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The Extracellular Matrix and the Control of Proliferation of Vascular Endothelial and Vascular Smooth Muscle Cells

D. Gospodarowicz, I. Vlodavsky, and N. Savion

Cancer Research Institute and the Department of Medicine, University of California Medical Center, San Francisco, California 94143

In this short review we describe the observations which have led us to conclude that one of the most important components involved in modulating cell proliferation in vitro, and probably in vivo as well, may be the extracellular matrix upon which cells rest.

Key words: extracellular matrix, FGF, vascular endothelial cells, vascular smooth muscle cells, aging, differentiation

The extracellular matrix (ECM), or basal lamina, produced by cells is the natural substrate upon which cells migrate, proliferate, and differentiate in vivo. Although the exact nature and composition of ECMs are still to be elucidated, they are composed in large part of different types of collagen, glycosaminoglycans, proteoglycan [1, 2], and glycoproteins, among which is fibronectin [3], which has been shown to be a ubiquitous component of various types of extracellular matrices.

Numerous studies have dealt with the tissue differentiation induced by the extracellular matrices and basal lamina, but few studies have dealt with their effect on cell proliferation. Since in most organs cell proliferation precedes cell differentiation, it is likely that both proliferation and differentiation could be controlled directly or indirectly by the substrate upon which the cells rest [4]. This is particularly true of tissues which have only one developmental option. Early in vitro studies done on the control of cell proliferation by fibroblast growth factor (FGF) have indicated that FGF could modulate, either directly or indirectly, the rate of production of various cell surface proteins and extracellular matrix components. This was observed in at least two cell models, the vascular and corneal endothelia. In this short review we describe the observations which have led us to conclude that, in vitro, one of the most important components involved in modulating cell proliferation is the extracellular matrix produced by the cells and upon which cells rest.

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FGF AND THE PROLIFERATION OF CULTURED ENDOTHELIAL CELLS

Significant limitations on the culture of vascular and corneal endothelial cells have been imposed by the slow doubling time of these cultures, which can be passaged only at a high cell density if precocious senescence is to be avoided [5–7]. In contrast, if one adds FGF to the cultures, no such limitations are encountered. When primary cultures of cells from the bovine aortic endothelium are initiated with few cells (3 cells/cm^2), the development of a monolayer will depend on the presence of FGF in the culture medium [8, 9]. In 10% calf serum alone, small colonies develop from cell aggregates during the first few days but the cells look unhealthy and proliferate slowly, overlapping each other and becoming vacuolated and considerably enlarged. However, if FGF is added to the cultures, the population doubling time is reduced to as little as 18 hours and the cultures soon become confluent, exhibiting both the morphology and functions characteristic of the vascular endothelium in vivo. These include the formation of a highly contact-inhibited cell monolayer (Fig. 1) composed of closely apposed and flattened cells characterized by a nonthrombogenic upper cell surface and an active secretion of a fibrillar basement membrane composed mostly of fibronectin, proteoglycans, and collagen types III, IV, and V as well as by the synthesis of Factor VIII antigen and prostacyclins (Fig. 2), both of which are produced specifically by the vascular endothelium in vivo. Such cultures maintained and propagated in the presence of FGF divide with an average doubling time of 18 hours when seeded at either a high (up to 1:1000) or low split ratio (Fig. 3A). Upon reaching confluence, the cells adopt a morphological configuration similar to that of the confluent culture from which they originated (Fig. 4D).

In contrast, seeding of the same cells in the absence of FGF, even at a low (1:6) split ratio, results in a much longer doubling time (60–78 hr) (Figs. 3A,C) and in a strikingly different morphology. When seeded at a high split ratio (1:128) and in the absence of FGF, the cells proliferated poorly. The alterations in growth behavior were best demonstrated after 3 to 4 passages (15–20 generations) in the absence of FGF (Fig. 3A). The cells, by then 4- to 6-fold larger in size, failed to adopt a non-overlapping monolayer configuration even after being split at a 1:4 ratio. Instead, at sparse density they were flattened and highly spread (Fig. 4A) and at confluence grew on top of each other, leaving intercellular spaces (Fig. 4B). These cells exhibited a short lifespan, as reflected by vacuolization and cell degeneration after 30 generations.

Endothelial cells maintained for 3 passages in the absence of FGF and beginning to show a greatly increased average doubling time were still capable of responding to the mitogen, since addition of FGF resulted in a greatly increased growth-rate of the cultures (Fig. 3C) which at confluence adopted the characteristic endothelial configuration of a cell monolayer composed of small, highly flattened and closely apposed cuboidal cells, rather than their prior appearance as overlapping and large cells. In the case of cultured vascular endothelial cells, therefore, FGF is not only a potent mitogen which acts on cell plated at a clonal density but, by inducing the cultures to proliferate actively, it also serves as a survival factor, as is reflected by a substantial delay in the ultimate senescence of the cells.

VASCULAR ENDOTHELIAL CELLS AND AGING

There has been considerable discrepancy in the results of various laboratories regarding the number of doublings that vascular endothelial cells maintained in vitro will undergo. Schwartz [10], using Waymouth medium and endothelial cells derived from the adult

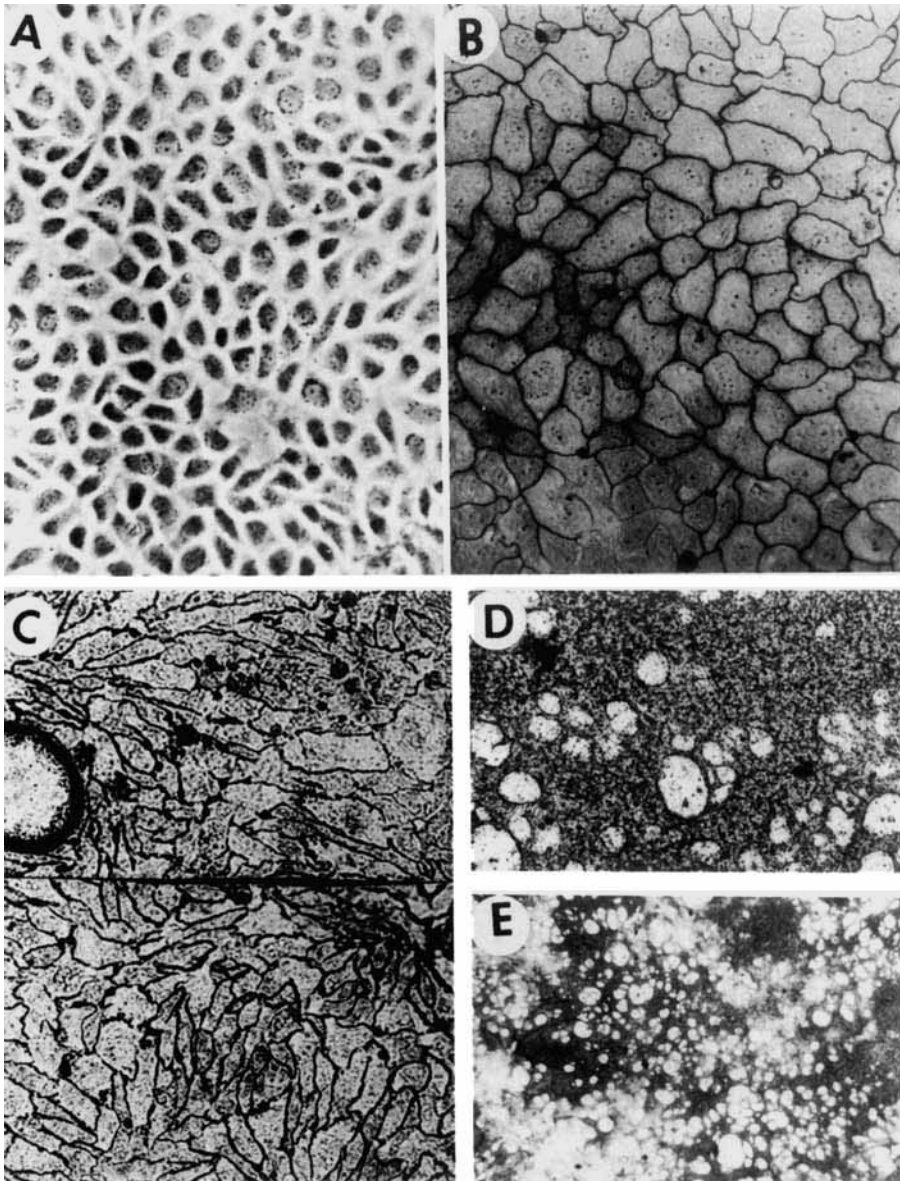


Fig. 1. A) Monolayer of vascular endothelial cells (13 passages, 65 generations) maintained in the presence of 10% calf serum and 100 ng/ml FGF. The cells are polygonal, closely apposed, and have an indistinct border (phase contrast, ×150). B) Same monolayer as in A, but stained with silver nitrate to show the cell borders (×210). The cells showed the same organization as did preparations of endothelium stained in situ. C) Adult bovine aortic endothelial cells maintained in culture for 3 weeks. The cultures were then exposed to alizarin red. The intercellular border stained bright red (×105). D) Adult bovine aortic endothelial cells were maintained in culture for a month and then exposed to 0.5% Triton X in PBS. After removal of the cell monolayer, the basement membrane was stained with alizarin red (×70). E) Same as D, but at a magnification of ×28; the basement membrane stained bright red.

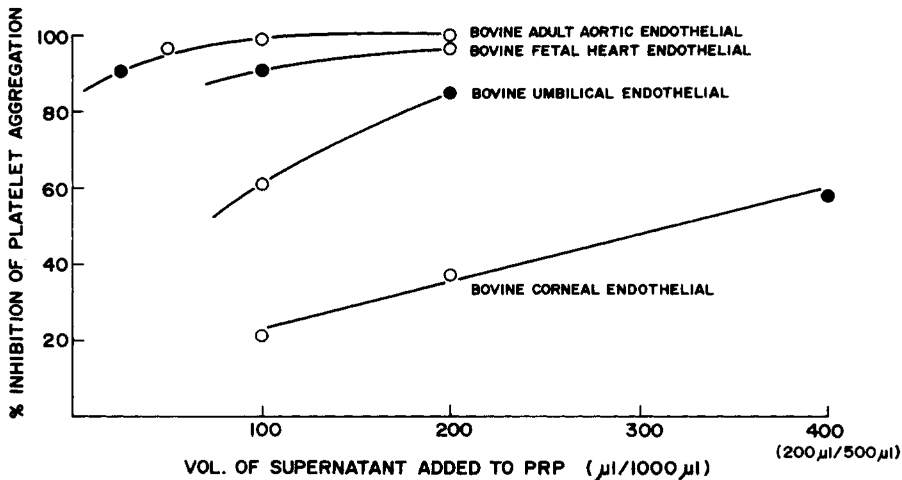


Fig. 2. Generation of PGI_2 activity by endothelial cell monolayer. Confluent vascular and corneal endothelial cell cultures were washed twice with DMEM, 15 mM Hepes and incubated in the presence of 1 ml of the same medium supplemented with arachidonic acid (1×10^{-4} M) for 5 min at 37°C before aliquots (200 µl, 25 µl) of the medium were added to stirred platelet-rich plasma (PRP). The PRP and medium were incubated together for 5 min at 37°C with stirring in a Payton Aggregation Module. Aggregation was induced by ADP ($5-8 \times 10^{-4}$ M) and was biphasic. The total reaction volume was 1 ml. Inhibition of the primary phase of the ADP-induced aggregation was monitored as an indication of the generation of prostacyclin-like activity. Aspirin (1×10^{-3} M) inhibited the generation of PGI_2-like activity by all four cell cultures. In addition, incubation of the cells with tranylcypromine (5×10^{-3} M), a known inhibitor of PGI_2 synthesis, also blocked the generation of inhibitory activity.

aortic arch, was able to passage cultures at a low split ratio (1:2). While the doubling time of early-passage cultures was 4 days, that of late-passage cultures was 7 days. Senescence was observed after 50 generations and could be speeded up if cultures were passaged at a split ratio of 1:4 [10]. Mueller et al [11], using MEM rather than Waymouth medium and cells derived from fetal rather than from adult aortic arch, have reported the development of clonal cell lines. However, the cloning efficiency was extremely low (approx. 1%) and could have led to great selective pressure. Cultures could be passaged at a split ratio of 1:11 for 80 generations, and early-passage cultures had an initial doubling time of 60 hours. This 60-hour doubling time is similar to that reported for ABAE cultures maintained in the absence of FGF [5]. In fact, when one corrects for the low split ratio used by Mueller et al, one discovers that the rate of proliferation of their cultures is the same as that reported by us earlier for vascular endothelial cells maintained in the absence of FGF (Fig. 5). Late-passage cultures had a doubling time of 72–96 hours. Similar results were claimed by McAuslan et al [12], who used medium 199 supplemented with thymidine to grow endothelial cells derived from adult bovine aortic arch. We have reported the cloning of various vascular endothelial cell lines using FGF. Using DMEM and FGF one can get a cloning efficiency as high as 50–75%. The risk of selecting special cell types is therefore greatly reduced. Such cultures maintained in the presence of FGF have been passaged at a high split ratio (1:64 every 5 days or 1:128 every 7 days) and can have average doubling time of 18–20 hours. Perfect contact inhibition in cultures derived from the adult aortic arch have been reported. Although the total generation number one can achieve with

