instrument for the study of a variety of medical and biological questions.

The authors wish to thank Dr. James G. Rheinwald for providing the rabbit antiserum against epidermal keratins, Mr. John Lye for assistance with indirect immunofluorescence, and Ms. Karen Brown for preparing the manuscript.

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0022-202X/83/8101S-0040s$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:40s–50s, 1983 Vol. 81, No. 1 Supplement
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The Control of Mammalian Cell Proliferation by Growth Factors, Basement Lamina, and Lipoproteins

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The effect of growth factors such as fibroblast growth factor on the production of a basement lamina by cultured endothelial cells has been investigated. The ability of these cells to grow and differentiate properly correlated with their ability to produce a basement lamina. The effect of such a substrate on the growth, differentiation, and aging of cells in vitro, as well as its use for the long-term culture of either normal diploid cells or tumor cells, is reviewed.

Cell migration and growth in vivo are the result of a complex balance of interactions among cells and between cells and their substrate. Those interactions which combine to modulate the cell shape may either permit or prevent cell proliferation and differentiation [1]. Following its original proposal by Grobstein [2], a role for cell-substrate interactions in the control of cell proliferation, morphogenesis, and gene expression has been amply demonstrated (for review, see [3]).

During the whole of embryogenesis, as well as in neonatal and adult life, an intact basement-lamina (BL) scaffold is required for the maintenance of orderly tissue structure [4]. By its presence it defines the spatial relationships among similar and dissimilar types of cells and between these cells and the space occupied by connective and supportive tissues. Replacement of cells which have died during their terminal differentiation or have been damaged by injury occurs in an orderly way along the framework of the BL [4]. This process seems to be aided by the polarity of the BL and by an apparent specificity for cell types. It enables multicellular organisms to reconstitute histologic structures of most tissues and organs to what they were prior to loss of cells. If the BL is destroyed, the healing in most tissues results in scar formation and loss of function [4].

In this short review, we outline our present knowledge of the effect of growth factors, fibroblast growth factor (FGF) in particular, on BL formation and the effect of the latter on cell

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This work was supported by Grants No. HL 20197, HL 23678, and EY 02186 from the National Institutes of Health.

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Abbreviations:
- BL: basal lamina
- EGF: epidermal growth factor
- FGF: fibroblast growth factor
- HDL: high-density lipoprotein
- LDL: low-density lipoprotein
- HMG CoA: 3-hydroxy-3-methylglutaryl coenzyme A
- NGF: neurite growth factor
- PDGF: platelet-derived growth factor
- Rat-1: normal rat fibroblasts
- SDS: sodium dodecyl sulfate

growth, differentiation, and aging. The use of such a substrate for the long-term culture of normal diploid cells or tumor cells is also described.

FIBROBLAST GROWTH FACTOR: EFFECT ON CELL PROLIFERATION, DIFFERENTIATION, AND SENESCENCE

The biological effects of FGF in vitro can be seen with respect to cell proliferation, differentiation, and senescence. Both pituitary and brain FGF have been shown to be mitogenic for mesoderm-derived cells [5] and have been used primarily to develop new in vitro endothelial cell lines, particularly of vascular and corneal origin.

FGF can also affect the phenotypic expression of some cell types. This is a particularly interesting characteristic of FGF, since it has made possible the long-term culturing of various cell types which otherwise rapidly lose their normal phenotypes in culture when passaged repeatedly at low cell density. This biological effect of FGF has been best studied using vascular endothelial cell cultures cloned and maintained in the presence of FGF and then deprived of it for various time periods [6,7].

Vascular endothelial cells grown and passaged serially in the presence of FGF adopt, upon reaching confluence, the morphology of a polarized-cell monolayer composed of tightly packed and flattened cells (Fig. 1). This cell layer shows, as it does in vivo, an asymmetry of cell surfaces. The apical cell surface is nonthrombogenic, while the basal cell surface is involved in the synthesis of a highly thrombogenic basal lamina composed of interstitial collagen type III and basement-membrane collagen types IV and V [7]. Two glycoproteins involved in cell adhesion, laminin and fibronectin, are associated with the BL produced by vascular endothelial cells [8] and are absent from their apical surface.

In contrast, when low-cell-density cultures are passaged repeatedly in the absence of FGF, they lose within three passages their ability to form a confluent monolayer of closely apposed and flattened cells. Instead, the cultures adopt a multilayer configuration consisting of large and overlapping cells that are no longer polarized [7]. Parallel to these changes in cell morphology, a loss of cell-surface properties and polarities is observed. Both the apical and basal cell surfaces become covered by an extracellular matrix composed predominantly of interstitial collagen types I and III together with lesser amounts of basement-membrane collagen types IV and V [7,8]. Fibronectin, which in cells expressing their proper phenotype cannot be detected in the apical surface of cells, became present when cells started to express an aberrant morphology [7,8]. These observations suggest that vascular endothelial cells maintained in the absence of FGF exhibit, in addition to a much slower growth rate, morphologic and structural alterations that primarily involve changes in the composition and distribution of their BL [7]. These changes result in no less than the loss of the expression of the differentiated phenotype, since cells are no longer contact-inhibited and have a thrombogenic apical cell surface. Vascular endothelial cell cultures can regain their characteristic phenotypic expression if sparse or subconfluent cultures previously maintained without FGF are reexposed to it [6]. It may therefore be concluded that FGF stabilizes and can reestablish the phenotypic expression of vascular endothelial cells.

FGF can also significantly delay the ultimate senescence of cultured cells, as well as their ultimate differentiation. Thus addition of FGF of myoblast cultures delays their fusion and myotube formation [11]. In the case of granulosa cells, addition of FGF to either a clonal strain of granulosa cells or to mass cultures can extend their lifespan in culture from 10 to 60 generations [11]. Adrenal cortex cell lines cloned in the presence of FGF show a similar dependence during their limited in vitro lifespan, and removal of FGF from the culture medium results not only in a greatly extended doubling time, but also in rapid senescence [11]. In the case of vascular and corneal endothelial cells, FGF has been shown to extend the lifespan of the cultures greatly. The effect is best observed with corneal endothelial cells passaged at low cell density, which have a lifespan of 20 to 30 generations in the absence of FGF and one of 200 generations in its presence [10].

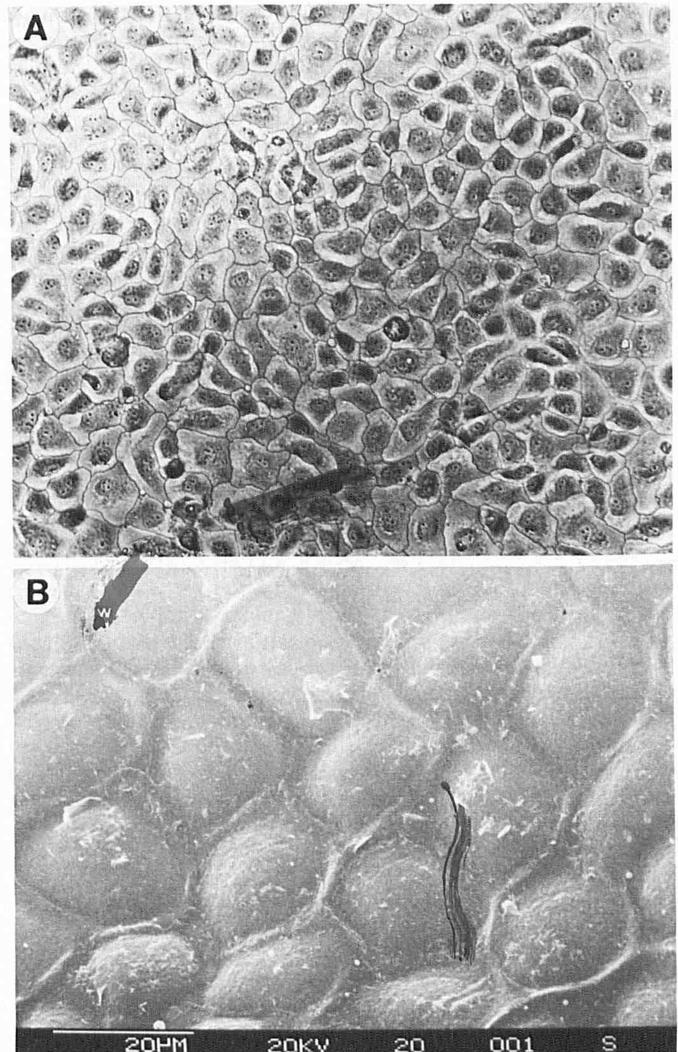

FIG 1. *A*, Monolayer of vascular endothelial cells grown in the presence of 10% calf serum and 100 ng/ml FGF. The cells are closely apposed and have their borders stained with silver nitrate (phase contrast; reduced from ×150). *B*, Morphologic appearance of confluent cultures of vascular endothelial cells as seen by scanning electron microscopy. The cell-surface area is 200 to 300 μm^2.

