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Cell-extracellular matrix interactions in morphogenesis: an in vitro approach

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Summary. We briefly review evidence from in vitro models that supports a role for the extracellular matrix in two essential steps of organogenesis: the establishment of appropriate three-dimensional cell-to-cell relationships, and the determination of a correct cell polarity.
Key words. Cell culture; organogenesis; collagen; endocrine pancreas; cell polarity; LLC-PK cells; endothelial cells; angiogenesis.

Formation of a precisely organized functional tissue or organ (organogenesis) is one of the major phases of embryonic development, and represents the culmination of a series of specific cellular events. Throughout the various stages of organogenesis, macromolecules present in the extracellular matrix provide structural support, and act as a glue that binds together the different cell types in a complex tissue or organ. Since the pioneering studies of

Grobstein[14], it has become increasingly clear that the extracellular matrix serves not only as an inert physical scaffolding for the developing tissues, but also provides environmental signals to the cells involved, thus playing an important instructive role in morphogenetic processes. In addition, the extracellular matrix represents the natural substratum through which various cell types migrate during embryogenesis, or in pathological conditions, such as inflammation and tumor spread.

In this brief review, we will summarize the recent contributions of this laboratory to the understanding of the role of cell-extracellular matrix interactions in organogenesis. It is not our purpose to survey the extensive literature existing in this field. For a full discussion of the morphogenetic role of matrix molecules, the reader is referred to the recent reviews by Hay[17], Hynes[19], Toole[34], Kleinman et al.[20], Heathcote and Grant[18], Aplin and Hughes[1], Bissell et al.[6], Bernfield et al.[5], and Reddi[30].

Since cell-extracellular matrix interactions are difficult to analyze and to manipulate experimentally in vivo, we sought a culture model in which to study the influence of specific extracellular matrix constituents on the morphogenetic behavior of various cell types. Hydrated collagen gels[9] proved useful for this purpose. A major advantage of this technique over conventional monolayer cultures is that cells can be embedded within a lattice of reconstituted collagen fibrils. Although collagen gels lack other extracellular matrix components (for example, proteoglycans), they mimic the three-dimensional organization of connective tissue matrices. Initially, collagen gels were used mainly to study the behavior of fibroblasts and other mesenchymal cells[2,9], and only in the last few years has it been realized that epithelial cells can organize into tissue-like structures when grown inside collagen gels[4,8,16,29,38,39]. We therefore used this culture method to investigate the role of cell-collagen interactions in morphogenetic processes. Such interactions may be mediated either by adhesive glycoproteins, such as fibronectin[19,32,37], or cell surface collagen receptors[25,31,37]. For experimental details concerning the preparation of three-dimensional gels of rat tail tendon type I collagen, see Montesano et al.[22-24].

The pancreatic islet model

We first addressed the question of whether cell-collagen interactions could contribute to the establishment of appropriate intercellular relationships in solid, heterocellular tissues. Pancreatic endocrine cells appeared to be particularly well suited for this study because in vivo they are associated into spheroidal aggregates, the islets of Langerhans, in which the four different endocrine cell types are not randomly distributed but have a characteristic topographical arrangement (i.e. a central mass of insulin-containing cells surrounded by a rim of glucagon-, somatostatin-, and pancreatic polypeptide-containing cells)[28]. In contrast, when pancreatic endocrine cells are grown in vitro under standard culture conditions[21], they form flattened clusters in which the characteristic cell distribution seen in intact islets is no longer recogniz-

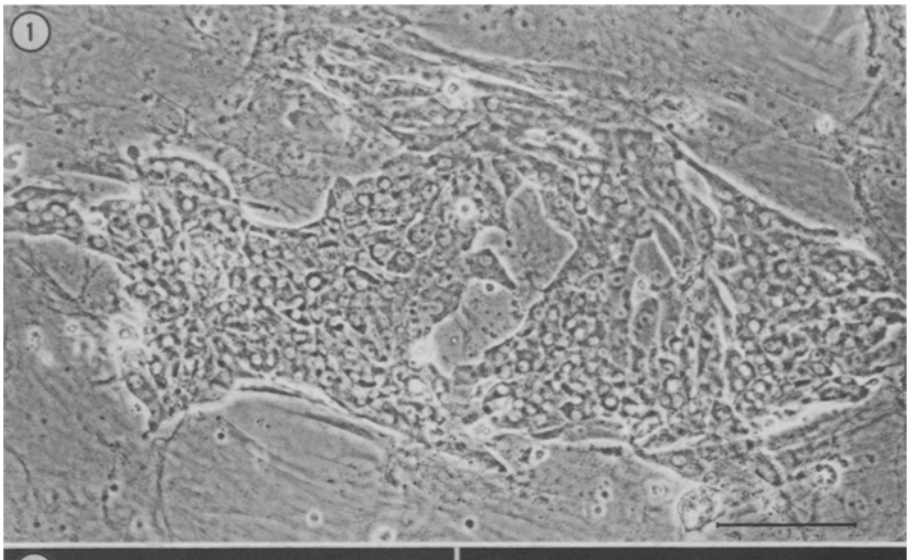

Figure 1. Pancreatic islet cells grown on the surface of a collagen gel (phase contrast). The culture appears as a monolayer consisting of irregularly-shaped, flattened clusters of endocrine cells. Bar, 100 μm. (From ref. 22, by courtesy of the Journal of Cell Biology).

