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The Effects of Laminin on the Growth and Differentiation of Embryonal Carcinoma Cells in Defined Media

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In this paper we have examined the growth and differentiation of the embryonal carcinoma cell line, F_9, in the defined medium EM-3 at low density. We show that the growth of F_9 and their differentiated cells (F_9-diff) in EM-3 is strongly density dependent. At low cell densities the growth of both cell types is severely limited and most of the cells do not survive. Although this poses a problem for working with F_9 and F_9-diff in EM-3, it provides a convenient assay for identifying molecules that support their growth at low density. Using this assay, we have determined that laminin, a newly isolated glycoprotein of basement membranes, significantly improves the growth and short-term survival of both F_9 and F_9-diff. However, addition of laminin to EM-3 is insufficient to promote the clonal growth of these cell types. Our findings also indicate that laminin promotes the attachment of F_9 and F_9-diff in defined media. On the basis of our results, we propose an attachment function for laminin during the early stages of mammalian development.

Key words: **embryonal carcinoma cells, laminin, extracellular matrices, basement membranes, retinoic acid, embryogenesis, parietal endoderm**

It is firmly established that embryonal carcinoma (EC) cells (the stem cells of teratocarcinomas) can colonize early mouse embryos and participate in normal development [1–4]. When one or a few mouse EC cells are injected into mouse blastocysts (64-cell stage) and these are placed into foster mothers, a proportion of the resulting offspring possess a wide range of normal differentiated cell types that are derived from the donor EC cells. In at least one case, the EC cells have given rise to viable sperm [2], thus, unequivocally demonstrating that EC cells are totipotent (ie, able to differentiate into any cell type). In contrast, when the same EC cells are injected subcutaneously into a syngeneic or nude mouse, they give rise to a teratocarcinoma or teratoma – highly differentiated but disorganized tumors. These results clearly demonstrate that the environment of

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the EC cell plays a decisive role in determining its pattern of differentiation. While it can be anticipated that a combination of factors is involved (cell–cell interactions and/or interactions with appropriate molecules, eg, hormones, growth factors, adhesion factors), their identity remains a mystery. Clearly, important insights into the control of mammalian embryogenesis can be gained by examining the role of the extracellular environment.

Little is known about the role of the extracellular environment (both the maternal and the microenvironment within the embryo) during the early stages of development – a time when important deterministic events are known to occur [5]. Studies of cultured mouse embryos have shed some light on this matter. Development in vitro to the early blastocyst stage has been shown to take place in simple media whose formulations include salts, glucose, pyruvate, lactate, and bovine serum albumin [6]. However, for development to proceed further, the culture medium must include amino acids and serum [7–10]. Although serum-containing media are quite unlike the uterine environment, development of the inner cell mass in vitro proceeds to at least the egg cylinder stage in a manner that is sequentially, morphologically, and biochemically very similar to development in vivo [11].

These findings clearly demonstrate that serum factors support the early stages of development. Hence, their identification should facilitate a better understanding of the role of the extracellular environment. Attempts to determine the serum factor requirements of developing embryos, using the embryos themselves, have had only limited success [10, 12]. Given the number of serum factors that must be examined, it is impractical to test each one directly with embryos, since embryo preparation is very time-consuming and expensive. Consequently, in order to pursue this matter, Rizzino and co-workers [13–16] have turned to the use of EC cells, which 1) as cell lines, can be produced in large quantities; 2) have been shown to mimic, both morphologically and biochemically, important stages of early mammalian development [17]; and 3) require serum for growth and differentiation.