In summary, the following effects of FGF have been observed with various cell types: It allows cells to survive and grow at the low densities required for cloning; it increases the rate of cellular proliferation; it stimulates cell migration; and it stabilizes their phenotypic expression and extends their "in vitro" lifespan. All these effects correlate with the ability of the cells to produce a BL or extracellular matrix.

THE BASEMENT LAMINA (BL) AND ITS EFFECT ON CELL PROLIFERATION

The BL is an organized complex of collagens, proteoglycans, and glycoproteins, all interacting to produce a highly stable structure of whose area only a very small portion is in contact with the cell surface. Elucidation of the components of the BL

involved in controlling cell proliferation either in vivo or in vitro has been made difficult mostly by its intricate nature. Because the correct in vitro reconstruction of the BL from its isolated native components into the highly ordered structure that it represents would be a formidable task, advantage was taken of the fact that cultured corneal endothelial cells have the ability to produce, underneath their basal surface, a thick BL that adheres strongly to plastic [12] (Fig. 2). This matrix consists of elastin, basement-membrane collagen types IV and V, and interstitial collagen type III. Also present are proteoglycans, composed of heparan sulfate and dermatan sulfate proteoglycans, and glycoproteins such as fibronectin and laminin [8]. This last glycoprotein has been reported to be a specific marker for BL [13]. Therefore, the matrix synthesized and secreted by endothelial cells in culture conforms to the chemical criteria of a BL, as well as to the biological criterion, which is to serve as the substratum upon which cells migrate and divide in vivo.

THE IDENTIFICATION OF GROWTH-PROMOTING AGENTS FOR NORMAL DIPLOID CELLS MAINTAINED ON BASEMENT LAMINA INSTEAD OF PLASTIC

Conventional tissue-culture techniques make use of falconized plastic dishes as a substrate for cultured cells. Since plastic could adversely affect the proliferation and phenotypic expression of various cultured cell types, we first sought to determine if maintaining normal diploid cells on BL-coated dishes could improve their growth rate and differentiation. Previous studies [9,10] have shown that corneal or vascular endothelial cells passaged repeatedly at low density in the absence of FGF have a declining growth rate. This correlates with morphologic and structural alterations caused primarily by changes in the composition and distribution of the BL [9,10,12]. The possibility therefore arose that the matrix produced by these cells could have an effect on their ability to proliferate and to express their normal phenotype at confluence.

This was analyzed by comparing the growth of vascular endothelial cells seeded at low density on plastic with that of those seeded on BL-coated dishes [14]. Regardless of the initial seeding density, cells maintained on BL and exposed to plasma-supplemented media divided extremely rapidly (average doubling time 18 hours) and expressed their proper phenotype once confluent, even in the absence of exogenously added FGF. In contrast, similar cultures maintained on plastic and exposed to plasma proliferated poorly (average doubling time 48 to 72 hours) and required FGF if they were to become confluent. A similar observation was made for various other cell types that required FGF in order to show an optimal growth rate when maintained on plastic and exposed to either serum or plasma (Table I).

Although one might have thought that the BL has a direct mitogenic effect on the cells similar to that of FGF, this was shown not to be the case, since the rate of proliferation of these various cell types maintained on BL-coated dishes was a direct

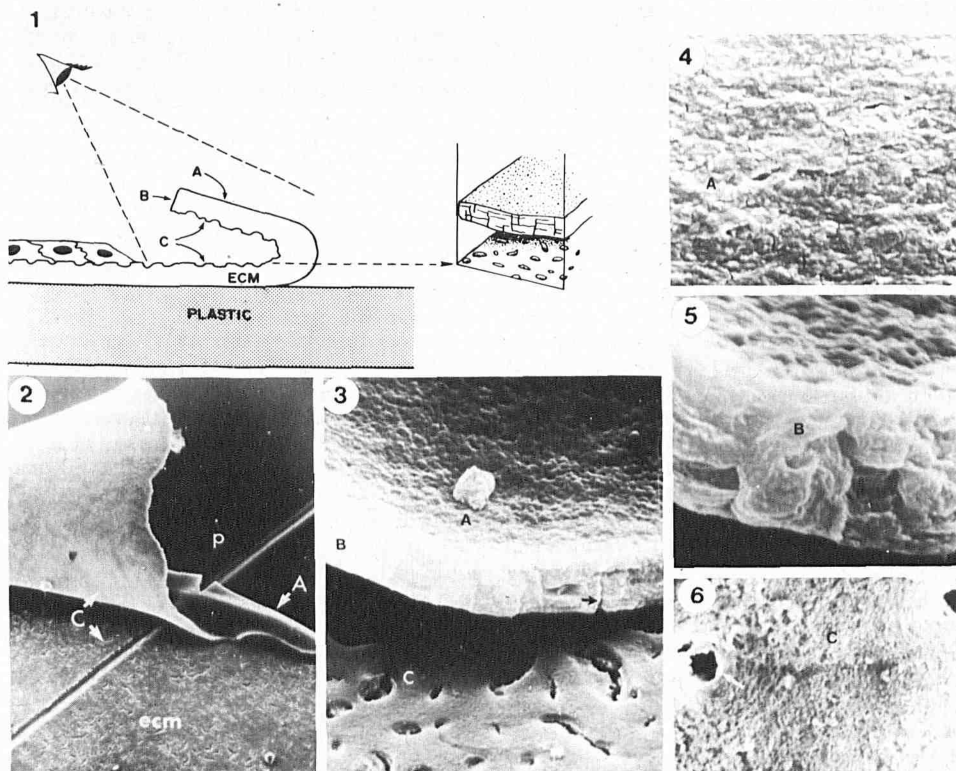

FIG. 2. Scanning electron micrographs of denuded basement lamina (BL) produced by confluent bovine corneal endothelial cells. (*1*) Schematic illustration of the BL after removal of the cell monolayer. The cell monolayer was removed following exposure to 20 m*M* NH_4OH, as described elsewhere [19, 40,41]. The plate was then scratched with a needle in order to detach the BL from the plastic locally. Scanning electron micrographs were taken in the area where the BL was folded: (*A*) was the side that was originally attached to plastic; (*B*) is the fracture edge; (*C*) is the side on which cells were originally attached to the BL. (*2*) Low-power (×200) scanning electron micrograph of the BL. The plastic (*p*) has been exposed, and the trace of the needle running through the BL (*ecm*) and the denuded plastic can be seen: (*A*) was the BL side originally attached to the plastic, while (*C*) was the side on which cells were originally attached. This side is pitted, the pits corresponding to cell processes entering into the BL. These pits were probably the sites where the cells were originally anchored to the BL. (*B*) The BL can be observed at a higher magnification (×3000). The fracture edge of the BL (*B*) can be seen, as can the transverse section (*arrow*) of a pit in which a cellular process was present before denudation. The BL side to which the cells were attached is covered with numerous small empty pits. (*4*) High magnification (×8000) of side of BL previously attached to plastic showing a corrugated pattern. (*5*) High magnification (×14,000) of the BL fracture edge, showing its lamellar structure. (*6*) High magnification (×16,000) of the BL side to which cells were previously attached, showing its fibrillar appearance. Two small empty pits can be seen distinctly.

