressed more strongly at leading edges (Fig. 7D). In PE outgrowths on VN, $\alpha_V\beta_3$ was found in a diffuse, punctate pattern throughout points of contact with the substratum (Fig. 7E).

The localization pattern of integrins differed somewhat in PE outgrowths on LN. In these rounder PE cells, α_5 integrin was found only in an evenly distributed punctate pattern and never in striations at edges (Fig. 7F). α_{6A} was found strongly expressed at the leading edges of PE cells at the periphery of the outgrowths, whereas more central cells expressed α_{6A} weakly in a punctate pattern (Fig. 7G). α_{6B} expression was low and diffuse, but was slightly stronger at the leading edges of PE cells (Fig. 7H). There was no immunofluorescent signal in nonimmune antibody controls (Fig. 7I,J).

Outgrowth on FN, LN or VN is perturbed by antibodies only to the integrins for which these ECM molecules are ligands

Although PE cells expressed multiple integrins in the absence of exogenous PTHrP or TE, they migrated from ICMs only on FN. However, PE cells secrete a variety of ECM molecules that modified the substratum (e.g., Fig. 2B). Was the induction of PE by FN mediated by the α_5 integrin? We plated ICMs on FN in the presence of function-perturbing antibody to the α_5 integrin subunit and found that ICM outgrowth was inhibited for both PE and other cell types (Fig. 8). Most of the ICMs (70%) exposed to anti-α_5 IgG remained unattached. These data show that $\alpha_5\beta_1$ supports ICM outgrowth and PE differentiation on FN.

PE cells growing out on FN expressed several integrins that can bind LN or VN, but on LN or VN these integrins were not functional as adhesive and inductive receptors except in the presence of PTHrP. Did PTHrP upregulate endogenous synthesis of FN, making substratum requirements less stringent? ICM cultures growing on LN or VN in the presence of PTHrP and anti-α_5 antibody did not show inhibited PE outgrowth (Table 3). ICMs cultured in the presence of function-perturbing antibodies to the α_6 and $\alpha_V\beta_3$ integrins, or appropriate nonimmune controls, showed that outgrowth of PE was specifically inhibited by anti-α_6 on LN and by anti-$\alpha_V\beta_3$ on VN (Table 3). Therefore, in the presence of PTHrP, outgrowth of PE can be mediated by any of the cognate receptors expressed, and not just by α_5.

DISCUSSION

We have investigated soluble and adhesive signals that govern the differentiation of PE in cultures of normal ICMs of early mouse embryos. The outgrowth of PE from ICMs onto ECM substrata mimics the outgrowth of PE along the blastocoelic aspect of the trophoblast basement membrane in vivo.

Table 3. PTHrP stimulates PE outgrowth on several ECM components

Substratum	Coating (μg/ml)	Day	Treatment	Number of ICMs in experiment	PE cells (mean ± s.e.m.)	Other cells (mean ± s.e.m.)
Fibronectin (FN)	50	3	Control	20	18±13 (1)	34±10
			PTHrP	20	43±11 (1*)	26±10
			NRtIg	20	14±2 (2)	59±16
			Anti-α_5 IgG	19	3±1 (2*)	9±4
	10	6	Control	20	7±3 (3)	14±6
			PTHrP	20	100±32 (3*)	5±3
Laminin (LN)	50	6	NRtIg	20	1±2	0
			PTHrP + NRtIg	20	117±22 (4)	0
			PTHrP + anti-α_5	19	104±23 (9)	0
			PTHrP + anti-α_6	18	18±6 (4*)	0
Vitronectin (VN)	50	5	Control	33	4±2 (5)	50±34
			PTHrP	33	40±12 (5*)	52±24
		6	PTHrP + NRbIg	17	71±16 (6)	9±5
			PTHrP + anti-$\alpha_V\beta_3$	15	2±1 (6*)	0
		7	PTHrP + NRbIg	17	73±17 (7)	0
			PTHrP + anti-α_5	18	47±10 (10)	16±16
			PTHrP + anti-$\alpha_V\beta_3$	11	27±11 (7*)	0
Type IV collagen (COL IV)	100	6	Control	19	1±1 (8)	5±5
			PTHrP	20	15±4 (8*)	51±19

ICMs were plated on FN, LN, VN or COL IV. For FN, both a 50 μg/ml coating concentration and a poorly adhesive low coating concentration (10 μg/ml) were used.