Recently, Rizzino and Crowley have developed a defined medium that permits indefinite growth of the EC cell line, F_9, without loss of its differentiative and tumorigenic properties [15]. In this medium, known as EM-3 (Embryonic Medium 3), serum has been replaced with insulin, transferrin, and the plasma form of fibronectin (cold-insoluble globulin – CIG). The F_9 EC cells can be induced to differentiate in EM-3 just as they do in serum-containing media when retinoic acid is added [15, 18–20]. A disadvantage of culturing F_9 and its differentiated cells in EM-3, instead of in serum-containing media, is that these cells do not grow or remain attached in EM-3 at low cell densities. Although this poses a technical problem for working with F_9 cells, it does provide a convenient assay for the identification of molecules that can promote the growth and attachment of the cells in EM-3 at low density. One of the molecules that we have examined is laminin. Laminin is a newly discovered glycoprotein (mol wt 850,000), which has been found in all basement membranes examined [21]. It is produced by epithelial cells [21] and can mediate the attachment of several epithelial cell lines in the absence of serum [22]. More recently, Leivo et al have determined that laminin is present in early mouse embryos, starting as early as the 16-cell stage [23].

In this report, we demonstrate that laminin can significantly promote the growth and attachment of F_9 and its differentiated cells at low density in EM-3. It is likely that our findings are relevant to early mammalian development, and they suggest an attachment function for laminin during embryogenesis.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: Dulbecco's modified Eagle's medium (DME) and nutrient mixture F-12 (Ham's F-12) from Gibco; fetal bovine serum and horse serum from Reheis; bovine insulin and human transferrin from Sigma; human plasma fibronectin (CIG) from Collaborative Research; all-trans-retinoic acid from Eastman Kodak Co.; plasticware for cell culture from Falcon.

Stock solutions of insulin and transferrin were prepared and stored as previously reported [13]. Stock solutions of retinoic acid (1 μM) were prepared in absolute ethanol (protected from light) and stored at -20°C.

Cell Cultures

Stock cultures of F_9 cells were grown as previously described [13]. Cells grown in defined media were cultured in EM-3, except where indicated in the text. EM-3 is composed of DME and Ham's F-12 (50:50) plus 15 mM Hepes buffer, 1 μg/ml insulin, 5 μg/ml transferrin and 1 $\mu g/cm^2$ CIG. The choice of basal medium is important. If DME is used alone, little or no growth occurs [A. Rizzino, unpublished data]. If Ham's F-12 is used alone, growth occurs, but at a significantly slower rate than when the DME:F-12 mixture is employed [A. Rizzino, unpublished data]. When CIG was used, the culture medium was added after the tissue culture dish had been coated with CIG. This was done as follows: CIG was added to culture dishes containing PBS, quickly mixed, incubated at 37°C for 15 min and washed once with PBS. When laminin and CIG were used together, the laminin was added after the culture dishes had been coated with CIG. In these cases, laminin was added to culture dishes containing medium. The dishes were then incubated for 30 min at 4°C, followed by 30 min at 37°C. 35 mm dishes were used for all experiments. Cells to be counted were removed from the culture dish with 0.1% trypsin PBS-EDTA. Medium containing serum was used to stop the action of trypsin. The cells were then collected and counted in a Coulter Counter.

Preparation of Conditioned Culture Dishes (CCD)

F_9 cells were plated at 2×10^4 cells/cm^2 and grown to high cell densities, $1.5-2 \times 10^5$ cells/cm^2, in EM-3. The cells were then removed with PBS containing 1 mM EDTA. This removes approximately 95% of the cells. The surface of each culture dish was then treated for 10 min with sterile water to remove the few remaining cells. Trypsin is not needed and, in fact, destroys the adhesive properties of CCD.

Preparation of Laminin

In the experiments presented here, laminin preparations of two different purities were used. Highly purified laminin was used in some experiments. It was prepared according to the procedures of Timpl et al [21] and was free of detectable contaminants, as determined by SDS polyacrylamide electrophoresis. In the text it is referred to as "highly enriched laminin." In other experiments, "partially purified laminin" was used. By comparison to the highly enriched laminin, the partially purified preparations were approximately half as active in promoting the attachment of epithelial cells in the absence of serum [V. Terranova, unpublished data]. These preparations were purified according to the procedures of Timpl et al up to the stage prior to DEAE-cellulose chromatography.