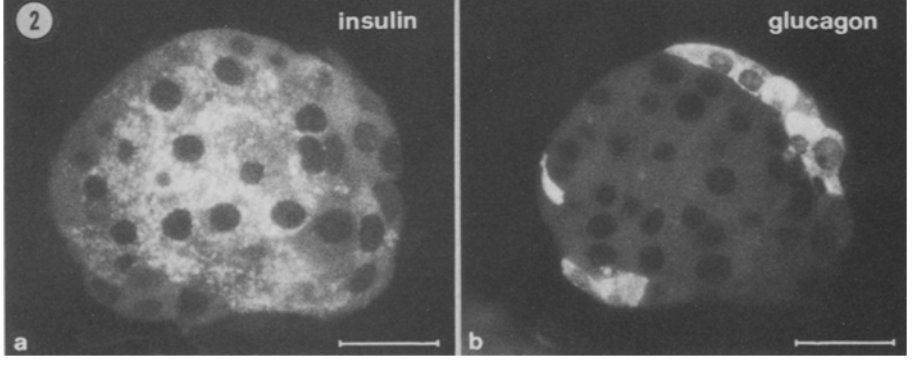

Figure 2. Consecutive semithin sections of a spheroidal islet cell aggregate formed inside a three-dimensional collagen matrix. The sections were stained by indirect immunofluorescence with anti-insulin and anti-glucagon antisera to visualize the topographical arrangement of the two endocrine cell types. Insulin-containing cells have a central location (a), while glucagon-containing cells are distributed at the periphery of the aggregate (b). Bar, 20 μm. (From ref. 22, by courtesy of the Journal of Cell Biology).

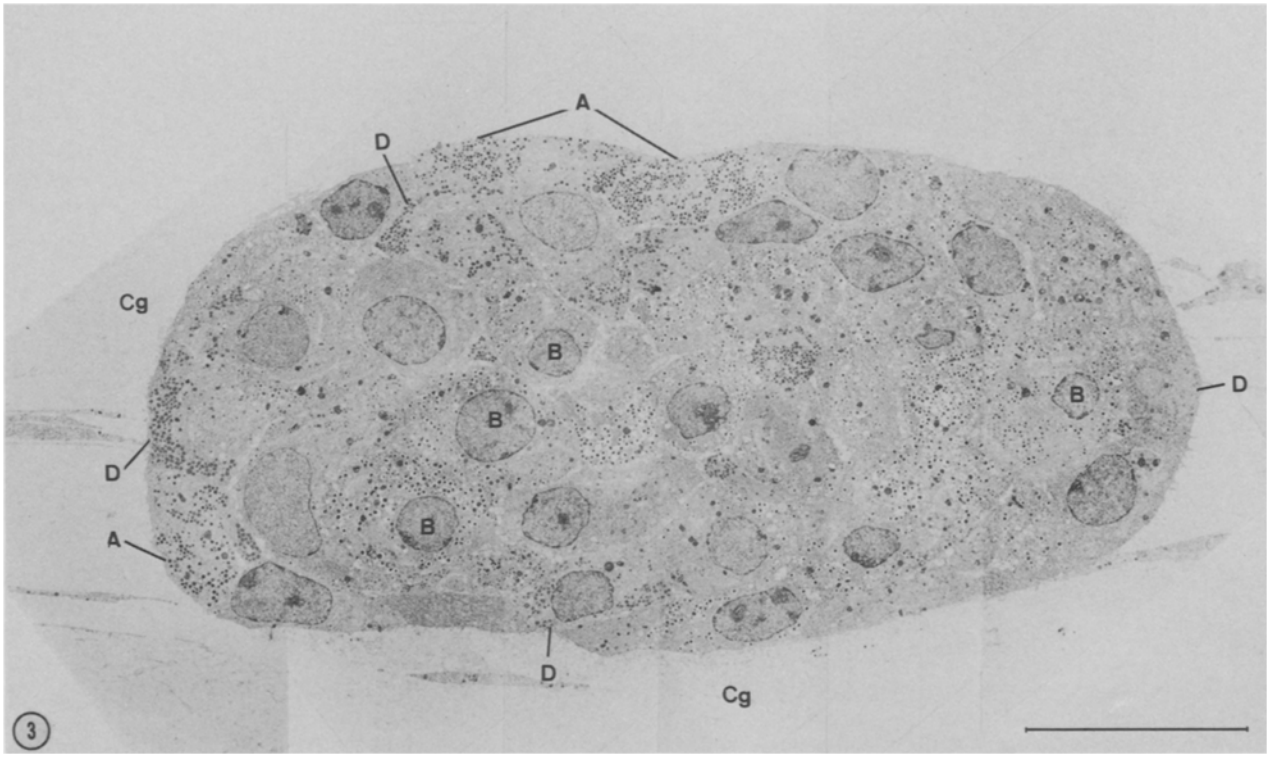

Figure 3. Thin section perpendicular to the culture plane showing a three-dimensional islet cell aggregate embedded in a collagen gel. B cells are concentrated in the central portion of the aggregate, whereas A and D cells are in a peripheral position. B, B cells; A, A cells; D, D cells; Cg, collagen gel. Bar, 20 μm. (From ref. 22, by courtesy of the Journal of Cell Biology).