Outgrowths were counted and fixed at indicated days of culture. PTHrP was used at a concentration of 100 nM. Anti-α_5 IgG and NRtIg were used at a concentration of 1 μg/ml.

Anti-α_6 antibody was used as a 1:10 dilution of GoH3 culture supernatant. Anti-$\alpha_V\beta_3$ IgG and NRbIg were used at a concentration of 200 μg/ml. In experiments with LN, an entactin-free preparation of LN (Collaborative Biomedical Products) was used. This preparation appears to be nonpermissive for VE outgrowths, unlike the LN/EN (Sigma) substrate used in Figs 1B and 3. Cells of the inner cell mass were not counted as outgrowth cells. For comparison of amounts of PE outgrowth, samples followed by a superscript were analyzed for significance by Student's *t*-test as follows; 1 versus 1*, $P<0.005$; 2 versus 2*, $P<0.001$: 3 versus 3*, $P<0.001$; 4 versus 4*, $P<0.001$; 5 versus 5*, $P< 0.005$; 6 versus 6*, $P<0.001$; 7 versus 7*, $P<0.05$; 8 versus 8*, $P<0.005$; 4 versus 9 and 7 versus 10 were not significantly different. Results are shown from a single representative experiment, but experiments were done 2-4 times.

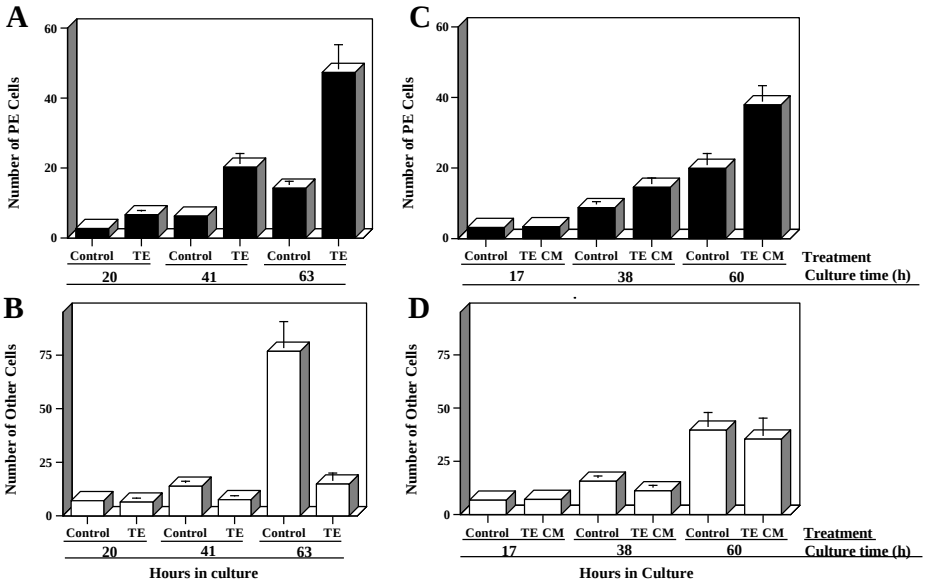

Fig. 5. TE cells stimulate PE differentiation. (A,B) ICMs were plated singly in 4 μl drops on spots prepared with 50 μg/ml FN in the absence or presence of 1 X-irradiated blastocyst (TE) (see Materials and Methods). The experiment was performed three times; data shown are from one representative experiment. (C,D) ICMs were plated in the absence or presence of 25% (by volume) TE-conditioned medium (prepared by culturing 1 X-irradiated blastocyst per microliter for 24 hours). The experiment was performed twice; data shown are from one representative experiment. The number of PE cells was increased significantly at day 3 by both TE cell co-culture (control, 14.3±2.0; TE cells, 47.3±7.9; mean ± s.e.m., n=20 ICMs, P<0.001, Student's t-test) and TE-conditioned medium (control, 20±4.2; TE-conditioned medium, 38±5.4; mean ± s.e.m., n=20 ICMs, P=0.011, Student's t-test). Cultures were scored daily for PE and other cells. CM, conditioned medium.

