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Extracellular Matrix Composition and Resilience: Two Parameters That Influence the In Vitro Migration and Morphology of Rat Inner Cell Mass-Derived Cells[1]

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ABSTRACT

Parietal endodermal (PE) migration along rat trophectodermal (TE) cells coincides with the deposition of Reichert's membrane between these two cell layers. In this study, we compared the influences of fibronectin and laminin, two components of Reichert's membrane, on the migration and replication of PE-like cells from cultured rat inner cell masses (ICMs). We also explored the role of substrate nondeformability by comparing cell translocation on gels versus coatings of Matrigel (a tumor cell-derived basement membrane preparation) or of collagen. ICMs, isolated by immunosurgery from Day 5 blastocysts, were cultured on coatings of collagen IV, laminin, fibronectin, collagen I, or Matrigel, or on gels of the latter two substrates. Minimal laminin or fibronectin coating concentrations of 2.5 μg/ml were required for ICM attachment and cell migration. Migration was similar during the first 48 h of culture on fibronectin and on laminin; however, by 72 h, the extent of cell translocation on fibronectin was greater (1.5- to 2-fold) than that measured on laminin. Fibronectin-cultured ICM-derived cell clusters also contained 1.5- to 2-fold more cells than those on laminin. Migration did not occur on undiluted gels of Matrigel but was supported by diluted (1:10 and 1:20) Matrigel coatings. Similarly, cell migration on coatings of collagen IV reached almost 3-fold that measured on collagen I gels. Most of the cells migrating on fibronectin or collagen (I or IV) were flattened and elongated. In contrast, a high proportion of the cells migrating on laminin or Matrigel coatings were tall and rounded, with thin cytoplasmic extensions. Fibronectin- and collagen IV-cultured cells stained strongly for both collagen IV and laminin, but contained no fibronectin. In contrast, laminin-cultured cells contained fibronectin but were less immunoreactive for laminin and collagen IV. These findings indicate that substrate composition and resilience influence the in vitro migration and morphology of ICM-derived PE-like cells. A role for the TE cells in anchoring Reichert's membrane during development of the PE cell layer within the blastocyst is postulated. Furthermore, the sensitivity of cell morphology and differentiation to individual basement membrane components provides a potential key mechanism whereby an emerging basement membrane can regulate cell migration and differentiation, two fundamental processes that occur throughout embryonic development.

INTRODUCTION

Basement membranes are extracellular matrix structures that not only provide structural support for epithelial cells, but also can influence such basic cell properties as morphology, proliferation, and differentiation. Within the rat embryo, an early developmental event following blastocyst formation is the differentiation into primitive endodermal cells of the inner cell mass (ICM) cells facing the blastocoelic cavity and the subsequent migration of a population of these primitive endodermal cells along the inner face of the trophectoderm (TE) to form the parietal endodermal (PE) layer [1]. Reichert's membrane is a basement membrane whose deposition on the inner surfaces of the TE cells of rat and mouse blastocysts begins at this time as well [1]. Structurally, Reichert's membrane has been shown to contain type IV collagen, fibronectin, laminin, entactin, and heparan sulfate proteoglycan [2–4]. Type IV collagen is unique to basement membranes and comprises a major proportion of their glycoprotein content [5]. Fibronectin and laminin can act as bridging molecules between cells and the rest of the basement membrane components because of their complement of specific binding sites for collagen IV, heparin sulfate, and a range of cell surface receptors [6–9]. Receptors for collagen IV have also been identified on some cell types [10, 11].

Studies with teratocarcinoma cells have provided evidence favoring a role for fibronectin (over collagen IV or laminin) in supporting the migration and differentiation of PE-like cells from embryoid bodies [12]. However, we have reported equivalent migration of laminin-containing PE-like cells from rat ICMs cultured on collagen IV or fibronectin as well as appreciable migration in response to laminin [13]. Interestingly, we found that migration did not occur when ICMs were cultured on a 1-mm slab of Matrigel, a tumor cell-derived basement membrane preparation (Collaborative Research Inc., Bedford, MA) that contains both collagen IV and laminin [13]. The present study was designed to further explore and compare the abilities of various components of Reichert's membrane to support the migration of ICM-derived cells and to provide some characterization of those migrating cells. The concentration-related abilities of laminin and fibronectin to support both cell migration and proliferation were compared. The influence of substratum

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deformability on cell translocation was assessed by comparing the abilities of coatings versus gels of either Matrigel or type I collagen to support cell migration in vitro. Finally, the influences of different culture substrata on the morphology of the migrating cells as well as their content of specific extracellular matrix glycoproteins was evaluated by means of scanning electron microscopy, morphometric analysis, and immunofluorescence.

MATERIALS AND METHODS

Animal Treatment and Blastocyst Collection

Follicular development was induced in immature female Sprague-Dawley rats (65–70 g body weight) by a single injection (i.p.) of 4 IU of eCG (Equinex; Ayerst Labs, Montreal, PQ) given on the morning (0900–0930 h) of Day −2 [14]. An injection 52 h later of an LHRH analogue (des-gly^{10}(D-ala^{6})LH-RH-ethylamide; Sigma Chemical Co., St. Louis, MO) was used to synchronize LH-induced ovulation, and the animals were mated overnight [15]. The rats were killed on Day 5 of gestation (Day 1 = day of copulatory plug; 6 animals per experiment), and each uterine horn was flushed with Opti-MEM 1 (GIBCO Laboratories, Mississauga, ON) to obtain blastocysts at the early hatching stage of development (14.3 ± 1.4 per female). Opti-MEM 1 is a HEPES-buffered modification of Eagle's Minimal Essential Medium and was used for all ICM handling and culture procedures. The base medium (used for flushing of uterine horns) was supplemented with $NaHCO_3$ (2.4 g/L), nonessential amino acids (0.1 mM), penicillin-streptomycin (50 000 U and 50 000 μg/L, respectively), and 2-mercaptoethanol (5.5×10^{-5} M); that used for ICM culture (BSA-Opti-MEM 1) was also supplemented (0.25%) with BSA (Fraction V; Sigma). For each experiment, blastocysts from 6 rats were pooled in BSA-Opti-MEM 1 and precultured overnight before ICMs were isolated.

Isolation of ICMs

ICMs were isolated using immunosurgery, as has been previously described for mouse blastocysts [16]. To obtain the necessary antibodies, two New Zealand White rabbits were injected with rat spleen cells (1 injection every 2 wk of 3 ml Dulbecco's PBS [D-PBS; calcium- and magnesium-free; GIBCO] containing 1.3×10^{8} spleen cells/ml; total of three injections) and then bled 10 days after the final spleen cell injection. Following heat inactivation of complement (30 min at 56°C), the serum was stored at −80°C until used for immunosurgery.

In order to lyse the TE cells, the blastocysts were incubated (30 min at 37°C) with rabbit antiserum to rat spleen cells (diluted 1:8 with BSA-Opti-MEM 1) and then with guinea pig complement (GIBCO; diluted 1:4 with BSA-Opti-MEM 1). They were then pooled in an organ culture dish containing 1 ml of this medium, and the adherent TE cellular debris was removed using a micropipette 45–50 μm in diameter. The cleaned ICMs were pooled in fresh BSA-Opti-MEM 1 and randomly allocated to substratum culture groups.

Staining of ICM Cell Nuclei with Hoechst 33342

ICMs [15] were stained with Hoechst 33342 [17] in order to determine the mean number of cells per ICM put into culture. Freshly isolated ICMs were incubated, first with 0.01% trypan blue in 2.3% sodium citrate dihydrate (1 min at room temperature) and then with Hoechst solution (0.75 ml of 2.3% sodium citrate dihydrate + 0.25 ml ethanol + 0.01 ml Hoechst 33342 stock [1 mg/ml in distilled water]); the latter staining procedure was performed for 4 min at 37°C. The ICMs were then mounted in PRO-TEXX mounting medium (American Scientific Products, McGaw Park, IL), and the number of fluorescing nuclei was determined for each ICM by means of epifluorescent optics.