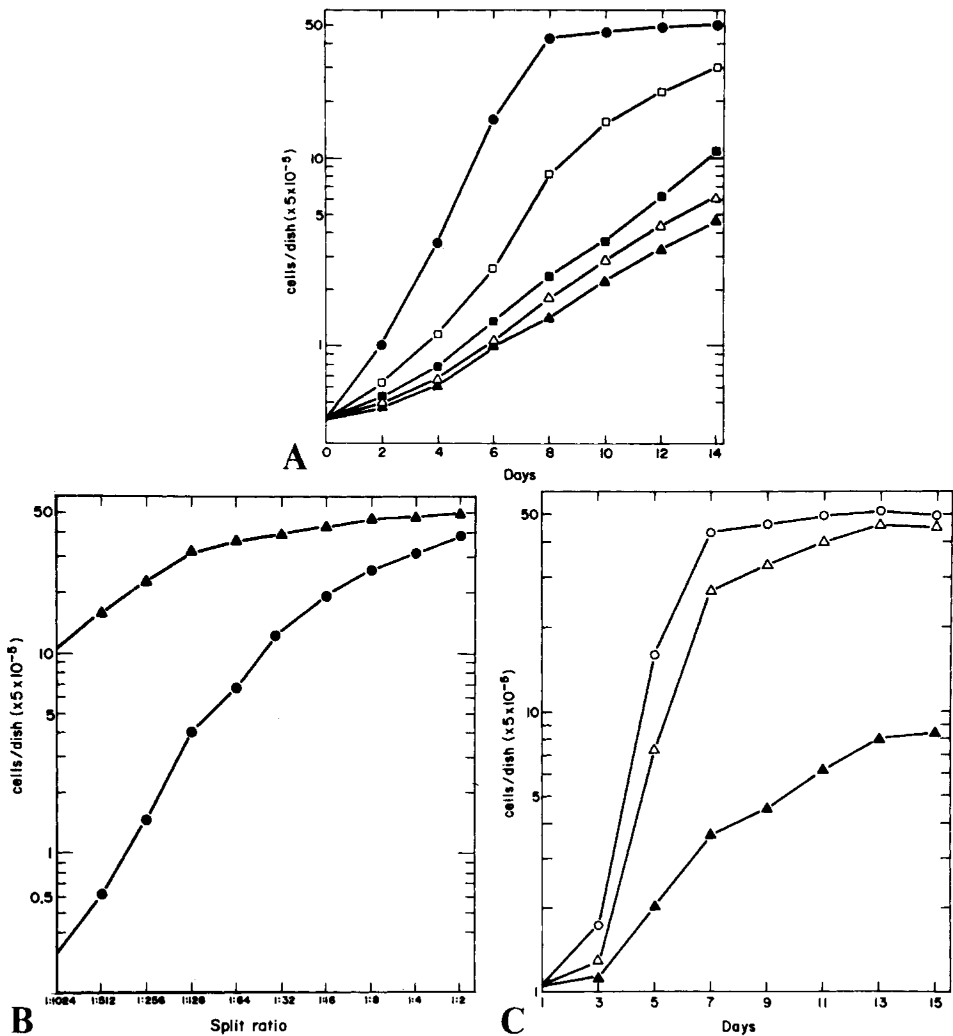


Fig. 3. Growth rate of vascular endothelial cells in the presence and absence of FGF. A) Bovine aortic endothelial cells (ABAE) maintained in the absence of FGF for 1 (□), 3 (■), 5 (△), and 6 (▲) cell passages (each passage representing 3–5 cell doublings and 6–8 days in culture) were seeded into 35-mm dishes (8 × 10^3 cells per dish) in DMEM supplemented with 10% bovine calf serum. The medium was replaced every four days, and duplicate dishes counted every other day. Control cultures (●) which were not subjected to FGF withdrawal were similarly seeded and counted, except that FGF was added every other day. These cells adopted at confluence a perfect monolayer configuration, whereas cells that were cultured (3 or more passages) in the absence of FGF grew on top of each other and formed an unorganized cell layer. B) Confluent endothelial cell cultures maintained with (16 passages, ▲) or without (4 passages, ●) FGF were split at various ratios and cultured in DMEM containing 10% calf serum in the presence and absence of FGF (added every other day), respectively. The medium was replaced after five days and triplicate dishes counted every other day. The number of cells after 9 days in culture is plotted as a function of the split ratio. The seeding level at a split ratio of 1:2 was 1.65 × 10^5 and 1.4 × 10^5 cells per 35-mm dish for cells maintained with and without FGF, respectively. C) Vascular endothelial cells derived from the adult aortic arch (32 passages; 160 generations) were plated into 35-mm dishes (2 × 10^4 cells per dish) in DMEM (H-16) supplemented with 10% calf serum. Duplicate cultures were counted every other day and the medium replaced every 4 days. ▲, Cells after 3 passages in the absence of FGF. No FGF was added. △, Cells after 3 passages in the absence of FGF. FGF was added on the third day after seeding and every other day thereafter. These cells adopted at confluence a perfect monolayer configuration indistinguishable from that of cells that were never subjected to FGF withdrawal. ○, Cells derived from endothelial cultures that were continuously maintained with FGF. FGF was added every other day.

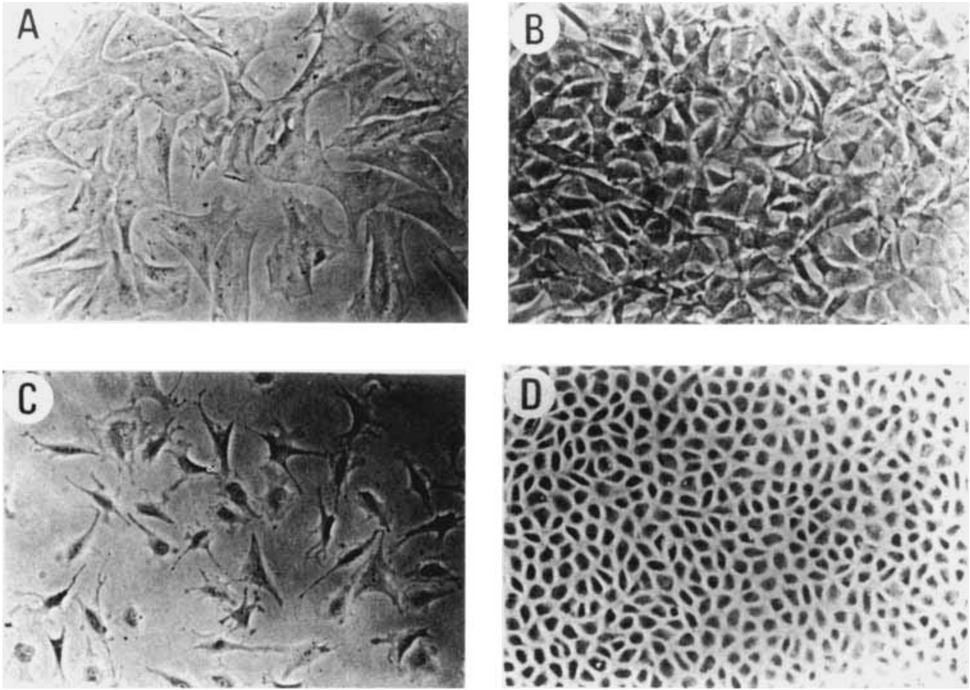


Fig. 4. Morphological appearance of sparse and confluent vascular endothelial cultures maintained with and without FGF (phase contrast, ×150). A) Sparse endothelial cells maintained in the absence of FGF. The cells are 4- to 6-fold larger and spread farther apart than sparse cells (C) seeded and maintained in the presence of FGF. B) Confluent endothelial cells after 4 passages (12 generations) in the absence of FGF. The cells grow on top of each other and in various directions. D) A confluent endothelial monolayer formed by cells (100 generations) maintained in the presence of FGF. The cells are highly flattened, closely apposed, and non-overlapping.

cultures originating from vascular territories as different in age as fetal versus adult and as different in origin as vein versus artery can itself differ greatly, our greatest success has been 390 generations. In all cases, cells maintained in the presence of FGF were capable of making Factor VIII antigen, as well as of producing high level of prostacyclins. Cultures were perfectly contact inhibited, as documented by their (^{3}H)thymidine and (^{35}S)methionine incorporation as a function of cell density and cell morphology. Therefore, in three different laboratories the number of generations that vascular endothelial cells will undergo is as different as 50 [10], 80 to 90 [11, 12], or 390 [13].

But is there really a difference? As shown in Table I, if one corrects for the average doubling time of the cultures, one arrives at the surprising result that, regardless of the number of generations the cells have undergone, the total time cultures have endured is very similar in all cases and averages 284 ± 29 days, despite the wide difference in the way the cultures were maintained (split ratio, medium, as well as vascular territory from which cells originated). Even more remarkable is that the average lifetime of the cultures is similar to that reported for WI-38 cultures passaged weekly at a split ratio of 1:2 for 50 weeks.

This simple observation seems to indicate that aging in culture is more a somatic problem than a fixed program of senescence imprinted in the DNA, since senescence of the cells correlates better with the *total lifetime* of the cultures than with the generation

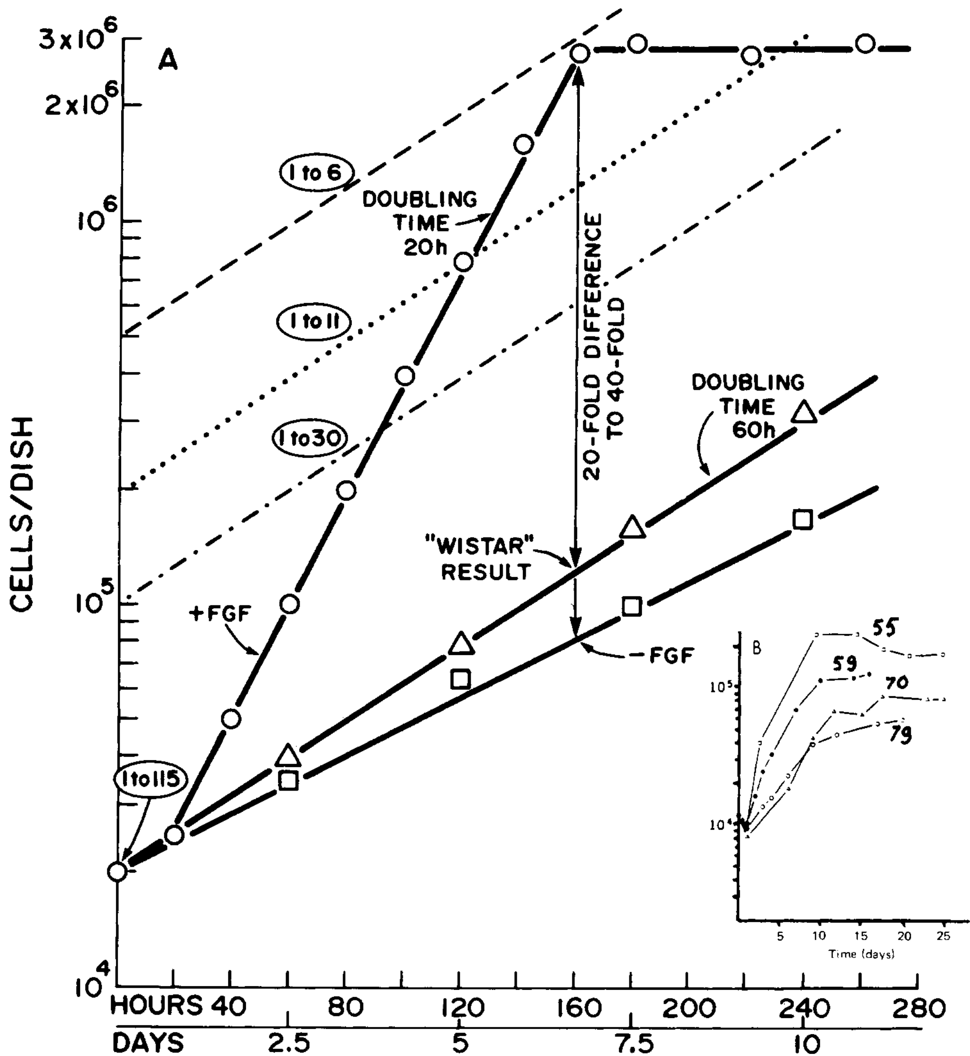


Fig. 5. Rate of proliferation of vascular endothelial cells maintained in the presence or absence of FGF. A) Vascular endothelial cells seeded at an initial cell density of 2×10^4 cells/6 cm dish and maintained in the presence of DMEM (H-16) supplemented with 10% calf serum. In the presence of FGF (○, average doubling time of the culture was 20 hours. In the absence of FGF, it was 72 hours (□) . This average doubling time in the absence of FGF is similar to that reported by Mueller et al [11]. Their data are shown in the insert (B). When the optimal proliferative rate of their cultures was recomputed according to the split ratio at which we transferred our cultures (1:115) (Wistar result, △), there was little if any difference in the growth rate of control cultures maintained in the absence of FGF (□). The main difference, however, was that confluence in the presence of FGF was reached *after 6 days* (○). In the case of Mueller et al [11], it would not have been reached *before 17 days.* To reach confluence within 5 days, these cultures would have to be passaged at *a split ratio of 1:6* (– – –). At a split ratio of 1:15, confluence is reached by day 10 (· · · · ·), and at 1:30 it is reached by day 13. B) Growth curves as a function of the cumulative population doubling (CPDL) for endothelial cell clone BFA-1c developed by Mueller et al [11]. Cells were inoculated into 25-cm^2 flasks at densities of 1.0×10^4 to 1.2×10^4 cells/cm^2 (8 ml medium per flask). Flasks were incubated under standard conditions and given fresh culture medium every 6 or 7 days. At various times after inoculation, duplicate cultures were counted with a Coulter counter to determine density. The CPDLs studied were 55 (□), 59 (●), 70 (△), and 79 (○). Their results are expressed as cell density per cm^2 as a function of time [11].

TABLE I. Lifetime in Culture of Bovine Vascular Endothelial Cells Maintained in Different Laboratories

	Days in culture	Generation	Day of passage	Split ratio	Doubling time (hr)	Cell number generated from 1 cell
Mueller et al [12]	260	80–90	10–15	1:10	60–72	10^{24-27}
Schwartz [11]	280	50	15	1:2 or 1:4	84–168	10^{15}
Gospodarowicz et al [13]	325	390	5	1:64	18–20	10^{117}
McAuslan et al [12]	270	–	–	–	60–72	–
WI 38	200–250	50	4–5	1:2	96–120	10^{15}

Mean value of lifetime in culture of vascular endothelial cells ± standard error = 284 days ± 28.

number. In this regard, it is interesting to note that if senescence in culture is due to an oxidation problem (generation of free radicals), it makes more sense to consider it a somatic than a genetic problem. The effect of time in culture on mitochondrial replication and the ability of cells to perform metabolic functions adequately has not been studied in this context. Yet mitochondrial replication is as likely a candidate as nuclei replication as a locus for senescence.

If one were to predict the maximal number of generations cells could undergo in vitro based on a lifetime of the cultures of 300 days and the shortest cycle cells can achieve (16 hours), it would be 495 generations. How does this compare with the in vivo situation? Not very well. The basal cell layer of the corneal epithelium renews itself every week. Therefore, based on an average human lifespan of 70 years, each basal cell will go through 3850 generations in vivo. Yet, when put in culture this cell type will go through 50–100 generations at most. One should further point out that clonal proliferation, except in the case of the hematopoietic system, is a rare event in vivo. When a cell divides, in most cases it replaces a dead or a terminally differentiated cell. Usually one of the daughter cells resulting from the replication will lose its ability to proliferate while the other cells will retain it. This leads to an apparent zero growth rate, although cells in some organs such as the gut go through thousands of replications during the lifespan of the individual.