able. To establish whether an appropriate extracellular matrix environment could induce islet cells to re-express the specific patterns of three-dimensional association found in vivo, we cultured pancreatic endocrine cells on the surface of a collagen gel (fig. 1), and then covered the culture with a second layer of collagen. This induced the monolayers of endocrine cells to reorganize, within a few days, into spheroidal aggregates of a size comparable to that of the islets of Langerhans[22]. When we examined the distribution of the various islet cell types, by either immunofluorescence with antibodies against islet hormones (fig. 2), or electron microscopy (fig. 3), we found that insulin-containing B-cells were concentrated in the central portion of the aggregates, while the other endocrine cell types had a preferential distribution at the periphery, exactly as observed in islets of Langerhans in situ. These results show that pancreatic endocrine cells, when cultured in a three-dimensional extracellular matrix environment, are able to self-associate in a well-defined topographical pattern reproducing the organization of the islet in vivo[22].

The kidney-derived LLC-PK$_1$ cells: a model for the study of epithelial polarity

The morphogenetic process leading to the formation of the various tissues and organs requires not only the ordered organization of individual cells into complex three-dimensional structures, but also the asymmetric spatial distribution of cellular components – that is, cell polarization. To investigate the role of the extracellular matrix in the establishment of cell polarity, we took advantage of the property of a kidney-derived epithelial cell line (the LLC-PK$_1$ cell line, clone D$^+$) to form monolayers of polarized cells in culture. These studies involved two successive experimental steps[36]. We first seeded the LLC-PK$_1$/D$^+$ cells in tissue culture dishes coated with agarose, a non-adhesive substratum, to prevent the attachment of the cells to the bottom of the dishes. In these conditions, the cells aggregated in suspension and formed, within about one week, free-floating hollow spheres or cysts (fig. 4a), lined by a monolayer of flattened cells (fig. 4b). Electron microscopy showed that the cells forming the wall of the cysts had a microvilli-rich surface oriented towards the outside, and a smoother surface facing the lumen (fig. 4c). These observations indicate that cell-to-cell contact, in the absence of attachment to a solid substratum, is a sufficient signal to induce the organization of D$^+$ cells into polarized cavitary structures[36]. However, the polarity of the cells lining the cysts was inverted with respect to that observed in kidney tubules (the proposed tissue or origin of LLC-PK$_1$ cells) as well as in other cavitary organs in vivo, in which the microvilli-rich apical surface is oriented towards the lumen of the cavity.

In the second step of this study, we examined the influence of the extracellular matrix on the polarity of the epithelial cysts. For this purpose the cysts were embedded in collagen gels, thus exposing the apical cell surface to extracellular matrix components normally associated with basal surfaces. This resulted in a rapid and dramatic