ICM Culture

The ICMs were cultured (37°C, 5% CO_2 in air) for up to 72 h in 8-chamber Lab-Tek chamber slides (GIBCO). Five different extracellular matrix components were examined and compared for their influence on ICM-derived cell migration and/or proliferation. They were applied to the wells of the chamber slides as either coatings or gels, as described below, before addition of the culture medium. Following preparation of the culture substratum, 300 μl BSA-Opti-MEM 1 supplemented (2%) with controlled process serum replacement-2 (CPSR-2; Sigma) was added to each well. CPSR-2 is a bovine plasma-derived serum substitute with low background mitogenic activity. We have also found (J.A. Carnegie, unpublished observation), using ELISA, that this serum substitute contains nondetectable (< 6 ng/ml) levels of plasma-derived fibronectin. In all cases, the medium was added to the matrix-pretreated wells at least 45 min before the addition of the ICMs.

Collagen IV. Each well was coated (18 h at 37°C) with type IV collagen (10 μg/ml; Collaborative Research) prepared in sterile distilled water containing 100 U and 100 μg/ml, respectively, of penicillin G and streptomycin sulfate [18]. The following morning, the collagen coating solution was removed and the wells were rinsed once with sterile water and then air-dried before the culture medium was added.

Fibronectin and Laminin. Chamber slides were incubated (18 h at 37°C) with various concentrations (0–40 ng/ml) of either fibronectin or laminin (Collaborative Research) prepared in D-PBS. Following removal of the coating solution, the wells were rinsed with D-PBS and the culture medium was added immediately.

Matrigel. Matrigel (a soluble basement membrane preparation obtained from Engelbreth-Holm-Swarm tumor cells; Collaborative Research) either was used undiluted (gel) or was applied following dilution (1:5 to 1:20) in unsup-

plemented Opti-MEM 1. For use as a gel, 75 μl of Matrigel was spread evenly in each well, allowed to gel (30 min at 37°C), and then overlaid with culture medium. This provided a coating density of 100 μl/cm^2. Additional wells were preincubated (1 h at 37°C) with various dilutions (1:5, 1:10, and 1:20) of Matrigel. At the end of the 1-h period, the residual solution was removed and the wells were washed once with unsupplemented Opti-MEM-1; the culture medium was then added. It should be noted that at a 1:5 dilution, the Matrigel formed a viscous gel and very little residual solution could be removed. Hence only the 1:10 and 1:20 dilutions of Matrigel provided true coatings of extracellular matrix material.

To ensure that all of the Matrigel components were adhering to the culture surface in a concentration-dependent manner, additional studies were performed using Matrigel-coated derivatized glass culture surfaces [19]. Briefly, round glass coverslips were treated (4 min at room temperature) with 3-aminopropyltriethoxysilane (Sigma), rinsed with sterile water, and then further incubated (30 min at room temperature) with freshly prepared 0.25% glutaraldehyde in D-PBS. The coverslips were then washed thoroughly with D-PBS, placed in 24-well plates, and coated with diluted Matrigel (1:10) as described above.

Collagen I. Type I collagen solutions were prepared from rat tail tendons as described by Foster et al. [20]. It should be noted that these solutions are crude preparations that may contain other extracellular matrix components and growth factors. Tendons were isolated from pre-sterilized (70% ethanol, overnight) tails of 30-day-old Sprague-Dawley rats. Following tendon digestion in 17.4 mM acetic acid (1 g tendons in 100 ml acetic acid solution; 4 days at 4°C with continuous stirring), the solution was centrifuged (1 h at 100 × *g*) to remove any residual debris. The crude collagen I solution was stored (4°C) in sterile bottles until required for culture.

To prepare collagen I gels, 10 ml of cold collagen I solution was neutralized with 1.3 ml of a 2:1 mixture of 10-strength MEM (GIBCO) and 0.33 M NaOH [21]. Immediately, and with minimal agitation, 75 μl (equivalent to 100 μg protein) of this solution was added to each well of a chamber slide, and gelation was allowed for 30 min at 37°C before addition of the culture medium.

To coat culture surfaces with collagen I, 5 or 10 μl (5 or 10 μg protein, respectively) of the acidic collagen solution was evenly spread over the bottom of each well of a chamber slide using a 200-μl pipette tip [22]. The collagen coating was allowed to air-dry before culture medium was added.

Evaluation of Responses of ICM-Derived Cells to Extracellular Matrix Substrata

Overall cellular migration was quantified by means of morphometric analysis to measure increases in the area of each ICM-derived cell cluster during culture on the different ECM substrata. Cellular differentiation patterns in response to these substrata were evaluated as follows. The morphology of the migrating cells was compared qualitatively via light and scanning electron microscopy. In addition, individual cell areas and shapes were quantitatively compared by means of morphometric analysis. Finally, cellular content of fibronectin, laminin, and collagen IV (the latter two of which are markers for mature PE cells) was determined by immunofluorescence. These procedures were carried out as specified below.

Morphometric Analysis

To quantitatively evaluate cell migration and proliferation, each ICM was photographed at 24-h intervals using a magnification that would accommodate the entire ICM-derived cell cluster in a single frame. Each frame was projected onto the monitor screen of a Videoplan image analysis system (Kontron Bildanalyse GmbH, Munich, Germany) by means of a Tamron Fotovix film video processor (Tamron Industries, Port Washington, NY), and the outlines of each ICM-derived cell cluster in its entirety and/or outlines of the individual migrating cells were traced. From these data, the areas of each ICM-derived cell cluster were calculated. For evaluation of individual cell morphologies, maximum cell length and width as well as total perimeter were determined for at least 100 cell projections within each treatment group. From these measurements, projected cell area and contour index (a size-independent measurement of the shape of a profile in which increases in profile irregularity are represented by higher numerical values [23]) were calculated for each cell, and mean values ± SEM were determined for each culture group. Finally, migrating cells (a migrating cell having been defined as one that had attached to the substrate and traveled sufficiently to separate itself from the original ball of cells placed in culture) were counted for each ICM-derived cell cluster every 24 h.

Light Microscopy

For examination of the ICM-derived cell clusters at the end of the culture period, the cells were fixed (overnight at 4°C) in 2% glutaraldehyde (0.1 M sodium cacodylate buffer, pH 7.4) and then stained using the Wright-hematoxylin stain for cultured cells (M.C. Léveillé, personal communication). Briefly, the fixed cells were incubated with 0.3% (in methanol) Wright's stain (BDH Chemicals, Toronto, ON) for 45 sec at room temperature. The stain was diluted 2-fold with distilled water, and staining was continued for an additional 45 sec. Following thorough rinsing with distilled water, the cells were counterstained (45 sec at room temperature) with Harris' Alum Hematoxylin (EM Diagnostic Systems, Gibbstown, NJ), washed with distilled water, air-dried, and mounted with PRO-TEXX mounting medium.

Scanning Electron Microscopy

Cells to be examined by scanning electron microscopy were cultured on 13-mm round coverslips that had been precoated with the appropriate matrix component(s). At the end of the culture period, the cells were prepared for scanning electron microscopy using a modification of the procedure described by Ouhibi et al. [24]. Following removal of the culture medium, the cells were fixed (overnight, 4°C) in 2% glutaraldehyde (sodium cacodylate buffer, pH 7.4) and dehydrated through a standard ethanol series. The cells were then critical point-dried, coated with gold palladium, and examined by means of an AMR Model 1000 scanning electron microscope.

Immunolocalization of Extracellular Matrix Glycoproteins

After 72 h of culture on coatings of fibronectin, collagen IV, or laminin, the ICM-derived cell clusters were prepared for the immunolocalization of each of these three glycoproteins as follows. All procedures were performed at room temperature. The cells were first fixed (5 min) with 3.7% paraformaldehyde in PBS and then permeabilized (20 min) with 0.1% PBS-Nonidet P-40 [25, 26]. The cells were rinsed with PBS and then incubated (45 min) with a specific antiserum to laminin or collagen IV (rabbit anti-mouse; Collaborative Research) or to fibronectin (goat anti-human; Terochem Laboratories, Toronto, ON). The cells were again rinsed with PBS and further incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (laminin or collagen IV) or swine anti-goat IgG (fibronectin) (all from Intermedico, Willowdale, ON). After final rinsing in PBS, the cells were mounted in 0.1% *p*-phenylenediamine in 50% glycerol (to reduce bleaching of fluorescence during ultraviolet illumination [27]) and observed using epifluorescence optics. The immunolocalization studies were repeated three times; a minimum of 8 ICM-derived cell clusters were observed for each treatment group. These studies included control ICM-derived cell clusters for which incubation with the first antibody was replaced by incubation with PBS or the appropriate preimmune serum. No fluorescence was seen in the control cells.

Statistical Analysis

Data in all studies, with the exception of those comparing paired treatment groups, were evaluated by analysis of variance. Where appropriate, post hoc comparisons between different treatment groups were made using Tukey's multiple range test [28]. The data presented in Tables 1 and 3 were evaluated using Student's *t*-test.

RESULTS

Cell Migration on Coatings of Individual Extracellular Matrix Components

The abilities of fibronectin and laminin to support cell migration were evaluated at coating concentrations ranging from 0.04 to 40 μg/ml (Fig. 1). The lowest concentration of either glycoprotein to support ICM attachment and the outward migration of PE-like cells was 2.5 μg/ml. Cell migration continued over the 72-h culture period on wells precoated with 2.5–40.0 μg/ml of fibronectin or of laminin, with significant ($p < 0.001$) increases in the mean areas of the ICM-derived cell clusters noted at each of the three time points examined (Fig. 1). However, for fibronectin (especially) and also for laminin, a dose-response relationship did not exist between coating concentration (higher than 2.5 μg/ml) and the extent of cell migration. The mean areas of the ICM-derived cell clusters were equivalent ($p > 0.5$) on each of the three highest coating concentrations of fibronectin throughout the culture period. For laminin, there was a trend toward greater migration with higher coating concentrations, but these differences were not significant ($p > 0.05$). The extent of cell migration measured on fibronectin and on laminin was similar during the first 48 h of culture. However, by the end of the culture period, it was found that the ICM-derived cell clusters on the laminin substrates occupied areas that were 50% (2.5 μg/ml) to 75% (40 μg/ml) of the mean areas of the fibronectin-cultured ICMs (Fig. 1; $p < 0.05$).

Proliferation of the migrating cells occurred on all those concentrations of fibronectin or laminin that were sup-

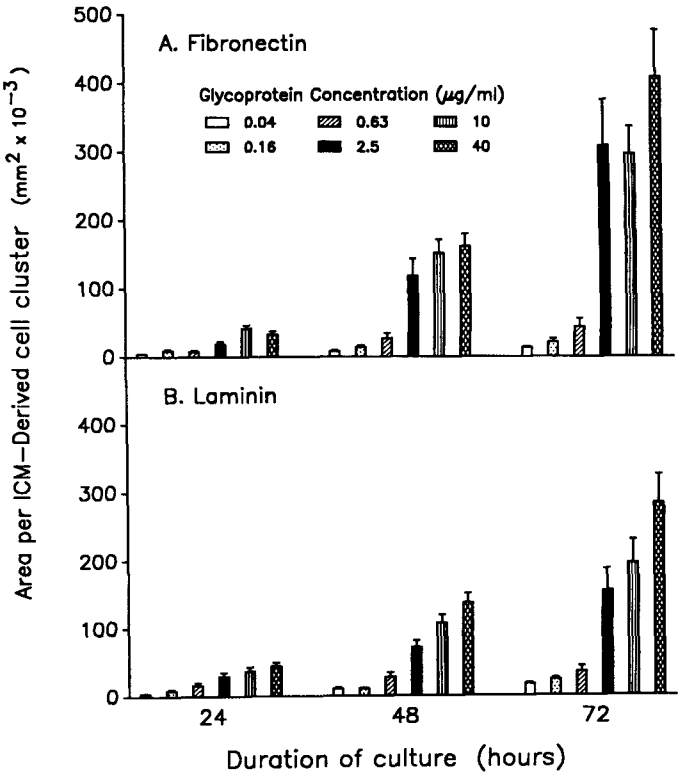


FIG. 1. Influences of various coating concentrations of fibronectin (A) or laminin (B) on the mean projected areas of ICMs following 24 to 72 h of culture. Mean ICM area at time 0 is 3.6 ± 0.3 × 10^{-3} mm^2 (n = 20). Each bar is the mean ± SEM of 13–18 ICMs from 3 experiments.

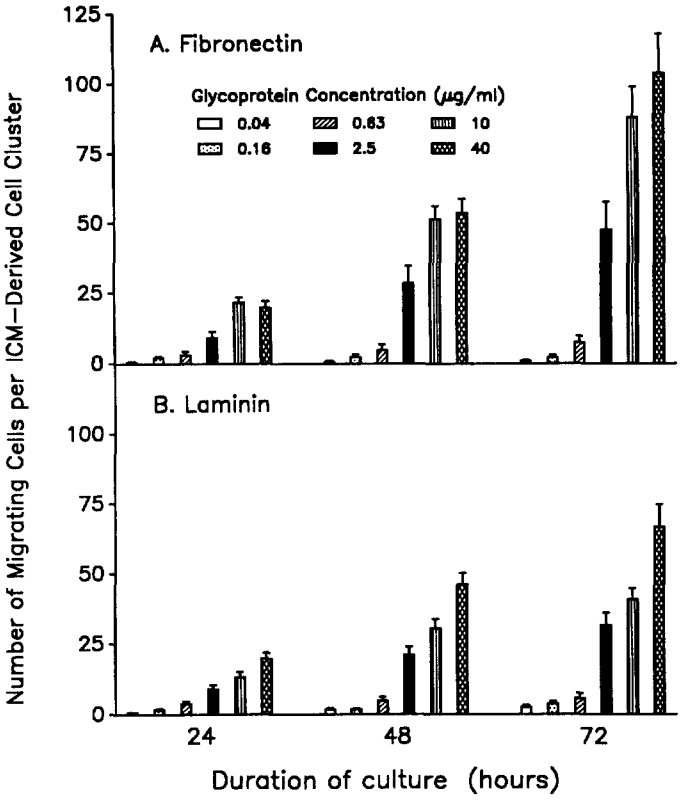


FIG. 2. Influences of different coating concentrations of fibronectin (A) or laminin (B) on the mean number of ICM-derived migrating cells after 24 to 72 h of culture. Mean ICM cell number at time 0 is 14.2 ± 1.3 (n = 15). Each bar is the mean ± SEM of 13–18 ICMs from 3 experiments.

portive of cell translocation (2.5–40.0 μg/ml; Fig. 2). At concentrations of 2.5 μg/ml or higher of each glycoprotein, all of the cells within each ICM-derived cluster had attached to the culture surface and could be counted. Differences between the numbers of migrating cells associated with the fibronectin- and the laminin-cultured cells became apparent as the duration of the culture period increased. By 72 h of culture, 1.5- to 2-fold more cells ($p < 0.001$) were associated with each fibronectin-cultured ICM-derived cell cluster than with cultures maintained on laminin.

TABLE 1. Outward migration of PE-like cells from ICMs cultured (72 h) on Matrigel (undiluted gel vs. 1:10 coating) applied to untreated or derivatized glass.

	Area/ICM-derived cell cluster ($mm^2 \times 10^{-3}$)*			
	Undiluted Matrigel		1:10 Matrigel coating	
Hours of culture	Uncoated glass	Derivatized glass	Uncoated glass	Derivatized glass
24	8.0 ± 1.9	4.8 ± 0.4	17.8 ± 3.1	13.2 ± 1.8
48	6.1 ± 0.8	7.3 ± 0.7	73.6 ± 14.0	56.1 ± 8.7
72	9.3 ± 1.2	8.9 ± 1.1	176.0 ± 32.0	160.0 ± 22.0

*Values are mean ± SEM; n = 11–18 (2 experiments).

Gels Versus Coatings

The culture of ICMs on a thin layer of undiluted Matrigel was associated with virtually no outward migration of PE-like cells (Fig. 3). Although some cell migration did occur on Matrigel diluted 1:5, the mean area occupied by the ICM-derived cell clusters after 72 h of culture was only 10% of that measured on a coating (10 μg/ml) of type IV collagen (Fig. 3). At a dilution of 1:5, Matrigel was still sufficiently concentrated that it formed a resilient gel on top of the culture surface to which each ICM then attached. However, when the Matrigel was further diluted to 1:10 or 1:20 there was no evidence of gel formation, and a thin coating could be applied to the culture surfaces. Under these conditions, cell migration was significantly improved ($p < 0.001$) and

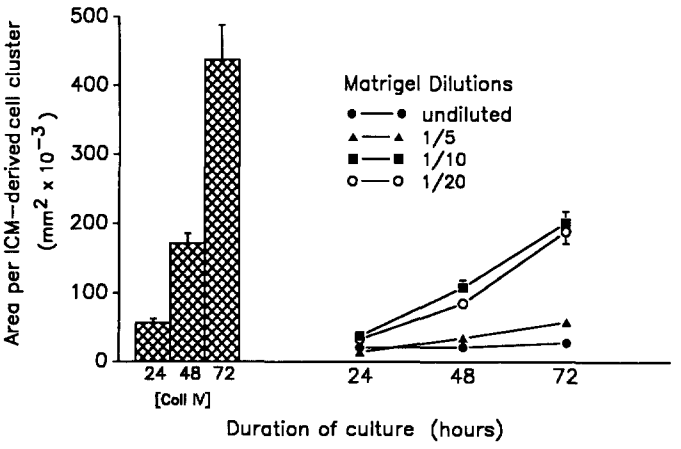


FIG. 3. Time-dependent increases in mean projected areas of ICMs cultured (24–72 h) on either gels or diluted (1:10 and 1:20) coatings of Matrigel. The extent of migration is compared with that of ICMs cultured on wells precoated with 10 μg/ml of collagen IV. Mean ± SEM of 12–18 ICMs from 3–4 experiments.

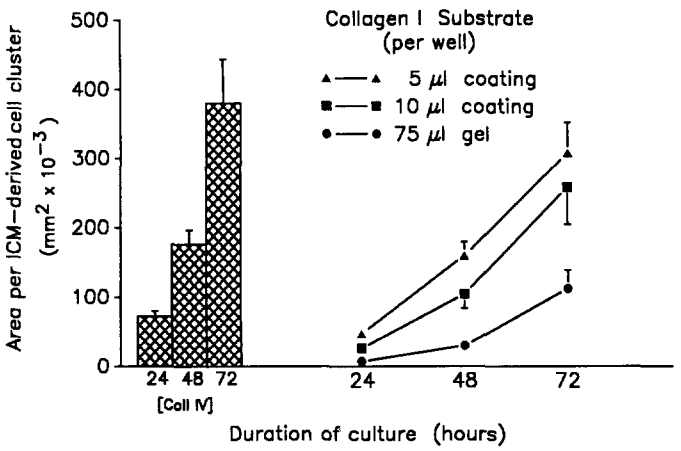


FIG. 4. Time-dependent increases in mean projected areas of ICMs cultured (24–72 h) on either gels or coatings of type I collagen. The cellular responses are compared with those of ICMs cultured on wells precoated with 10 μg/ml of collagen IV. Data represent the mean ± SEM of 10–16 ICMs from 3 experiments.

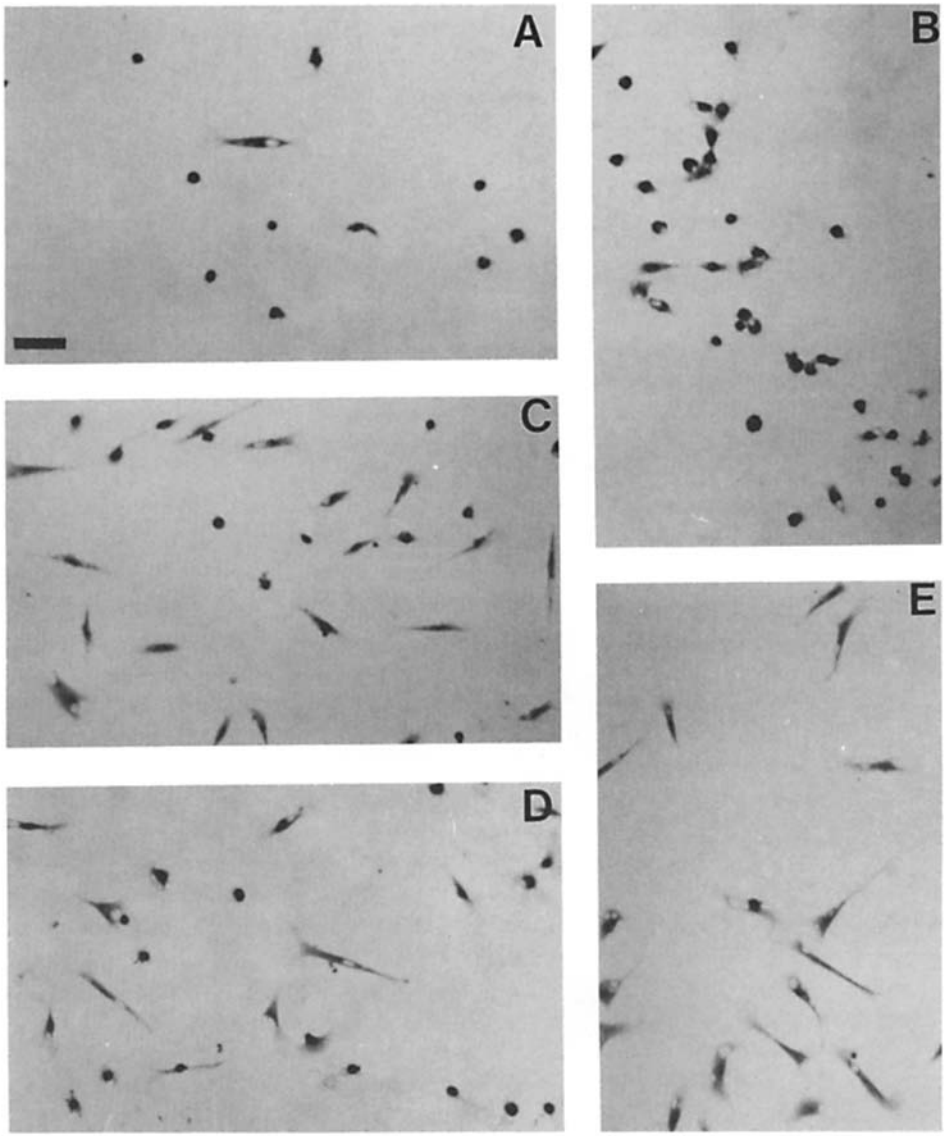


FIG. 5. Light microscopic appearance of migrating cells following 72 h of culture of ICMs on surfaces precoated with laminin (A; 10 μg/ml), Matrigel (B; 1:10 dilution), fibronectin (C; 5 μg/ml), collagen IV (D; 5 μg/ml), or collagen I (E; 5 μl/well). The bar represents 25 μm.

in both cases reached about 40% of that measured on collagen IV by the end of the 72-h culture period (Fig. 3).

To ensure that the Matrigel coating still contained all of the components present in the undiluted gel, cell migration was also evaluated on Matrigel applied to derivatized glass (Matrigel applied to untreated glass used as controls). Proteins within a mixture bind to derivatized glass in a concentration-dependent manner [29, 30], and this property of activated glass has previously been utilized for application of diluted Matrigel to culture surfaces [19]. The responses of the cultured ICMs were remarkably similar between the derivatized glass and the untreated glass treatment groups (Table 1) and were not different from those observed when Matrigel was applied to tissue culture plastic (Fig. 3). Specifically, culture on undiluted Matrigel was associated with virtually no cell translocation, whereas culture on a 1:10-diluted coating of Matrigel supported appreciable and equivalent extents of cell migration (10-fold or more by 72 h) in both the untreated and derivatized glass culture groups (Table 1).

A similar influence of substratum mechanics on cell translocation was noted when the ICMs were cultured on gels versus coatings of type I collagen (Fig. 4). The migration of PE-like cells occurred on both substrate preparations throughout the 72-h culture period, with significant increases ($p < 0.01$) in the mean areas of the ICM-derived cell clusters recorded at each 24-h interval. However, cell migration on the 1-mm-thick collagen gels reached only 30%

TABLE 2. Means of individual cell areas* following 72 h of culture on coatings of each of the extracellular matrix substrata under study.

Culture substratum	Number of cells measured	Mean area (μm^2 ± SEM)
Collagen IV (5 μg/ml)	150	539 ± 21
Fibronectin (5 μg/ml)	165	310 ± 15
Collagen I (10 μl)	119	225 ± 16
Laminin (10 μg/ml)	119	223 ± 15
Matrigel (1:10)	141	162 ± 9

*Area defined by each cell on the culture surface.

TABLE 3. Means of the individual cell areas, perimeters, and contour indices for the collagen I- and laminin-cultured cells presented in Table 2.

Culture substratum	Area (μm^2)	Perimeter (μm)	Contour index*
Collagen (10 μl)	225 ± 16	85.7 ± 4.4	6.1 ± 0.2
Laminin (10 μg/ml)	223 ± 15	71.3 ± 3.3	5.0 ± 0.1

*A cell in the shape of a perfect circle has a contour index of 3.54.

of that measured on collagen IV coatings (72 h of culture), whereas that on the collagen I coatings reached 70–80% of the collagen IV-associated cell translocation (Fig. 4). Indeed, a comparison of the mean projected areas of the ICM-derived cell clusters following 72 h of culture on coatings of either collagen I (5 or 10 μl) or collagen IV (10 μg/ml) revealed that they were not significantly different ($p > 0.05$).

Substratum and Cell Morphology

The morphology of the migrating cells was influenced by the composition of the extracellular matrix substratum (Fig. 5). The cells migrating on all of the extracellular matrix coatings were a heterogeneous population, with some undergoing extensive flattening and elongation and others being more rounded. Presumably some of the rounded cells were preparing to undergo mitosis. However, coatings of fibronectin and collagen (both at 5 μg/ml; Fig. 5, C–E) were associated with a higher proportion of very spread cells than were coatings of laminin (10 μg/ml; molecular weight twice that of fibronectin or collagen IV) or diluted Matrigel (Fig. 5, A and B). When the individual projected areas of 119–165 randomly chosen migrating cells were measured for each of the five culture substrata, it was found that the most cell spreading ($p < 0.05$) occurred on type IV collagen (Table 2). A coating of fibronectin was also associated with a high proportion of elongated cells, whereas cells migrating on laminin or a diluted Matrigel coating had smaller projected areas ($p < 0.05$; Table 2). Interestingly, the mean projected areas of the cells migrating on collagen I and laminin were not different ($p > 0.05$), despite our visual observations that many of the collagen I-cultured cells appeared to be quite elongated while the laminin-cultured cells were, in general, taller and more rounded (compare Fig. 5A with Fig. 5E). A further comparison of mean cell perimeters and contour indices confirmed this difference between the two culture groups of ICMs (Table 3). The mean perimeters of the collagen I-cultured cells were 20% higher ($p < 0.01$) than those of the cells maintained on laminin. This observation suggested that despite the similar cell areas, the cells on collagen I had more tortuous or extended cell outlines than those on laminin. In support of this notion, the collagen I-cultured cells had a mean contour index value of 6.1 ± 0.2, while that of the cells migrating on laminin was 5.0 ± 0.1 ($p < 0.01$). Since a perfect circle has a contour index of 3.54, a mean contour index of 5.0 reflects a cell population having a higher proportion of more rounded cells of regular outline than does a mean contour index of 6.1. This difference in cell shape between the two culture groups is compatible with the less motile nature of the rounded, laminin-cultured cells versus the collagen I-cultured cells, whose more active translocation would require the coordinated extension and retraction of cytoplasmic regions.

Figure 6 summarizes the appearance, by scanning electron microscopy, of representative cells cultured on fibronectin (A-C), collagen IV (D-F), and collagen I (G-I). It should be noted that with the exception of the two cells that were just completing mitosis (Fig. 6B, small arrows), the fibronectin-cultured cells had a broad, flattened appearance. In some instances, the direction of migration was suggested by a peripheral region of tremendous spreading and extension of the cytoplasm (Fig. 6, B and C, large arrowheads). Similarly on collagen IV, considerable cell flattening and extension occurred, although a variety of cell shapes were observed (Fig. 6, D-F). Variable degrees of cell extension were seen on type I collagen (Fig. 6, G-I). Some cells were quite narrow (Fig. 6, G and H), and others appeared very rounded (Fig. 6I). In some cases, cells had extended processes under collagen I fibrils (Fig. 6, G-I, arrowheads).

In contrast, the majority of cells migrating from ICMs cultured on coatings of laminin (Fig. 7, A-C) or of Matrigel (Fig. 7, D-F) remained considerably more rounded and tall. Some long and narrow cells were seen on these substrata. This is more readily apparent in Figure 7, A and D; each of these low magnification micrographs shows an entire ICM-derived cell complex. Thin cytoplasmic processes were often seen, extending from many of the laminin- and Matrigel-cultured cells (Fig. 7, B and F, arrowheads). However, these processes represented only a small proportion of each cell's cytoplasm; the rest was contained within a tall, rounded cell body. The processes extending outward from the two daughter cells just completing mitosis (Fig. 7C, arrowheads) were especially prominent.

Substratum and Cellular Immunoreactivity for Extracellular Matrix Glycoproteins

Studies using tissues obtained at midgestation have shown that collagen IV and laminin are two markers of mouse and rat parietal endodermal cells [3, 31, 32]. Figure 8 summa-

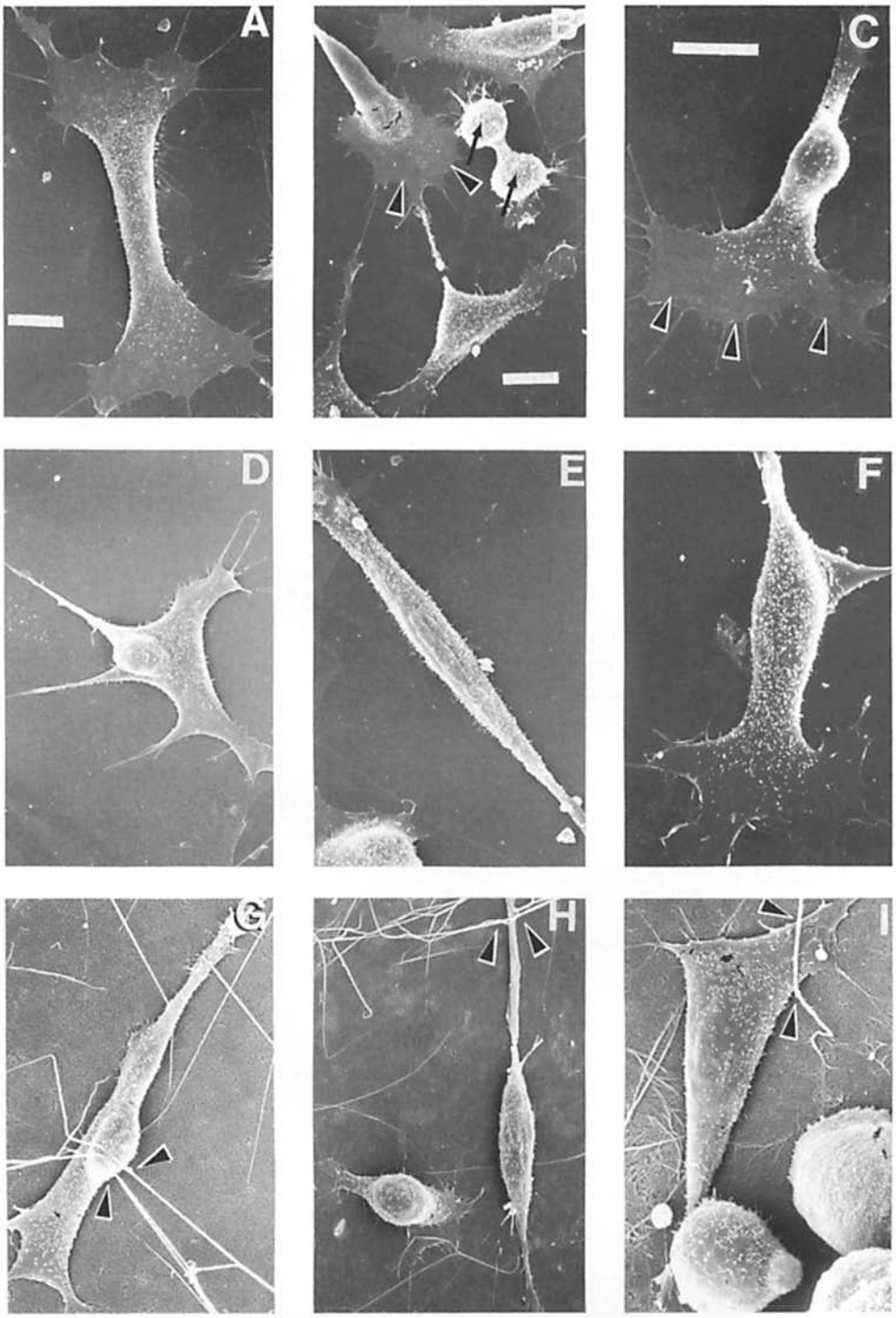


FIG. 6. Representative cells migrating from ICMs that have been cultured (72 h) on surfaces precoated with fibronectin (A-C; 5 μg/ml), collagen IV (D-F; 5 μg/ml), or collagen I (G-I; 5 μl/well). The small arrows in B indicate two cells completing mitosis, while the large arrowheads in B and C indicate peripheral regions of extensive cytoplasmic spreading by these translocating cells. Collagen I fibrils are indicated by the arrowheads in G-I. The bars in A, B, and C indicate 10 μm; they are representative of the magnifications of the each of these figures and of the two figures that appear below each, respectively.

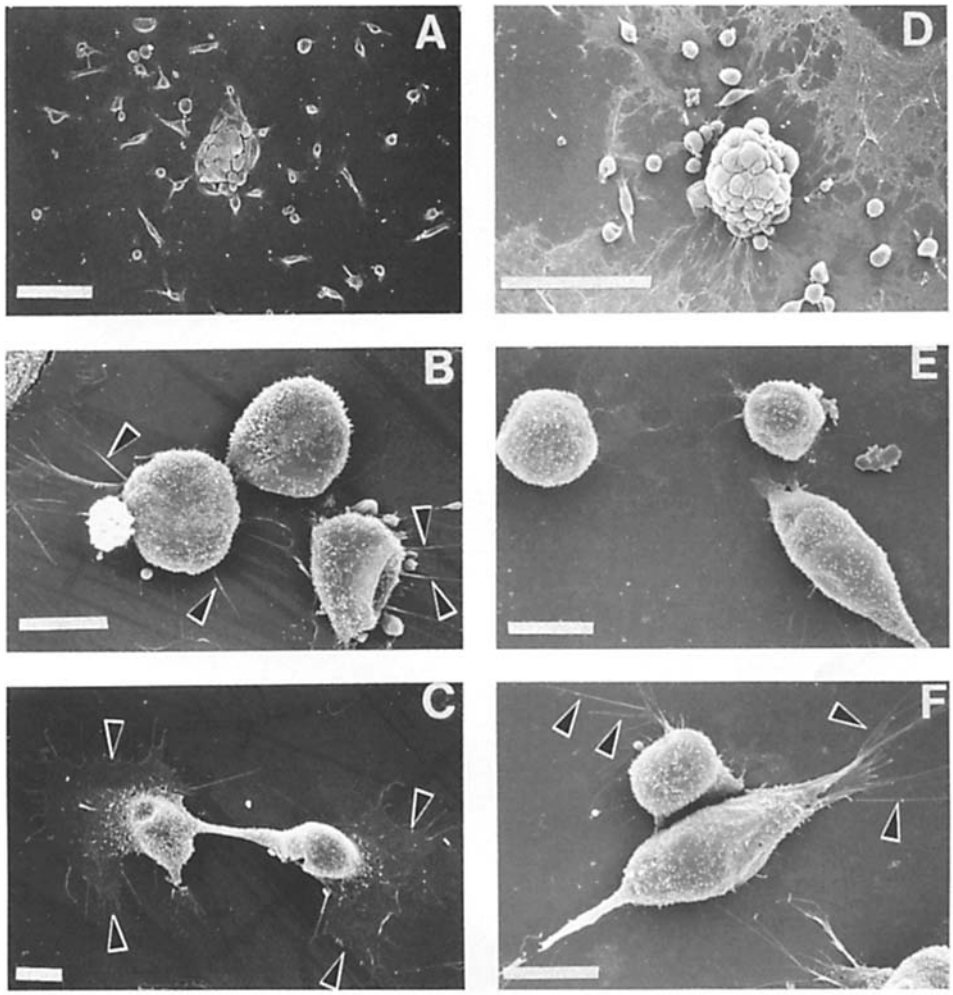


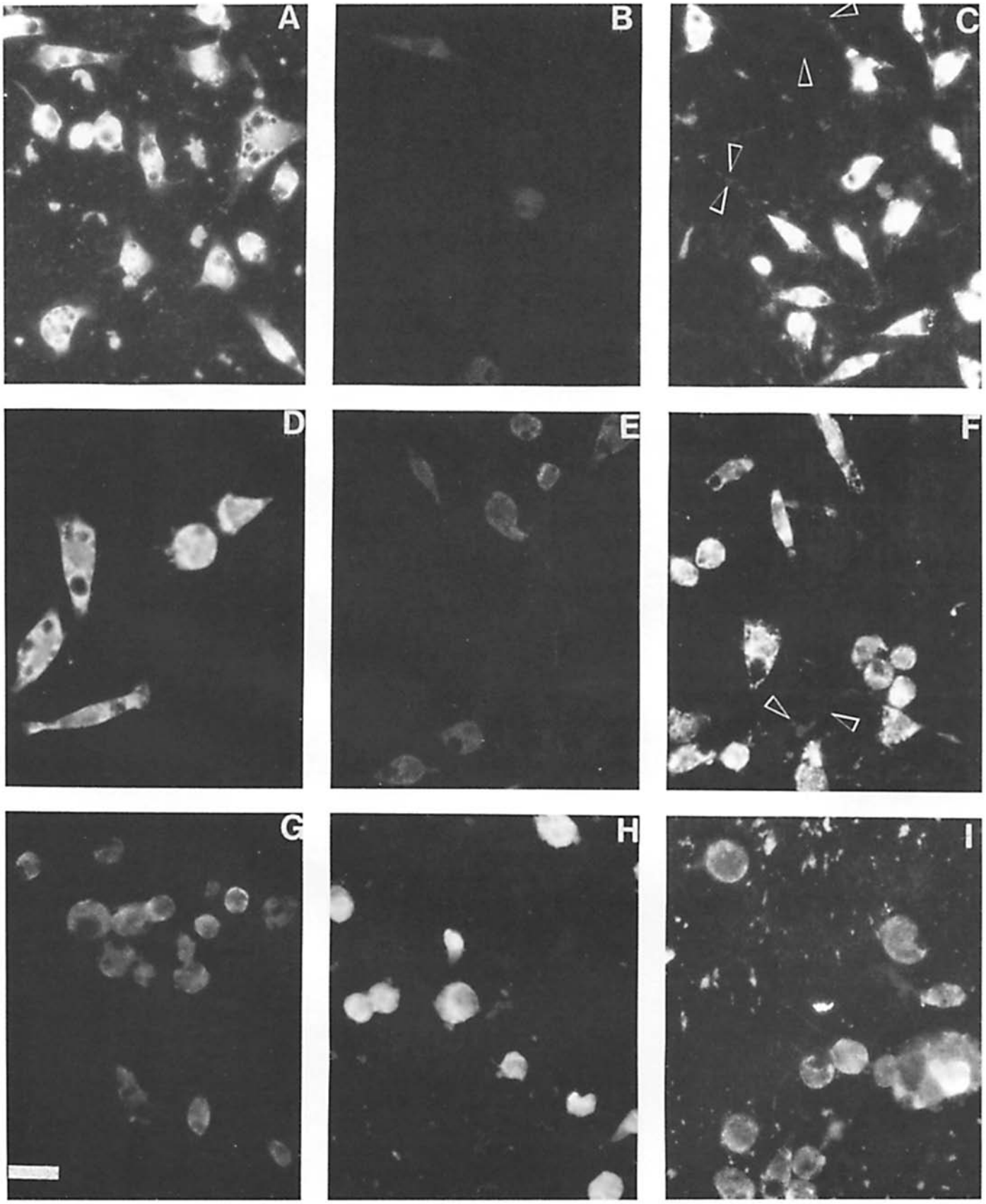
FIG. 7. Typical morphology of migrating cells following 72 h of culture of ICMs on surfaces precoated with laminin (A-C; 10 μg/ml) or Matrigel (D-F; 1:10 dilution). The arrowheads in B and F show long, thin cytoplasmic processes extending from rounded cells, while those in C indicate the extensive peripheral cytoplasmic flattening of two cells on the laminin substrate that are completing mitosis. The bars in A and D each indicate 100 μm; those in B, C, E, and F each represent 10 μm.

rizes the immunostaining patterns for fibronectin, laminin, and collagen IV within representative ICM-derived cells following 72 h of culture on each of these three extracellular matrix components. Cells on either collagen IV or fibronectin stained strongly for the presence of both collagen IV (Fig. 8, A and D) and laminin (Fig. 8, C and F). In addition to the high level of laminin immunoreactivity associated with each cell, substratum-bound laminin was observed between cells (Fig. 8, C and F, arrowheads; identified by comparing phase-contrast and immunofluorescence images of these cells) and presumably represents trails deposited by the migrating cells. Substratum-associated laminin was not found in the areas between entire ICM-derived cell clusters. In contrast, immunostaining for fibronectin was virtually nonexistent within the ICM-derived cells migrating on fibronectin or on collagen IV (Fig. 8, B and E).

Cells migrating on laminin demonstrated a different immunostaining pattern for all three glycoproteins (Fig. 8, G-I). These cells also tended to be less adhesive; whole ICM-derived cell clusters remained attached less frequently throughout the permeabilization and washing procedures associated with immunolocalization (Carnegie, unpublished observation). Immunostaining for collagen IV (Fig. 8G) and for laminin (Fig. 8I), while still present, was less intense than that observed in the fibronectin- and collagen IV-cultured cells. Immunostaining for fibronectin revealed a mixed cell population. In general, the staining for fibronectin was more intense than that seen in the collagen IV- and fibronectin-cultured ICM-derived cell clusters; fibronectin immunoreactivity was especially prominent in those laminin-cultured cells having an overall rounded morphology (Fig. 8H).

DISCUSSION

Reichert's membrane is a basement membrane containing type IV collagen, fibronectin, laminin, entactin, and he-



paran sulfate proteoglycan [2–4, 33]; its deposition between the TE cells and the emerging PE layer begins soon after blastocyst formation [1]. The role(s) that various components of this basement membrane play in facilitating and/or directing the migration of ICM-derived PE cells within the developing embryo remains to be defined. However, the involvement of these components is suggested by the presence of basement membrane material (notably fibronectin) on the inner surfaces of and/or within the TE cells of rat, mouse, and pig blastocysts at the time that this developmental event is beginning [34–36]. We have previously reported that the in vitro migration of ICM-derived PE-like cells occurs on fibronectin, collagen IV, and laminin [13]. Indeed, a collagen IV coating concentration as low as 160 ng/ml is sufficient to support ICM attachment and the outward migration of laminin-containing PE-like cells [13].

In the present study, the concentration-related support of migration by fibronectin and laminin was evaluated. As previously observed for collagen IV [13], ICM attachment to fibronectin and the migration of PE-like cells showed an "all-or-none" rather than a dose-response relationship. A coating level of 2.5 μg/ml was required for cell translocation, and further increases in the coating concentration did not elicit more cell displacement. A trend toward a dose-response relationship was observed, however, for the three highest coating concentrations of laminin. A minimal laminin coating level similar to that of fibronectin was required for cell translocation; this coating concentration is 15-fold higher than the concentration required when collagen IV is used as the substrate [13]. Interestingly, in studies evaluating the outgrowth of PE-like cells from PSA-1 teratocarcinoma cell-derived embryoid bodies, it was found that cell migration is supported 10-fold more effectively by fibronectin than by collagen IV or laminin, and that collagen IV coating concentrations as high as 40 μg/ml have been associated with only 25% endoderm outgrowth [12]. We have also found the outgrowth of PE-like cells from F9 teratocarcinoma cell-derived embryoid bodies to preferentially occur on fibronectin or laminin over collagen IV (Carnegie, unpublished observation). These differences in cell-matrix glycoprotein interactions between the different sources of PE-like cells are worthy of further study. Comparisons of extracellular matrix receptor expression by different sources of migrating PE-like cells (ICMs versus teratocarcinoma cells), and during culture on various basement membrane components, may provide additional insight into the influences of these extracellular matrix glycoproteins on cell translocation and morphology.