FGF AND THE DIFFERENTIATION OF CULTURED VASCULAR ENDOTHELIAL CELLS

As already pointed out, vascular endothelial cells grown in the presence of FGF adopt, upon reaching confluence, the morphology of a cell monolayer composed of tightly packed and flattened cells. This cell layer, as in vivo, shows an asymmetry of cell surfaces. While the apical cell surface is a nonthrombogenic surface to which platelets do not bind (Fig. 6), the basal cell surface is involved in the synthesis of a highly thrombogenic extracellular matrix which, when examined by immunofluorescence, is composed of collagen type III and, to a lesser extent, of collagen type IV [11] (Fig. 7). Also noteworthy is the redistribution or de novo appearance of new cell surface proteins which correlate with the organization of subconfluent cells not yet organized into a highly contact-inhibited cell monolayer. Sparse, actively growing vascular endothelial cells contain fibronectin associated with both their apical and basal cell surfaces as well in the areas of cell-cell contact. Upon reaching confluence, a dramatic redistribution of that cell surface protein occurs which results in its disappearance from the apical cell surface and its secretion into the basal cell surface, where it eventually becomes an integral component of the extracellular matrix [14, 15]. Parallel to the redistribution of fibronectin as the cells reorganize into a nonoverlapping cell monolayer, the appearance of a new cell surface protein called CSP-60 can also be observed [16]. This protein was found to be exposed to iodination by lactoperoxidase only in endothelial cells that have adopted a monolayer configuration. It was not detected either in actively growing or in unorganized endothelial cell cultures. Likewise, CSP-60 was no longer exposed for iodination in disorganized endothelial cell monolayers and was not present in sparse or confluent cultures of fibroblasts or vascular smooth muscle cells that grow in multiple layers [16]. CSP-60 has now been observed with all types of vascular endothelium studied to date, whether they be fetal or adult in origin or from territories as diverse as the endocardium, aortic arch, umbilical vein, or lung arteries (Fig. 8).

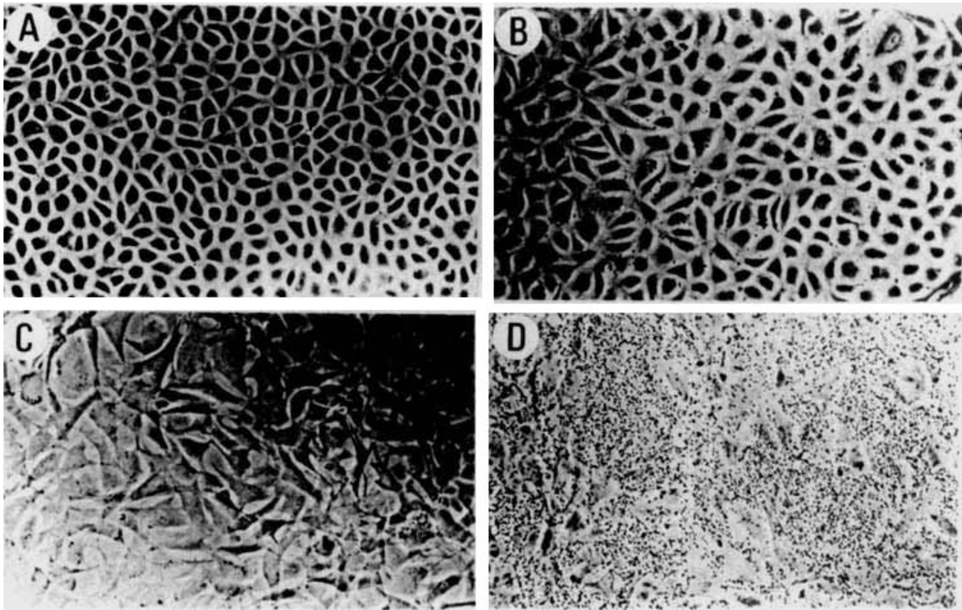


Fig. 6. Adherence of platelets to confluent vascular endothelial cultures maintained with or without FGF. Confluent endothelial cultures maintained in the absence (3 passages) or presence (during the phase of logarithmic growth) of FGF were incubated with human platelets (2 × 10^8/ml, 30 min, 37°C), washed, and observed by phase microscopy (×150). A, B) Cells maintained with FGF. Very little or no platelets can be seen (B) attached to the upper surface of cells that adopt a monolayer configuration as in (A). In contrast, most of the upper surface of unorganized cultures (C) maintained in the absence of FGF is covered with platelets which attached singly and do not form aggregates (D).

In contrast to cultures grown in the presence of FGF, cultures maintained in its absence lose within 3 passages their ability to form at confluence a monolayer of closely apposed and flattened cells. Instead, the cultures adopt a multilayer configuration consisting of large and overlapping cells which are no longer contact inhibited [5, 17]. Parallel to these changes in cell morphology, a loss of cell surface polarity is observed. Both the apical and basal cell surfaces are now covered by an extracellular matrix which fluoresces strongly when analyzed by immunofluorescence with purified antibodies against collagen types I, III, and IV [17] (Fig. 7). Parallel to this loss of orientation in the secretion of extracellular matrix, the apical cell surface becomes thrombogenic, as reflected by an increase in platelet-binding capacity [5, 17]. Likewise, marked changes in the distribution and appearance of cell surface proteins such as fibronectin and CSP-60 can be observed. Fibronectin, which in confluent and highly organized cultures grown in the presence of FGF is detected only in the basal cell surface, now appears in both basal and apical cell surfaces [5]; CSP-60 is no longer exposed for iodination, even late at confluence [5, 17].

The production of fibronectin by sparse and confluent endothelial cultures maintained with or without FGF has been further studied by exposing the cells to (^{35}S)methionine and subjecting both the cell layer and tissue culture medium to SDS slab gel electrophoresis before and after immunoprecipitation with anti-fibronectin antiserum. Although anti-fibronectin precipitated less than 3% of the total (^{35}S)-labeled proteins that were secreted into the culture medium of cells maintained and actively growing in the presence

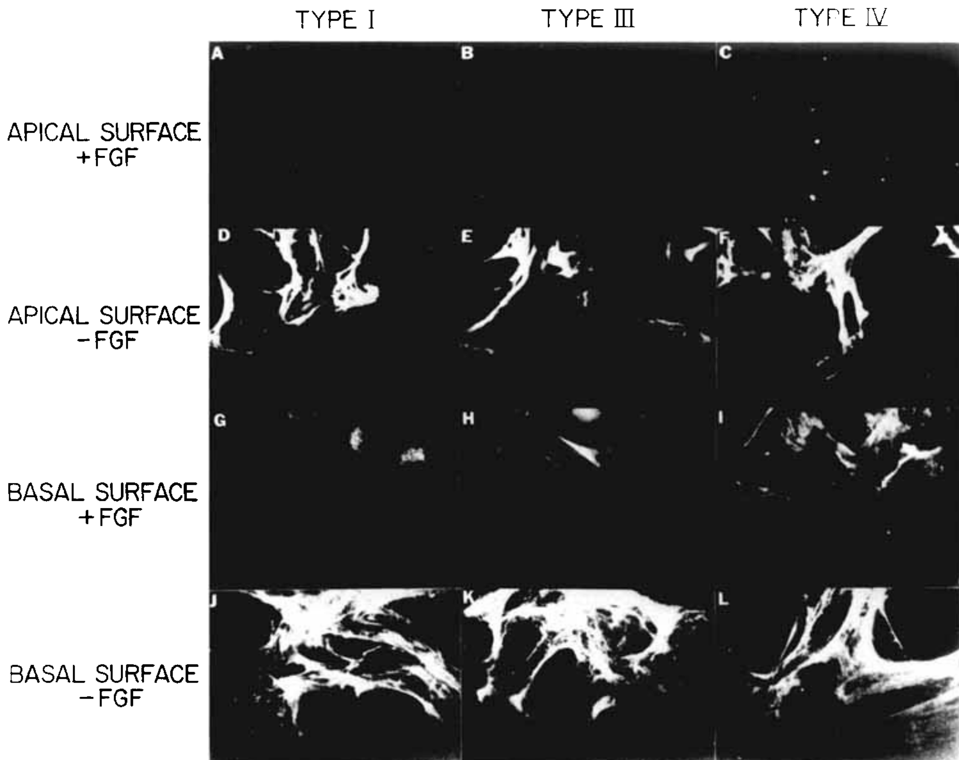


Fig. 7. Indirect immunofluorescent localization of collagen in vascular endothelial cells maintained in the presence (ABAE) or absence (ABAE-F) of FGF. Cultures were stained with affinity purified antisera (kindly provided by Drs. G. Martin and J. M. Foidart, Dental Institue, NIH, Bethesda, MD). ABAE cell monolayers were stained with antibodies directed against (A) collagen type I, (B) collagen type III, and (C) collagen type IV. Focusing was on the apical cell surface. ABAE-F cell monolayers were stained with antibodies directed against (D) collagen type I, (E) collagen type III, and (F) collagen type IV. Focusing was on the apical cell surface. The basal cell surface was studied by first permeabilizing the cells in acetone (10 min at –20°C) prior to staining. The ABAE basal cell surface was examined with antibodies directed against (G) collagen type I, (H) collagen type III, and (I) collagen type IV. The basal cell surface of ABAE-F was examined with antibodies directed against (J) collagen type I, (K) collagen type III, and (L) collagen type IV. In all cases, non-immune rabbit serum was non-reactive with either ABAE or ABAE-F cells (X200).

of FGF, 20–25% of the total (^{35}S)-labeled proteins were precipitated from the culture medium of cells that were maintained in the absence of FGF. When analyzed on SDS polyacrylamide gels, more than 90% of the immunoprecipitated radioactivity comigrated with fibronectin [5]. On the basis of the immunoprecipitation values, it can be calculated that sparse and confluent cultures maintained in the absence of FGF secreted into the medium 30- and 50-fold more fibronectin per cell than sparse and confluent cells cultured in the presence of FGF, respectively [5].

Endothelial cells previously maintained in the absence of FGF showed a normal proliferative response to readdition of FGF when reseeded at a low density (split ratio 1:16), as indicated by a 4-fold decrease in doubling time (18 hr versus 72 hr) and increased in final cell density at confluence (900 cells/mm^2) [5] (Fig. 3C). In terms of morphological

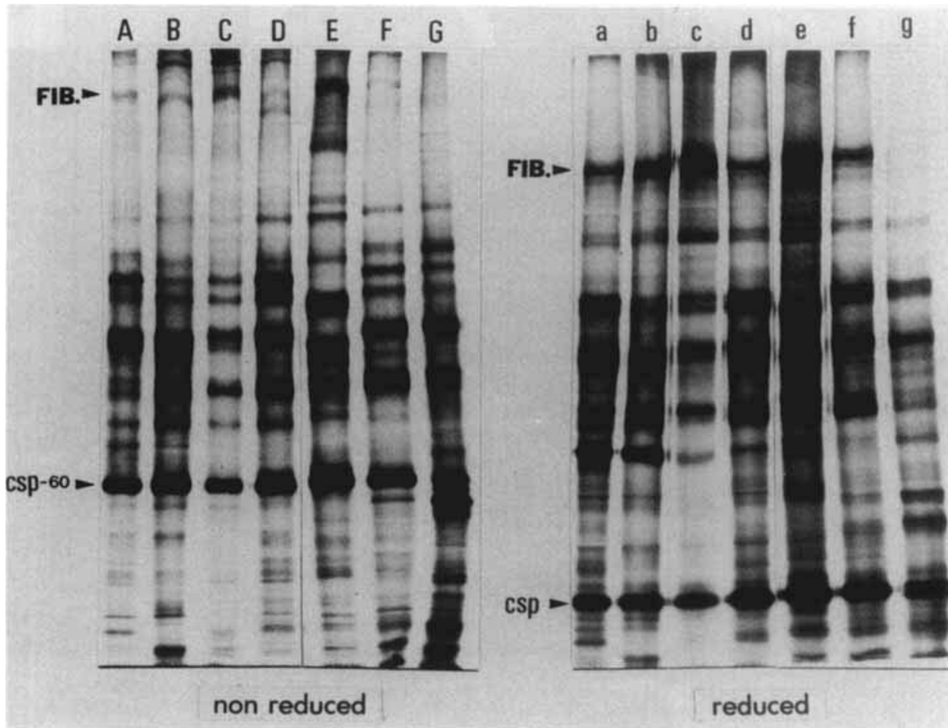


Fig. 8. Appearance of CSP-60 in various types of cultured vascular endothelial cells. Vascular endothelial cells of various origins were obtained, cloned, and maintained in culture as described [5, 21, 22]. Confluent cell monolayers (5 to 7 days after reaching confluence) were radioiodinated (lactoperoxidase/glucose oxidase) and analyzed by a gradient (5–16%) PAGE before (lanes A–G) or after (lanes a–g) reduction with 0.1 M DTT. Vascular endothelial cells of the following origins were studied: A, a) Bovine pulmonary artery; B, b) adult pig aorta; C, c) fetal bovine aorta; D, d) adult bovine aorta; E, e) fetal bovine heart; F, f) bovine umbilical vein; and G, g) calf bovine aorta. Arrows mark the positions of fibronectin (460K and 230 K) and CSP-60 (60K and 30K) before and after reducing the samples with DDT, respectively.

appearance, the cells became smaller and formed at confluence a highly organized monolayer in which little or no cell overlapping was observed. When the confluent cultures were tested for surface iodination pattern (Fig. 9D, I) [5], CSP-60 appeared as a major protein, whereas fibronectin was detected in a much smaller amount, as in a confluent monolayer of endothelial cells that were continuously maintained with FGF (Figs. 9A, F). Similarly, extracellular matrix was produced toward the basal part of the cells and no longer on top of the cell layer. The cells which reverted also showed the normal low rate of fibronectin synthesis and secretion as well as a nonthrombogenic apical surface to which platelets cannot adhere. Endothelial cells that were maintained for over 40 generations (7 passages) in the absence of FGF underwent cell senescence and hence no longer responded to FGF [5].