TABLE I. *Growth factors requirement for the proliferation of normal diploid and transformed cells maintained on basement-membrane-coated dishes and exposed to defined medium*

	HDL (μg protein/ml)	Insulin or somato C (ng/ml)		FGF or EGF (ng/ml)		Transferrin (μg/ml)	Reference
Normal diploid cells:							
Vascular endothelial cells	500	—	—	—	—	10	[16]
Corneal endothelial cells	250	2500	100	100	50	10	[17]
Vascular smooth-muscle cells	250	2500	100	100	50	10	[19]
Granulosa cells	30	1000	100	100	50	10	[20]
Adrenal cortex cells	30	50	10	100	—	5	[21]
Lens epithelial cells	250	2500	100	100	—	10	[18]
Kidney tubule cells	500	—	—	—	—	10	[30]
Embryo fibroblasts (Rat-1)	500	5000	—	—	25	25	[34]
Transformed cells (Tumor):							
A-431 Carcinoma	500	—	—	—	Toxic	10	[11,33]
Colon carcinoma cells	500	—	—	—	—	10	[11,33]
Ewing sarcoma cells	500	—	—	—	—	10	[11,33]
Rhabdomyosarcoma	500	—	—	—	—	10	[11,33]
MDCK (kidney-derived)	500	—	—	—	—	10	[11,30]
B-31 cell line	1000	—	—	—	—	25	[34]

function of the plasma concentration to which cells were exposed [12,14]. It is therefore likely that the final effect of the BL on cell proliferation is a permissive one that results in an increased cell sensitivity to plasma factor(s).

Among the plasma factors that could be held directly or indirectly responsible for the active proliferation of these various cell types are plasma lipoproteins [12], insulin or somatomedin C, and epidermal or fibroblast growth factor. Preliminary studies demonstrated that transferrin, the main iron-carrying protein in the bloodstream, had to be present, as with most cell types studied to date, if cells were to respond to plasma factors. This absolute requirement for transferrin could either reflect its role in delivering iron to the cells or its ability to detoxify the medium by removing toxic traces of metals [15].

In addition to transferrin, the plasma-factor requirements of the various normal diploid cells analyzed fell into three broad categories. The first category, represented by vascular endothelial cells, required only the presence of high-density lipoproteins (HDL) in order to proliferate actively [16]. The second category, to which corneal endothelial cells, lens epithelial cells, and vascular smooth-muscle cells belong, required, in addition to HDL, the presence of insulin or somatomedin C, as well as that of EGF or FGF [17-19]. For both these categories, HDL are the main mitogenic factor, since in their absence, cells could not be triggered to divide when exposed to transferrin, insulin, somatomedin C, and FGF, or EGF added alone or in combination. The third category was represented by steroid-producing cells, such as adrenal cortex or ovarian granulosa cells. Both these cell types showed only a minor response to HDL, which at concentrations above 50 μg protein per milliliter became cytotoxic [20,21]. Instead, insulin was the main mitogenic factor for both granulosa and adrenal cortex cells, and although addition of HDL and EGF or FGF improved the final cell density of the cultures exposed to a defined medium, the improvements were slight.

The substrate upon which cultures were maintained was found to be of crucial importance if a significant response to HDL or insulin (depending on the cell type) was to be observed, since it was not observed with cells maintained on plastic dishes. This suggests that in vivo, the integrity of the BL upon which cells rest and migrate is an important factor in determining the cells' response to lipoproteins and insulin-like activity present in plasma [12,16].

Although FGF had only a minor mitogenic effect on cells maintained on BL and grown in defined media, its biological effect on cell longevity could still be observed. This was particularly evident in the case of corneal endothelial cells, which, when exposed to HDL, insulin, and transferrin, had a lifespan in culture of 20 generations. This could be increased to 85 generations by the single addition of FGF [16]. Similar observations were made with lens epithelial cells [17].

One important conclusion from these studies is that cells in culture seem to respond to growth-promoting agents that govern their proliferation early in their ontogeny. This conclusion can be reached on the basis of a comparison of the growth requirements of bovine *corneal endothelial* cells and *vascular endothelial* cells maintained and passaged in total absence of serum. While one might expect that both cell types would have the same growth requirements (HDL and transferrin), corneal endothelial cells additionally required the presence of insulin and EGF in order to grow an optimal rate (Table I). Their growth requirements are therefore similar to those of vascular smooth-muscle cells and demonstrate that endothelial cells from different organs do not necessarily have similar growth requirements. This may reflect their different embryologic origins. Indeed, while the vascular endothelium is the first tissue to develop in the embryo and is derived from the primary mesenchyme [22], *both* vascular smooth-muscle cells from the aortic arch [23] and corneal endothelial cells [24] develop later and originate from the neural crest.

THE MODULATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A (HMG CoA) REDUCTASE ACTIVITY BY HDL: IMPLICATIONS FOR THE GROWTH-PROMOTING ACTIVITY OF HDL

In the absence of HDL, vascular endothelial cells do not divide, and both vascular smooth-muscle cells and corneal endothelial cells no longer respond to the mitogenic stimuli provided by insulin or somatomedin C and EGF or FGF. For all three cell types, therefore, HDL can be described as the main mitogen.

One possible mechanism through which HDL acts to support cell growth could be mediated by its effects on the cholesterol biosynthetic pathway. Cholesterol synthesis is primarily regulated at the level of HMG CoA reductase, the enzyme responsible for the synthesis of mevalonic acid [25]. The metabolism of mevalonate occurs through three recognized synthetic pathways in addition to that resulting in cholesterol formation [25]. A branch point exists at the level of the isopentenyl pyrophosphate intermediate, which serves as the source of isopentenyl tRNA. Downstream in the mevalonate pathway, at the level of farnesyl pyrophosphate, another major branch point exists, giving rise to pathways for the synthesis of ubiquinone, a component of the mitochondrial electron-transport chain, and the dolichols, long-chain polyisoprenols that participate in the process of protein glycosylation. These three pathways together account for only a quantitatively minor fraction of the total flux of mevalonate metabolism, but they could well be important in the control of cell proliferation. The role of mevalonate, rather than of cholesterol itself, in cell proliferation was first recognized when it was found to substitute for acetate in an otherwise

acetate-requiring strain of *Lactobacillus acidophilolus* [26]. Interestingly, bacteria neither require nor synthesize cholesterol, and the mevalonate, therefore, was used for the synthesis of a nonsterol product.

Quesney-Huneeus and coworkers [27] found that compactin could block the transit of synchronized BHK cells through the S phase of their cell cycle (as measured by [^{3}H]thymidine incorporation into DNA). The compactin block could be reversed by the addition of mevalonate, but not cholesterol, in the form of low-density lipoprotein (LDL). Later studies [28] showed that isopentenyl adenine could duplicate the effect of mevalonate, but not that of cholesterol donated by LDL. Thus, although cholesterol itself is clearly of importance in cell proliferation, products of the nonsterol branching pathways of mevalonate metabolism may also be crucial.