Removing trophoblast, and restoring factors secreted by these cells, allowed us to mimic signals that alter the differentiation and behavior of endoderm. We suggest that interaction of $\alpha_5\beta_1$ integrin with FN, together with low levels of PTHrP produced by ICM (van de Stolpe et al., 1993), can initiate the delamination and differentiation of PE. Addition of TE-secreted factors or PTHrP enhanced PE differentiation and changed the expression or functional properties of integrins to allow non-permissive substrata to mediate outgrowth of PE.

FN is a signalling molecule that promotes differentiation and outgrowth of PE from the surface layer of endoderm on isolated ICMs

As ICMs attached to the various ECM molecules, only FN induced the separation of 25% of the cells into PE. FN promotes the migration of many cell types, including avian precardiac mesoderm (Linask and Lash, 1988), neural crest cells (Bronner-Fraser, 1986) and primordial germ cells (ffrench-Constant et al., 1991). Perturbation of the interaction of these cells with FN by using RGD peptides or antibodies to FN or FN receptor resulted in loss of migration. Conversely, the stable overexpression of $\alpha_5\beta_1$ in sarcoma S180 cells enhances motility on FN and facilitates migration in vivo along neural crest pathways not available to the parent cell (Beauvais et al., 1995). We have demonstrated that disruption of FN receptor function results in reduced outgrowth of PE.

LN mediates attachment of ICMs but is not permissive for outgrowth of PE

The interaction of LN with α_6 integrin on endoderm mediated ICM adhesion and allowed outgrowth of pavements of AFP-positive VE, but did not generate PE. In mixed substrata, LN suppressed the ability of FN to generate PE. Nevertheless, mature PE cells adhered to LN; interaction with LN may

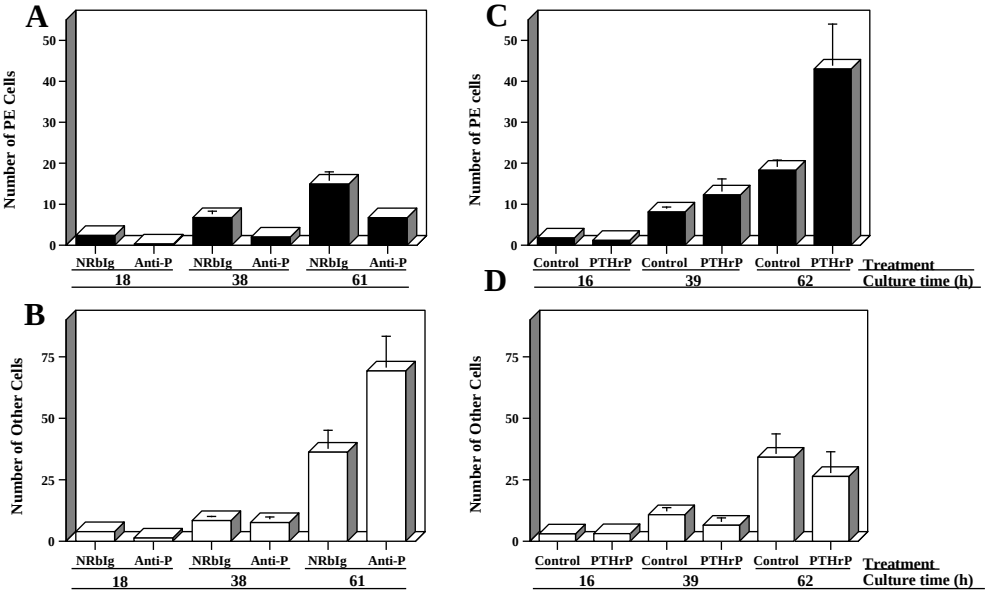

Fig. 6. PTHrP stimulates the differentiation of PE in ICM outgrowths. (A,B) ICMs were plated singly in 4 μl drops on 50 μg/ml FN with 67 nM anti-PTHrP IgG (Anti-P) or nonimmune rabbit IgG (NRbIg). (C,D) ICMs were plated in the presence or absence of 100 nM PTHrP. At 3 days the number of PE cells was decreased 55% by anti-PTHrP IgG (NRbIg, 14.9±3.0; anti-PTHrP IgG, 6.7±0.9; mean ± s.e.m., n=20 ICMs, P=0.007, Student's t-test), and the proportion of PE cells in the outgrowth was decreased 70% (P<0.001). The number of PE cells was increased 2.4-fold by PTHrP (control, 18.3±2.5; PTHrP, 43±11; mean s.e.m., n=20 ICMs, P=0.004, Student's t-test). Cultures were scored daily for PE and other cells.