When FGF was added to sparse or subconfluent cultures previously maintained in its absence, it was found that as the cell density and degree of overlapping increased, the cells became less able to regain their normal differentiated properties. The reorganization of cells into a closely apposed monolayer of cells required at least 1 to 2 cell doublings and was always associated with a reappearance of CSP-60, a decreased synthesis and secre-

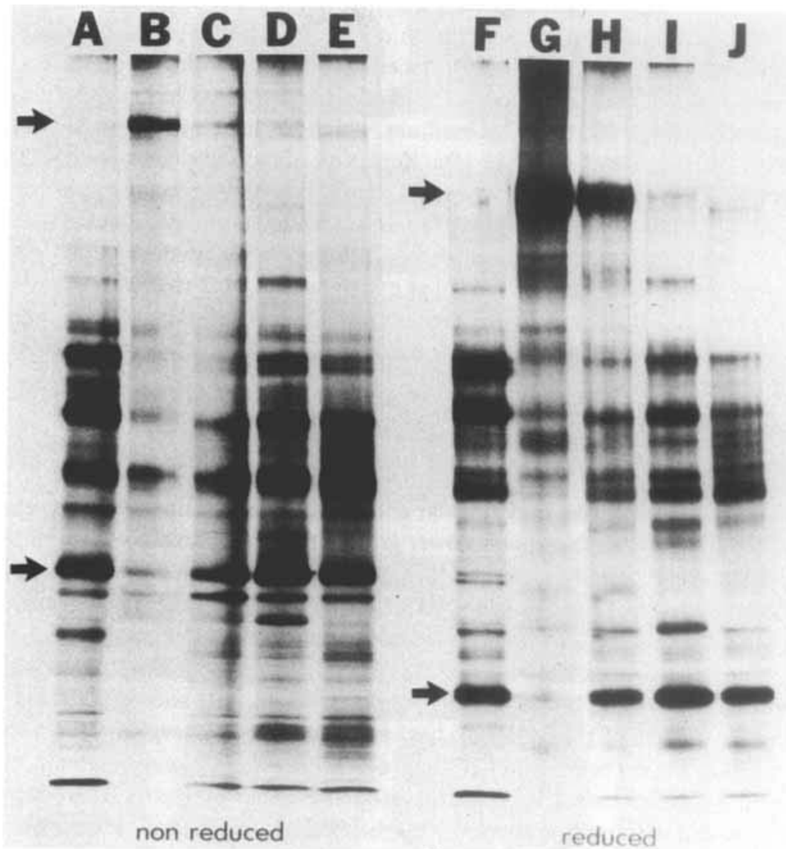


Fig. 9. Cell surface iodination pattern of overlapping and reorganized endothelial cells before and after being reexposed to FGF. Endothelial cells maintained for 3 passages in the absence of FGF were seeded at a split ratio of 1:8 and FGF was added (every other day) starting on day 2 or day 7 after seeding. Control cultures (not exposed to FGF) and reorganized cultures (reexposed to FGF) were iodinated at confluence (14 days after seeding) and analyzed by gradient (5–16%) polyacrylamide slab gel electrophoresis before (lanes A–E) and after (lanes F–J) reduction with 0.1 M DTT. A, F) A confluent monolayer of endothelial cells that were continuously maintained in the presence of FGF. CSP-60 appears as a major band. B, G) Confluent but unorganized endothelial culture maintained for 4 passages without FGF. Fibronectin appears as a major band; CSP-60 is missing. C, H) Same cells as in (B) and (G) exposed to FGF at a subconfluent density (7 days after seeding) and labeled 7 days later. Most of the cultures adopt a monolayer configuration, although some unorganized areas (about 20% of the culture) are still present. Both fibronectin and CSP-60 are exposed for iodination. D, I) The same cells as in (B) and (G) exposed to FGF at a sparse density (3 days after being split at a 1:8 ratio) and labeled 12 days afterwards, when the cells were highly organized and closely apposed. CSP-60 appears as a major band, whereas fibronectin is detected in small amounts as in cells that are maintained continuously with FGF. E, J) (-FGF) culture exposed when subconfluent to a medium conditioned by a confluent monolayer of endothelial cells and iodinated 7 days afterwards.

tion of fibronectin into the tissue culture medium, and the acquisition of non-thrombogenic properties. On the other hand, fibronectin already present on the apical cell surfaces disappeared only partially [5]. Limited reversion, if any, was obtained when unorganized cultures were reexposed to FGF late at confluence, suggesting that the spatial organization might, among other factors, determine whether or not and to what extent cells will respond

to FGF. Unlike the limited degree of reversion obtained with FGF, total reversion of highly overlapping endothelial cell cultures was induced without promoting cell proliferation by a conditioned medium taken from confluent endothelial cells that have adopted a monolayer configuration (Figs. 10, 11). This medium, which has little mitogenic activity, is fully potent even in the absence of serum or after depletion of its fibronectin content by affinity chromatography on a gelatin-Sepharose column [17]. It is now being analyzed for the presence of proteolytic activity and various components of cellular origin which might be responsible for the induced changes in cellular morphology and growth characteristics. Therefore, endothelial cells that are seeded at a high density (1:2 to 1:10 split ratio), unlike cells that are seeded at a low clonal density, may, by conditioning the medium, adopt a cell monolayer configuration and the associated differentiated properties even in the absence of an added factor such as FGF [17].

THE EXTRACELLULAR MATRIX (ECM) AND CELL PROLIFERATION

The above results suggest that vascular endothelial cells maintained in the absence of FGF exhibit, in addition to a much slower growth rate, morphological as well as structural alterations which mostly involved changes in the composition and distribution of the ECM. This raises the further possibility that the ECM produced by these cells could have an effect on their ability to proliferate and to express their phenotype once confluent.

The importance of the ECM for normal growth and development in vivo has long been recognized [18]. It has been demonstrated by Dodson [19] and by Wessels [20] that the basal cell layers of the epidermis have to be in direct contact with the ECM upon which they rest in vivo in order to retain their normal orientation and to remain mitotically active in organ culture. This substrate can be produced either by the mesenchyme which is closely associated with most epithelia or by the epithelia themselves, after they interact with the ECM produced by other tissues. Such is the case with the isolated corneal epithelium which can recreate its own stroma if cultured in vitro on isolated lens capsule but not if cultured on a non-collagenous stroma [21]. Recent transfilter experiments have shown that direct contact by epithelial cells with a collagen substrate is required if they are to produce their own ECM and that the extent of cell surface area in contact with the substrate is directly proportional to the stimulation of stroma production [21–23]. This newly produced ECM could in turn be held responsible for the control of proliferation of the basal epithelial cell layer, possibly by affecting the cell shape [21]. Investigation of the role of extracellular materials at the epithelial-mesodermal interface has shown that glycosaminoglycans present as major molecular species at the junction of interacting tissues could be implicated in epithelial morphogenesis [24]. Likewise, evidence that the substrate upon which cells rest when maintained in tissue culture is important for their proliferation is now plentiful. The pioneering work of Ehrmann and Gey [25] has shown that various tissues demonstrate enhanced growth and differentiation when cultured on collagen gels. Recent studies have also shown that collagen is important in promoting cell attachment [26–30], cell migration [31], and cell proliferation [27, 32, 33]. Of particular interest is the study of Liotta and his colleagues [32] on the growth of fibroblasts in culture which indicates that, even when grown on plastic, the cells deposit a collagen substrate which is required for proliferation. This was demonstrated using the proline analog, cis-hydroxyproline, which decreases the amount of newly synthesized procollagen secretion when incorporated into collagen [34]. Cultures maintained on plastic and exposed to cis-hydroxyproline did not produce collagen and did not proliferate, while cultures exposed to cis-

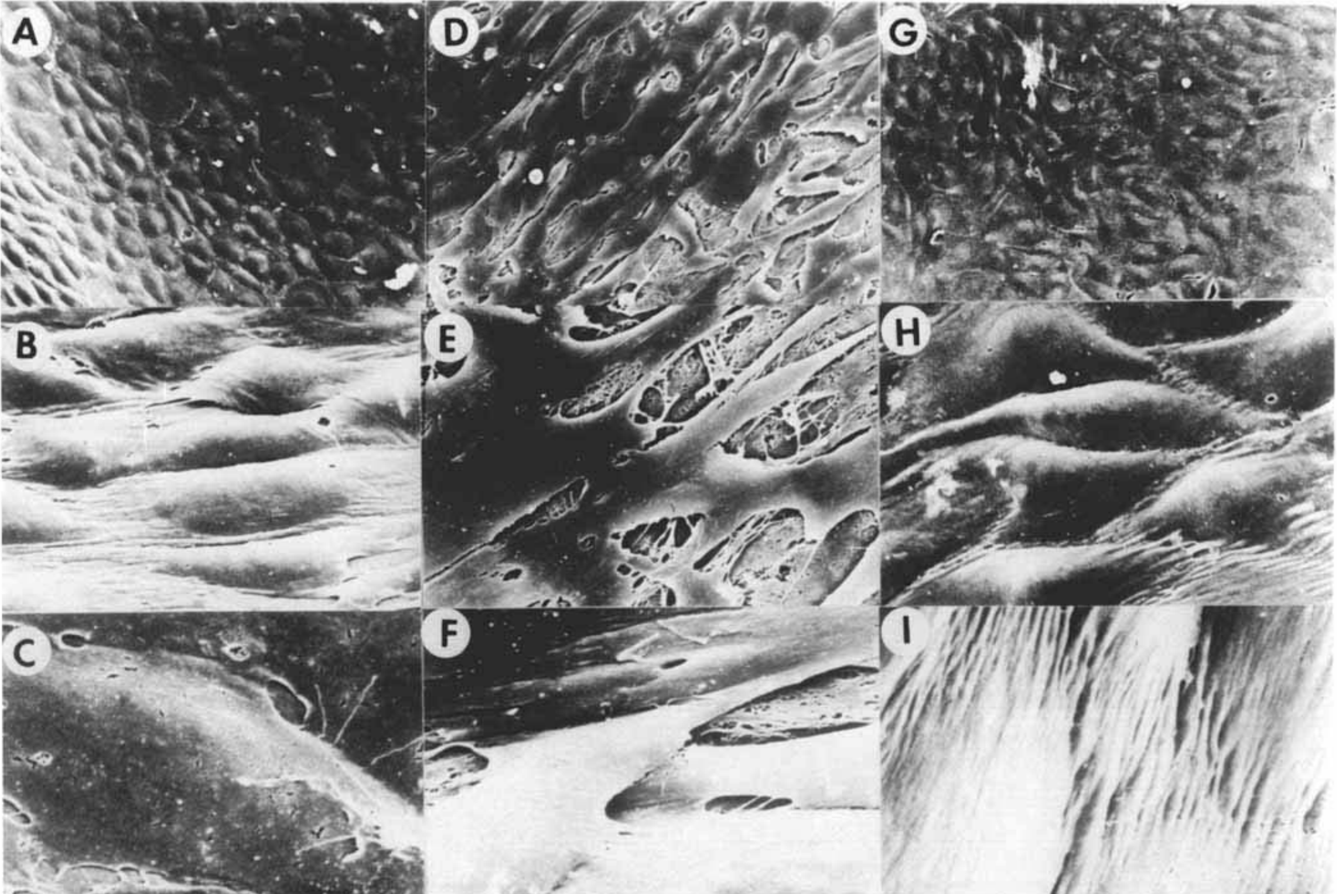


Fig. 10. Restoration of the normal phenotypic expression of ABAE-F cells by conditioned medium (scanning electron microscopy). A, B, C) Confluent ABAE cells maintained with FGF do not overlap each other, are closely apposed, and have a prominent nucleus. D, E, F) ABAE-F cells maintained without FGF for 4 passages (1:8) are larger, disorganized, and form multiple layers. The cell nucleus is not prominent and the underlying extracellular matrix is exposed in some areas. G, H, I) When 600,000 ABAE-F cells are maintained for 8 days in conditioned medium, the non-overlapping monolayer is reestablished. The cells become smaller, organized, and non-overlapping, thus forming the typical "cobblestone pattern" (X2,000).

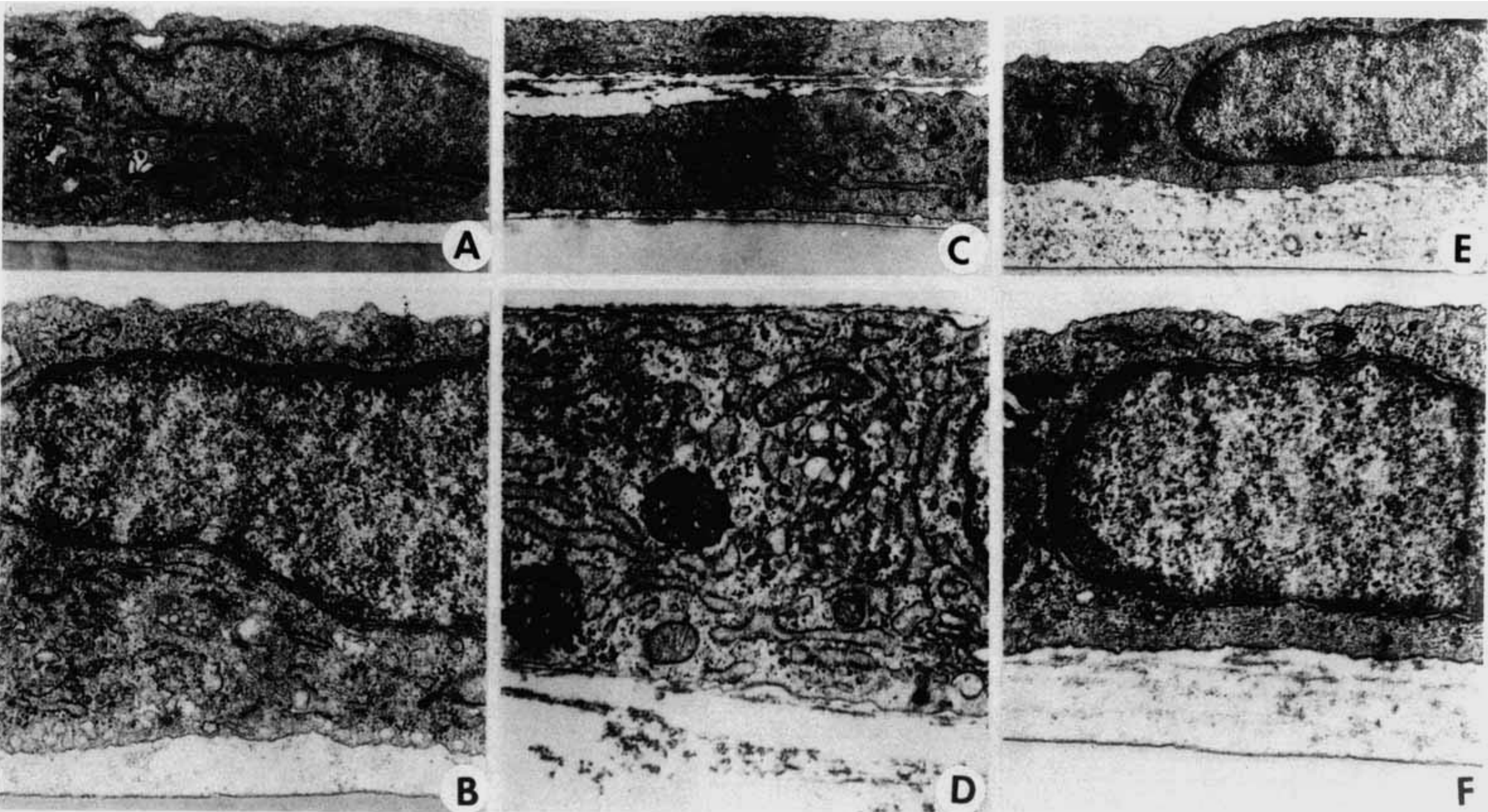


Fig. 11. Transmission electron microscopy of ABAE, ABAE-F, and ABAE-F maintained in conditioned medium. Sections were made in cross-section with the cell monolayer (A, C, E at ×20,000 and B, D, F at ×50,000). A, B) ABAE cell monolayer showing the presence of an amorphous basement membrane underlying the cell with no material on the apical cell surface. C, D) ABAE-F cells can be seen to overlap, and the presence of extracellular matrix on the basal and apical cell surfaces is illustrated. E, F) ABAE-F cells maintained for 8 days in conditioned medium have regained their characteristic morphology with the concurrent redistribution of extracellular matrix to the basal cell surface. No matrix can be seen on the apical cell surface.

hydroxyproline and provided with an artificial collagen substrate did proliferate [32]. This observation therefore linked collagen and ECM production to cell proliferation. It is not known, however, if the main effect of collagen is to promote cell attachment, thereby indirectly allowing the cells to proliferate, or if it has a direct effect on both cell attachment and cell proliferation. In either case, the possibility exists that cells which do not adapt and grow readily in tissue culture could be limited in their collagen production and that providing them with an ECM produced by other cell types could be one way to circumvent this limitation.