When the effect of HDL on the vascular endothelial cell HMG CoA reductase activity was analyzed, it was found to affect this enzyme activity strongly [29]. In confluent cultures exposed to optimal concentration of HDL, the basal activity of HMG CoA reductase was increased 10-fold. This effect was time- and HDL concentration-dependent and could be observed as early as 3 hours after the addition of HDL to the cultures [30]. HDL's stimulation of HMG CoA reductase could be prevented and reversed by LDL. This suggests that the stimulation of HMG CoA reductase by HDL does involve HDL-mediated cholesterol efflux, although other mechanisms may be involved as well.

The HMG CoA reductase activity of sparse, actively growing cultures was 50-fold greater than the enzyme activity of confluent, nondividing cells. The enzyme activity of proliferating cells, despite its unstable baseline, which declines with increasing cell density, was stimulated by HDL and inhibited by LDL. Thus both sparse and confluent cells respond to HDL by increasing their HMG CoA reductase activity, and they thereby gain a greater capacity to synthesize mevalonate.

To study the possible coupling between HDL's ability to stimulate mevalonate proliferation and cellular proliferation, the effects of compactin on sparse cells exposed to HDL and to LDL were compared. HDL-treated cells were found to be 30-fold more "resistant" to the toxic effects of compactin and were capable of proliferating actively even when exposed to compactin concentrations as high as 8 μM. In contrast, cells exposed to both LDL and a low concentration (0.1 μM) of compactin died, despite the ability of LDL to donate cholesterol to the cells. The relative lack of cytotoxicity of compactin when cultures were exposed to HDL correlated with a much higher HMG CoA reductase activity than that observed in cultures exposed to both LDL and compactin [29]. Similar effects of HDL on HMG CoA reductase activity were also observed with MDCK cells. The increased HMG CoA reductase activity of the cells in response to HDL correlated with an even higher increase (100-fold) in resistance to the cytotoxic effect of compactin (Fig. 3) over the level seen with cells exposed to both LDL and compactin. MDCK cells proliferate to a similar extent when exposed to either HDL and transferrin or serum-supplemented mediums and the addition of compactin to cultures already exposed to HDL had no deleterious effect on the cells [30] (Fig. 4). In contrast, when cells were exposed to LDL and transferrin-supplemented medium, the addition of compactin killed the cells, despite the availability of cholesterol donated to the cells by LDL (Fig. 3).

One could therefore conclude that a relationship exists be-

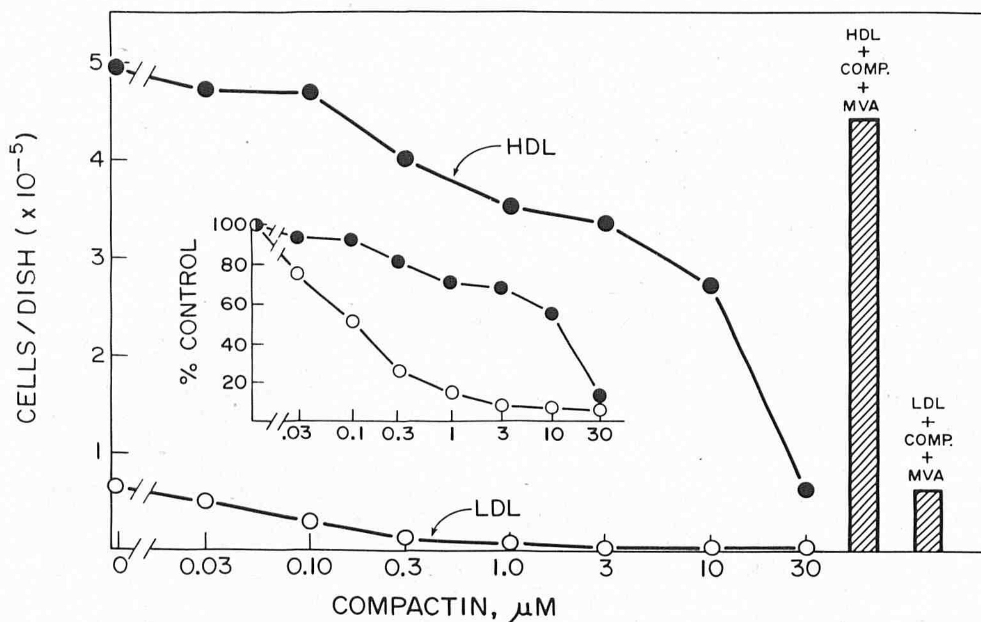

FIG. 3. Effects of increasing concentrations of compactin on the viability and proliferation of MDCK cells exposed to HDLs and LDL. MDCK cells (4 × 10^4 cells/35-mm dish) were seeded onto ECM-coated dishes in DME supplemented with transferrin (10 μg/ml) and either HDLs (750 μg protein/ml; ●) or LDL (250 μg protein/ml; ○). Cultures in both groups were also exposed to compactin, at concentrations of 0.03 to 30 μM, or to compactin plus MVA (10 mM). Cultures were counted 4 days after seeding. The inset shows the same data normalized to 100 percent of the control (i.e., without compactin) values for cultures exposed to HDLs and LDL, respectively. Cultures exposed to HDLs in the absence of compactin reached a final density of 4.9 × 10^5 cells per dish. Cultures exposed to HDLs and increasing concentratons of compactin showed a dose-related decrease in final cell density. At the 30 μM concentration of compactin, the final cell density was 6.5 × 10^4 cells per dish. The concentration of compactin that halved the final cell density was approximately 10 μM (*see inset*). The addition of MVA to cultures exposed to 10 μM compactin increased the final cell density from 55 to 90 percent of the control value. Cells exposed to LDL reached a final cell density of 6.5 × 10^4 cells per dish, and final cell densities declined sharply as a function of exposure to increasing concentrations of compactin. The concentration of compactin that effected a 50 percent decrease in final cell density was approximately 0.1 μM (*see inset*). MVA increased the final cell density of cultures exposed to 10 μM compactin from 9 to 67 percent of the control value.