RESULTS

Growth and Differentiation in Defined Media

F_9 EC cells can be grown indefinitely in a defined medium (EM-3) composed of equal amounts of DME and Ham's F-12 supplemented with insulin (1 μg/ml), transferrin (5 μg/ml), and CIG (1 μg/cm^2) [15]. When retinoic acid (10 nM) is added to EM-3, the F_9 cells undergo an irreversible cellular transition [15]. The differentiated cells that appear (to be referred to as F_9-diff) exhibit at high density a morphology characteristic of parietal endoderm [24], similar to that of the parietal endoderm-like cell line PYS-2 and unlike that of F_9 in either EM-3 or serum-containing media (Fig. 1). Furthermore, the differentiated cells formed in EM-3 secrete plasminogen activator [15], as do the cells formed in media containing serum and retinoic acid [18].

There are three prominent differences between the growth of F_9 cells in EM-3 and in serum-containing media. 1) The extent of cell spreading is reduced in EM-3, which causes their morphology to differ from that of F_9 in serum-containing media (compare Fig. 1A to 1B). 2) The cells grow more slowly in EM-3. When the F_9 cells are initially switched from serum-containing media to EM-3, they grow for several generations at a rate equivalent to that in serum-containing media. However, after growth in defined medium for 10 generations, when all traces of serum have been eliminated, the generation time is increased from approximately 16 to 30 h (Fig. 2). As mentioned in Materials and Methods, the composition of the synthetic portion of the medium is important in determining the growth rate. If Ham's F-12 is used in place of the DME:F-12 mixture, the generation time is significantly increased [A. Rizzino, unpublished results]. 3) The last, and most significant, difference between the use of EM-3 and serum-containing media is the failure of F_9 cells to grow in EM-3 at clonal densities.

Growth and Differentiation in Defined Media at Low Cell Density

F_9 cells will grow indefinitely in EM-3 if the cells are maintained at densities of 2×10^4 cells/cm^2 or higher and the medium is changed daily [15]. However, when the cell density was reduced below this level, growth was significantly affected, and many of the cells did not survive. These numbers relate to cells that have been cultured in EM-3 for more than 5 generations. By comparison, when cells were initially switched from serum-containing media to EM-3, they grew and survived at slightly lower densities (approximately 5-fold lower) due to carry-over of trace amounts of serum. Under these circumstances, growth was not significantly affected at densities as low as 5×10^3 cells/cm^2, but at 10^3 cells/cm^2 the cells did not survive for more than a few days. At the latter density, greater than 50% of the cells attached [A. Rizzino, unpublished observation], and there was a transient increase in cell number by day 2, but by day 4 the majority of these cells had detached and subsequently lysed (Table I). Similarly, when F_9 cells in EM-3 were exposed to retinoic acid at low cell densities, differentiation occurred as shown previously [15], but the F_9-diff exhibited limited growth and did not survive if the initial cell density was below 5×10^3 cells/cm^2. Once again, the cells attached at low density but did not remain attached (Table I; compare cell numbers on day 2 to those on day 4). These results demonstrate that the growth of both F_9 and F_9-diff in defined media is strongly density dependent. This poses a technical problem when working with F_9 and F_9-diff in EM-3 but provides a convenient assay for identifying molecules that support the growth of these cells at low density (see below).