inhibit the initial differentiation and migration of PE. F9 PE cells exhibit reduced adherence to LN (Tienari et al., 1989). Similarly, LN arrests the migration of some human breast cells (Coopman et al., 1991). LN inhibits keratinocyte migration in culture; in wounded skin, basal cell keratinocytes migrate away from the LN-rich lamina lucida onto the FN-rich wound bed (Woodley et al., 1988). In contrast, LN promotes the migration of neuronal cells, perhaps by being anti-adhesive (Calof and Lander, 1991). The type of interaction with LN may depend on the state of PE maturity or the integrin activation state.

PE differentiation can be induced in cultures of ICMs by trophoblast

X-irradiated TE cells from a single blastocyst co-cultured with an ICM on FN increased the number of PE cells, suggesting that TE promotes PE formation and suppresses VE outgrowth. VE generates PE at sites of contact with TE in vivo (Hogan and Tilly, 1981; Gardner, 1982), probably by transdifferentiation (Hogan and Newman, 1984). Whether the PE directly differentiated from primitive endoderm has properties distinct from those of PE transdifferentiated from VE is not known.

It is likely that differentiation induced in co-cultures is multi-factorial. PTHrP is expressed at low levels by endoderm and at high levels by TE and raises cellular cAMP in EC and embryonic stem cells (Chan et al., 1990; van de Stolpe et al., 1993). In our study, PTHrP stimulated differentiation and outgrowth of PE on LN, VN and COL IV, and on concentrations of FN not usually permissive for outgrowth. We found that outgrowths on LN and VN were not mediated by a FN bridge and were sensitive only to function-perturbing antibodies to the α_6 LN receptor or $\alpha_V\beta_3$ VN receptor, respectively. F9 cells undergo a switch between the α_{6B} subtype in undifferentiated endoderm to α_{6B} and α_{6A} in differentiated PE-like cells (Hierck et al., 1993; Jiang and Grabel, 1995). This is consistent with our observation of strong expression of α_{6A} and weak expression of α_{6B} in migrating PE. Interestingly, monocytes migrate on LN more efficiently with α_{6A} than with α_{6B} (Shaw and Mercurio, 1994).

TE cells do not always support PE outgrowth in vivo; during implantation delay, PE cells do not migrate onto mural TE (Gardner et al., 1988), although a TE-derived basal lamina is present (Schlafke and Enders, 1963). Thus, the signal depends on activation of (or relief of suppression of) TE by interactions with the maternal environment.

Comparison of the molecular determinants of PE outgrowth in vivo and in vitro

TE and PE share the synthesis of several components of Reichert's membrane. However, some proteins are expressed predominantly by one cell type or the other (Hogan et al., 1980). FN, LN and PTHrP are all expressed during PE development in mouse blastocysts; these proteins, which are bioactive in vitro, could fulfill these functions in vivo. FN is synthesized by TE cells and is prominent in the basal lamina of TE in the blastocyst (Wartiovaara et al., 1979; Carnegie, 1991; Thorsteinsdottir, 1992). It is tempting to speculate that