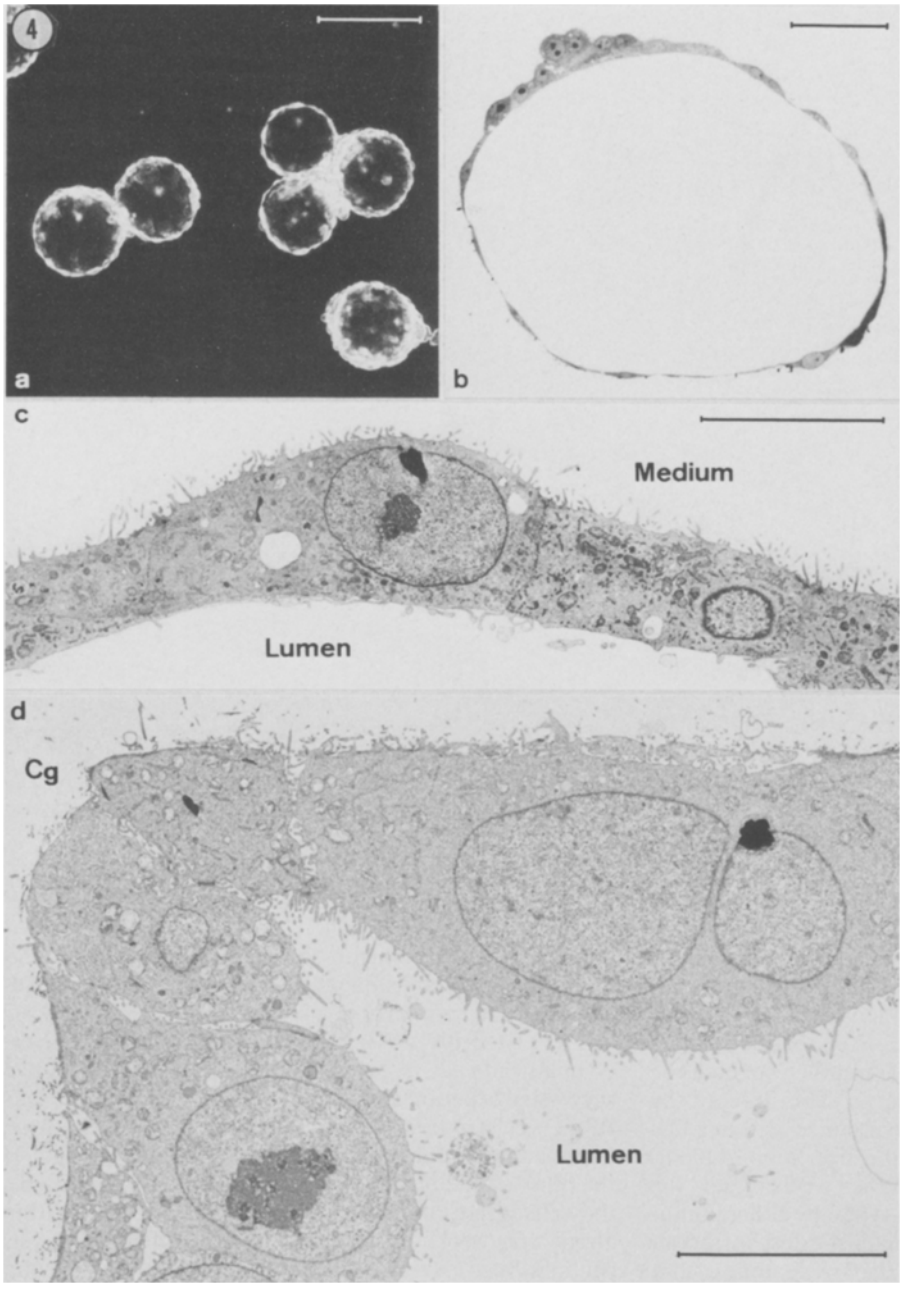

Figure 4. Reorganization of LLC-PK_1/D^+ cysts following embedding in collagen gels. *a* LLC-PK_1/D^+ cells after 4 days of culture in suspension. The cells have organized into free-floating, spherical, hollow cysts (dark field illumination). Bar, 200 µm. *b* Semithin section of a cyst. Bar, 50 µm. *c* Thin section through the wall of a cyst after 8 days of culture in suspension. The cells have a microvilli-rich surface oriented towards the outside and a smoother surface that faces the lumen of the cyst. Bar, 10 µm. *d* Inversion of cell polarity 42 h after embedding a cyst in a collagen gel. The cells have a microvilli-rich surface oriented towards the lumen of the cyst. Cg, collagen gel. Bar, 10 µm. (From ref. 36, by courtesy of the Journal of Cellular Physiology).

reorganization of the cyst structure, leading to an inversion of the original cell polarity: microvilli progressively disappeared from the outer surface of the cysts, while new microvilli formed on the surface facing the lumen (fig. 4d). Thus, the new polarity induced by the contact with the collagen matrix corresponds to that observed in vivo. Similar results have been reported previously with thyroid follicular cells[8].

The formation of blood capillaries (angiogenesis)

The experiments performed with pancreatic endocrine cells and the kidney-derived cell line demonstrate that cell-matrix interactions play a crucial role in two essential steps of organogenesis: 1) the organization of epithelial cells into complex three-dimensional structures, and 2) the establishment of a correct cellular polarization. These encouraging results prompted us to investigate the role of cell-matrix interactions in a clinically-important process of organogenesis, i.e. the formation of capillary blood vessels or angiogenesis.

Angiogenesis takes place during embryonic development, but also occurs in a wide range of important biological processes in adult life[11]. For example, wound healing could not take place without angiogenesis. However, there are conditions in which angiogenesis is deleterious to the organism. Folkman and coworkers (reviewed in ref. 11) have shown that angiogenesis is necessary for the continuous growth of tumors: if a tumor cannot receive new capillary blood vessels from the surrounding host tissues, it stops growing. Therefore, therapeutic inhibition of the angiogenic process has been proposed as a strategy for the control of tumor growth[11]. There are other pathological situations in which angiogenesis has

an adverse effect. For example, proliferating capillaries invade the vitreous humor of the eye in diabetes, leading to blindness, or the cartilage of the joints in rheumatoid arthritis, thereby participating in the destruction of the cartilage. The important role played by angiogenesis in tumor growth and other pathological processes has stimulated an increasing interest in this phenomenon over the past decade[11].

However, in spite of the identification of angiogenesis-inducing factors derived from either tumoral or normal cells[10, 11, 33, 35], the mechanism of angiogenesis is still poorly understood. It is well known that, in response to angiogenic stimuli, the endothelial cells of blood capillaries start to invade, and to migrate through, the extracellular matrix of the surrounding connective tissue, eventually forming a new capillary network[11]. However, we do not know how the sprouting endothelial cells organize into new capillary tubes, nor how they can migrate through a dense matrix of collagen fibrils. Obviously, experiments aimed at answering these questions cannot be done in vivo. The recent development of techniques for the isolation and culture of microvascular endothelial cells[13] has provided an opportunity for the in vitro study of endothelial cell properties that are relevant to the process of angiogenesis[12, 15]. Despite this, conventional culture methods suffer from two major limitations; namely, loss of cell-matrix interactions, and two-dimensional growth. To approximate as closely as possible the in vivo situation, we cultured capillary endothelial cells in contact with a collagen matrix.