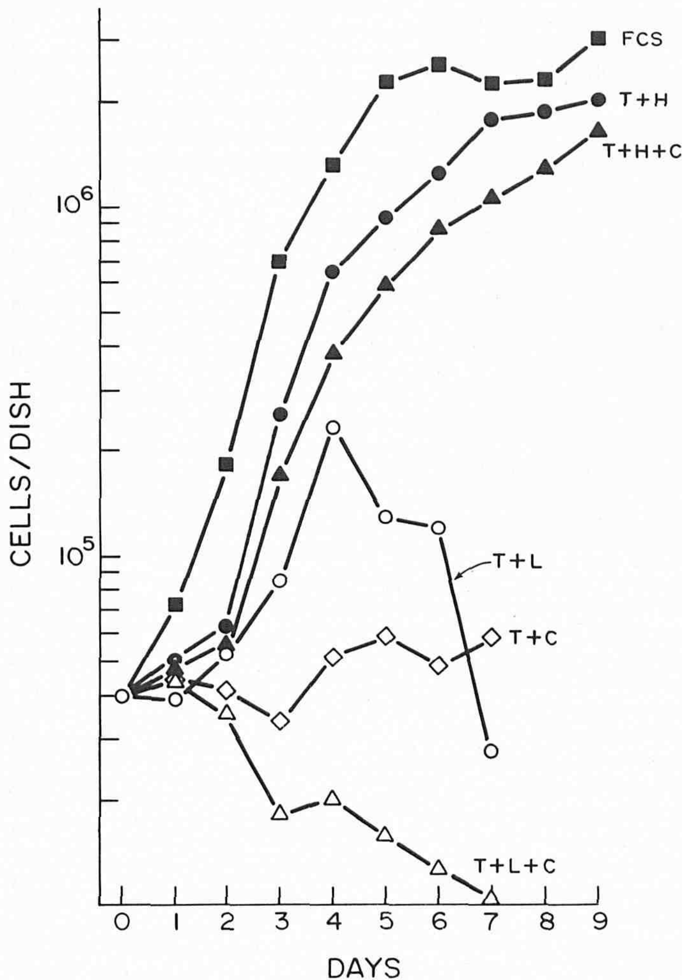

FIG 4. Effect of a single concentration of compactin alone, or in combination with HDLs or LDL, on the growth of MDCK cells. MDCK cells (4×10^4 cells/35-mm dish) were seeded on ECM-coated dishes in DME supplemented with 5% fetal calf serum (FCS; ■), transferrin (10 μg/ml) plus HDLs (800 μg protein/ml) (T + H; ●), transferrin, HDLs, and 1 μM compactin (T + H + C; ▲), transferrin plus LDL (200 μg protein/ml) (T + L; ○), transferrin, LDL, and compactin (T + L + C; △), or transferrin and compactin (T + C; ◇). Cultures from each group were counted daily for 7 to 9 days. Cell growth and final cell density were optimal in the presence of fetal calf serum, but nearly optimal growth was attained by cultures exposed to transferrin and HDLs. The presence of 1 μM of compactin had a minimal effect on the growth of cultures exposed to HDLs. The final cell density of cultures exposed to T + H + C was 85 percent that of cultures exposed to T + H. Cultures exposed to T + L proliferated for 4 days and then showed progressive cell death. Cultures exposed to T + L + C never proliferated, and progressive cell death was evident by day 2. Cultures exposed to T + L + C never proliferated, and progressive cell death was evident by day 2. Cultures exposed to T + C did not grow and showed a relatively constant cell density throughout the course of the experiment.

tween the ability of HDL to activate HMG CoA reductase and its ability to support proliferation. It would also appear that the significance of the increased rate of mevalonate synthesis does not lie solely in an increased provision of cholesterol to the cells, since cholesterol provided by LDL does not relieve compactin's toxicity, while HDL does. Therefore, the mevalonate-derived metabolic products most crucial to proliferation could be the products of the branching isoprene pathways that yield dolichol, ubiquinone, and isopentenyl adenine.

EFFECT OF BASEMENT LAMINA ON TUMOR CELL ATTACHMENT, MIGRATION, AND PROLIFERATION

A central issue in tumor biology is the understanding of the interactions between tumor cells and their environment. It is of interest in this regard to study the tumor cells' interaction with the BL, since this could throw light on the need for stromal and fibroblastic support for tumor cell growth, on the ability of tumor cells to reorganize their local environment in order to grow and invade, and on the mechanisms through which various artificial substrates that are introduced in vivo into an animal result in the production of malignant mesothelioma and fibrosarcoma.

When the attachment of colon carcinoma cells and Ewing tumor cells to BL-coated dishes was compared with that observed on plastic, these cells were observed to attach firmly and rapidly to the BL, but not to plastic dishes [31] (Fig. 5). The rapid attachment of colon carcinoma cells and Ewing sarcoma cells was shown in subsequent studies [32] to be mediated by different adhesion factors present in the BL. While colon carcinoma cells attached through their interaction with laminin, the Ewing cells interacted with fibronectin. The presence of specific adhesion factors in BL, as well as the ability or inability of tumor cells to produce and/or interact with such factors, may therefore determine the localization of given tumor-cell types to specific BLs.

After their initial attachment to the BL surface, cells were observed to migrate rapidly out from cellular aggregates [*31*]. This rapid migration of cells that are in contact with a BL could be relevant to tumor growth and invasiveness. Contact of the tumor cells with a BL may favor migration of tumor cells along defined routes, and it is also likely that cell movement and its velocity are controlled by alterations in the extracellular environment and not solely by regulation of the locomotive machinery itself. Migration of cells from aggregates may simply reflect the much stronger adhesive forces between the cells and the BL versus those existing between cells and plastic or among cells associated in an aggregate. This migratory effect might also be responsible in part for the stimulation of cell growth observed in dishes coated with a BL versus plastic alone.

Since cell shape has been shown to be a major factor in regulating cell growth, the growth rate of tumor cells plated on plastic (spherical configuration) was compared with that of cells plated on BL, which adopt a flattened morphology. Although differences in growth rate were observed, both the Ewing and colon carcinoma cells proliferated in either configuration [31]. This demonstrated that adhesion of the cells to the BL and their subsequent flattening did not impose a restriction on their ability to proliferate. On the contrary, when cells flattened out, a stimulation of cell growth that depended on the cell type and culture conditions was observed (Fig. 6*A*, *B*). This was best seen with Ewing sarcoma cells, since active proliferation of attached cells was observed only in dishes coated with a BL. This resulted in an infinite increase in the final density of cells attached to such a substrate versus those attached to plastic [31] (Fig. 6*B*). Cultured tumor cells can therefore proliferate either when they are highly flattened and firmly attached to the substrate or when they are in suspension. Cell adhesion to the BL, however, results in a stimulation of cell growth, which is best observed with cells growing in the anchorage-dependent configuration, but not in the floating configuration, thereby indicating a need for an actual contact between the cells and the BL.

The observation that tumor cells will divide actively when maintained on a BL triggered our interest in their growth requirements. In most cases examined thus far (Table I), we found that tumor cells show a unique requirement for HDL alone in order to proliferate actively [33]. Two mammary carcinoma cell lines (MCF-7 and ZR-75-1) were exceptions to this rule in that they required insulin in addition to HDL in order to proliferate actively [33]. Since it would be expected that most of the tissues from which these tumor cells are derived would have a sophisticated growth requirement, it is possible that when cells become transformed, they can revert to a primitive type of growth control in which hormones play a minor part and HDL becomes the main plasma factor involved in their proliferation. This was examined by looking at the

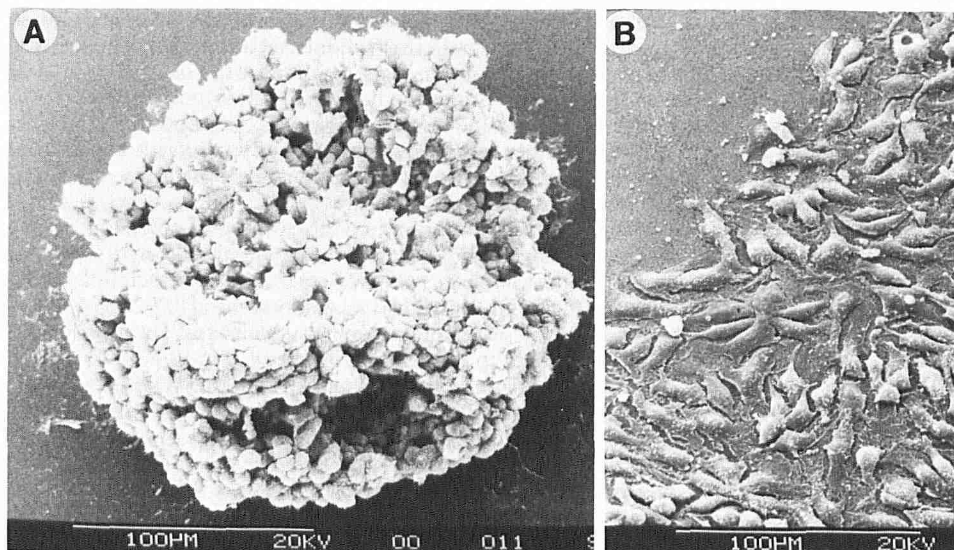

FIG 5. Scanning electron microscopy of colon carcinoma cells maintained in tissue culture on either a plastic substratum (*A*) or basement-lamina-coated dishes (*B*). While on plastic, cells grew as an aggregate that did not adhere to the substratum (*A*); when maintained on basement lamina, the cells flattened and grew as a monolayer (*B*).

