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Role of laminin in endothelial cell recognition and differentiation

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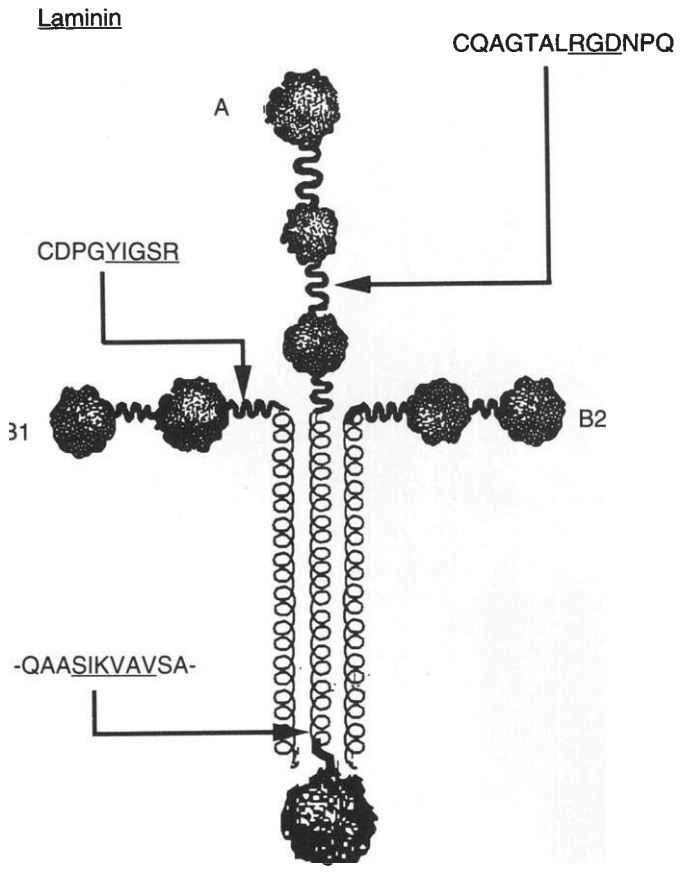
Role of laminin in endothelial cell recognition and differentiation. The vascular endothelium normally is maintained in a quiescent state, but under certain conditions it is induced to undergo marked changes in behavior and form new vascular structures. A complex interaction among various growth and differentiation factors and the extracellular milieu regulates this behavior. One series of signals affecting endothelial behavior is provided by laminin, a major structural protein of basement membrane. These signals have been studied using Matrigel, a reconstituted basement membrane preparation from the murine Englebreth-Holm-Swarm sarcoma, in an in vitro assay of endothelial cell differentiation. Three biologically-active sequences from the laminin molecule have been evaluated. Synthetic peptides that include the sequences -RGD-, -YIGSR-, and -SIKVAV- mediate, respectively, cell binding to Matrigel, alterations in cell morphology, and induction of migration and collagenase activity. Preliminary data indicate that observations made with this system may be relevant to endothelial function in vivo. Endothelial cell differentiation on Matrigel may thus be a useful in vitro model for the study of certain steps in angiogenesis.

The vascular endothelium shows a markedly low turnover rate in vivo under normal conditions [1]. It is comprised of polarized epithelioid cells with distinct apical and basal cytoplasmic domains [2, 3]. These cells are usually in a quiescent state, reflecting the stability and integrity of the vascular wall. They form complex cell-cell junctions, and secrete a basement membrane [4, 5]. This homeostasis is altered in vessel injury, such as that which occurs in physical trauma [6], atherosclerosis [7], or inflammation [8]. In addition, the presence of a tumor [9] or abnormal production of regulatory growth factors [10] may stimulate changes in endothelial cell behavior leading to angiogenesis. Endothelial cells also become activated in the vasculogenesis of embryonic development [5, 11–13]. Formation of new blood vessels is a desirable event in wound healing, but is deleterious in, for example, the neovascularization of the eye in diabetes mellitus. The understanding of key events and factors that regulate the angiogenic process is a prerequisite for developing and targeting the pharmacological means to control it for therapeutic purposes.

De novo physiologic or pathologic vessel formation requires five steps: (1) basement membrane surrounding the vessel is degraded, allowing the endothelial cells to penetrate the connective tissue stroma. Then, (2) endothelial cells migrate into the interstitium, where (3) they proliferate. Subsequently, (4) the new cells realign to form a capillary structure. Finally, (5) complex junctions form between the cells, and new basement membrane is secreted basally [13]. The signals that regulate vessel formation are currently under investigation. Many cell- and tissue-specific factors, including lymphokines [14, 15], heparin-binding growth factors [acidic fibroblast growth factor (FGF), basic FGF, transforming growth factor (TGF)-β and tumor necrosis factor (TNF)] [12, 13, 16], and cytokines [16–19] have been shown to stimulate cell migration and proliferation in vitro [12, 16, 20–25]. Although the relationship of these observations to the cascade of events that results in endothelial cell differentiation in vivo is uncertain, initial efforts to develop in vitro models of blood vessel formation suggest that these factors do play a significant role in modulating angiogenic behavior of endothelium. When endothelial cells are maintained in a superconfluent state for an extended duration, capillary-like structures form spontaneously [24, 26, 27]. Deprivation of growth factors also initiates this behavior [28]. Similar structures form when endothelial cells are cultured on extracellular matrix substrata such as collagen I, fibronectin and gelatin, or on a fragment of aorta in a fibrin clot [11, 17, 29–31]. In general, this differentiation from actively growing cells to quiescent cells in a capillary-like structure requires from two days to eight weeks. These in vitro models, however, only partly duplicate in vivo events. Not all endothelial cells in these models contribute to the formation of tubes, and the "vessels" may be inside out, secreting basement membrane substances into the lumen [27].

Numerous studies have demonstrated that type IV collagen is involved in endothelial cell attachment and migration [23, 25, 27]. The ability of this and other basement membrane substrata to partially support endothelial cell differentiation suggests that components of the extracellular matrix (ECM) play an important part in angiogenesis. Indeed, the period of time required for tube formation in the absence of exogenous basement membrane may relate to the need for the endothelial cells to secrete a critical amount of basement membrane (tube formation can be blocked by inhibitors of protein syntheses). Further, the occurrence of vessel proliferation in injury, which likely deprives the cells of their normal milieu, and in diseases such as diabetes mellitus when basement membrane composition is altered [32], suggests that abnormality or absence of basement membrane matrix may in some way allow endothelial cell proliferation to occur. The normally quiescent state of endothelium in the



Bioactivity of laminin peptides

	YIGSR	RGD	SIKVAV
Adhesion	++	+++	++
Morphology	+++	+	+++
Migration	+	+	++++
Growth	0	+	0
Differentiation	+++	0	++
Collagenase	++	0	++++

Fig. 1. *Schematic model of the laminin molecule.* Globular and helical domains are indicated. The location of sequences used to construct synthetic peptides, and relative activities of these peptides for several biological assays, are shown.

Table 1. Factors affecting tube formation by HUVEC on Matrigel

Substance added	% Control
Protein synthesis, activation or cytoskeletal assembly	
Cycloheximide *2 μg/ml*	50
Cytochalasin D *10 μg/ml*	4
Colchicine *10 μg/ml*	40
Phorbol *10 nM*	250
H-7 *10 μM*	50
Cytokines	
Interferon alpha *100 U*	250
Interferon gamma *100 U*	5
Matrix components	
Cis-hydroxyproline *50 μg/ml*	61
Ascorbic acid *50 μg/ml*	145
Collagen IV *10 μg added to the Matrigel*	140
Anti-collagen IV antiserum (1:10)	70
Anti-laminin antiserum (1:50)	<10
Anti-entactin antiserum (1:10)	<10
Anti-fibronectin antiserum (1:10)	100
Anti-heparan sulfate proteoglycan (1:10)	65
Synthetic laminin-derived peptides	
CTFA**LRGD**NP *250 μg*	40
CD**PYIGR**C *250 μg*	60
CSARKQAA**SIKVAV**SADR *250 μg*	40

presence of basement membrane also supports the notion that ECM may play a role in regulating endothelial cell function.

This possibility has been studied in vitro using Matrigel, a reconstituted matrix prepared from the Englebreth-Holm-Swarm (EHS) tumor [33]. Except for a relative decrease in the amount of type IV collagen present, Matrigel contains similar components to those found in most basement membranes. Matrigel has been found to induce differentiation by a variety of cell types. For example, mammary carcinoma cells form gland-like structures that secrete casein into their lumens [34], and bone cells form canaliculi [35]. When human umbilical vein endothelial cells (HUVEC) or microvascular endothelial cells are seeded onto Matrigel, they form a network of capillary-like structures within 18 hours [4]. Almost all of the cells in the culture form tubes, and cross sections of these tubes indicate that the cells are physiologically polarized with apical microvilli, secretory granules, and basal nuclei [36]. This process mimics the endothelial cell alignment, cell-cell adhesion, lumen formation, and basal secretion of basement membrane that occurs during formation of new microvessels. Thus, culture of HUVEC or other endothelial cells on Matrigel may serve as a useful, rapid model for in vitro study of endothelial cell activity in certain steps of angiogenesis.

Matrigel contains multiple potential signals for endothelial cell differentiation. The growth-regulatory factors TGF-β, platelet-derived growth factor (PDGF), basic FGF, epidermal growth factor (EGF), and insulin-like growth factor (IGF)-1 are present [37]. The highly anionic heparan sulfate proteoglycans in ECM bind some of these factors and probably serve as a

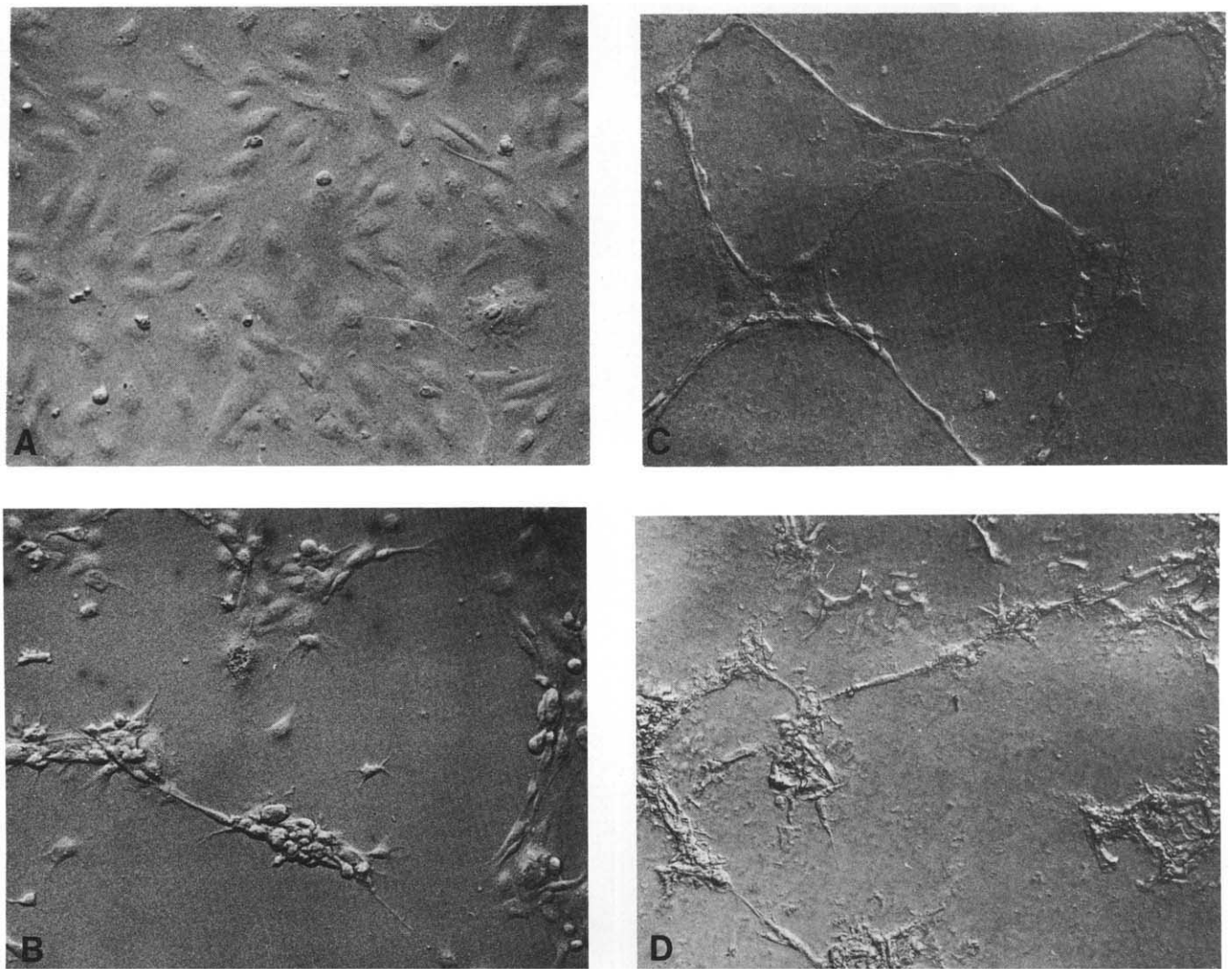


Fig. 2. *Effect of the -SIKVAV- peptide on endothelial cell morphology.* All photographs are 200×, taken through a Hoffman system. **A.** Appearance of cells cultured on plastic. **B.** Endothelial cells cultured on plastic in the presence of 200 μg/ml -SIKVAV-. **C** and **D.** Tube formation by HUVEC plated on Matrigel without (C) and in the presence of (D) exogenous -SIKVAV- peptide.

reservoir for them [38, 39]. FGF is known to promote angiogenesis in a variety of in vitro and in vivo model systems. It is not known if the FGF present in basement membranes is active in the bound form or must be freed.

In addition to the effects of recognized growth factors, it is likely that individual extracellular matrix proteins themselves play a direct role in regulating endothelial behavior. One of the most extensively studied of these is laminin. This protein is composed of three chains designated A (M_r = 400,000), B1 (M_r = 210,000) and B2 (M_r = 200,000) (Fig. 1). While the intact molecule has been shown to affect differentiation in several systems [40, 41], our laboratory has focused more recently on three specific peptide sequences within laminin. These have previously been found to affect the behavior of other types of cells. A portion of the laminin A chain, containing the -RGD- (arginine-glycine-aspartic acid) sequence found in a large number of protein-cell attachment sites, mediates cell attachment to laminin [42]. A second sequence, on the short arm of the laminin B1 chain, includes -YIGSR- (tyrosine-isoleucine-glycine-serine-arginine) and is active for adhesion to laminin and to Matrigel [43]. Most recently, a sequence near the carboxy-terminal end of the laminin A chain containing -SIKVAV- (serine-isoleucine-lysine-valine-alanine-valine) has been found to induce neurite outgrowth in neuronal cells [44] and facilitate metastatic behavior by tumor cells [45]. Thus, laminin contains multiple active sites for regulating cell behavior. These sites also induce similar changes in endothelial cells [4] which will be discussed further below.

Modulation of endothelial cell tube formation on Matrigel

A quantitative assay for tube-forming activity on Matrigel has been developed. Matrigel, prepared from the EHS tumor by 2 M urea extraction [46], is maintained frozen until use. When thawed and maintained at 4°C, it remains in a liquid state. It polymerizes into a gel when warmed to 37°C. To perform the assay, 300 μl Matrigel is plated into wells on a 24-well culture plate. After it has been allowed to gel for 30 minutes at 37°C, 40,000 endothelial cells (previously grown on plastic dishes) in

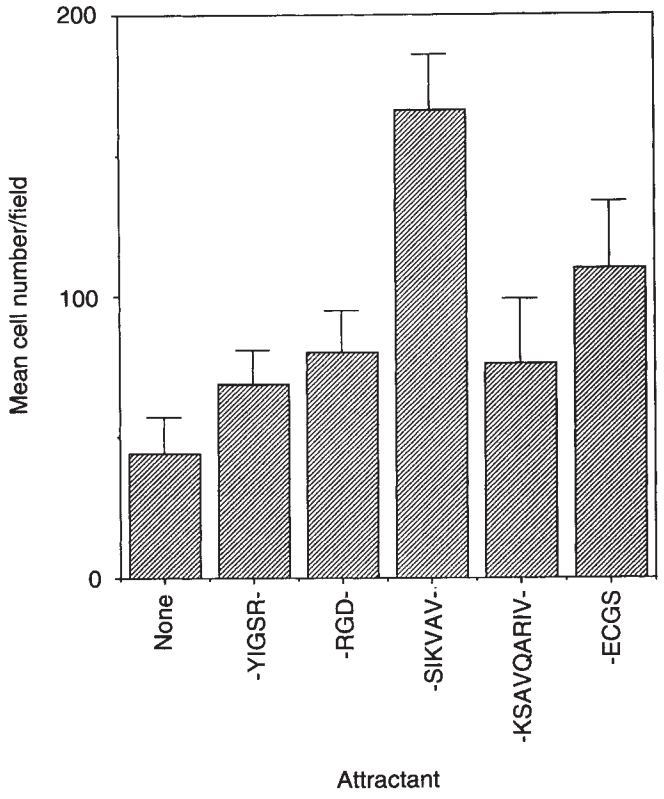


Fig. 3. *Chemoattraction by the SIKVAV-containing peptide.* Cells were placed in the upper well of a Boyden chamber; in the lower well were control medium, biologically active, laminin-derived synthetic peptide (-RGD-, -YIGSR-, or -SIKVAV-), a permutation of the -SIKVAV- peptide sequence, or endothelial cell growth supplement (ECGS). See text for details of the experimental methods.

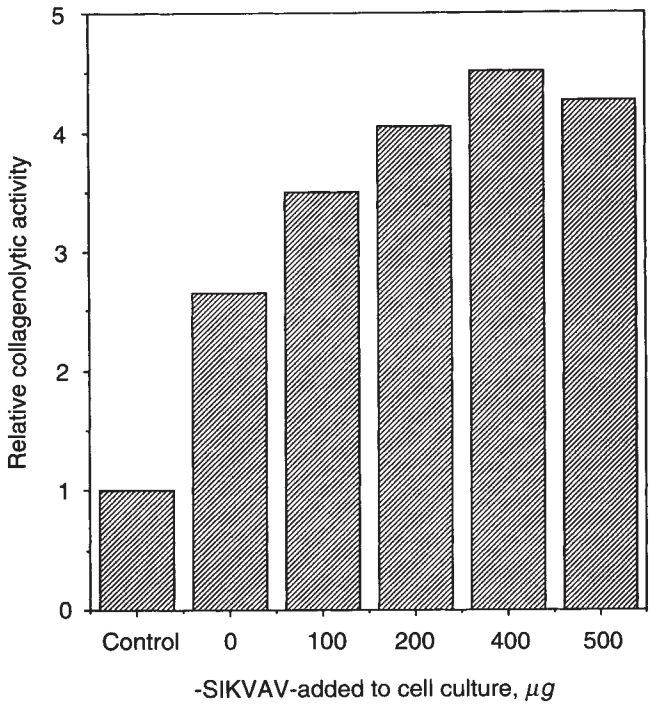


Fig. 4. *Release of radiolabel from type IV collagen by serum-free supernates of cultured HUVEC.* Cells (1×10^5) were cultured in 1 ml serum-free medium in the presence of varying doses of -SIKVAV- peptide. After 6 hours, the supernates were transferred to wells coated with radiolabeled collagen IV. Results are expressed as fold increase over release induced by unconditioned medium (control).

1 ml of culture medium are plated onto the Matrigel substratum. Eighteen hours later, the culture is fixed and stained, and the area of tubes formed on the surface of the Matrigel is quantitated utilizing an Optomax imaging system [39]. This assay has been used to investigate the effect of a number of agents on tube formation (Table 1). Cycloheximide inhibits tube formation, indicating that this process requires new protein synthesis. Because of the requirement for structural change and formation of tight junctions, it is not surprising that cytochalasin and colchicine decrease tube-forming activity [36]. Phorbol esters enhance tube formation, whereas the kinase inhibitor H-7 inhibits this process. Type I and Type II interferons have opposite effects, with IFNα increasing and IFNγ decreasing tube area.

Cis-hydroxyproline is inhibitory in the assay, while ascorbic acid moderately enhances tube formation, suggesting that collagen synthesis is important in tube formation. When additional type IV collagen is added to the Matrigel, tube formation is also somewhat enhanced. Antisera to heparan sulfate proteoglycans, collagen IV or fibronectin have partial or no effect compared to the profound inhibition caused by antisera raised against either laminin or entactin. Taken together, these data suggest that protein synthesis and an intact cytoskeleton are required for tube formation, and that endothelial cells interact with multiple components of Matrigel during this process. These results also indicate that this in vitro assay system may provide a quick, reliable assay for testing certain angiogenic properties of biological and pharmacological agents.

Because of the known biological activities of the three laminin-derived peptide sequences, their activity was tested in the tube assay. The peptide CTFAL**RGD**NP decreases tube formation by blocking attachment of the endothelial cells to the Matrigel. CDPG**YIGSR**C inhibits tube alignment and other morphological changes associated with tube formation. The effects of the CSARKQAA**SIKVAV**SADR peptide are more complex. Photography of endothelial cells plated on plastic in the presence of -SIKVAV- peptide shows increased extension of cytoplasmic processes (Fig. 2), and time-lapse photography (D. Grant and L. Thompson, unpublished observation) shows greater movement across the culture plate. On Matrigel, the cells treated with SIKVAV-containing peptide sprout multiple, spike-like projections and invade the surface of the substratum. Clumps of cells that form the "hubs" of the tube network may extend more processes than usual, but many of these appear to terminate prematurely, perhaps after entering the Matrigel (Fig. 2). The overall result is a decrease in tube area as measured by the Optomax system [47].

Effects of the -SIKVAV- peptide on endothelial cell function

The biological activity of the SIKVAV-containing, laminin-derived synthetic peptide on endothelial cells is not confined to the tube-forming assay. In the experiment shown in Figure 3, endothelial cells were evaluated for migratory activity across a Millipore filter in a Boyden chamber assay. The upper chamber contained 2×10^5 HUVEC. After five hours at 37°C, 5% CO_2,

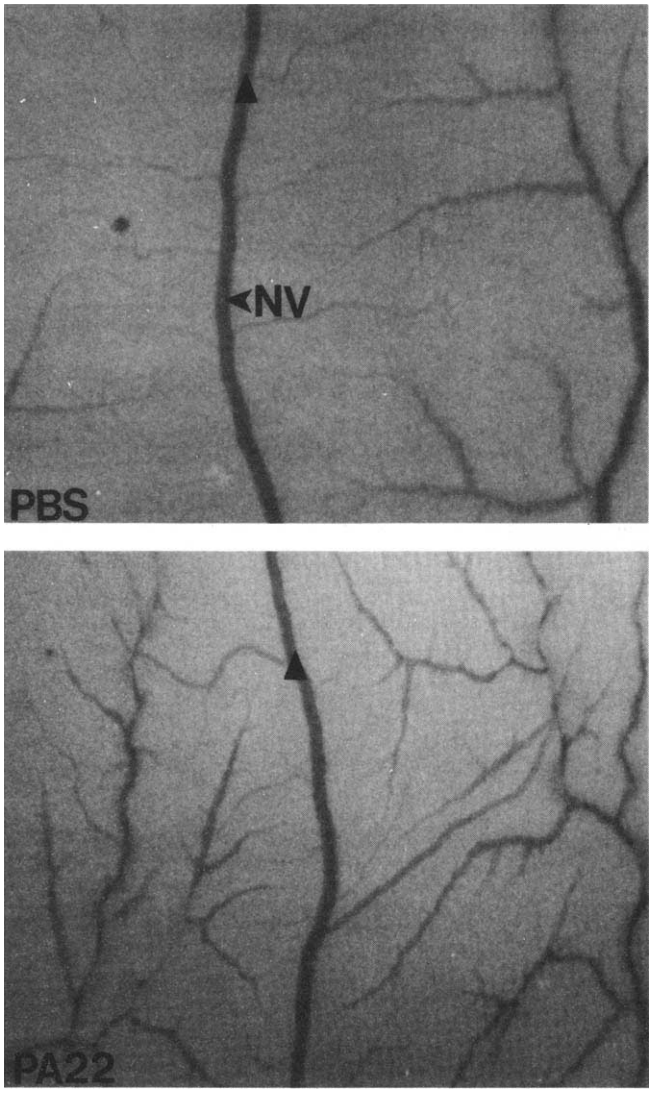


Fig. 5. *Effects of -SIKVAV- on vascularization of chick yolk sac membrane.* The assay was prepared as described in the text. Peptide was added at initiation of the assay and photographs were taken 24 hours later. NV, central vascular branch from embryo; PBS, phosphate-buffered saline; PA22, -SIKVAV- peptide.

the filters were removed and stained, and the number of cells present on the underside of the filter were quantitated visually through an inverted microscope. In this chemoattraction assay, peptides incorporating the sequences -RGD- and -YIGSR-, when placed in solution in the lower chamber, caused slight increases in migration compared to controls. Some migration was stimulated by endothelial cell growth supplement (Collaborative Research, Bedford, Massachusetts, USA), an agent necessary for culturing HUVEC that is prepared from bovine brain and contains a variety of growth factors, including FGF. The greatest increase in migration was caused by the peptide containing SIKVAV. A control peptide including -KSAVQARIV-, representing a "scrambled" version of the SIKVAV sequence, induced little migratory behavior. These findings confirm the studies made with time-lapse photography and demonstrate that the -SIKVAV- sequence stimulates endothelial cell migration.

The observation that endothelial cell processes appear to enter the Matrigel suggested that these cells may be producing proteolytic activity. To test this hypothesis, supernates of cells treated with the -SIKVAV- peptide were tested for the presence of a substance that degrades type IV collagen, a substrate chosen because it is relatively specific for basement membrane. In the experiment shown in Figure 4, collagenolytic activity was measured by release of radioactivity from a solid-phase, radiolabeled collagen IV substrate. Degradation was slightly above background in the presence of supernates from cells not exposed to this peptide, indicating that a small amount of activity was secreted constitutively by cultured endothelial cells. The -SIKVAV- peptide had a dose-dependent stimulatory effect on secreted proteolytic activity, with maximum effect at 400 μg/ml. Thus, this peptide stimulates degradation of type IV collagen by endothelial cells.

In vivo effects of the -SIKVAV- peptide

To determine whether the effects of the -SIKVAV- sequence on activity of cultured endothelial cells might be extended to the process of angiogenesis in vivo, a chick yolk sac membrane (CYSM) assay is employed. Fertilized chicken eggs are removed from their shells under sterile conditions and vascularization of the CYSM is followed during embryogenesis. As shown in Figure 5, when the -SIKVAV- peptide is placed near the main vessel growing out into the CYSM, a large collateral circulation develops in the area of the peptide. The vessels involved are also more prominent and more tortuous than usual. This finding suggests that, in addition to its activities in vitro, the -SIKVAV- sequence appears to be at least partially responsible for laminin-induced angiogenic activity in vivo.

Summary

A variety of stimuli appear to coordinately regulate the process of endothelial cell differentiation on Matrigel. In addition to cytokines and other intercellular mediators, the structural proteins of basement membrane appear to have their own regulatory potential. At least three bioactive peptide sequences have been identified as participating in laminin-mediated effects of Matrigel. The sequence containing -RGD- mediates cell attachment, -YIGSR- mediates morphological changes, and -SIKVAV- activates or enhances several biological processes that may be important in endothelial cell differentiation. Preliminary experiments suggest that observations made regarding endothelial behavior on Matrigel may provide data relevant to endothelial function in vivo. Further studies are in progress to determine how this in vitro model can be applied in characterizing diseases of neovascularization and in developing approaches to therapeutic manipulation of angiogenesis.

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