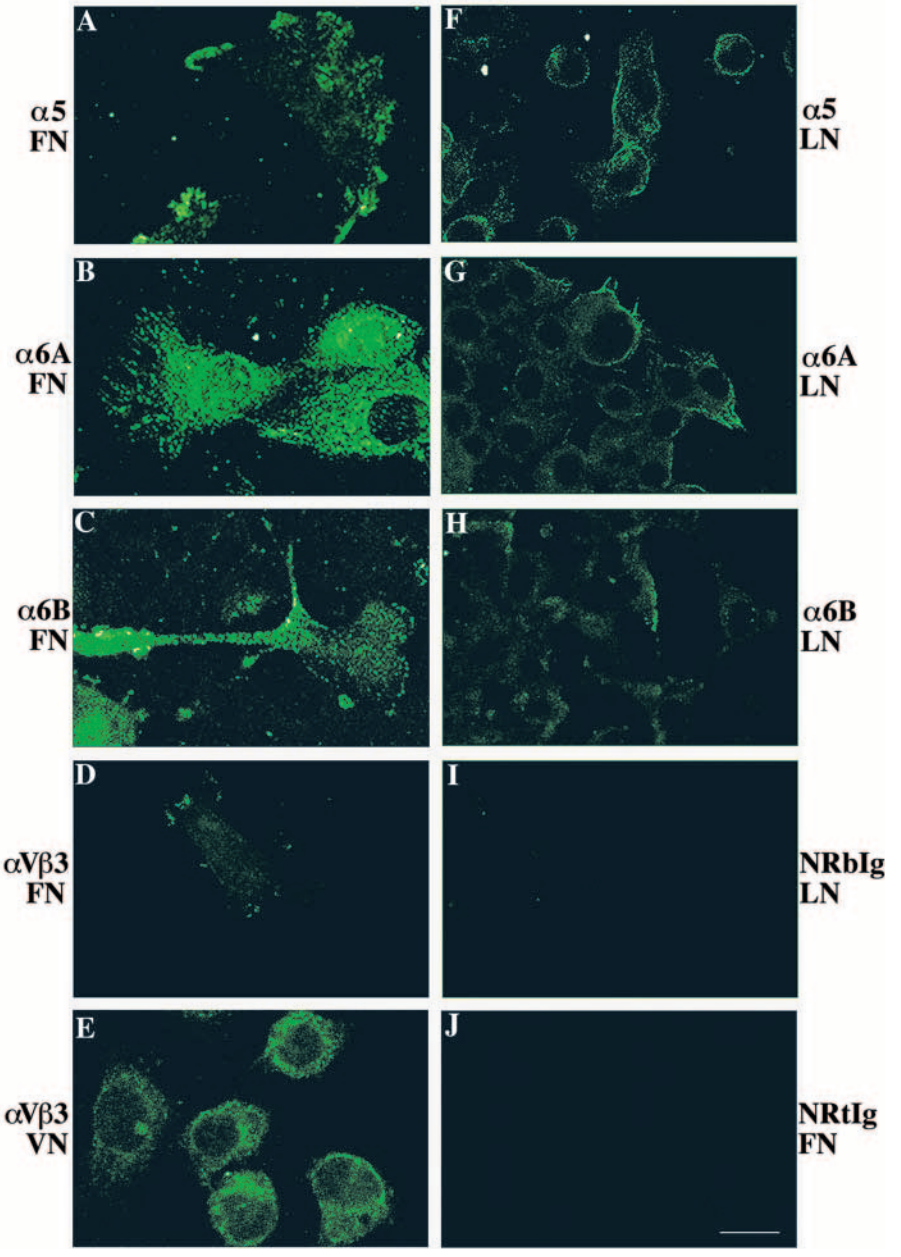

Fig. 7. Integrin localization on various substrata. Chambers of 16-chamber slides (Nunc) were coated with FN, LN or VN at 50 μg/ml. 7-10 ICMs were plated in a volume of 50 μl in each chamber. Outgrowth on LN and VN required the presence of 100 nM PTHrP. Outgrowths were fixed at 6 days with acetone-methanol and probed with antibodies to α_5 integrin (A, F), α_{6A} integrin (B, G), α_{6B} integrin (C, H), $\alpha_V\beta_3$ integrin (D, E), or with nonimmune rabbit IgG (NRbIg) (I) or nonimmune rat IgG (NRtIg) (J). Bar = 6 μm.

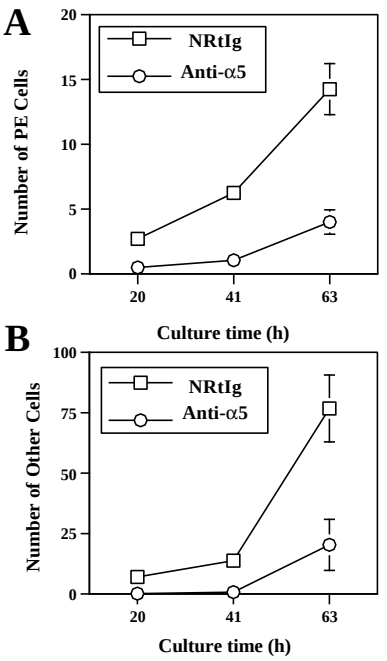

Fig. 8. Antibody to α_5 integrin inhibits ICM outgrowth. ICMs were plated singly in 5 μl drops on spots prepared with 50 μg/ml FN in the presence of 7 nM anti-α_5 integrin IgG (anti-α_5 IgG) or nonimmune rat IgG (NRtIg). Cultures were scored daily for (A) PE cells and (B) other cells. All forms of outgrowth were reduced by 70% at 3 days (NRtIg, 14.3±2.0 PE cells and 76.8±13.9 other cells; anti-α_5 IgG, 4±0.9 PE cells and 20.3±10.6 other cells; mean ± s.e.m., n=20 ICMs, P<0.001, Student's t-test). The experiment was performed three times; data shown are from one representative experiment.

FN is a primary factor in PE outgrowth. However, in mice containing null mutations in FN, or in the α_5 integrin, PE does develop (George et al., 1993; Yang et al., 1993). The second wave of PE cells migrates at least in part on the additional LN and modified ECM secreted by the first wave.

The development of PE in ICMs cultured on LN, VN and COL IV was delayed compared with that on FN; this allows the differentiation of a VE rind on the ICM surface. Thus, it is possible that this delayed PE may arise by transdifferentiation from VE, whereas PE migrating on FN may develop directly from primitive endoderm. One clue that such a mechanism may be operating comes from the observation that PE migrating on LN does not express high concentrations of LN although it still expresses the anti-adhesive protein SPARC. Clearly, PTHrP or other factors are needed in the process, because VE does develop from ICMs on LN/EN and VN substrata, but PE development does not ensue.

The evolution of integrin function may reflect a temporal change normally going on in vivo that exists to cope with the changing face of the ECM, activating novel integrin-mediated signaling pathways in PE differentiation. Mice with a targeted mutation in PTHrP survive until birth (Karaplis et al., 1994). We do not know whether PE forms; however, there may be enough maternally derived PTHrP, as for animals with a null mutation in the transforming growth factor (TGF)-β gene (Letterio et al., 1994). Other factors of maternal or TE origin also may be involved. Blastocysts express fibroblast growth factor (FGF)-4, insulin-like growth factor-2, TGF-α, TGF-β, platelet-derived growth factor and activin (reviewed by Rappolee and Werb, 1994; van de Stolpe et al., 1993; Albano et al., 1993), and FGF-4 has been found to stimulate PE differentiation from ICMs (Rappolee et al., 1994). Leukemia inhibitory factor inhibits ICM differentiation to PE in ICM cultures (Newman-Smith and Werb, 1995). The epidermal growth factor (EGF) receptor mediates ICM survival in some genetic backgrounds (Threadgill et al., 1995). Moreover, TGF-β1 induces the de novo expression of $\alpha_V\beta_6$ integrin and upregulates $\alpha_5\beta_1$, $\alpha_V\beta_5$ and $\alpha_2\beta_1$ integrins in keratinocyte colonies, stimulating migration toward FN and VN (Zambruno et al., 1995), and EGF enhances migration of squamous carcinoma cells (Fujii et al., 1995) and keratinocytes (Chen et al., 1993) on type I collagen by upregulating expression of $\alpha_2\beta_1$ integrin. EGF has also been shown to activate $\alpha_V\beta_5$-dependent motility of cells without an increase in expression of $\alpha_V\beta_5$ (Klemke et al., 1994).

Comparison of the induction of PE in ICMs and in EC cell lines

In F9 cells, PTHrP is expressed in significant quantity and PE arises mainly from VE (van de Stolpe et al., 1993). Differentiation of PE from VE precursors in PSA-1 or F9 EC cell lines also requires contact with an appropriate substratum, such as FN (Casanova and Grabel, 1988; Grabel and Watts, 1987). By comparing integrin expression, PTHrP secretion and ECM synthesis for these cell types, it should be possible to establish new strategies for studying the process of PE differentiation in ICM or whole embryo systems.

In summary, the addition of PTHrP to cultures of ICMs modulates cellular response to ECM ligands. When PTHrP is limiting, PE differentiation is critically dependent on the presence of FN. With higher PTHrP concentrations, any of at least three integrins can mediate the morphogenesis of PE. We suggest that as intracellular cAMP concentrations rise, the functionality of the integrins is affected. These observations suggest specific cytokines will change cellular responses to a given ECM molecule.

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