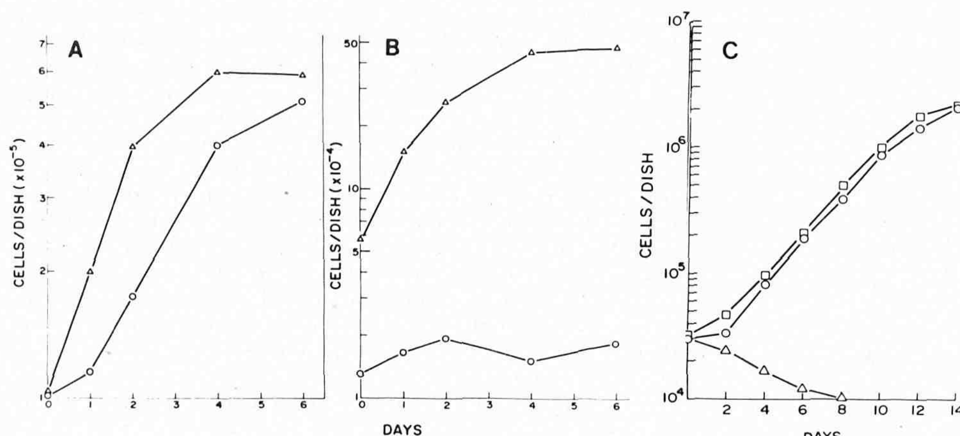

FIG 6. Proliferation of Ewing's sarcoma cells when maintained on plastic versus basement-lamina-coated dishes. *A*, *B*, 10^5 cells in 2 ml DMEM containing 0.5% calf serum were seeded into each plastic (○) or basement-lamina-coated (△) 35-mm dishes. Duplicate cultures were counted every day to determine the number of floating cells (present in the tissue-culture medium) and of cells that are firmly attached (dissociated with STV). *A*, Total cell number (floating plus attached). *B*, Firmly attached cells. The number of firmly attached cells was higher than 80 percent of the total cell number found in dishes coated with basement lamina and lower than 10 percent of the total cell number obtained on plastic. *C*, Ewing sarcoma cells (4×10^4 cells) were seeded in total absence of serum on 35-mm basement-lamina-coated dishes and exposed to DME alone (△) or to DME supplemented with transferrin and HDL (○) or 10% fetal calf serum (□) The concentration of transferrin was 10 μg/ml, while that of HDL was 750 μg protein per milliliter. The standard deviation in different determinations did not exceed 10 percent of the mean.

growth control of normal rat fibroblasts (Rat-1) before and after transformation (B31 cells) by an avian sarcoma virus. While the Rat-1 cells do require HDL, insulin, and EGF in order to proliferate at an optimal rate, in the process of transformation, their requirement for insulin and EGF was nearly lost and HDL alone was able to support their proliferation at an optimal rate [34] (Table I). The substrate upon which cells were maintained was also an important factor to consider if tumor cells were to respond to HDL. This was best seen in the case of Ewing cells, which when maintained on BL-coated dishes responded to HDL by proliferating as actively as when exposed to serum (Fig. 6*C*). Yet when the same cell type was maintained on plastic and exposed to serum, the cells that adhered loosely to plastic did not proliferate actively (Fig. 6*B*). This suggests that in this configuration, they no longer responded to the mitogenic stimuli provided by serum HDL.

EFFECT OF BASEMENT LAMINA ON THE ATTACHMENT, MIGRATION, PROLIFERATION, AND DIFFERENTIATION OF NERVE CELLS

The role of cell-substrate adhesion in the initiation, elongation, and branching of axons from either embryonic neurons or from neuronal tumor cell lines has been the object of numerous in vitro studies that have led to the conclusion that there is a strong correlation between adhesion and enhanced neuronal morphogenesis. Thus the adhesive interaction between the nerve cells and their microenvironment could be a crucial part of the initiation and elongation of neurons. Regulation of neuronal morphogenesis may therefore be expressed in part through the physicochemical properties of the interacting cell surface and its extracellular environment [35]. However, in most cases, the substrates considered have been artificial in

nature, consisting of plastic, chemically modified surfaces (palladium, polyornithine, or polylysine-coated), collagen gels, and the remains of killed nonneuronal cells and the substrate-associated material produced by them.

The ability of cultured corneal endothelial cells to produce a matrix similar to those found in vivo provides an opportunity to study the properties of a natural substrate with regard to nerve cell adhesion and the subsequent neurite initiation and outgrowth. It also allows the study of how the response of nerve cells to a trophic and chemotactic agent such as nerve-growth factor (NGF) may differ from the response previously observed when cells were maintained on various artificial substrates.

The rat pheochromocytoma cell line PC12, which shares many properties with cultured sympathetic ganglion cells, was selected to study the role of cell-substrate interaction in regulating nerve cell attachment and neurite outgrowth. The advantage of using PC12 cells instead of normal sympathetic cells is that although these cells do not require NGF in order to survive, they depend on it in order to initiate neurite outgrowth.

When the attachment of PC12 cells to BL-coated dishes and plastic or collagen-coated dishes was compared, it was observed that in contrast to collagen-coated or plastic dishes, cells attached rapidly and tenaciously to BL-coated dishes. Following cell attachment, cell spreading was observed, reflecting the functional association of the cells with their substratum [36,37].

After this initial cell spreading, neurite outgrowth occurred within 24 hours. Even in the absence of NGF, cells were capable of extending neurites for 5 to 10 days. In contrast, no neurite outgrowth occurred on either plastic or collagen-coated dishes unless NGF was present. It is therefore likely that the cell-substratum interaction between PC12 cells and the BL is solely responsible for this initial neurite outgrowth. However, the presence of NGF is still required for the long-term maintenance (over 10 days) and expression of new neurite outgrowth [36,37]. This also suggests that NGF may be required not so much for initiating neurite outgrowth as for stabilizing it.

The morphologic appearance of neurites from PC12 cells maintained on BL-coated dishes in the presence or absence of NGF was similar, with varicosities, branching, some fascicles, and growth cones (Fig. 7). Even after 1 or 2 days, some neurites in cultures not exposed to NGF could have a length greater than 100 μm. Particularly interesting was the disposition on both sides of some neurites of microspikes, which extended from the neurite at regular intervals and appeared to staple it to the substrate. A similar configuration of microspikes or branchlets has been observed by Roberts [38] for actively growing axons in vivo that move along the inner surface of an epidermal BL.

Since most of the BL components can be found in soluble form in the tissue-culture media of various cell types capable of producing such a matrix, the cell adhesion and neurite outgrowth are similar to those found in the conditioned media of various cell types. It is interesting to note in this regard that the components of conditioned medium from heart cells, which stimulate neurite extension, do so primarily when bound to the substratum [39]. The mechanism of stimulation is unknown, but it may be mediated by an enhanced adhesion between the cell surface and the substratum.

Therefore, the adhesive interaction of nerve cells with the BL produced by cultured corneal endothelial cells may not only provide a model with which to study the trophic and chemotactic effects of NGF on neurites developing on a natural substrate, but may also prove to be useful for the development in culture of neurons from the central nervous system, which require intimate contact with glial cells for survival and growth. Although the role of glial cells may be to provide trophic factor(s), it is equally possible that they could provide a suitable surface (such as extracellular material) for neuronal cell adhesion and axon outgrowth.

NATURE OF THE BASEMENT-LAMINA FACTOR(S) INVOLVED IN CELL ATTACHMENT AND CELL PROLIFERATION AND DIFFERENTIATION

Little is presently known about the BL factor(s) that have a permissive effect on cell proliferation and could restore the cell response to plasma factors. These factor(s) could be either intrinsic components of the BL or, since cells producing it are grown in presence of medium supplemented with serum or plasma and FGF, they could consist of as yet unidentified serum or plasma factor(s), as well as of growth factors, such as FGF or platelet-derived growth factor (PDGF), which have become part of the structure during its formation of the BL, cellular growth-promoting factors could be adsorbed onto it and be responsible for its growth-promoting properties.