Among the other components of the ECM which have been studied in regard to cell attachment and proliferation in vitro is fibronectin. Like collagen, it has been shown to promote cell attachment, migration, and proliferation [35–37]. Whether fibronectin directly mediates these effects or acts by stimulating the production of ECM from cells which are exposed to it has not been analyzed.

The effect of the ECM on the proliferation of cells maintained in cultures has not been studied. This is mostly due to the fact that, with the exception of lens capsule, it is difficult in vivo to isolate such material from neighboring tissues. In vitro the reconstitution of an ECM from its separate elements (collagens, proteoglycans, glycosaminoglycans, and glycoproteins) may be difficult, if not impossible. Not only must the correct ratio of components constituting the ECM be respected, but they must also be linked in such a way that the resulting tri-dimensional structure will be like that of the extracellular scaffolding in vivo. The problem in reconstituting an ECM in vitro is made even more difficult by the fact that collagen types IV and V, which in vivo are the main components of basement membrane collagens, can only be extracted from tissue following proteolysis. This could result in structural alterations and prevent their proper polymerization in vitro. One must also consider that our knowledge of the ECM components is limited. Components such as laminin [38] have only been isolated recently, and the number of these which remain to be identified can only be guessed.

Corneal endothelial cells maintained in tissue culture retain their ability, in contrast to most cell types, to synthesize and secrete an ECM found underneath, but not on top of the cells [39]. As shown in Figure 12A, corneal endothelial cells upon reaching confluence form a monolayer of small, highly flattened, tighly packed (1,100 cells per mm^2), and nonoverlapping cells. Secretion of an ECM takes place only underneath the cell layer [15, 40], and the underlying matrix is revealed after exposing the cell layer to 0.5% Triton X-100 and subsequent washing with PBS to remove remaining nuclei and cytoskeletons [41]. The matrix then appears as a uniform layer of amorphous material coating the entire area of the dish (Figs. 12B, C). The chemical composition of the ECM produced by corneal endothelial cells in vitro is currently being analyzed [C. Tseng, N. Savion, R. Stern, and D. Gospodarowicz, manuscript in preparation]. Based on immunofluorescence studies (Fig. 13), collagen types III and IV appear as the major collagen components of the matrix, forming an evenly distributed fibrillar meshwork (Fig. 13). Fibronectin and laminin are also present [15, 39]. This ECM, whose appearance has been shown to correlate with the acquisition by cultured corneal endothelial cells of their normal "in vivo" morphology, cell surface polarity, and function [15, 39], could substitute for the ECM produced by other cell types. The ability of corneal endothelial cells in tissue culture to produce an extensive ECM could, therefore, provide us with a tailor-made ECM with which to test the proliferation and response of other cell types to growth factors. We have, therefore, compared the rates of proliferation of vascular endothelial cells maintained on plastic versus an ECM.

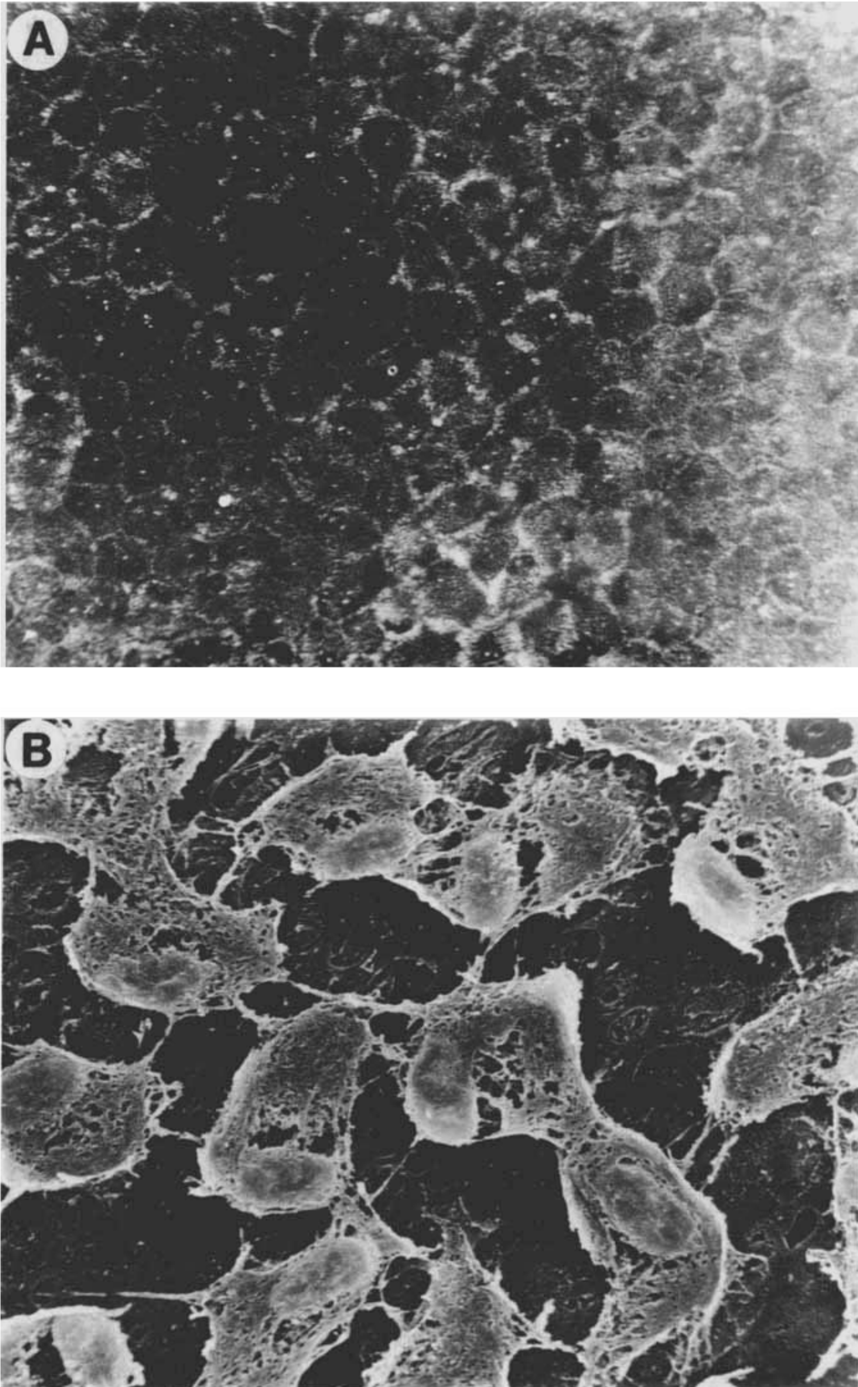
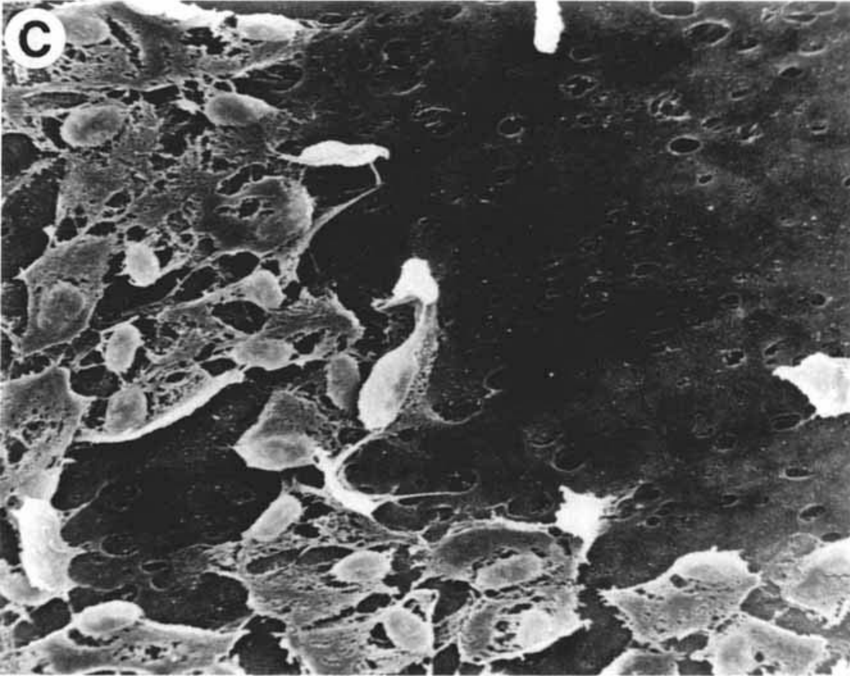
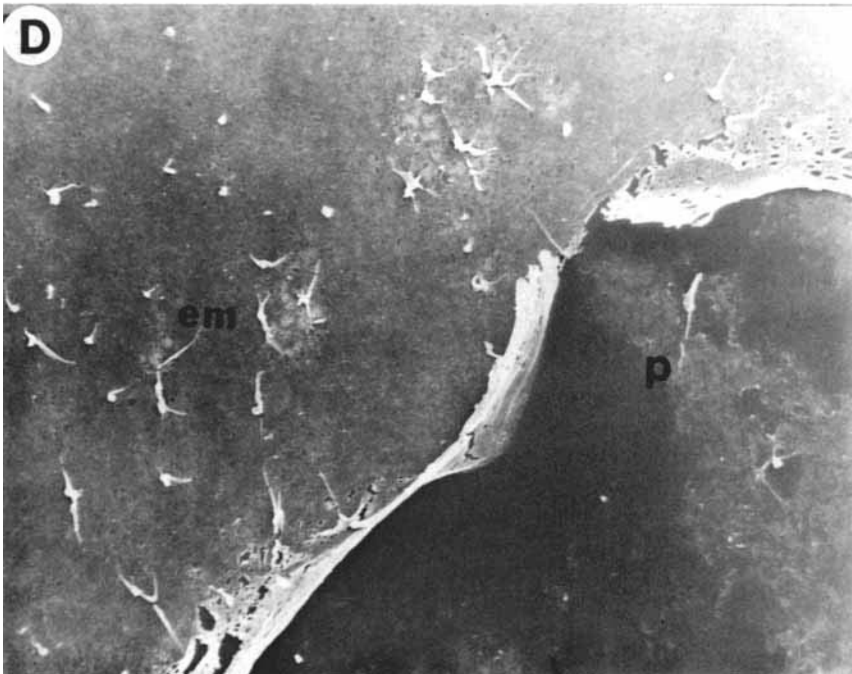


Fig. 12. Scanning electron microscopy of a monolayer of bovine corneal endothelial cells before and after exposure to Triton X-100. A monolayer composed of polygonal, highly flattened, and closely apposed cells can be seen in A (×600). After the monolayer has been treated with Triton X-100, it is composed of nuclei and cytoskeletons which no longer attach firmly to the extracellular matrix (B,





×200). In some areas the extracellular matrix has been exposed (C, ×600). Washing the dishes with PBS removed the cytoskeleton and exposed the extracellular matrix present underneath the cells (D, ×200). The plate has been scratched with a needle to expose the plastic (P) to which the extracellular matrix (em) strongly adheres.

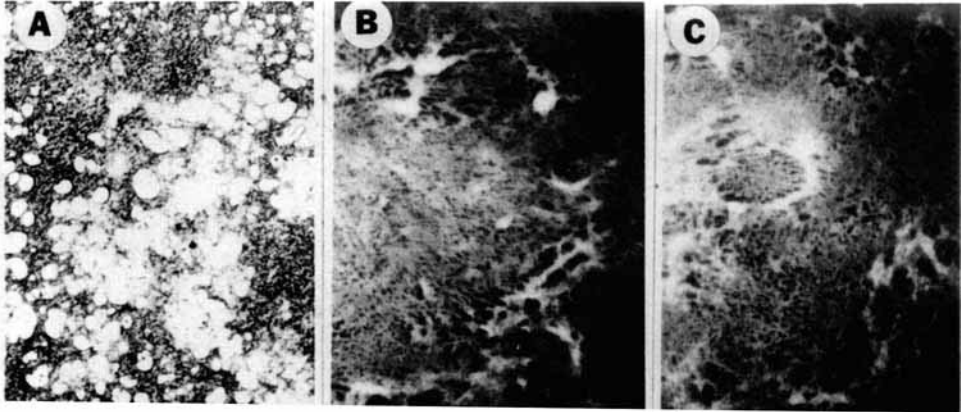
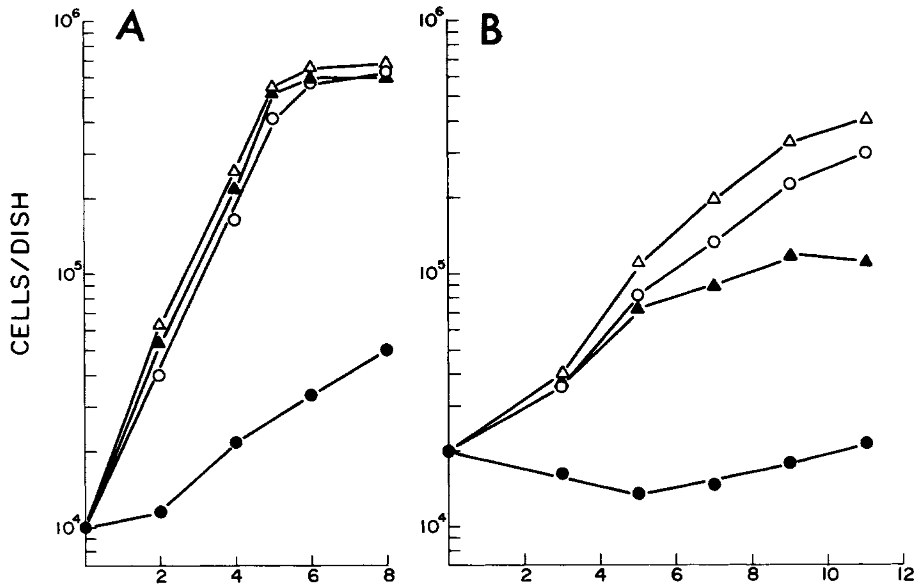


Fig. 13. Immunofluorescence of the extracellular matrix produced by corneal endothelial cell cultures. Confluent corneal endothelial cell cultures were treated (60 min, 24°C) with 0.5% Triton X-100 to remove the cell layer and to expose the underlying extracellular matrix shown in (A) stained with alizarin red.B–C) show the immunofluorescence pattern of the extracellular matrix when exposed to anti-collagen type III (B), or anti-collagen type IV (C) followed by incubation with fluorescein-conjugated anti-rabbit IgG. Collagen type IV (C) was present in lower amounts, as indicated by a 3- to 5-fold longer exposure time of the automatic camera.