Collagen matrix promotes the organization of endothelial cells into capillary-like tubes

The first question we asked was the following: Does the extracellular matrix promote the organization of endothelial cells into capillary-like tubes? To answer this question, we seeded capillary endothelial cells onto the surface of a collagen gel, and subsequently covered the culture with a second layer of collagen. Under these conditions, the culture underwent a dramatic change; collagen overlay was followed by a progressive reorganization of the monolayer, which resulted, within about 2 days, in the formation of a network of branching and anastomosing cords of endothelial cells. By phase contrast microscopy, most of these cords showed a central, translucent cleft along their axis, which suggested the formation of a lumen. That this was indeed the case was demonstrated by sections perpendicular to the culture plane. Both semithin (fig. 5, a, b) and thin sections (fig. 5c) clearly showed that the endothelial cells were organized so as to form tubular structures resembling blood capillaries. In addition, electron microscopy revealed the presence of a basal lamina at the interface between the endothelial cells and the collagen matrix – that is, in the same location as observed in vivo. These results demonstrate that an appropriate interaction of the endothelial cells with collagen fibrils plays a critical role in the organization of these cells into correctly-polarized capillary-like tubes[24].

Tumor-promoting phorbol esters induce angiogenesis in vitro

As mentioned above, during angiogenesis in vivo, new capillary blood vessels form by a process of active invasion of the extracellular matrix by sprouting endothelial cells. Therefore, the next question we addressed was the following: What changes occur in a quiescent endothelial cell that cause it to become invasive? Our approach to this problem has been to culture capillary endothelial cells on the surface of a collagen gel, with the purpose of determining what factors (if any) could induce the endothelial cells to invade the underlying collagen matrix. It has been proposed that cellular invasiveness in various biological processes, including angiogenesis, requires the elaboration of proteases capable of degrading the extracellular matrix (for a recent review see Mullins and Rohrlich[26]). Capillary endothelial cells produce low levels of collagenase and plasminogen activator under normal conditions, but the secretion of these proteases is markedly stimulated by the tumor promoter, phorbol myristate acetate (PMA)[15]. We therefore treated capillary endothelial cells on collagen gels with PMA to establish whether the increased secretion of proteases by these cells was accompanied by the invasion of the underlying collagen matrix.

Endothelial cells grown in normal medium formed a confluent monolayer at the fluid/matrix interface, and did

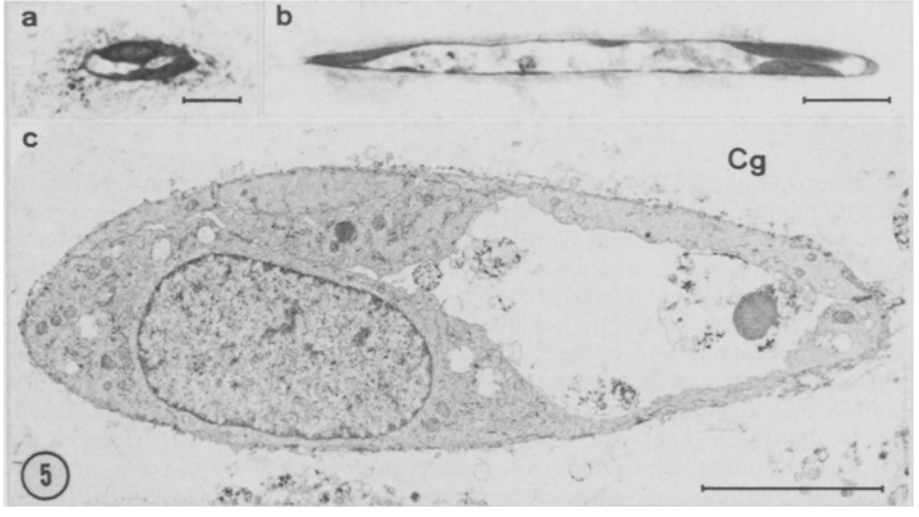

Figure 5. Semithin (a and b) and thin (c) sections perpendicular to a culture of capillary endothelial cells grown on a collagen gel and subsequently overlaid with a second collagen layer. The endothelial cells are organized around narrow lumina so as to form capillary-like tubular structures. Cg, collagen gel. *a* bar, 10 μm; *b* bar, 20 μm; *c* bar, 5 μm. (From ref. 24, by courtesy of the Journal of Cell Biology).

