Vitronectin: Effects on Keratinocyte Motility and Inhibition of Collagen-Induced Motility

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Epibolin, a plasma protein, was initially purified on the basis of its ability to enhance spreading of keratinocytes. It is now known that epibolin is identical to serum spreading factor, S protein, and vitronectin, and the current name for the protein is vitronectin. Studies of vitronectin on cultured keratinocytes showed that it caused spreading and epiboly but not cellular adhesion to the substratum. In studies with other types of cells, vitronectin increased migration of several types of cells in a Boyden chamber. Because some agents that enhance spreading and adhesion, such as collagen and fibronectin, also increase motility, we tested whether vitronectin increased motility of keratinocytes. By photographing and quantitating motility of keratinocytes plated on a bed of colloidal gold particles, we determined that vitronectin increased local movement of keratinocytes in a concentration-dependent fashion, resulting in clearing of gold particles in a circular pattern around the cells, but did not cause the production of tracks found in cultures plated on collagen or fibronectin. The small increases in clearing of the gold particles that occurred in the presence of vitronectin were abolished by

antibody to vitronectin. Furthermore, the marked increase in motility produced by type I collagen was significantly reduced when the keratinocytes were treated with vitronectin. Antibody to vitronectin also abrogated the vitronectin-induced reduction in collagen-stimulated motility, confirming that this action was specific for vitronectin. Serum, which contains vitronectin, stimulated motility in a fashion identical to purified vitronectin, but serum lacking vitronectin was inactive. These studies show that vitronectin causes a localized increase in movement associated with spreading resulting in a halo around individual cells, that vitronectin does not enhance directional motility of keratinocytes in this assay but in contrast antagonizes such motility produced by collagen, and that vitronectin is the factor in serum responsible for this effect. The findings with vitronectin and collagen show that these agents stimulate different types of motility. The roles in wound healing of agents stimulating different types of motility are unclear and require further study. *J Invest Dermatol* 96:724-728, 1991

Vitronectin was first studied in keratinocytes because of its effect on growth of skin in organ culture, in which the epithelium rapidly epibolized (or covered the nonepithelialized surfaces). It was for this property that the activity in serum was named epibolin when it was purified to homogeneity by Stenn [1]. This protein was also found to increase the epithelial outgrowth from skin organ explant cultures and to cause spreading of freshly isolated guinea pig keratinocytes. More recently, it has been found to be immunologically cross reactive with serum spreading factor [2,3], now called vitronectin [4,5], an adhesion factor for fibroblasts. Molecular cloning data show that vitronectin is also identical with S-protein, initially described as an inhibitor of complement-mediated cytotoxicity [6]. It is now clear that epibolin, S-protein, serum spreading factor, and vitronectin are all the same serum protein.

Vitronectin induced rapid spreading of keratinocytes [7] and en-

hanced mobility of the epithelium in vitro. In studies of "serum spreading factor," vitronectin enhanced migration of some tumor cells in a Boyden chamber [8]. In order to compare the action of vitronectin with agents known to stimulate keratinocyte motility, such as fibronectin or collagen [9], we examined the effect of vitronectin on cultured keratinocytes in an established assay for motility [9].

MATERIALS AND METHODS

Vitronectin prepared according to Stenn [1] was used for most of these studies; the partially purified material used in these studies has been characterized in an earlier study [7]. Purified vitronectin, indicated as "highly purified vitronectin" in the text below, was purchased from Calbiochem (La Jolla, California). Plasma was depleted of vitronectin by absorption with antibody [10]. Anti-vitronectin antibody used in this study has been characterized previously [7].

Human keratinocytes derived from foreskins of neonates were cultured in supplemented MCDB 153 medium in 0.1 mM Ca^{++} as previously described [11], suspended with trypsin, and counted in a Coulter particle counter prior to assay for motility. Keratinocyte motility was assessed in petri dishes (35 mm) coated with particles of colloidal gold prepared according to Albrecht-Buehler [12] as described [9]. Type I collagen (Vitrogen, Flow Laboratories, McLean, VA) was added to dishes in indicated amounts in 2 ml of Hanks buffered saline solution for 60 min at 37°C, and the dishes were washed with Hanks buffered saline solution. Cells (10,000) were then added in supplemented MCDB 153 medium with or

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Abbreviation:

EGF: epidermal growth factor

without various dilutions of vitronectin in Dulbecco's buffered saline solution with 1 mg bovine serum albumin per ml and allowed to attach and move for 24 h at 37°C. In some experiments, highly purified vitronectin was added to dishes in buffered saline solution before the cells and medium, as in the method described above for collagen.

Supplemented MCDB 153 medium contained hydrocortisone (0.4 µg/ml), insulin (5 µg/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine pituitary extract (150 µg/ml), epidermal growth factor (EGF, 5 ng/ml), and 0.1 mM Ca⁺⁺. The cultures were washed gently to remove nonadherent cells and the cells were then fixed with 3% buffered formaldehyde and viewed in a dark field microscope. Photographs of multiple fields were projected onto paper and tracks were traced and the areas computed with a digitizing pad as described [13]. At least three fields per point were digitized. Comparison of fixed and unfixed cells indicated that fixation with formaldehyde caused no change in the apparent clearing of gold particles or in the morphology or size of the cells comparable to the fixation artifact produced when tissues are fixed, dehydrated, and embedded.

Extensive studies with vitronectin have shown that vitronectin increases spreading of keratinocytes whether added to dishes before cells or after cells are already plated, whereas collagen and fibronectin must be present in medium (and presumably on the plastic surface) or else on the plastic surface alone before cells are added, for spreading to occur [7]. Collagen, however, can be added before cells and has been shown to stimulate motility of keratinocytes when added in this fashion [9]. In these studies of vitronectin we plated cells in vitronectin-containing medium, as this was the way in which vitronectin was known to be effective on keratinocytes when added in this fashion, and because it was not known whether our

partially purified vitronectin would remain on the dish if the vitronectin-containing medium was removed before cells were added. In additional experiments designed to treat collagen and vitronectin identically, highly purified vitronectin was added to dishes in buffered saline, the solution was removed, and cells and medium were added. Unless stated otherwise, however, cells and vitronectin were added simultaneously.

RESULTS

Keratinocytes plated on a bed of gold particles did not alter the bed of particles under control conditions, in the absence of matrix or vitronectin, in the motility assay (Fig 1A). When vitronectin was incubated with keratinocytes, spreading-associated movement of the cells resulted in localized clearing of gold particles around the cells (Fig 1B) not apparent in controls, but linear tracks observed in cultures plated on collagen (Fig 1C) were not present. The vitronectin-treated cells when viewed under phase contrast microscopy did not occupy all of the cleared area (Fig 1D), suggesting that the clearing was related to movement of the cell or its border and did not simply reflect the area over which the cell was spread. No qualitative difference in motility, such as the formation of linear tracks, was produced by increased quantities of vitronectin. Unlike type I collagen, vitronectin induced distinctive local movement producing circular cleared areas around individual cells. Digitization of the clearing of gold particles showed that these small increases in area depended on the amount of vitronectin added (Fig 2).

Because we used a preparation of partially purified vitronectin for these studies, it was important to determine whether the effect we were measuring was caused by vitronectin or a contaminant in the preparation. We therefore incubated antibody specific for vitronectin [7] with the cells in the presence of vitronectin and measured

Figure 1. Vitronectin stimulates keratinocyte motility in a manner distinct from that produced by collagen. Keratinocytes cultured in 0.1 mM Ca⁺⁺ in supplemented MCDB 153 medium (see *Materials and Methods*) were plated on gold particles and incubated for 24 h at 37°C, fixed with 3% formaldehyde, and photographed under dark field (A–C) or phase contrast (D) optics. Keratinocytes plated on (A) plastic, (B,D) plastic in the presence of vitronectin (1:100) in the medium; or (C) type I collagen (40 µg/35 mm dish). Bar, 100 µm.

Figure 2. Keratinocyte motility is stimulated by vitronectin in concentration-dependent fashion. Keratinocytes were incubated on gold particles for 24 h in the presence or absence of vitronectin in various dilutions or on type I collagen-coated plastic.

motility. Antibody to vitronectin was able to abolish the effect of vitronectin on motility, and the reduction was greater when increased amounts of antiserum were added (Fig 3). Nonimmune rabbit serum had no effect (not shown).

Because the action of vitronectin appeared to be qualitatively different from that of collagen, we determined whether the effects on quantitative motility were additive. In contrast to the expected result, when keratinocytes were plated on collagen and also incubated with vitronectin, motility was reduced in comparison with that of cells plated on collagen without vitronectin; the reduction was greater in the presence of increased concentrations of vitronectin (Fig 4). This ability of vitronectin to inhibit collagen-induced motility was independent of the amount of collagen on the dish, because it was demonstrable with a range of amounts of collagen added to the dishes (Fig 5). To verify that this action of vitronectin was not caused by a contaminant in the partially purified vitronectin, we examined the effect of anti-vitronectin antibody in this experimental setting. The reduction by vitronectin in the collagen-stimulated directional motility of keratinocytes was prevented by addition of anti-vitronectin antibody (Fig 6). In the same experiment, anti-vitronectin was also shown to be able to reduce the enhancement of quantitative motility produced by vitronectin alone, in the absence of collagen, as expected (Fig 6). In control experiments, nonimmune serum was without effect (not shown).

These effects on keratinocytes were produced by vitronectin purified from serum. In order to determine whether similar effects were produced by the protein in the presence of other plasma proteins, as

Figure 4. Vitronectin reduces collagen-stimulated increases in keratinocyte motility. Keratinocytes were incubated on gold particles for 24 h in the presence or absence of vitronectin at various dilutions with or without type I collagen.

it is found in vivo, the effect of human serum was compared with that of serum depleted of vitronectin by passage over an antibody affinity column. As is shown in Table I, human serum, like partially purified vitronectin, increased the motility of keratinocytes, but serum depleted specifically of vitronectin was without effect. Like vitronectin, serum increased the size of the areas cleared around keratinocytes in a circular pattern but did not alter directional motility (not shown). These data demonstrate that vitronectin is the agent in serum that increases the cleared area around keratinocytes in a characteristic manner.

Because the studies described above used vitronectin added to cells, whereas collagen was added to the plastic surface before the cells, we also tested the effect of vitronectin added in a fashion parallel to that used with collagen. Highly purified vitronectin added to plastic dishes before the cells had identical effects on cells with respect to the clearing of gold particles as did partially purified vitronectin added with the cells; clearing was increased in a circular pattern around the cells, but no lateral mobility producing "tracks" was produced as was the case with collagen (data not shown).

DISCUSSION

The complex functions of the vitronectin molecule are poorly understood. It circulates in plasma in concentrations of 0.1 to 0.4 mg per ml [8], and the presence of the 65- or 75-kd form, or both, defines the vitronectin phenotype, which is based on a genetic polymorphism [14]. Vitronectin reduces the cytolytic activity of complement by interacting with the membrane attack complex [15] and also interacts with the coagulation pathway and prevents accelera-

Figure 3. Antibody to vitronectin abrogates the effect of vitronectin on keratinocyte motility. Keratinocytes were incubated on gold particles for 24 h in the presence or absence of vitronectin with various concentrations of antibody to purified vitronectin.

Figure 5. Vitronectin reduces collagen-induced motility at various concentrations of collagen. Keratinocytes were incubated on gold particles for 24 h in the presence or absence of vitronectin with or without various concentrations of type I collagen.

Figure 6. Reversal of vitronectin-induced reduction in collagen-stimulated motility by anti-vitronectin. Keratinocytes were incubated on gold particles for 24 h in the presence or absence of vitronectin, type I collagen, and antibody to purified vitronectin as indicated.

tion by heparin of antithrombin III inactivation of thrombin [16]. Furthermore, it is a cell surface-associated protein [5], contains the sequence arginine-glycine-aspartic acid characteristic of the cell recognition site of many extracellular matrix proteins, and binds the cell surface protein integrin [17]. It also has heparin and collagen binding sites [14]. It has been reported to bind keratin filaments in pathologic aggregates of keratin in epidermis [18] and appears to be deposited from late childhood on in the skin in association with elastic fibers [19]. In addition to enhancing spreading and adhesion of fibroblasts, for which it was called serum spreading factor, it has effects on motility. Vitronectin has been shown to increase directional motility of several types of tumor cells through filters in a Boyden chamber in a fashion indicating haptotaxis [8]. It is not known whether a preparation of vitronectin would similarly affect cultured keratinocytes; the assay for motility and the type of cell used by Basara et al [8] differed from those in our study. Interleukin-1 and conditioned medium from smooth muscle cells are known chemoattractants for keratinocytes in a Boyden chamber [20]. Further studies of keratinocyte motility comparing the effects of different proteins enhancing motility in these various assays may help elucidate the distinct types of motility they stimulate.

Because vitronectin enhances motility in an epithelial sheet, we sought to determine whether vitronectin could increase motility in an assay in which matrix factors such as collagen and fibronectin are known to dramatically enhance motility. Although the action of these factors is thought to play a role in wound healing, the precise role each agent serves in vivo is uncertain. The results of our studies with vitronectin further complicate this picture, because vitronec-

tin, which is known to affect epithelialization in vitro, has an entirely different action, and even antagonizes the action of type I collagen in our assay. Although the experiments reported here were performed in medium containing EGF and bovine pituitary extract, the effects of collagen and vitronectin were not dependent upon these additives; similar results were obtained in medium lacking one or both.

In keratinocytes treated with vitronectin, the borders of the cells do not correspond with the areas cleared of particles, indicating that the cleared areas record movement of the cell border associated with spreading. The type of movement produced by vitronectin apparently interferes with lateral migration. Examination of the patterns of motility in cultures with both collagen and vitronectin indicated that tracks were shorter in comparison with those found in cultures with collagen alone, but there was no apparent qualitative alteration in the tracks. The results suggest that the localized membrane movement produced by vitronectin interferes with the actions required by the cell for directional motility. Thus, the movement of the cell border produced by vitronectin is apparently a different type of movement from that giving rise to lateral directed migration, and apparently both types of movement cannot take place simultaneously. Real-time or time-lapse photography of these two types of movement may be of interest in this regard.

Different types of motility have been observed previously in epithelial cells. Tchao [21] found that epithelial cells in small groups were capable of rotatory migration when plated on glass but migrated independently, i.e., as isolated cells, in a directional fashion on collagen-coated glass. Barrandon and Green [22] showed that migration accounted for a substantial part of the expansion of stratified keratinocyte colonies and was stimulated by EGF or transforming growth factor- α . This growth factor-stimulated alteration in colony size was apparent within 15 min. Similarly, when isolated keratinocytes [23] or A-431 cells [24] are treated with EGF, the cells undergo dramatic spreading and ruffling within minutes. EGF does not stimulate lateral migration of isolated keratinocytes in the assay used in the studies described here, and it does not produce clearing of gold salts around colonies or individual cells (not shown), suggesting that the type of motility produced by EGF is different from that produced by vitronectin or the matrix proteins fibronectin, type I collagen, or type IV collagen (see [9]).

The biologic functions of these different types of motility is unclear. Because it accelerates epiboly, vitronectin may enable epithelial sheets to spread over a non-epithelialized surface, an action similar to that proposed by Barrandon and Green [22] for EGF. A protein as large as IgG ($M_r = 150,000$) can penetrate the basement membrane and bind to epidermal cells, in that it is detectable in the epidermis in the autoimmune disorder, pemphigus vulgaris. Vitronectin is a plasma protein of $M_r = 75,000$ and may be able to penetrate the basal lamina and reach epithelial cells in the absence of wounding, but vitronectin has not been described in the epidermis, only in the dermis [25]. It is not known whether epidermal keratinocytes are normally exposed to vitronectin, and it is unclear how its action might be regulated. Freshly obtained epithelial cells do not readily spread in basal-unsupplemented medium or on fibronectin-coated surfaces but spread in medium with vitronectin [7], suggesting that keratinocytes in vivo may be responsive to vitronectin.

Contrasting findings have been reported for fibronectin. Freshly isolated epidermal keratinocytes do not spread on fibronectin-coated dishes but gain the ability to do so several days after the cells are introduced into culture. The ability to spread corresponds with expression of the receptor for fibronectin, and responsiveness to fibronectin is regulated by modulation of expression of the receptor [26]. Keratinocytes may not normally be in contact with fibronectin and can apparently become responsive to it only under special conditions. Thus, although keratinocytes are constitutively responsive to vitronectin, they are only facultatively responsive to fibronectin. It is possible that the different types of motility stimulated in vitro by vitronectin or matrix proteins are in some way complementary and that these different responses are regulated during wound healing.

Table I. Effect of Control Serum or Serum Depleted of Vitronectin on Keratinocyte Motility^a

Additions	Percent of Area Cleared	
	Experiment 1	Experiment 2
None	0.08 ± 0.01 ^b	0.18 ± 0.02
Control serum	0.58 ± 0.07	0.60 ± 0.1
Depleted serum	0.14 ± 0.09	0.27 ± 0.08
Vitronectin (1:50)	0.53 ± 0.1	ND ^c
Collagen ^d	3.70 ± 1.1	ND

^a Keratinocytes were plated in collagen-coated or plastic dishes with or without vitronectin, 1% serum, or 1% serum depleted of vitronectin (10) for 24 h at 37°C and assayed for motility.
^b Mean ± SD.
^c ND, not done.
^d 30 µg per dish.

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