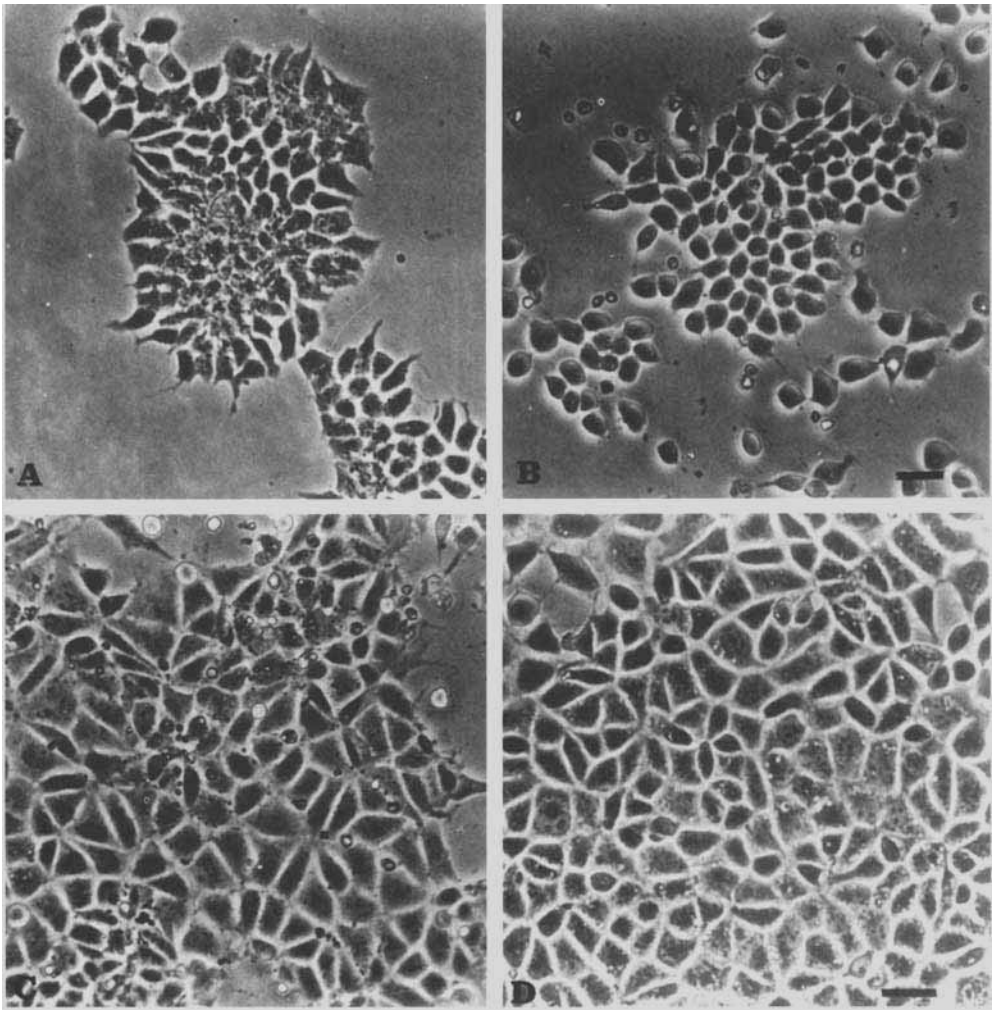

Fig. 1. The morphology of F_9 cells and PYS-2 cells in defined and serum-containing media. A) F_9 cells cultured in serum-containing medium (7.5% fetal calf serum). B) F_9 cells cultured in EM-3. C) Cells formed after F_9 cells were induced by retinoic acid to differentiate. The F_9 cells were exposed to retinoic acid in EM-3 for 48 h. At that time the medium was replaced with EM-3 without retinoic acid, and on day 5 the cells were photographed. D) PYS-2 cells growing in serum-containing medium.

The ability of the cells to grow at high, but not low, cell density strongly suggests that the cells condition their culture environment. Believing that the poor survival at low density was due to inadequate attachment, we examined the possibility of using "conditioned culture dishes" (CCD) to grow F_9 and F_9-diff at low density. To prepare CCD, F_9 cells were grown to high cell densities ($1.5–2 \times 10^5$ cells/cm^2) in EM-3, and the cells were removed as described in Materials and Methods. The effectiveness of CCD was tested by plating F_9 cells at a density of 2×10^3 cells/cm^2 onto CCD and control culture dishes containing EM-3 plus 10 nM retinoic acid. In both cases, differentiation occurred