GROWTH AND MORPHOLOGICAL APPEARANCE OF CULTURED BOVINE AND HUMAN VASCULAR ENDOTHELIAL CELLS MAINTAINED ON PLASTIC VERSUS ECM AND EXPOSED OR NOT TO FGF

When the growth of bovine vascular endothelial cells maintained on plastic versus ECM was compared, cells maintained on an ECM, regardless of whether they were exposed or not to FGF, reached a final cell density within 5 days which was 10- to 12-fold that of cultures maintained on plastic alone (Fig. 14A). Addition of FGF to cultures maintained on an ECM did not decrease their mean doubling time, which was already at a minimum (18–20 hr), nor did it result in a higher final cell density, which was already at a maximum (700–1000 cells/mm^2). When the morphology of confluent cultures of bovine vascular endothelial cells maintained on plastic and exposed to FGF was compared to that of confluent cultures maintained on ECM, it was found to be similar (Figs. 14C, D). In contrast to bovine vascular endothelial cells, human umbilical vein endothelial cell cultures did not proliferate when seeded at low cell density on tissue culture dishes (Fig. 14B). As previously reported [13, 42], addition of FGF to the cultures induced the cells to divide actively and within 11 days a 15-fold increase in cell number was observed. When cells were seeded on ECM instead of on plastic, a 6-fold increase in cell number was observed over the same period of time. In contrast, with bovine endothelial cell cultures, FGF was still required if cultures maintained on ECM were to reach confluence (Fig. 14B). Addition



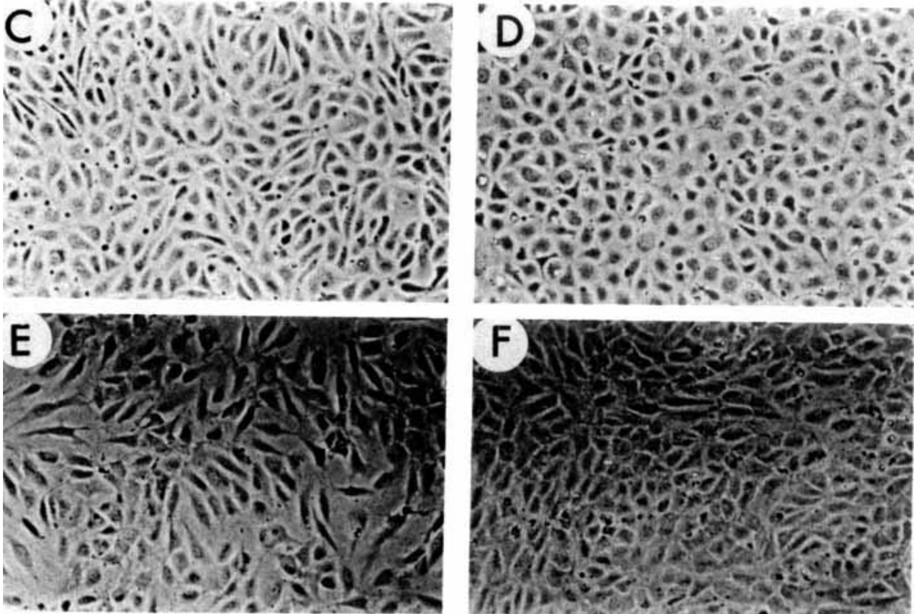


Fig. 14. Proliferation and morphological appearance of bovine aortic endothelial cells and human umbilical vein endothelial cells when maintained on plastic versus extracellular matrix (ECM) and exposed or not to FGF. A) Bovine vascular aortic endothelial cells were plated at an initial density of 1×10^4 cells per 35-mm dish coated (△, ▲) or not (○, ●) with an ECM. Cultures were maintained in the presence of DMEM supplemented with 10% calf serum and with (△, ○) or without (▲, ●) FGF (100 ng/ml) being added every other day. B) Human vascular endothelial cells were plated at an initial density of 2×10^4 cells per 35-mm dish coated (△, ▲) or not (○, ●) with an ECM. Cultures were maintained in the presence of DMEM supplemented with 10% calf serum and with (△, ○) or without (▲, ●) FGF (100 ng/ml) being added every other day. The morphological appearance of bovine vascular endothelial cells maintained on plastic and grown in the presence of FGF or maintained on an ECM and grown in the absence of FGF is shown in (C) and (D), respectively. The morphological appearance of human umbilical vein endothelial cells maintained on plastic or on an ECM and grown in both cases in the presence of FGF is shown in (E) and (F), respectively. Pictures were taken once the culture reached confluence (phase contrast, ×100).

of FGF to cultures maintained on ECM induced an optimal growth rate and the cultures became confluent within 11 days. The final cell density of the confluent cultures was 20-fold higher than that of cultures maintained on plastic and not exposed to FGF. When the morphological appearance of confluent cultures maintained on plastic (Fig. 14E) versus ECM (Fig. 14F) and exposed in both cases to FGF was compared, the cultures maintained on ECM were composed of cells more closely apposed and tightly packed than those maintained on plastic.

The rate of proliferation of bovine vascular endothelial cells maintained on plastic and exposed to FGF (Fig. 15B) or maintained on an extracellular matrix and not exposed to FGF (Fig. 15A) was a strict function of the serum concentration to which the cultures were exposed. While cells maintained on an extracellular matrix and exposed to serum concentration as low as 1% proliferated actively even in the absence of FGF (Fig. 15A), cells maintained on plastic, when exposed to a serum concentration as high as 10%, proliferated poorly (Fig. 15B). In contrast, if FGF was added to such cultures, then active proliferation resumed (Fig. 15B).

It can, therefore, be concluded that when the proliferation of bovine vascular endothelial cells maintained on plastic versus an ECM is compared, low-density cell cultures maintained on plastic proliferate poorly. FGF is, therefore, needed in order for the cultures to become confluent within a few days. In contrast, when the cultures are maintained on ECM, they proliferate actively and no longer require FGF in order to become confluent. In both cases (either maintained on plastic and exposed to FGF or maintained on an extracellular matrix), the rate of proliferation was a direct function of the serum concentration to which cultures were exposed. It is, therefore, likely that the effect of the ECM is more a permissive than a direct mitogenic one, since cells still required serum in order to proliferate.

To test the possibility that collagen or fibronectin alone could be the component of the ECM responsible for the increased rate of proliferation of cells plated on it, we have compared the growth of bovine vascular endothelial cells plated on dishes coated with purified collagen types I, II, III, and IV or with fibronectin. In no case did the cultures significantly increase their rate of growth when maintained on these different substrates (Fig. 16). In all cases, an aberrant morphological appearance was observed, the cultures being composed of large cells of which a high proportion are binucleated. Only cells maintained on an ECM proliferated actively (Fig. 16), reaching confluence within 5 days. This observation, therefore, excludes the possibility that the component in the ECM produced by corneal endothelial cells which could have a permissive effect on their proliferation is either collagen or fibronectin alone.

Proliferation of cells in culture is not only a function of the medium, serum, or growth factor(s) to which cells are exposed. It is also a function of cell density. While at high cell density, cells can rapidly condition their medium, thereby compensating for the nutrient deficiency of the medium when plated at clonal density they can no longer do so. Therefore, factors or nutrients required for cell survival and proliferation may be more readily apparent when cells are maintained at low rather than at high cell density. In particular, requirements for a proper substrate could become apparent. While cells maintained at high density could readily make a basement membrane, thereby facilitating further proliferation, at clonal density, even if every cell were to produce a basement membrane, it would be extremely difficult for them to cover the whole dish in a reasonable period of time. We have, therefore, analyzed the proliferation of vascular endothelial cells plated at clonal density on plastic versus plates coated with an extracellular matrix. As can be seen

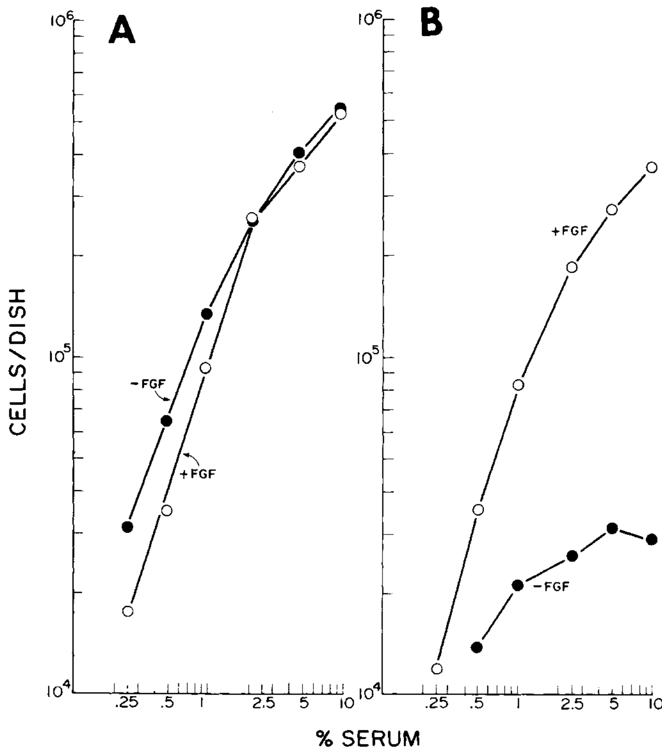


Fig. 15. Comparison of the rates of proliferation of bovine vascular endothelial cells maintained on plastic versus an extracellular matrix as a function of the serum concentration to which they are exposed. Bovine vascular endothelial cells were plated at 10^4 cells per 35-mm dish coated either with an extracellular matrix (A) or not (B). Cultures were maintained for 8 hr in the presence of DMEM, H-16 supplemented with 10% serum, 50 μg/ml Gentamycin, and 2.5 μg/ml Fungizone. After 8 hr, the medium was removed and the cultures washed once with DMEM, H-16. DMEM, H-16 supplemented with 2.5 μg/ml Fungizone, 50 μg/ml Gentamycin, and various concentrations of serum was then added to the dishes (○, •). To half of the dishes FGF (100 ng/ml) was added every other day (○, +FGF). After 5 days the cultures were trypsinized and counted.

(Fig. 17), when cells were plated at low cell density on plastic, the plating efficiency was extremely poor or cells died rapidly, since no clones were visible after 10 days. If FGF was present in the medium, 25% of the cells gave rise to individual clones. In contrast, when cells were plated at low density on an ECM, not only was a 90% plating efficiency observed at all cell densities (from 0.012 cells/mm^2 to 1.2 cells/mm^2) but, in addition, *all* cells gave rise to clones even in the absence of FGF. This demonstrates that the substrate upon which cells rest, even at clonal density, is crucial to insure both their survival and proliferation in response to serum or plasma factors.

Since cultures maintained on ECM no longer require FGF in order to proliferate actively, we have also investigated its effect on the lifetime of vascular endothelial cells. Cultures which had been maintained on plastic in the presence of FGF for 50 generations and which had been shown to increase their average doubling time rapidly as soon as FGF was no longer added to the media [5] were maintained in the absence of FGF on dishes coated with an ECM (Fig. 18). When maintained on such a substrate the cells proliferated

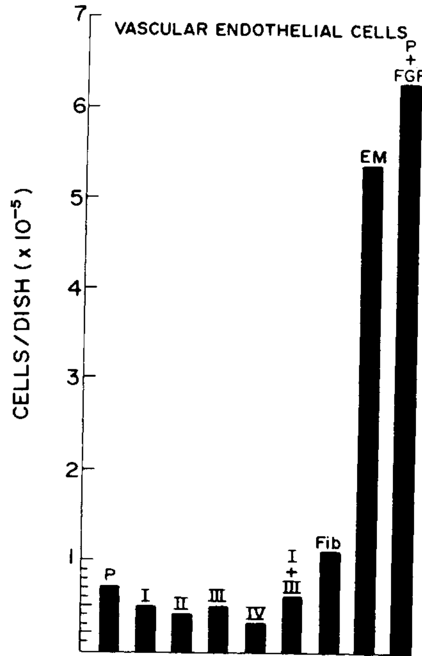


Fig. 16. Proliferation of bovine vascular endothelial cells maintained on different substrates. Bovine vascular endothelial cells (2×10^4 cells per 35 mm dish) were seeded on dishes coated with collagen types I, II, III, and IV, with collagen types I and III, with fibronectin (Fib), or with an extracellular matrix (EM). Cultures were maintained in the presence of DMEM, H-16 supplemented with 10% calf serum, 50 μg/ml Gentamycin, and 2.5 μg/ml Fungizone. After 5 days the cultures were trypsinized and the cells counted. The final cell density was compared to that of cultures maintained for the same period of time on plastic (P) and exposed or not to FGF (P + FGF).

actively and could be passaged weekly at a split ratio of 1:95. It was not until 50 generations later that a detectable increase in their average doubling time was observed (Fig. 18). It is, therefore, demonstrated that cells maintained on ECM have a much longer lifespan in culture than do cells maintained on plastic alone when passaged at a high split ratio (compare Figs. 3A and Fig. 18).