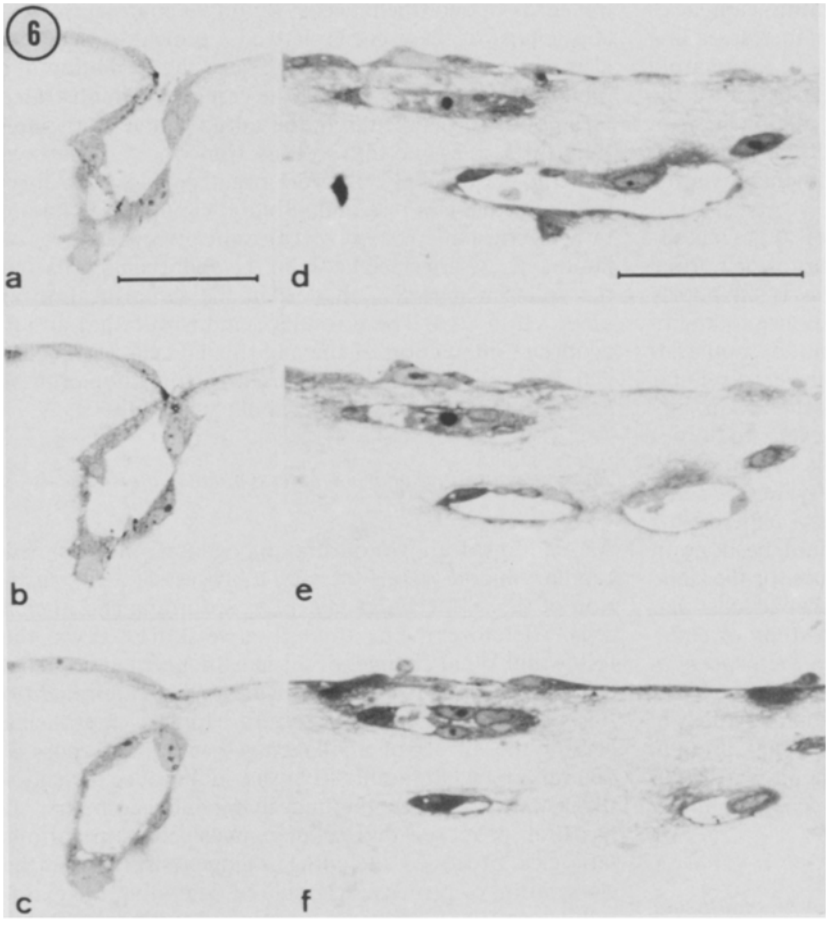

Figure 6. Invasion of collagen gels and formation of vessel-like structures by PMA-treated capillary endothelial cells (semithin sections perpendicular to the culture plane). *a–c* Consecutive serial sections showing the continuity between the endothelial cells forming the surface monolayer and those delimiting a tube-like structure inside the collagen matrix. *d–f* Serial semithin sections showing the dichotomy of a vessel-like structure into two smaller tubes that progressively separate from one another; *d* and *e* are consecutive sections, whereas *f* is three sections farther. Bars, 50 µm. (From ref. 23, by courtesy of Cell).

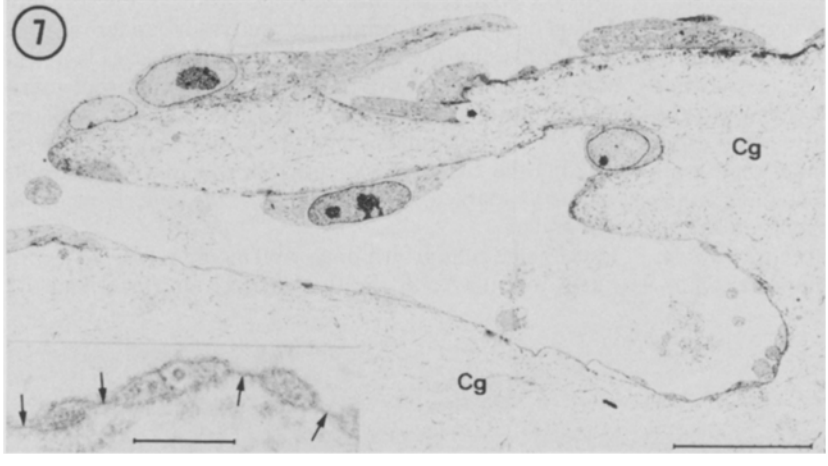

Figure 7. Thin section showing the continuity between the endothelial cells that line a capillary-like tube formed inside the collagen gel (Cg) and those present at the surface of the gel. Bar, 20 µm. Inset: endothelial pores with fenestral diaphragms (arrows). Bar, 0.2 µm. (From ref. 23, by courtesy of Cell).

not infiltrate the underlying collagen lattice. Within 2–6 h after the addition of 20 ng/ml PMA, the endothelial cells became more refractile and took on an irregular shape with long cell processes. After about 18 h of treatment, scattered cells with an elongated or dendritic morphology and whose plane of focus was obviously beneath the surface monolayer could be recognized. Examination with a high power phase contrast objective (40 ×) clearly showed that these cells had invaded and migrated through the three-dimensional collagen matrix. Within 2–3 days after the addition of PMA, the invading cells formed branching cords which tended to anastomose with one another. These endothelial cell cords frequently had translucent clefts along their axes, and many of them enclosed large, clear spaces, indicating the formation of capillary-like tubular structures. Therefore, PMA treatment of endothelial cell cultures resulted in the formation of a monolayer of irregularly-shaped cells at the surface of the collagen gel and a network of tube-like cords extending throughout the collagen matrix, as demonstrated also by sections perpendicular to the culture plane (fig. 6). These sections also revealed the continuity between the endothelial cells delimiting the tubular structures and those at the surface of the gel (figs 6a–c, and 7)[23].

To determine whether the above effects of PMA required de novo protein synthesis, cultures were preincubated for 2 h with 1 μg/ml cycloheximide, then treated with PMA in the continuous presence of cycloheximide. Cycloheximide did not inhibit the early morphological effect of PMA – that is, the change of cell shape – but completely prevented its late effect – that is, the invasion of the collagen matrix and the formation of capillary-like tubes. Endothelial cells treated with both PMA and cycloheximide had an altered morphology, similar to that induced by PMA alone, but remained entirely confined to the surface of the collagen gel. This inhibition was reversible: within 2 days after removal of cycloheximide, PMA-treated endothelial cells started to infiltrate the underlying matrix, and a network of tube-like structures was formed within 3–5 days[23]. These results indicate that PMA has two distinct, morphologically recognizable effects on cultured capillary endothelial cells: an early shape change which appears to be independent of protein synthesis, and a late effect, the invasion of the collagen gels, which is first detectable after about 18 h of treatment and requires de novo protein synthesis. This suggests that PMA modifies endothelial cell behavior by inducing the synthesis of specific proteins that are essential for invasion to take place.

The role of proteolytic enzymes

The experiments described so far demonstrated that treatment with PMA induced capillary endothelial cells to invade the underlying collagen matrix, where they formed an extensive network of capillary-like tubular structures, and that this process required protein synthesis[23]. However, they still did not provide information as to the involvement of proteases in this phenomenon. We therefore performed further experiments specifically aimed at elucidating the following questions: 1) is the PMA-induced invasion of collagen gels accompanied by collagen degradation?; 2) does the invasive behavior of PMA-treated endothelial cells require protease activity? To answer the first question, we cultured the endothelial cells on gels prepared form collagen radiolabeled with [^{3}H]acetic anhydride. The collagenolytic activity of control and PMA-treated endothelial cells was determined by measuring the release of radioactivity from the collagen matrix into the supernatant culture medium (table). Control cultures released only small amounts of label. In contrast, PMA-treated endothelial cells caused a markedly enhanced amount of radioactivity to be released between the 2nd and 4th days of culture, concomitant with extensive invasion of collagen gels as seen by phase contrast microscopy. Between the 4th and 6th days, PMA-treated endothelial cells induced the release of up to 30 times more label than did control endothelial cells (table). These data indicate that PMA-induced invasion of collagen gels is associated with the degradation of collagen fibrils[23].

To answer the second question – that is, whether the invasive behavior of PMA-treated endothelial cells requires protease activity – we studied the effect of a wide spectrum of protease inhibitors. Proteases are usually classified into 4 groups on the basis of the chemical nature of the active site responsible for the catalytic activity of the enzyme: serine-proteases (which include plasmin and plasminogen activator), thiol-proteases, carboxy-proteases, and metallo-proteases (which include collagenase)[3]. The following inhibitors, directed against each of these 4 classes of proteases, were added to the cultures: benzamidine, soybean trypsin inhibitor, ε-aminocaproic acid, Trasylol, chymostatin, p-nitrophenylguanidinobenzoate and α_1-proteinase inhibitor (α_1-antitrypsin), all inhibitors of serine proteases; antipain and leupeptin, which inhibit some serine proteases as well as thiol proteases; pepstatin, an inhibitor of carboxyproteases; the metallo-protease inhibitor, 1,10-phenanthroline; and α_2-macroglobulin, a physiological broad spectrum protease inhibitor which reacts with the majority of proteases from all 4 groups[3]. We found that inhibitors active against serine-, thiol- and carboxy-proteases did not prevent the effect of PMA. Although a relative resistance of membrane-bound proteases to inhibitors present in the extracellular fluid cannot be ruled out, these results suggest that the activity of proteases from these three classes may not be required for endothelial cells to invade, and to migrate through, a lattice of collagen fibrils. In contrast, the effect of PMA was prevented by the metalloprotease inhibitor 1,10-phenanthroline, a result which is compatible with the involvement of a metalloprotease (e.g. collagenase) in the invasive process[23].

These results show that PMA induces capillary endothelial cells to mimic a well defined step in the process of angiogenesis – that is, the invasion of the perivascular extracellular matrix, in which type I collagen is a major constituent. However, during angiogenesis, this step is

Collagen degradation associated with invasion of collagen gels and formation of capillary-like tubes by PMA-treated capillary endothelial cells**

Day	Total cpm recovered in the medium			Net cpm released by the cells (= collagen degradation)	
	Gels without cells (background)+	Control endothelial cells	PMA-treated endothelial cells	Control endothelial cells	PMA-treated endothelial cells
2	3,382 ± 46	4,250 ± 88	6,247* ± 130	868	2,865
4	2,592 ± 31	3,055 ± 105	10,810* ± 347	463	8,218
6	2,152 ± 109	2,455 ± 11	12,135* ± 357	330	10,010

Capillary endothelial cells were grown to confluency on three-dimensional gels prepared from collagen radiolabeled with [^{3}H]acetic anhydride, and further incubated with 500 μl of either control medium or medium containing PMA (20 ng/ml) for 6 days. The culture medium was changed at 2-day intervals, at which time the total radioactivity recovered in the medium was measured. The radioactivity recovered in the medium overlying gels without cells (background non-specific release) was also determined, and this value subtracted from the corresponding value obtained from gels with cells to calculate the net release of radioactivity due to the presence of the endothelial cells. Each value in the table represents the mean ± SEM of 4 replicate cultures.

+ The release of radioactivity from cell-free gels was not modified by the addition of PMA. A total of 2.4×10^5 cpm was recovered from each 200 μl collagen gel after complete dissolution with bacterial collagenase.

* $p \ll 0.001$ versus control endothelial cell values (Student's t-test). ** From ref. 23, by courtesy of Cell.

preceded by the local destruction of the endothelial basement membrane and the hydrolysis of type IV collagen. Whether PMA-treated endothelial cells are also able to degrade type IV collagen and/or to cross basement membranes remains to be investigated.