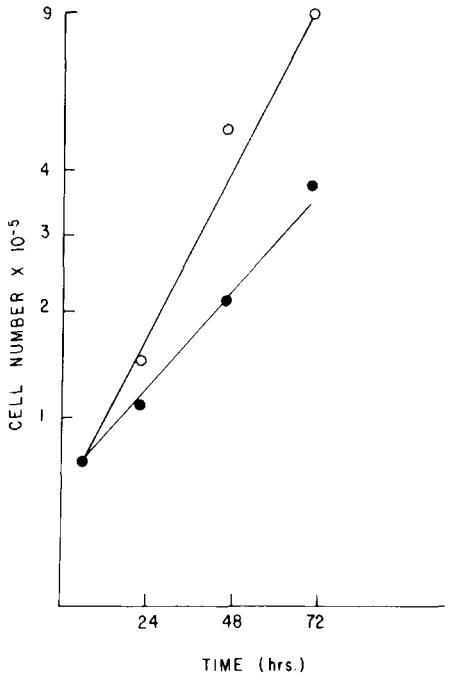

Fig. 2. The growth rate of F_9 in EM-3 and serum-containing media. At time zero 10^5 F_9 cells, which had been grown in EM-3 for 10 generations, were plated into either EM-3 (-•-) or into medium containing 7.5% fetal bovine serum (-o-). At the indicated times the cells were removed and counted with a Coulter Counter. Each point represents the average of 3 samples.

TABLE I. EC Cell Growth and Differentiation in EM-3: Effect of Cell Density*

	Day 0 cells/cm^2	Day 2 cells/cm^2	Day 4 cells/cm^2	Day 4:Day 0
F_9 cells	$1.0x10^4$	$5.0x10^4$	$7.7x10^4$	7.7
	$5.0x10^3$	$2.2x10^4$	$3.7x10^4$	7.4
	$2.0x10^3$	$5.7x10^3$	$4.5x10^3$	2.25
	$1.0x10^3$	$2.1x10^3$	$5.5x10^2$	0.55
	$5.0x10^2$	$1.0x10^3$	$1.9x10^2$	0.38
F_9-diff	$1.0x10^4$	$3.1x10^4$	$4.1x10^4$	4.1
	$5.0x10^3$	$1.1x10^4$	$7.2x10^3$	1.4
	$2.0x10^3$	$2.4x10^3$	$2.4x10^2$	0.12
	$1.0x10^3$	$9.0x10^2$	$1.4x10^2$	0.14
	$5.0x10^2$	$4.1x10^2$	$8.1x10^1$	0.16

*F_9 cells were plated at the indicated density in EM-3, or in EM-3 plus retinoic acid to induce differentiation. After 48 h or 96 h the cells were removed and counted with a Coulter Counter. Each value represents the average of two samples. On day 2 the medium was changed with EM-3.

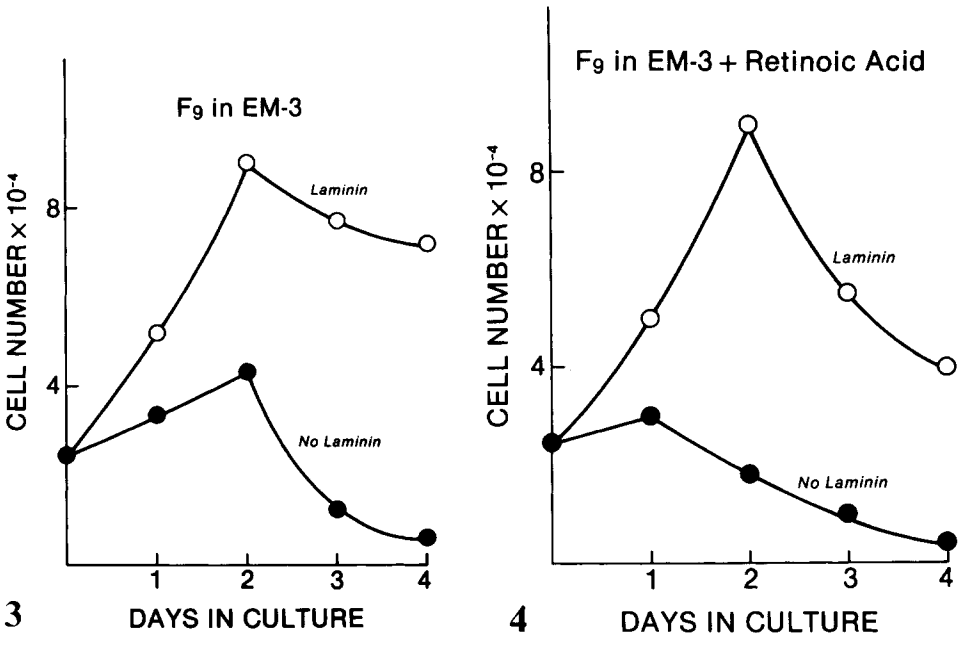

Fig. 3. The effect of laminin on the growth and survival of F_9 cells plated in EM-3 at low density. 2.5×10^4 F_9 cells were plated onto 35 mm culture dishes containing either EM-3 (-•-) or EM-3 plus 10 μg/ml of partially purified laminin (-o-). At the indicated times the cells were collected and counted with a Coulter Counter. Each value is the average of two samples. On day 2 the medium was changed, and the laminin cultures were refed with laminin.

Fig. 4. The effect of laminin on the growth and differentiation of F_9 cells plated at low density in EM-3 plus retinoic acid. 2.5×10^4 F_9 cells were plated onto 35 mm culture dishes containing either EM-3 plus 10 nM retinoic acid (-•-) or EM-3 plus 10 nM retinoic acid and 50 μg/ml of partially purified laminin (-o-). At the indicated times the cells were collected and counted with a Coulter Counter. Each value represents the average of two samples. On day 2 the medium was changed with EM-3 lacking retinoic acid, and the laminin cultures were refed with laminin.

(as determined by altered morphology) but, in direct contrast to cells plated onto untreated culture dishes, which did not survive (Table I), the differentiated cells continued to grow. After 6 days in culture on CCD, most cells exhibited the morphology of F_9-diff, and the cell number had increased to 2.1×10^4 cells/cm^2.

The Effect of Laminin on Low-Density Cultures

In order to determine the identity of the factors responsible for this result, we attempted to substitute laminin, a major component of basement membranes, for the CCD. When preparations enriched in laminin (see Materials and Methods) were added to EM-3, the growth and survival of both F_9 and F_9-diff were substantially improved. After 2 days the cell densities were 2- and 4-fold higher, respectively, in the presence of laminin (Figs. 3 and 4), but thereafter the cell numbers began to decrease, indicating that the addition of laminin to EM-3 does not allow indefinite growth of these cells at low den-

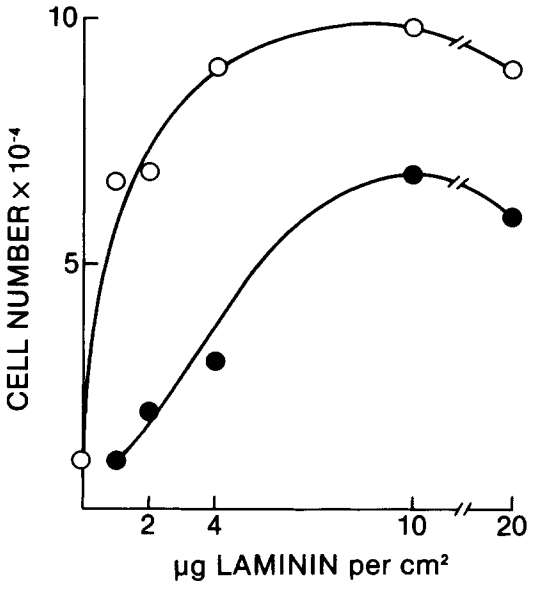

Fig. 5. The response of F_9 and F_9-diff to laminin. 2.5 X 10^4 F_9 cells were plated onto 35 mm culture dishes containing partially purified laminin at the indicated levels in EM-3 (-○-) or EM-3 plus 10 nM retinoic acid (-●-). After 48 h the medium was changed with EM-3 plus the indicated amounts of laminin. On day 4 the cells were collected and counted with a Coulter Counter. Each value represents the average of two samples. The amount of laminin added is expressed as $\mu g/cm^2$. The surface area of a 35 mm culture dish is approximately 10 cm^2.