The present results, therefore, raise the possibility that although FGF is clearly mitogenic for a number of mesoderm-derived cells [43], its action on some of the cell types could be indirect. It could either replace the cellular requirement for a substrate such as the ECM and thereby make the cells fully responsive to growth factors present in serum and plasma even when the cells are maintained on plastic, or, alternatively, it could control the synthesis and secretion of the extracellular matrix produced by the cells. Such control could in turn make the cells sensitive to factors present in serum or plasma. That the latter alternative could occur finds support in our previous observation that FGF can control the production by vascular endothelial cells of extracellular and cell surface components such as fibronectin and various types of collagen [5, 17]. Since sparse cultures of endothelial cells proliferate poorly when maintained on plastic but not when maintained on an ECM, it may be that low density cell cultures maintained on plastic are unable to produce enough extracellular material to support further growth. The mitogenic effect of FGF on these cells could be the indirect result of an increased synthesis of the ECM by vascular endothelial cells. These possibilities are currently being tested.

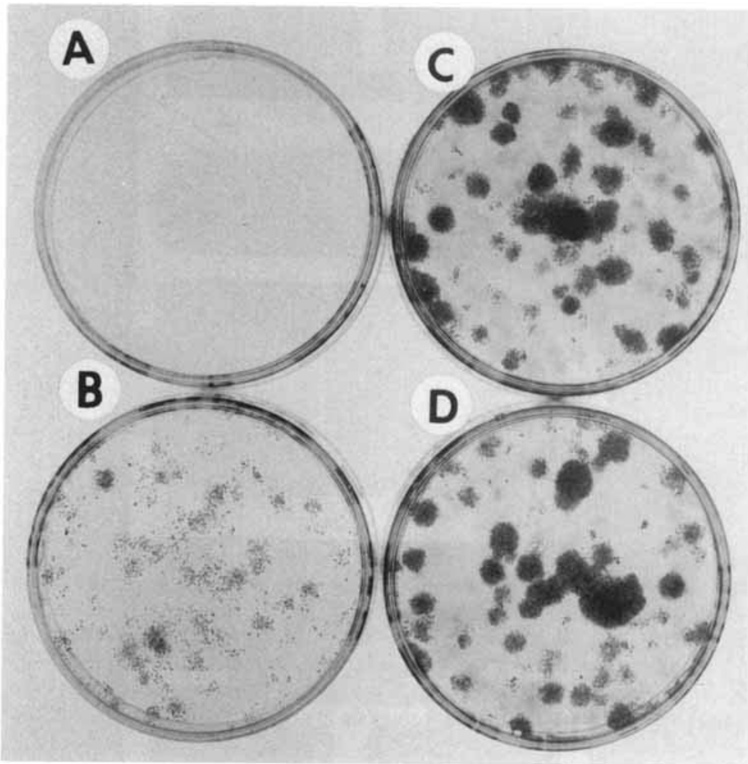


Fig. 17. Proliferation at a clonal density of bovine vascular endothelial cells maintained on plastic versus an extracellular matrix and exposed or not to FGF. Bovine vascular endothelial cells (50 cells per 35-mm dish) were seeded either on plastic dishes (A, B) or dishes coated with an extracellular matrix (C, D). Cultures were maintained in the presence of DMEM, H-16 supplemented with 10% calf serum, 50 μg/ml Gentamycin, and 2.5 μg/ml Fungizone. To half of the dishes (B, D) FGF (100 ng/ml) was added every other day. After 10 days (with a medium change on day 5), the medium was removed and the cultures were washed once and fixed with 10% formalin in PBS. Cultures were then stained with 0.1% crystal violet.

The ways in which the ECM exerts its permissive effect on cell proliferation can only be the object of speculation. One possible effect is, as pointed out earlier, 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. Recently, Folkman and Moscona [44], using vascular endothelial cells maintained on tissue culture dishes coated with an agent which modifies the adhesiveness of the cells to the dish, were able to control precisely the cellular shape in morphologies ranging from highly flattened to almost spheroidal. When the extent of cell spreading was correlated with DNA synthesis or cell growth, it was found to be highly coupled. Whereas highly flattened cells responded to serum factors, spheroidal cells no longer responded and intermediate degrees of response could be observed, depending on how flattened the cells were. Likewise, with corneal epithelial cells, changes in cell shape which depend on the substrate upon which the cells are maintained correspond to drastically altered sensitivities of the cells to EGF versus FGF [40, 45, 46].

The results presented above emphasize how drastically one can modify the proliferative response of a given cell type to serum factors depending on the substrate upon which the cells are maintained. It is possible that the lack of response of different cell types main-

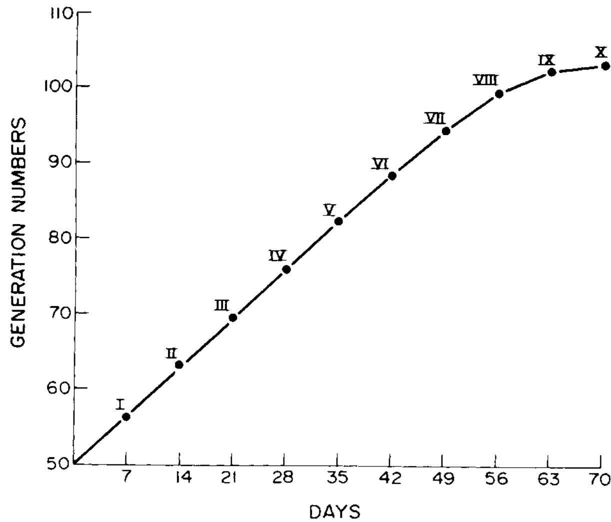


Fig. 18. Effect of the extracellular matrix on the culture lifetime of bovine vascular endothelial cells. Bovine vascular endothelial cells previously maintained on plastic tissue culture dishes and grown in the presence of FGF (100 ng/ml added every other day), as described in [8, 9, 13], for 50 generations were maintained and passaged weekly on dishes coated with an ECM. FGF was no longer added to the cultures. The number of generations was determined from the initial cell density 8 hours after seeding and the number of cells harvested at each transfer. Each point represents a single transfer. The average growth rate is given by the slope. Roman numbers indicate the passage number.

tained under tissue culture conditions to agents responsible in vivo for their proliferation and differentiation could be attributed to the artificial substrate, whether plastic or glass, upon which the cells rest and which limit their ability to produce an ECM.

PLASMA VERSUS SERUM. IS THERE A DIFFERENCE IN THEIR ABILITIES TO PROMOTE CELL GROWTH?

Culture of most cells in vitro requires the presence of serum [47]. Consequently, investigators have spent much effort in a search to identify the various factors in serum that stimulate cell growth in vitro. An important step in the search for serum growth factors has been the finding that one of the most potent mitogenic factors present in serum is derived from platelets. Such a possibility, first postulated by Balk [48], was based on studies of the growth of chick embryo fibroblasts in medium supplemented with plasma versus serum. While chicken fibroblasts do not proliferate in plasma-containing medium [48], when they are exposed to serum they proliferated actively. It was, therefore, concluded that serum contained growth-promoting activity which is lacking in plasma [49]. These studies were followed by reports which demonstrated that platelets are the source of a potent mitogen present in serum but not in plasma. While plasma was unable to support the growth of aortic smooth muscle cells [50] or that of BALB/c 3T3 cells [50], serum made from the same pool of blood stimulated their proliferation. Addition of a platelet extract to cell-free plasma-derived serum restored the growth-promoting activity [50–52]. One could, therefore, conclude that one of the principal mitogens responsible for the induction of DNA synthesis present in whole blood serum is derived from platelets [50–52]. The difference in the proliferative ability of cells exposed to plasma versus serum

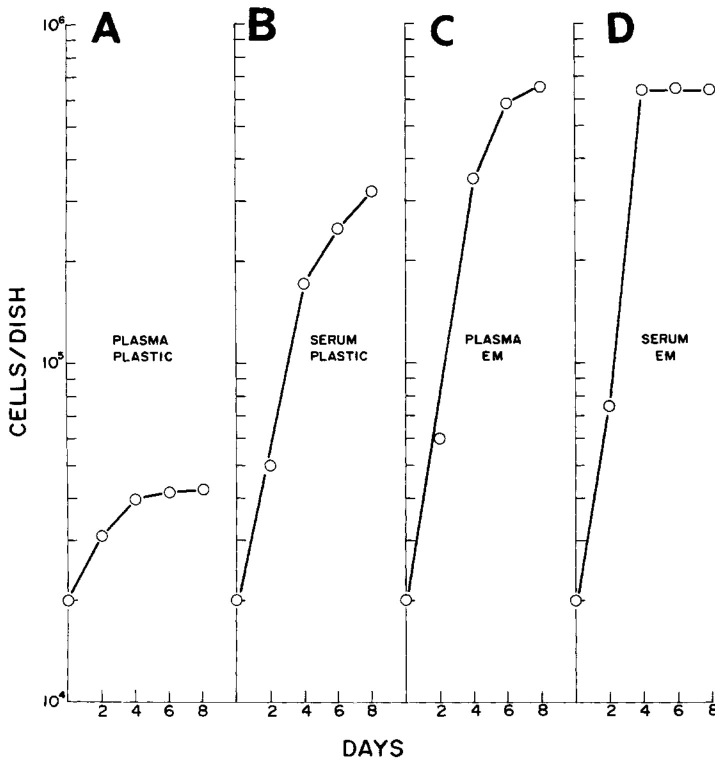


Fig. 19. Proliferation of bovine vascular smooth muscle cells when maintained on plastic versus extracellular matrix (ECM) and exposed to either plasma or serum. Vascular smooth muscle cells were seeded at 2×10^4 cells per 35-mm dish and maintained in the presence of DMEM supplemented with either 10% plasma (A, C) or serum (B, D). The cells were maintained on either plastic (A, B) or ECM (C, D).

results from the absence of the platelet factor in the former. However, all studies have, thus far, used cells maintained on plastic rather than on a basal lamina or an ECM. This difference in the substrate upon which the cells are maintained could have prevented their response to physiological factors present in plasma, thereby creating the difference in mitogenic activity between plasma and serum.

To explore the possibility that the serum factors to which cells maintained on ECM become sensitive are also present in plasma, we have compared the mitogenic activity of plasma versus serum, using as target cells vascular smooth muscle cells maintained on either plastic or an ECM.

Vascular smooth muscle cells maintained on plastic and exposed to plasma (10%) proliferate poorly. Within 4 days the cells go through one doubling and afterwards cease to proliferate (Fig. 19A). When the morphological appearance of such cultures was observed by phase contrast microcopy, the cells were considerably enlarged (Fig. 20A). When the same cultures were exposed to serum (10%) instead of plasma, the cells proliferated actively over a period of 6 to 8 days and underwent a 15-fold increase in cell number (Fig. 19B). During the logarithmic growth phase the mean doubling time of the cultures was 30 hours. These results, therefore, confirm previous results showing that when cells are maintained on plastic and exposed to plasma, they proliferate poorly or not at all,

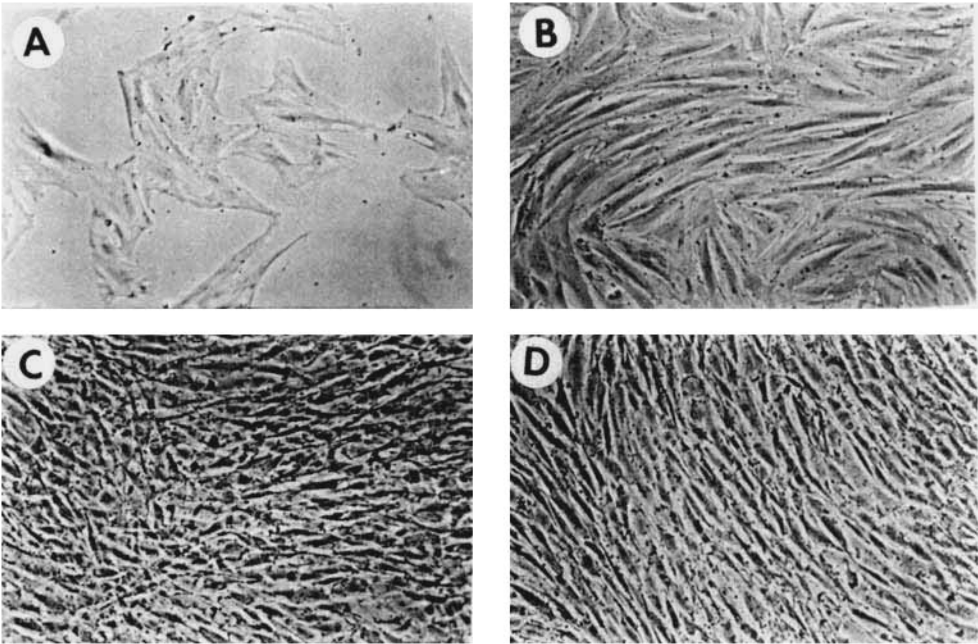


Fig. 20. Morphological appearance of vascular smooth muscle cells maintained on plastic (A, B) or an extracellular matrix (C, D). Cultures were plated and maintained as described in Fig. 19 and exposed to either 10% plasma (A, C) or 10% serum (B, D). Pictures were taken on day 8 with a phase contrast microscope (×100).

whereas they actively proliferate when exposed to serum [50, 51]. In contrast to the above results, when cells were maintained on an ECM and exposed to plasma, they proliferated actively (Fig. 19C). Within 6 days the cell number increased by 30-fold, and during the logarithmic growth phase the mean doubling time of the cultures was as low as 15 hours (Fig. 19C). Plasma was, therefore, even more mitogenic for cells maintained on an ECM than was serum for cells maintained on plastic. When the growth rate and the final cell density of cultures maintained on an ECM and exposed to either plasma (Fig. 19C) or serum (Fig. 19D) were compared, they were found to be the same. The differences between plasma and serum in their abilities to support cell growth, differences which are evident when the cells are maintained on plastic, therefore, vanish when the cells are maintained on an ECM. In Fig. 20, the morphological appearance of a culture maintained on plastic and exposed to serum (Fig. 20B) can be compared to that of a culture maintained on an ECM and exposed to either plasma (Fig. 20C) or serum (Fig. 20D). The difference between such cultures is apparent when one compares their respective average cell sizes. Cultures maintained on an ECM and exposed to either plasma or serum were composed of small, spindly, overlapping, and tightly packed cells which were on an average 3- to 5-fold smaller than the mean cell size of cultures maintained on plastic and exposed to serum (Figs. 20C, D). When the mean cell size of cultures maintained on ECM versus plastic and exposed in both cases to plasma was compared (Figs. 20A, C), cells maintained on plastic and exposed to plasma (Fig. 20A) had on an average a 10-fold larger size than cells maintained on an ECM (Fig. 20C).