The influence of matrix composition on migration has been widely studied and, in some cases, cells do not appear to differentiate between the matrix glycoproteins provided to them in culture. In a study in which fibronectin-laminin boundaries were presented to 16 different types of cells, 14 cell types migrated equally well on fibronectin and on laminin, while each of the remaining two preferred one substrate over the other [37]. Cell-specific migratory responses to various matrix components have also been reported. For example, murine skeletal myoblasts migrate more rapidly on laminin than on fibronectin [22], whereas aortic endothelial cells do not migrate at all on laminin but show appreciable translocation in response to collagen IV [38]. We found that the migration of PE-like cells occurred when ICMs were cultured on coatings of fibronectin, laminin, or collagen IV. However, differences in cell responses to these substrates were observed in that migration was less extensive on laminin, and was most sensitive to the presence of collagen IV.

Coating concentrations of laminin or of fibronectin permissive of cell migration were also associated with proliferation of the migrating cells. This is not to say that cell replication did not occur within the ICMs that remained free-floating when subsupportive coating concentrations of either glycoprotein were used. Indeed, nuclear counts obtained using Hoechst 33342 [17] revealed 4- to 5-fold increases in cell numbers over the 3-day culture period within the free-floating ICMs (Carnegie, unpublished observation). However, of interest to us in the present study was the possible existence of matrix-associated effects on the replication of PE-like cells in vitro. As for migration, the rate of cell replication was higher on each attachment-supporting concentration of fibronectin compared to laminin. It is of interest that thymidine incorporation by human keratinocytes was also higher when these cells were cultured on fibronectin or collagen IV than on laminin or plastic [39].

These differential influences on cell migration and proliferation appear to be glycoprotein-specific rather than to reflect different coating efficiencies of the three basement membrane components. We have previously compared the coating efficiencies of these glycoproteins and found them to be remarkably similar for collagen IV and fibronectin, and higher by about 50% for laminin [13]. Hence, the reduced extent of cell migration and proliferation on laminin appears not to be due to decreased availability of this glycoprotein to the cells.

Even more than composition, the physical properties of the matrix exerted dramatic effects on the migration of the ICM-derived PE-like cells. Cultured cells, whether they are spreading on a substratum or translocating across it, exert measurable forces as they extend processes, form attachments, and displace cytoplasm [40]. For cell spreading and/or migration to be permitted, the substratum has to be suf-

FIG. 8. Immunolocalization of collagen IV (A, D, G), fibronectin (B, E, H), and laminin (C, F, I) within ICM-derived cells following 72 h of culture on surfaces precoated with collagen IV (A-C), fibronectin (D-F), or laminin (G-I). The cells were incubated with appropriate primary and FITC-labeled secondary antibodies (see *Materials and Methods*) after fixation in 3.7% paraformaldehyde and permeabilization with 0.1% Nonidet P-40. The arrowheads (C, F) indicate substratum-associated laminin present between the migrating cells. The bar represents 10 μm.

ficiently rigid to resist these cell-induced physical stresses. Ingber [41] reported dose-related support of capillary endothelial cell spreading and proliferation by substratum-bound fibronectin. However, if the fibronectin was instead presented to the cells as soluble glycoprotein or fibronectin complexed to microcarrier beads, this also bound to cell surface receptors, but did not support cell spreading or DNA synthesis [41]. Similarly, we found that if ICMs were cultured on gels of collagen I or of Matrigel, the extension of cytoplasmic processes and the translocation of the ICM-derived cells either did not occur (Matrigel) or did so to a very limited extent (collagen I). On the other hand, PE-like cells did migrate out from ICMs cultured on the more firmly attached coatings of either of these extracellular matrices. Similarly, the outgrowth of PE-like cells from teratocarcinoma bodies plated on coatings of collagen I has been reported [42].

On the other hand, the development and/or maintenance of a differentiated phenotype is associated with the culture of cells such that their morphology remains tall and rounded rather than spread. By varying only substratum deformability, Opas [19] demonstrated that the differentiated traits of retinal pigmented epithelial cells are fully maintained only when the substratum is sufficiently pliable to prevent cell spreading. Furthermore, the release of gels of collagen I or Matrigel from their attachment to the culture dish results in higher levels of expression of the β-casein gene by mouse mammary epithelial cells than do culture of these cells on substratum-bound gels [43].

We found that the morphology of PE-like cells migrating from rat ICMs was influenced by the composition of the culture substratum. Cells migrating on coatings of laminin or of Matrigel were, in general, taller and more rounded than those translocating on collagen IV or fibronectin. As mentioned previously, laminin was also the matrix component associated with reduced cell migration and proliferation. Studies involving other cell types have linked laminin with differentiation-promoting rather than migration- and proliferation-supporting roles. As well as being less supportive of keratinocyte replication than either fibronectin or collagen IV [39], laminin also reduces cell migration [44]. Laminin maintains β-casein mRNA levels within cultured mouse mammary epithelial cells, whereas fibronectin and collagen IV do not [43]. Furthermore, the organization of intestinal epithelial cells into hollow tubes within Matrigel is inhibited by antibodies to laminin and a laminin-specific peptide, but not by antibodies to collagen IV [45]. Finally, the culture of rat Sertoli cells on laminin versus plastic has been associated with the assumption of a cuboidal to low-columnar, rather than a squamous, morphology as well as with an increased amount of plasma membrane-associated $G_{s\alpha}$ protein [46].

In the current study, ICM-derived cells migrating on laminin contained more fibronectin but less collagen IV or laminin than cells migrating on either collagen IV or fibronectin. Even though the presence of collagen IV and laminin and the absence of fibronectin are characteristic of midgestation PE [3, 31, 32], some evidence does exist for fibronectin within mouse PE at an earlier developmental stage [34]. The latter observation involved Day 7 embryos; both Reichert's membrane and the attached PE cells stained strongly for this adhesive glycoprotein. The role of this transient expression of fibronectin by the PE layer and its regulation remain to be determined. However, using individual basement membrane components, we have shown that differentiation-related characteristics of the ICM-derived cells can be influenced simply by the substratum-bound glycoprotein with which they are in contact. In addition to PE cells, rat TE cells have also been shown to contain collagen IV, fibronectin, and, subsequently, laminin [36, 47]. Hence, the potential exists for the composition of Reichert's membrane to be varied by both PE and TE throughout the course of PE cell migration and differentiation. The time-related appearance of or changes in the proportions of different basement membrane components could allow the basement membrane to sequentially support cell migration and various aspects of cell differentiation as the PE layer and Reichert's membrane itself develop.

In conclusion, while ICM attachment and the outward migration of PE-like cells occurred on coatings of fibronectin, collagen (I and IV), laminin, and Matrigel, cell translocation and replication were supported best by either collagen IV or fibronectin. The in vitro displacement of PE-like cells appeared to occur when the substratum was sufficiently rigid to resist the physical forces transmitted by the migrating cells. This latter property implies the existence of an important role for the TE in anchoring Reichert's membrane components during PE cell migration in the implantation-stage blastocyst. Finally, and perhaps most importantly, PE cell morphology and content of extracellular matrix glycoproteins were differentially affected by the basement membrane components used for culture. This observation, combined with findings that both PE and TE cells can synthesize various components present within Reichert's membrane, suggests the potential means through which this basement membrane can progress from supporting cell migration and proliferation to promoting various aspects of PE cell differentiation during the early stages of embryonic development in the rat.

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