sity. The laminin concentrations used in these experiments, 10 and 50 µg/ml, were optimal for F_9 and F_9-diff, respectively (Fig. 5), and it is noteworthy that F_9-diff requires approximately 5-fold more laminin than F_9 does to achieve a half maximal increase in cell number. In an attempt to improve the effectiveness of laminin, collagen-coated culture dishes were used. However, in preliminary experiments, the use of either type I or type IV collagen did not improve upon the above results.

Since partially purified laminin was employed in the above studies, we have begun to examine the effects of highly enriched laminin (free of contaminants, as determined by SDS polyacrylamide electrophoresis). In these experiments, CIG was omitted from the defined medium (EM-3). Under these conditions, the F_9 cells attach within 60 min and then most detach within 16 h, unless an attachment factor is present [15]. At high cell densities, highly enriched laminin can substitute for CIG and, like CIG, promotes both attachment and growth (Table II). At low cell densities, highly enriched laminin also improved the growth and survival of both F_9 and F_9-diff in defined media supplemented with only insulin and transferrin. As with partially purified laminin (Figs. 3 and 4), during the first 2 days the cell numbers increased 2- to 4-fold beyond those observed in culture dishes coated with CIG [A. Rizzino, unpublished data]. However, after day 2 the cell numbers decreased faster than in those cultures containing partially purified laminin, indicating that the cells respond to both laminin itself and to other molecules present in the partially purified preparations. These observations make two important points: 1) highly enriched laminin mediates attachment (CIG was not used and its use did not improve the results), and 2) it improves the growth of F_9 and F_9-diff at low density.

TABLE II. The Effect of Highly Enriched Laminin on the Growth of F_9 EC Cells*

Additions to basal medium	Final cell#:initial cell#
I + TF	0.5
I + TF + CIG	5.6
I + TF + laminin	6.3
7.5% FBS	5.9

*10^5 F_9 EC cells were plated onto 35 mm culture dishes containing basal medium (DME:F-12) plus the indicated additions. I = insulin (1 μg/ml); Tf = transferrin (5 μg/ml); CIG = cold-insoluble globulin (1 μg/cm^2); laminin = highly enriched laminin (5 μg/ml). After 48 h the cells were removed and counted with a Coulter Counter. Each value represents the average of two samples.

DISCUSSION

In this paper we have examined the growth and differentiation of F_9 EC cells in the defined medium EM-3 at low density. In contrast to their ability to proliferate indefinitely at high density [15], F_9 cells do not survive at densities lower than 2×10^4 cells/cm^2. This is also true for the differentiated cells (F_9-diff), which are formed when retinoic acid is added to EM-3. This density dependency strongly suggests that these cells condition their culture environment. We have observed that F_9 cells grown at high density in EM-3 do condition their substratum. Presumably, this occurs by the secretion of attachment factors onto the surface of the culture dishes, which then mediate the attachment and permit the growth of F_9 and F_9-diff at low density. In our attempts to identify the active molecules, it was determined that laminin promotes the attachment, growth, and short-term survival of both cell types in defined media at low density.

Our results also indicate that addition of laminin to EM-3 is insufficient to permit long-term growth or continued attachment at low density. This suggests that other molecules are necessary. The fact that purer laminin preparations were less effective argues that these molecules may be basement membrane components – eg, type IV collagen and proteoglycans. In experiments with laminin and culture dishes coated with type IV collagen, we observed that the attachment of F_9 was substantially less than when laminin alone was used. Since laminin binds to type IV collagen [22], this finding was unexpected. However, this result might be explained by competition between type IV collagen and F_9 cells for the available laminin. Thus, pitfalls of this nature must be considered when attempting to identify molecules that will complement laminin.