The possibility that adsorbed growth factors such as FGF or PDGF are the active agents was eliminated on the basis of inactivating treatment. Treatment of PDGF in the soluble or attached configuration with reducing agents such as DTT results in its complete inactivation. Likewise, heat treatment of

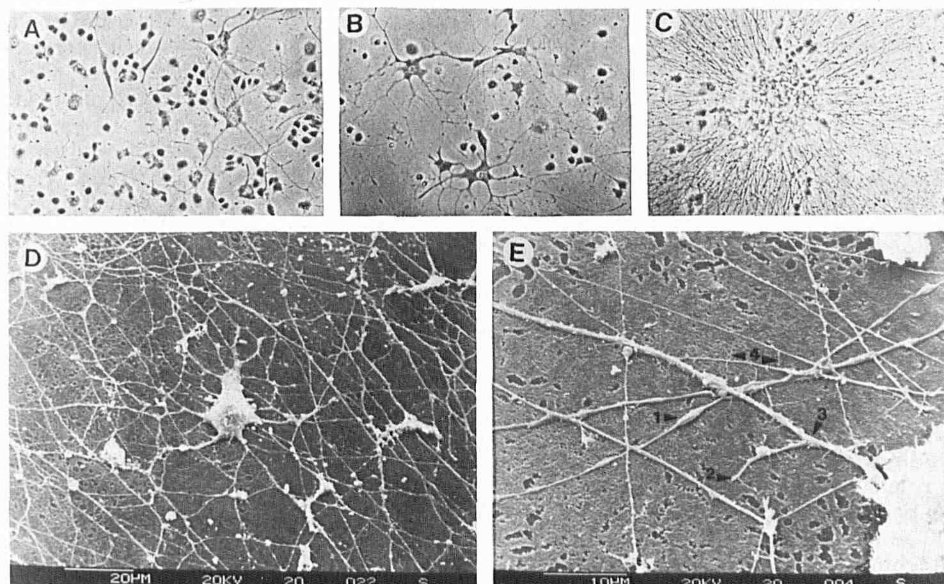

FIG 7. Morphologic appearance of PC12 cells maintained on BL in the presence or absence of NGF. *A–C*, Phase-contrast micrographs of unfixed PC12 cells cultured on basement-lamina-coated dishes in the absence of NGF for 3 days (*A*) and in the presence of NGF for 3 days (*B*) and 13 days (*C*). *D, E*, Scanning electron micrographs of PC12 cells on basement lamina in the presence of NGF for 12 days. *E*, Arrows point to (1) a varicosity in a neurite, (2) the end of a neurite, (3) a branching point, and (4) fine projections extending from a neurite.

FGF also results in its total inactivation. Since neither reducing agents nor heat treatment of the BL affects its ability to support cell growth [40], it is unlikely that its growth-promoting ability is due to traces of these factors adsorbed onto the BL.

Likewise, cytoplasmic factors that would have adsorbed to the BL during the lysis process are not likely to be involved, since denudation using treatment with 2 *M* urea, which results in little or no cell death, yields BLs that are as potent in supporting cell growth as those prepared by using either detergent or weak alkali treatment [41]. The possibility that the factors involved are derived from serum or plasma can also be eliminated by the demonstration that BLs produced by corneal endothelial cells growing in defined medium supplemented with HDL, transferrin, and insulin are as potent in supporting cell growth as BLs produced by cells growing in serum or plasma-supplemented medium [41]. The sole remaining possibility is that the factors involved in permitting cell growth are intrinsic components of the BL. Among the various components that have been analyzed for their ability to support cell growth are interstitial (types I, II, and III) and basement-membrane (type IV) collagen, as well as fibronectin. None of these components alone promoted an increased rate of cell proliferation, and in all cases, an aberrant morphologic appearance was observed, the cultures being composed of large cells of which a high proportion were binucleated.

The possibility exists that although no single BL component is active in supporting cell growth, a cooperative effect between components could result in such an effect. The intricate nature of the BL is exemplified by the complex interaction of its known components (collagen, glycoproteins, proteoglycans, and glycosaminoglycans), which form a highly stable scaffolding upon which cells rest in vivo. This complexity makes the reconstitution of this unique structure from its known constituents a formidable task. The role that the various BL components play in cell proliferation has therefore been investigated by indirect methods that rely on selective inactivation by chemical, enzymatic, or heat treatments. Of all the treatments used, only three were effective in inactivating the ability of BL to support cell proliferation. Exposure of BL-coated dishes to 14 *M* NH_4OH (pH 13.8), which results in the cleavage of proteoglycoproteins or glycopeptides at the *O*-glycosidic bond between the protein and carbohydrate moieties, inhibited the rate of proliferation of vascular endothelial cells by 97 percent. Likewise, treatment of BL-coated dishes with 4 *M* guanidine-HCl, which extracts up to 80 percent of the glycosaminoglycan, caused a 92 percent reduction in cell growth. Treatment of BL-coated dishes with nitrous acid (HNO_2), which results in the degradation of heparan or heparan sulfate into sulfated disaccharides and nonsulfated oligosaccharides, affected cell proliferation by 90 percent and led to the release of $^{35}SO_4$-labeled macromolecules, 50 percent of which were disaccharides. Although the effect of HNO_2 on intact BL is not known, its specific degradation of isolated heparan and heparan sulfate could indicate that it has a similar effect on BL, and this may correlate with its adverse effect on the ability of that structure to support cell growth. Chemical treatment of the BL with a reducing agent such as dithiothreitol (from 0.1 m*M* to 100 m*M*) did not affect its ability to support growth, nor did treatment with SDS (2%) or urea (2 *M* or 8 *M*). Specific enzymatic treatment with collagenase, pepsin, trypsin, chymotrypsin, or neuraminidase did not inhibit cell proliferation. It is therefore doubtful that any of the substrates of these enzymes plays a direct role in cell proliferation. The lack of effect of either hyaluronidase or chondroitinase ABC, as well as the presence of chondroitinase-resistant material in extracts of BL that affect cell proliferation, would tend to rule out a role for hyaluronic acid or chondroitin sulfates in the permissive effect of the BL on cell proliferation.

The importance of cell shape in proliferation suggests that attachment factors present within the BL, such as fibronectin and laminin, because of their direct contact with the cell membrane, could modify cell shape and could therefore play a dual role in controlling cell proliferation. To investigate the relationship between growth and attachment, the ability of vascular endothelial cells to attach to BL-coated dishes following treatment by alkaline pH and heat has been compared. In the absence of serum, only 10 percent of the cells attached to plastic after 1 hour, and maximal cell attachment (40%) was not reached before 18 hours. In contrast, cells seeded on BL-coated dishes rapidly attached and spread, and most (90%) have attached by 1 hour. No significant difference is seen in the rate of cell attachment after treatment of BL-coated dishes at alkaline pH, although it did destroy 97 percent of their ability to support cell proliferation. In fact, initial cell attachment (1 hour) was slightly enhanced by alkaline treatment. It therefore appears unlikely that the component of the BL that is removed or destroyed by high pH treatment is involved in cell attachment [40].

In contrast to the results obtained after treatment of the BL with alkaline pH, thermal treatment of the collagen bed, which results in its denaturation, had the opposite results. Treatment of the coated dishes at 70°C, which has no effect on cell proliferation, greatly reduced the rate of cell attachment. The rate of cell attachment was reduced even further after treatment at 90°C, although cell growth was reduced by only 60 percent. This indicates that the BL component(s) implicated in cell growth is probably distinct from those involved in cell attachment. Thermal disruptions of the BL also revealed the importance of the spatial geometry of the component responsible for cell proliferation [40].