Taken together, the experiments with PMA demonstrate that a well-defined chemical signal can switch the behavior of capillary endothelial cells from non-invasive to highly invasive. Although phorbol esters are not substances which occur physiologically, they cause a variety of effects in cultured cells, many of which are also elicited by hormones, growth factors, or other endogenous mediators[7,27]. The next step will be to establish whether phenomena similar to those induced by PMA can also be triggered by angiogenesis factors isolated from either normal or neoplastic tissues[10,11,33,35].

As previously emphasized, angiogenesis plays a crucial role in a wide range of physiological and pathological processes, and is necessary for the continuous growth of solid tumors[11]. Therefore, achieving the ability to control the proliferation of blood vessels may have important therapeutical implications in a variety of malignant and non-malignant diseases. Stimulating the growth of capillaries would improve wound healing and speed the recovery from myocardial ischemia, burns and other types of tissue damage. Conversely, inhibition of angiogenesis could represent a powerful means of blocking neoplastic growth and perhaps prevent diabetic retinopathy[11]. Unfortunately, progress in this direction has been hampered by our still limited knowledge of the mechanism of angiogenesis. With the in vitro model we have developed, the angiogenic process is now amenable to experimental manipulation and biochemical analysis. A better understanding of how this phenomenon is regulated at the molecular level may ultimately lead to pharmacological strategies for modulating the growth of blood vessels.

In conclusion, the results we have obtained by growing three different cell types (pancreatic islet cells, kidney-derived epithelial cells, and capillary endothelial cells) within three-dimensional collagen gels provide significant experimental support for the concept[5,6,14,17,20,34] that the extracellular matrix plays a crucial role in morphogenetic processes. We are confident that further studies of cell-matrix interactions using in vitro models such as those we have described will improve our understanding of phenomena that are too difficult or impossible to investigate in vivo.

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Stable isotopes of lithium: dissimilar biochemical and behavioral effects

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Summary. Lithium, which is used routinely in the treatment of mania, is composed of two stable isotopes, lithium-7 (92.58%) and lithium-6 (7.42%). Usually there is minimal physiological or biochemical differentiation between isotopes of an element, but lithium is an exception. Data derived from a variety of biochemical and behavioral experiments are reviewed to support this idea. Additionally, the clinical implications of this work are presented.
Key words. Lithium; isotopes; lithium-6; lithium-7; mania.

Use of isotopes of an element has been invaluable in studies of a variety of problems including metabolic pathways, enzyme reaction mechanisms and the distribution of administered drugs. Isotopes of lighter elements have significant mass differences, which could be manifested by unexpected differences in biological systems. However, when isotopes are incorporated into comparatively large molecules, differences between their masses (i.e. hydrogen, deuterium and tritium) are reduced because of high overall molecular weights. In the case of lithium (Li) it is the ion, Li^+, that is biologically active.

As a consequence of its small mass, Li^+ possesses physicochemical properties different from the other alkali metal ions, sodium (Na^+), potassium (K^+), rubidium (Rb^+) and cesium (Cs^+), with respect to solubility, formation of complexes and magnitude of the radius of hydration. Li exhibits properties intermediate between the alkali and alkaline earth elements; it is similar to magnesium (Mg) with respect to ionic radius and calcium (Ca) with respect to charge density. The uniqueness of Li^+ is primarily a result of the small size of its ionic radius and a capability of polarization superior to the other alkali metal ions, which leads to solvation and covalent bond formation[7]. As would be expected Li^+ has both the biggest charge/radius ratio and radius of hydration of the alkali metal ions; these combined effects have a profound influence on the transport of Li^+ ions across the cell membrane.

Naturally occurring lithium (Li-N) is composed of two stable isotopes: lithium-7 (Li-7) is the major (92.6%) and lithium-6 (Li-6) is the minor constituent (7.4%). Nuclei of Li-6 contain three protons and three neutrons, Li-7 three protons and four neutrons. Radii of hydration of $(Li\text{-}6)^+$ and $(Li\text{-}7)^+$ are not the same because of differences in the charge/mass ratios of the unhydrated ions. The associated changes in the electrostatic interaction between the isotope ions, water molecules and negatively charged membrane species could contribute to dissimilarities in transport of the two Li isotopes across membranes. Isotopically pure Li-6 and Li-7 salts are commercially available.

Previous references to the utilization of Li-6 in biological systems are not abundant. Thellier et al.[21] used Li-6 to determine the distribution patterns of Li in the rat with a technique related to conventional autoradiography. Li-6 was also used as a tracer for Li-7 in a pharmacokinetic study by Birch et al.[4]. A limited number of reports is found in the literature dealing with the chronic toxicology of the stable Li isotopes and their possibly different effects. Rats maintained on small quantities of Li-6 showed no obvious signs of toxicity and four human subjects received Li-6 without experiencing any ill effects[4,5]. However, recent studies have demonstrated differential effects of the Li isotopes in a variety of systems using biochemical, pharmacological, behavioral and toxicological approaches. An internal consistency is apparent with respect to the data obtained in these studies, regardless of the specific methodology used and an isotope effect is clearly indicated.

Until recently it has been tacitly assumed that toxicity of the two Li isotopes was equivalent primarily because isotopically pure Li-6 administered for a year in drinking