Before we discuss our data further, we will summarize what is known about laminin. Laminin has recently been purified from a transplantable tumor that produces a basement membrane [21]. It is a glycoprotein and consists of at least two polypeptide chains (mol wt 200,000 and 400,000). Antibodies produced against laminin do not react with fibronectin but do react with basement membranes of placenta, kidney, and skin [21]. This suggests that laminin, or a close relative, is present in each of these basement membranes. Recently, Chung et al have isolated a glycoprotein from an extracellular matrix secreted by an EC-derived cell line [25]. This glycoprotein, GP-2, reacts with antibodies made against laminin [21], and thus the two appear to be closely related. Similarly, it has been demonstrated that another EC-derived cell line, PYS, produces

laminin [21]. More recently, Leivo et al have detected the presence of laminin (or an immunologically related molecule) in mouse embryos as early as the 16-cell stage [23]. At later stages, the antibody to laminin reacts with the inner cell mass and, still later, reacts very strongly with Reichert's membrane, which is produced by parietal endoderm. This suggests that parietal endoderm secretes large amounts of laminin, a possibility that is supported by studies with EC cells in vitro. When F_9 cells are induced by retinoic acid to differentiate, the differentiated cells (which exhibit properties of parietal endoderm) produce a basement membrane that strongly reacts with an antiserum prepared against the basement membrane of embryoid bodies [26]. Since this antiserum specifically precipitates both subunits of laminin [Linney and Oshima, personal communication], it would appear that the differentiated cells, in contrast to F_9, secrete large amounts of laminin. This might explain why F_9-diff requires more laminin than F_9 does in EM-3; ie, their synthesis of laminin may be a reflection of their needs.

Although the above findings demonstrate that laminin is a prominent component of extracellular matrices during early development, they do not suggest a function for laminin. Our results suggest that laminin is involved in cell adhesion. This proposition is most strongly supported by our observation that highly enriched preparations of laminin enable F_9 EC cells at both high and low density to attach to culture dishes in the absence of serum or CIG. Moreover, experiments in progress indicate that other mouse EC cell lines – PCC-3 [27] and 1003 [28] – also attach to culture dishes in the absence of serum when highly enriched laminin is present [A. Rizzino, unpublished results].

The studies reported here with laminin, and those reported earlier with fibronectin [15], demonstrate that both can mediate the attachment of EC cells in defined media. EC cells and the cells of the inner cell mass at the time of endoderm formation are believed to be very similar [29–31]. Thus, it seems highly probable that laminin and fibronectin (both of which are present in Reichert's membrane) play important roles in cell adhesion during early embryogenesis. However, their functions may not be limited to the mere attachment of cells to extracellular matrices and/or other cells. It is becoming increasingly clear that the hormonal and growth factor requirements of cells are affected by their substratum. Gospodarowicz et al have shown that corneal epithelial cells, when grown on plastic tissue culture dishes, respond to fibroblast growth factor (FGF) but not to epidermal growth factor (EGF). In contrast, when grown on collagen-coated dishes, these cells respond to EGF and not to FGF [32]. More recently, Gospodarowicz has reported that 4 different endothelial cell types, when grown on untreated tissue culture dishes in the presence of serum, require FGF to proliferate. This requirement is eliminated when these cells are grown on a corneal extracellular matrix [33]. If the implications of these findings extend to early embryonic cells, then anchorage to appropriate extracellular matrices may regulate their proliferation. Thus an interplay between hormones, growth factors, and extracellular matrices may play a prominent role during early embryogenesis. Hence, further studies with laminin and other components of basement membranes, in hormone-supplemented defined media, are likely to provide important insights into the role of the extracellular environment.

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