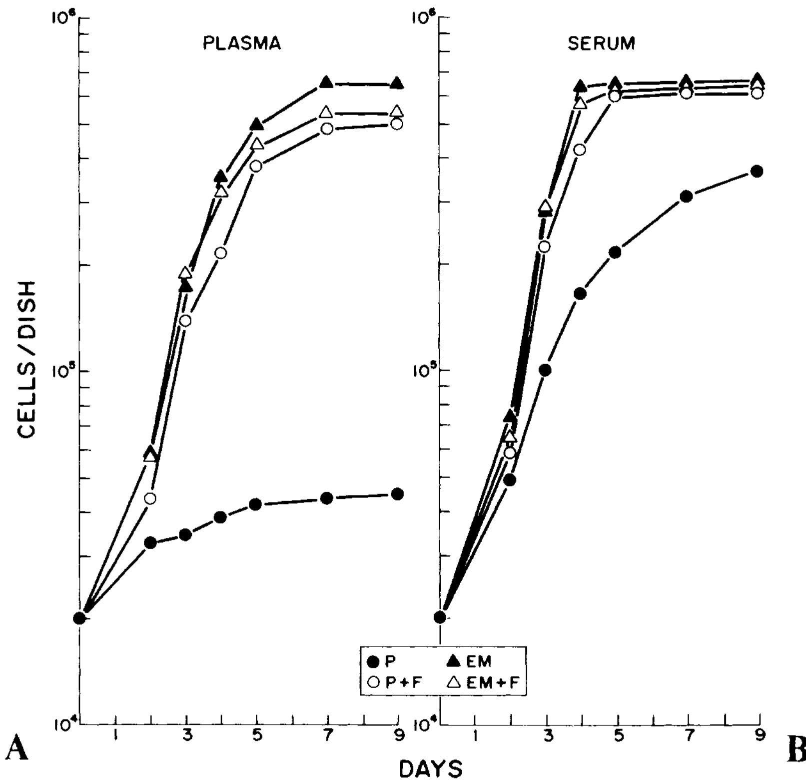


Fig. 21. Effect of FGF on the growth rate of vascular smooth muscle cell cultures maintained on either plastic or an extracellular matrix and exposed to 10% plasma (A) or 10% serum (B). Vascular smooth muscle cell cultures (2nd passage) were plated at 20,000 cells per 35-mm dish on plastic (○●, P) or an extracellular matrix (△▲, EM). The cultures were then maintained with DMEM, H-16 supplemented with 2.5 μg/ml Fungizone, 50 μg/ml Gentamycin, and with either 10% plasma (A) or 10% serum (B). FGF (100 ng/ml) was added every other day to half of the cultures maintained on plastic (○, P + F) or on an extracellular matrix (△, EM + F).

Earlier studies have shown that FGF, which has many similarities to the platelet-derived growth factor (same molecular weight and isoelectric point) [52], is a mitogen for vascular smooth muscle cells [9]. We have, therefore, compared its effect on cells maintained on plastic versus an ECM and exposed to either plasma or serum. As shown in Figure 21, cells maintained on plastic and exposed to plasma hardly proliferated. In contrast, when FGF was added to the cultures, the cells rapidly divided. After 7 days there was a 25-fold increase in cell number (Fig. 21A). The final cell density of cultures maintained on plastic and exposed to plasma plus FGF was higher than that of cultures maintained on plastic and exposed to serum alone. This demonstrates that the addition of FGF to the medium of cells maintained on plastic can make up for the difference in mitogenic activity between plasma and serum. When the growth rate of cultures maintained on plastic and exposed to plasma and FGF was compared to that of cultures maintained on an ECM and exposed to plasma but not to FGF, they were found to be similar. A noticeable but small difference was that the final cell density of cultures maintained on an ECM and ex-

posed to plasma alone was 50% higher than that of cultures maintained on plastic and exposed to plasma and FGF. Addition of FGF to cultures maintained on an ECM and plasma (Fig. 21 A) did not affect their growth rate. It resulted instead in a final cell density slightly lower than that observed with cultures not exposed to FGF (Fig. 21A). When the growth rate and final cell density of cultures exposed to serum and FGF were compared as a function of the substrate upon which cells were maintained, addition of FGF to cultures maintained on plastic and exposed to serum resulted in a decrease in the mean doubling time of the cultures (from 30–16 hr) as well as in a 2- to 3-fold increase in the final cell density (Fig. 21B). When FGF was added to cultures maintained on ECM, it did not affect their growth rate, which was already maximal (15-hr mean doubling time), nor did it affect their final cell density (Fig. 18B). Similar results were obtained when PDGF, instead of FGF, was used. One could, therefore, conclude that although FGF greatly increases the growth rate of cultures exposed to plasma and, to a lesser extent, that of cultures exposed to serum when the cultures are maintained on an ECM, it does not affect their growth rate since it become optimal when cultures are exposed to either plasma or serum.

The increased rate of proliferation of cells maintained on ECM and exposed to plasma or serum could either be the result of a direct mitogenic effect on the part of the ECM, the plasma or serum having a permissive role, or, conversely, the result of a direct mitogenic effect of plasma or serum, the ECM having a permissive role. To distinguish between these two possibilities, cells maintained on ECM were exposed to increasing concentrations of plasma or serum and the final cell densities were compared (Fig. 22). If the ECM should be the mitogen and the plasma or serum has a permissive effect, one would expect little difference in the rates of proliferation between high (10%) and low (0.5%) plasma or serum concentrations. When the final cell density of cultures maintained on an ECM and exposed to either plasma or serum was analyzed as a function of the serum or plasma concentration (Fig. 22) to which they were exposed, it was found to be a direct function of the serum or plasma concentration. It is, therefore, likely that the proliferation of vascular smooth muscle cells maintained on an ECM is controlled by factor(s) already present in plasma and that the ECM has a permissive role.

One could, therefore, conclude that, depending on the substrate upon which vascular endothelial cells are maintained, there are drastic differences in their requirements for growth factors. While cells maintained on plastic do not respond to plasma factor(s) [50, 53] and require serum [52], FGF [9], or PDGF [52] in order to proliferate when in close contact with an ECM the same cell types will respond to plasma factor(s) and no longer require serum for proliferation.

If in the case of vascular smooth muscle cells one were to extrapolate to the in vivo situation, maintaining them on an ECM and plasma is clearly a closer approximation to physiological conditions than exposing them to plastic and serum. Since vascular smooth muscle cells proliferate at a maximal rate when maintained on an ECM and exposed to plasma, it is likely that they are responding to mitogen(s) already present in plasma rather than to FGF or to mitogen(s) generated during the coagulation process.

THE COMMITMENT AND THE PROGRESSION FACTORS

Soluble factors which control the production of the ECM have not previously been reported. Until now, production of ECM was thought to be an automatic process which was mostly a function of the substrate upon which cells rest and of cell density. Although it is quite possibly that in vivo the production of an ECM is not under any control other

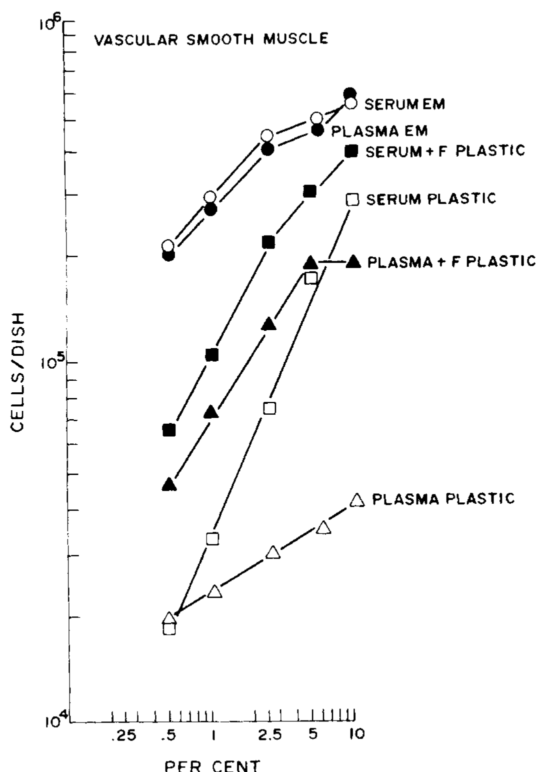


Fig. 22. Dependence for proliferation of vascular smooth muscle cells on plasma when the cells are maintained on an extracellular matrix. Vascular smooth muscle cells (2nd passage) were plated at 2 × 10^4 cells per 35-mm dish on plastic or on extracellular matrix (EM) . Cultures were maintained for 8 hr in the presence of DMEM supplemented with either 10% plasma or serum. After 8 hr, the media were removed and the cultures washed once with DMEM, H-16. DMEM, H-16 supplemented with 2.5 μg/ml Fungizone and 50 μg/ml Gentamycin and containing different concentrations of serum or plasma (from 0.5% to 10%) was then added to the dishes. FGF (100 ng/ml) was added every other day to some of the dishes. After 5 days, the cultures were trypsinized and counted. Symbols are as follows. Cultures maintained on plastic and exposed to plasma (△) or to plasma and FGF (▲); cultures maintained on plastic and exposed to serum (□) or to serum and FGF (■); cultures maintained on an extracellular matrix and exposed to plasma (●) or to serum (○).

than that provided by the substrate upon which cells migrate during the early embryological phase, our results suggest that at least in vitro, factors such as FGF could influence the formation of an ECM scaffolding. We do not know the extent of this influence. Our observation that FGF could control the ECM production thereby making cells sensitive to plasma factor(s), is consistent with the findings of others that there could be two sets of growth factors in vitro, one of which, the commitment factor(s), could be involved in the formation of the ECM.

Earlier studies done on the control of cell cycle by growth factors have developed the concept that it could be controlled by 2 independent sets of factors present in serum, each of which controls a different phase of the cell cycle [54, 55]. One set is composed of heat-stable (100°C) factor(s) that are released into serum during the clotting process and induces BALB/c 3T3 cells to become capable of synthesizing DNA [56]. A second set

of components, found in defibrinogenated platelet-poor plasma allows competent cells to progress through G_0G_1 and to synthesize DNA [54–56]. Stiles and his colleagues have further developed this observation by looking at the dual control of cell growth by the somatomedins, PDGF, and FGF [57]. Quiescent BALB/c 3T3 cells exposed briefly to PDGF or FGF become "competent" to replicate their DNA but do not "progress" into S phase unless exposed to somatomedin C, which is required for progression [57]. Since neither FGF nor PDGF is required to commit vascular smooth muscle cells to proliferation when they are maintained on an ECM and exposed to plasma, it is likely that competence factors would no longer be required if cells are maintained on an ECM.

THE IMPLICATIONS OF GROWING CELLS ON AN ECM INSTEAD OF ON PLASTIC

If one starts with a primary culture, it is likely that cells are selected which in the subsequent passages retain their ability to produce an ECM. Alteration in their phenotypic expression could be the direct result of an alteration in the type of ECM produced. This is best seen in the case of vascular endothelial cells, which express their normal phenotype when producing an ECM composed of collagen type III versus types IV and V at a ratio of 3:1 [58]. In contrast, when these cells no longer express their normal phenotype, the type of collagen produced is altered. Collagen type I begins to be synthesized, while the B and C chains of collagen type V are no longer produced [58]. This results in aberrant ECM production parallel with a loss of phenotypic expression [5, 17]. 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 ECM. 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 aging, it is generally recognized that senescent cells stop making an ECM [59]. Whether this is the result or the cause of senescence has not been investigated. It is to be suspected, however, that alterations in production or a loss of ability to produce a normal ECM could be directly linked to cell senescence, since this will result in a loss of their proliferative ability.

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 grown in tissue culture is their loss of anchorage-dependence, as reflected by their ability to grow either in soft agar or in suspension when maintained on plastic. Yet in vivo, tumor cells from solid tumors, although they can adhere loosely to one another, can adhere tenaciously to the substrate which is provided by the host tissue or which they themselves produce. This is best reflected in the phenomenon of metastasis, where tumor cells which are carried away by the bloodstream can stick to basement membrane, infiltrate through it, and form secondary tumors in organs located far from the original tumor. If tumor cells were not anchorage-independent, one would expect them to proliferate freely in the bloodstream, but this rarely happens. It is, therefore, likely that if one provides tumor cells in culture with an adequate substrate, they could totally shift their pattern of growth. This is in fact what we have observed with hepatocarcinoma cells, Ewing's tumor cells or melanoma tumor cells [61]. It should also be pointed out that maintaining active proliferation of epithelial cell cultures of either normal or tumoral origin is a challenge. For example, in the case of carcinoma cells, less than 5% of the original cells put in culture give rise to cell lines [60]. Using an ECM as a natural substrate could change the figures and a higher percentage of either normal or tumor epithelial cells could be established in culture. It has also been shown by others that the ECM can control the morphogenetic and phenotypic expression of the tissue associated with it [60]. Use of ECM as a natural substrate for culturing the epithelial cells could,

therefore, provide an opportunity to study not only their proliferation and the physiological factors controlling it but their differentiation as well. Although in the present study the ECM produced by corneal endothelial cells was used, other extracellular matrices produced by other cell types could also be used and would allow one to study the effects of various extracellular matrices on cell migration, proliferation, and differentiation.

Since cells maintained on an ECM now respond to plasma growth factors instead of to serum factors, one may wonder what these factors are. They could possibly be physiological agents such as trophic hormones which modulate cell proliferation in vivo but are inactive in vitro. Alternatively, they could be factors which have gone undetected because of the lack of sensitivity to them of cultured cells maintained on plastic. If this were so, one would now have an ideal substrate for restoring the normal growth response of many tissues to these naturally occurring factors. One might also suspect that conclusions concerning the mechanisms and controls of cell proliferation and cell migration of normal cells maintained on plastic may somehow differ from those which can be reached when cells are maintained on ECM.

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NOTE ADDED IN PROOF

Since submission of this manuscript, the factors present in plasma that regulate the proliferation of vascular endothelial cells and vascular smooth muscle cells have been identified. In the case of vascular endothelial cells, high density lipoprotein (HDL) is the major factor involved in the proliferation of vascular endothelial cells, since it can fully replace plasma [1, 2]. This is best shown by the observation that cells maintained in serum-free or plasma-free medium can be repeatedly passaged at low cell density and proliferate at an optimal rate if HDL is added to the medium. In the case of vascular smooth muscle cells, although HDL is also needed, somatomedin C and epidermal growth factor are required in order to induce optimal growth rate when cultures are maintained in plasma-free medium [3].

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