These studies provide evidence regarding the nature of the components of the BL that are responsible for conveying its permissive effect on cell proliferation. It is likely that the active component is a sulphated glycoprotein or proteoglycan that is susceptible to extraction by 4 *M* guanidine-HCl, is degraded by nitrous acid, and contains an *O*-glycosidic bond and either glucosamine and/or galactosamine. Its resistance to hyaluronidase and chondroitinase ABC suggests that neither hyaluronic acid nor chondroitin sulfates are involved. Identification of the active component, however, must await a detailed analysis of the material extracted from the BL under conditions that inhibit cell proliferation.

CONCLUSION

Although the final effect of FGF is that of a mitogen, its action could be indirect. It could in some way replace the requirement of cultured cells for a substratum such as the BL and thereby render cells, even those maintained on plastic, more responsive to plasma growth factors. Alternatively, FGF could direct the endogenous synthesis and secretion of the BL produced by cultured cells, which would in turn make the cells responsive to plasma factors.

Because the various cell types studied thus far respond to plasma factor(s) when maintained on a natural substrate and no longer require growth factors such as FGF in vitro, it is clear that the proliferative response of cells to plasma factors can be modified dramatically by the substrate upon which the cells are maintained. It is possible that the lack of response of different cell types in vitro to agents responsible for their proliferation and differentiation in vivo could be attributed to the artificial substrate (whether plastic or glass) upon which the cells rest. Maintenance on such a surface could limit both the cells' response to mitogenic factors and their ability to produce a basal lamina, either of the correct composition or in adequate amounts [12,14].

The observation that the basement lamina upon which cells rest can replace the need for competence factors such as FGF or PDGF has far-reaching implications, since it could make in vitro systems more representative of the in vivo situation. It also helps to explain why cells which in vivo rest in close association with their natural substrate respond differently to various growth-promoting agents than the same cells when they

rest on plastic. A better understanding of the role the basement lamina plays in cell proliferation and differentiation could affect the way in which we view various problems of cell biology currently being explored using in vitro techniques. For example, it is likely that if one starts with a primary culture, cells are selected which in the subsequent passages retain their ability to produce basement lamina. Alteration in their phenotypic expression could be the direct result of an alteration in the type of basement lamina produced. It is therefore possible that the widely acknowledged instability of the phenotypic expression of cultured cells could be due to their inability, when maintained on plastic, to continue to produce a normal basement lamina. If this should be the case, providing the cells with an artificial substrate closely resembling that produced in vivo should stabilize their phenotypic expression.

In the field of tumor-cell biology, the growing recognition that the substrate upon which cells are maintained could modify their phenotypic expression is also important. One of the main characteristics of tumor cells in tissue culture is their loss of anchorage-dependence. This is reflected in their ability to grow either in soft agar or in suspension. Yet tumor cells from solid tumors, which can adhere loosely to one another in vitro, in an in vivo site can adhere tenaciously to the substrate provided by the host tissue or which they themselves produce. This is best reflected in the phenomenon of metastasis, in which tumor cells carried through the bloodstream attach tenaciously to basement membrane, infiltrate through it, and form secondary tumors at sites located far from the original tumor. It is therefore likely that if tumor cells in culture are provided with an adequate substrate, they could shift their pattern of growth from anchorage-independence to anchorage-dependence. This could, in turn, result in an increased responsiveness to factors to which cells growing in suspension are not sensitive.

The ways in which the basement lamina exerts its permissive effect on the proliferation of normal diploid cells can only be the object of speculation. One possible effect is to modify the cell shape in order to make it responsive to factor(s) to which the cells do not respond unless they adopt an appropriate shape.

The author would like to thank Mr. Harvey Scodel for his invaluable assistance in the preparation of this manuscript.

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0022-202X/83/8101S-0050s$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:50s–55s, 1983
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Vol. 81, No. 1 Supplement
Printed in U.S.A.

Potassium Mediation of Calcium-Induced Terminal Differentiation of Epidermal Cells in Culture

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Epidermal cells cultured in low-calcium medium (0.02–0.1 m*M*) grow as a monolayer, in contrast to the stratified pattern of growth in medium with standard calcium levels (1.2–1.8 m*M*). These low-calcium cells lack desmosomes and maintain a high proliferation rate. Raising the extracellular calcium to >0.1 m*M* induces rapid desmosome formation followed by stratification, inhibition of proliferation, formation of cornified envelopes, and sloughing of the cells from the culture dish. This calcium-induced terminal differentiation program is characterized by an increase in the intracellular levels of sodium and potassium at 12 to 24 hours and is not blocked by inhibitors of calcium or sodium flux. Of 40 to 50 agents tested as inhibitors of calcium-induced epidermal differentiation, only ouabain, harmaline, A23187, and 8(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) were effective. These agents did not block the earliest calcium-induced effect (desmosome formation), but they did inhibit later stages in the program of terminal differentiation. Their detailed mechanism of action is unclear, although ouabain inhibits the sodium pump (Na^+K^+ATPase), lowering potassium and elevating sodium in the cells. The other inhibitors also prevented the calcium-induced elevation of intracellular potassium with no common effect on intracellular sodium. Reduction of potassium in the medium from the usual level of 6.5 m*M* to 0.1 m*M* lowers intracellular potassium by 60 to 70 percent and prevents calcium-induced differentiation. This result, along with the inhibitor studies, suggests that potassium plays an important role in epidermal terminal differentiation.

The usual pattern of epidermal cell growth in culture, characterized by limited proliferation, keratin synthesis, cell connections by desmosomes, stratification, and terminal differentiation, can be altered remarkably by lowering the calcium concentration in the medium from the usual 1.2 to 2.0 m*M* to 0.02 to 0.1 m*M*. In this low-calcium medium, desmosome formation and stratification are prevented and epidermal cells grow for several months as a monolayer with a high proliferation rate. The synthesis of keratin proteins continues, but differentiating cells are shed into the medium instead of forming multilayers, as they do in medium with >0.1 m*M* calcium [1–3].

Attached cells maintain distinct intercellular spaces, with physical communication between cells only by microvilli and occasional gap junctions and tight junctions; desmosomes are absent. Bundles of tonofilaments are arranged perinuclearly [1,2,6]. Low-calcium growth selects for epidermal cells with many of the characteristics of basal cells: small polygonal cells with a rapid proliferation rate that contain the pemphigoid antigen characteristic of basal cells, but lack the pemphigus antigen characteristic of superficial differentiating cells [4].

Basal cells can be induced to terminally differentiate by increasing the extracellular calcium concentration to >0.1 m*M*. The sequence of events following the addition of calcium to low-calcium cells has been characterized in some detail [1,2,5–8]. Desmosome formation begins within 5 minutes and is complete by 2 hours [6]. Proliferation decreases after 5 hours, reaching minimal levels by 24 to 36 hours. This decrease can be reversed by returning the switched cells to low calcium within 2 days. However, by 3 days, all the cells are committed to terminally differentiate [7]. Protein and RNA synthesis decrease gradually to about 50 percent of normal by 3 days [1,2]. No major changes in protein-synthetic patterns were detected at 6 hours, with no changes in synthesis of the major keratin proteins in the first 42 hours. However, the synthesis of leucine-labeled nonkeratin proteins in the molecular weight range of 35 to 45 kd is increased after the calcium switch [1,2]. Epidermal transglutaminase activity increases within 6 hours of the calcium switch and remains elevated for 1 to 2 days. The resulting $\epsilon(\gamma$-glutamyl)-lysine cross-links were demonstrated at 24 and 48 hours [5]. A 40- to 50-fold increase in attached cornified cells is seen at 24 hours [7]. Detachment of most cells from the culture dish occurs at 3 to 4 days.

The study of a variety of agents as potential modifiers of calcium-induced terminal differentiation has suggested that the process does not depend on changes in calcium or sodium flux

This work was supported by Grant No. AM 21557 from the National Institutes of Health.

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Abbreviations:
FBS: Fetal bovine serum
TMB-8: 8(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride