Newt epidermal cell migration over collagen and fibronectin involves different mechanisms

DONALD J. DONALDSON*, JAMES T. MAHAN
Department of Anatomy and Neurobiology, University of Tennessee, Memphis, The Health Science Center, Memphis, TN 38163, USA

and GERALD N. SMITH, Jr
Section of Rheumatology, Indiana University, School of Medicine, Indianapolis, IN 46223, USA

* Author for correspondence

Summary

Effects of the synthetic peptides, Arg-Gly-Asp-Ser (RGDS), the amino acid sequence representing the fibroblast attachment site in fibronectin, and Arg-Gly-Glu-Ser (RGES), on collagen- and fibronectin-mediated migration in newt epidermal cells were compared. When RGDS at 50 μg ml⁻¹ was included in the incubation medium of skin explants, migration in fibronectin-coated dishes was almost totally blocked. In type I collagen-coated dishes, this concentration of RGDS also inhibited migration, but to a lesser degree than on fibronectin. With 250 μg ml⁻¹ of RGES in the medium, the reverse was true. Here, migration on collagen was practically non-existent, while migration on fibronectin was affected only moderately. Collagen-mediated migration was sensitive to RGDS even when the peptide was added after migration on the coated substratum was well underway.

At a coating concentration of 10 μg ml⁻¹ CB3, a cyanogen bromide fragment of the collagen alpha 1(I) chain, which contains no RGD sequences, was as good a migration substratum as intact collagen applied at the same coating concentration. At lower concentrations intact collagen was somewhat better than equivalent concentrations of CB3. The presence of RGDS in the medium throughout an experiment inhibited migration in CB3-coated dishes in a manner similar to its effect in collagen-coated dishes.

Monensin, an ionophore known to block secretion and endocytosis inhibited migration on fibronectin and collagen to a small, but statistically insignificant degree. A more pronounced, statistically significant effect was seen in CB3-coated dishes. Electrophoretic analysis of the cell extract and the medium of cells migrating overnight on CB3 in the presence of [³⁵S]methionine showed no evidence of fibronectin secretion by the migrating cells.

These results indicate that newt epidermal cells use different mechanisms as they migrate over fibronectin and collagen. The data could be explained by the existence of two classes of extracellular matrix receptors on epidermal cells or one class of receptor that binds to the active site(s) in fibronectin and collagen with different affinities. Our inability to detect fibronectin secretion by cells migrating on CB3 and the collagen-like sensitivity of CB3-mediated migration to RGDS suggests that some epidermal receptors involved in migration are capable of recognizing more than just the RGD sequence. The effect of monensin in CB3-coated dishes may be an indication that receptor recycling occurs during migration over some substrata.

Key words: epidermal migration, wound closure, extracellular matrix, collagen, fibronectin, synthetic peptides.

Introduction

Where it rests on the basement membrane, the plasmalemma of epidermal basal cells in non-wounded skin is in close proximity to a number of extracellular matrix (ECM) proteins. These include type IV collagen and the cell attachment glycoproteins, laminin and fibronectin (Briggaman, 1982; Couchman et al. 1979;
Animals

Adult male newts (Notophthalmus viridescens) were obtained from Connecticut Valley Biological, Southhampton, MA. Details of animal maintenance have been described (Donaldson & Mahan, 1983).

Migration assay

For every animal used, a rectangular (1.5 mm × 3.0 mm) piece of skin was removed from the dorsal surface of each hind limb between the knee and ankle, and the pieces were placed on the bottoms of separate plastic dishes previously coated with fibronectin, type I collagen or alpha 1(1) CB3. Usually pieces of skin from seven animals were explanted into two dishes, one dish serving as a control. Explants were incubated in CEM 2000 (Scott Laboratories, Fiskeville, RI), a serum-free medium that, with the exception of insulin, contains no growth factors and is also devoid of all the known cell attachment proteins. A 5 ml sample of medium supplemented with a working concentration of 60 units ml⁻¹ of penicillin G, 60 μg ml⁻¹ of streptomycin, and 0.15 μg ml⁻¹ of amphotericin B (all from Sigma Chemical Co., St Louis, MO), was added to each dish and the dishes were incubated at 23°C for the desired period. In some experiments, monensin (sodium salt, Sigma) was added to the culture medium immediately prior to use at a final concentration of 2 μM from a 100-fold concentrated stock solution in ethanol. The same amount of ethanol was added to the control medium.

When we wished to record the amount of migration, the dishes were placed on the stage of a dissecting microscope equipped with a drawing tube with which the magnified image of the original explanted piece of skin and its surrounding halo of epidermal cells were drawn. Later, the area of each epidermal halo on the drawings was determined with a planimeter. The actual area covered by the halo was obtained by dividing the planimeter-derived figure by the appropriate magnification factor. The resulting values (in mm²) were used to compare the extent of migration in the various groups.

The extent of control migration varied considerably from day to day, even on outwardly identical prepared substrata. This is perhaps not surprising in view of the outbred nature of the species involved. The use of contralateral limb skin as the controls in each experiment minimized the impact of this variability. All ± values accompanying a mean in this report are standard errors. Stated P values were determined using Student’s t-test.

Proteins and peptides used

Human fibronectin was obtained from Collaborative Research Inc. (Lexington, MA). Bovine type I collagen was kindly provided by Dr M. Dabbous, Department of Biochemistry, University of Tennessee at Memphis. The synthetic peptides, Arg-Gly-Asp-Ser (RGDS), Arg-Gly-Glu-Ser (RGES), Lys-Gly-Asp-Ser (KGDS), Val-Gly-Ser-Glu (VGSE) and Thr-Pro-Arg-Lys (TPRK), were purchased from Peninsula Laboratories (Belmont, CA). To obtain alpha 1(1) CB3, type I collagen was extracted from rat tail tendons with 0.5 M acetic acid and purified by precipitation with 0.7 M NaCl. The alpha 1(1) chains were isolated by CM-cellulose chromatography (Piers et al. 1963) and digested with cyanogen bromide (Scott et al. 1976; Smith et al. 1976). The resulting peptides were chromatographed on CM-cellulose in formate buffer, pH 3.7 (Lichtenstein et al. 1975). The peak corresponding to CB3 was collected and lyophilized, and then further purified by chromatography on agarose A 0.5 m in 1m-Cl₂, 50 mM-Tris-HCl, pH 7.5. The purification process was monitored by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and only the central regions of the chromatographic peaks were used for experiments.

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Coating procedure

The central region of 35 mm plastic dishes received 300 μl of fibronectin, collagen or CB3 each diluted to the desired concentration in either phosphate-buffered saline (fibronectin) or 0.1 M acetic acid (collagen and CB3). The region of the dish covered by the applied solution was marked on the outside of the dish bottom and the dishes were placed at 45°C to dry. Before use all dishes were washed four times (5 min each) in distilled water and dried again at 45°C. Since there is practically no migration on uncoated plastic (Donaldson et al. 1987) counter coating with bovine serum albumin (BSA) was not necessary.

Metabolic labelling of cultured explants

Newt skin explants in dishes coated with collagen or CB3 were incubated for 18 h in 60% CEM containing 25 μCi ml⁻¹ [³⁵S]methionine (ICN Radiochemicals, Irvine, CA). The medium was removed and placed in a tube containing phenylmethylsulphonyl fluoride (PMSF, final concn 1 mM) and centrifuged at 2500 revs min⁻¹ for 10 min at 4°C to remove insoluble aggregates. Soluble proteins in the medium were collected by precipitation with 25% trichloroacetic acid for 3 h at 4°C followed by centrifugation as above. The protein was resolubilized by gentle sonication in 100 μl of 1% SDS.

Samples of proteins in the epidermal cells were prepared by lifting the epidermis from each explant after 20 min in 2 M NaBr. The pooled epidermal sheets from seven explants were sonicated in 100 μl of 1% SDS and then analysed (along with samples of the medium) by SDS-PAGE followed by gel fluorography.

Gel electrophoresis and fluorography

Samples of the medium and epidermal cells were analysed by SDS–polyacrylamide vertical slab gel electrophoresis using a 7.5% separating gel. Following electrophoresis the slab gels were stained with Coomassie Blue, treated with FluorHance (Research Products International, Mt Prospect, IL), and dried onto filter paper under vacuum. Fluorography was performed by exposing Kodak X-omat AR film to the dried gel at −70°C for 3–7 days.

Densitometry of SDS–gel fluorography

The relative amount of protein in each band on the fluorograph was determined with a Joyce Loebl Magiscan 2A image analyser (Nikon, Garden City, NY). This instrument measures the integrated density, which is a function of both the grey level and the area of a selected band. From these values the amount of protein in a given band as a percentage of the total newly synthesized protein in that sample was calculated.

Results

As we have shown earlier, when the peptide, Arg-Gly-Asp-Ser (RGDS), is included in the medium bathing explants in fibronectin-coated dishes, migration is severely inhibited at concentrations at which other peptides are without effect (Donaldson et al. 1987). Thus, in fibronectin-coated dishes migration is almost totally inhibited by 50 μg ml⁻¹ of RGDS. However, when the coating is type I collagen, this same peptide concentration is much less inhibitory. Increasing the peptide concentration 10-fold in collagen dishes produces no additional effect. (The previously published curves for these findings are included in Fig. 1 to permit comparison with the data that follow.)

From the results just described it appears that there might be different mechanisms operating when newt epidermal cells migrate across fibronectin as opposed to collagen. This notion is supported by data in Fig. 2 showing that when Arg-Gly-Glu-Ser (RGES) is added to the medium under the same conditions as when RGDS was added, in Fig. 1, the epidermal response is the reverse of what it was in RGDS. Thus, at 250 μg ml⁻¹ of RGES, migration on collagen was practically obliterated, while on fibronectin it was only mildly affected. At this same concentration, two other peptides (VGSE and KGDS), had no significant effect on collagen-mediated migration. (For VGSE, the experimental mean was 6.9 ± 0.6 mm², for the control, 6.9 ± 0.8; for KGDS, the experimental mean was 3.3 ± 0.4, for the controls, 3.9 ± 0.6, for all means, n = 6.) The weak effect of 250 μg ml⁻¹ of RGES in fibronectin-coated dishes and the lack of any effect of
Fig. 2. Effect of continuous RGES treatment on epidermal migration over substrata coated with various proteins. Except for the use of RGES, the protocol was the same as for Fig. 1. Each point represents the mean ± s.e. of at least seven explants.

This same concentration of two other peptides on collagen-mediated migration indicates that the RGES effect in collagen-coated dishes has functional significance. At 500 µg ml⁻¹, another control peptide (TPRK) severely inhibited migration on collagen, an indication that the complete inhibition of motility on fibronectin at this concentration of RGES (Fig. 2), may not be meaningful.

The collagen data above were obtained from dishes coated with 10 µg ml⁻¹ of protein. When the coating concentration of collagen was raised to 100 µg ml⁻¹ staining of the dishes with Coomassie Blue revealed a considerable increase in the amount of collagen bound. This was accompanied by a significant decrease in the inhibitory effect of 250 µg ml⁻¹ of RGES. (At 10 µg ml⁻¹ of collagen, migration in RGES-treated dishes was only 10 ± 3% of the control mean, n = 11. At 100 µg ml⁻¹ of collagen, it was 37 ± 12% of the controls, n = 7, a difference in sensitivity that was statistically significant, with P < 0.01.) The ability of substrate-bound collagen to modify the RGES response competitively in this way suggests that RGES interferes specifically with receptor-mediated recognition of active sites on the substratum.

In the experiments discussed above, all peptides were present throughout the incubation period and were therefore present before any epidermal cells moved off the explant. Since migration on collagen could be blocked more completely by RGES than RGDS, we wondered if the sensitivity to RGDS might be nothing more than an interference with an RGDS-sensitive first step in migration, such as the utilization of explant-derived fibronectin that might have to be crossed before the cells could interact directly with the collagen coating. To examine this question, we allowed skin explants to incubate in collagen-coated dishes until a halo of migrating cells could be seen around each explant. RGDS was then added to the medium. Fig. 3 shows that while the epidermal sheet continued to spread after addition of the peptide, a significant reduction in its rate of expansion was evident after only an hour. This indicates that RGDS does not simply interfere with an RGDS-sensitive first step, but that the spreading sheet is sensitive to this peptide even after migration is well underway.

Both fibronectin and collagen contain RGD sequences (Pierschbacher et al. 1985; Fietzek & Kuhn, 1976). To determine if the epidermal response to RGDS and RGES in collagen-coated dishes was related to the RGD sequence in collagen we examined the effect of these peptides on epidermal migration in dishes coated with CB3, a fragment of the alpha 1 chain of type I collagen that does not contain this sequence (Fietzek & Kuhn, 1976). At low protein concentrations intact collagen was a better migration substrate than CB3 (Fig. 4). At 10 µg ml⁻¹ they were approximately equal. Exposure of explants in CB3-coated dishes (10 µg ml⁻¹) to 50 µg ml⁻¹ of soluble RGDS throughout the incubation period severely inhibited migration (Fig. 5). Four other peptides of similar molecular weight (TPRK, VGSE, KGDS and RGES) at molar concentrations equivalent to 50 µg ml⁻¹ of RGDS, had no effect. The RGDS influence on CB3-mediated
Fig. 4. Epidermal cell migration on collagen and CB3. Skin explants were incubated for 18 h in plastic dishes coated with the indicated protein concentrations. Each point represents the mean ± S.E. of at least six explants.

Fig. 5. Effect of continuously treated peptide substrates on epidermal cell migration over CB3-coated substrata. Skin explants were placed in plastic dishes previously coated with CB3 (10 μg ml⁻¹). Medium alone, medium containing RGDS (50 μg ml⁻¹) or medium containing one of the other indicated peptides at a molarity equal to 50 μg ml⁻¹ of RGDS, was added; 18 h later the amount of migration was determined in each group. Each bar represents the mean ± S.E. of at least eight explants. *Significantly different from its control, P < 0.05.

Fig. 6. Effect of monensin on epidermal migration over substrata coated with the indicated proteins. Protocol essentially the same as for Fig. 1, except that the experimental medium contained 2 μM-monensin. Each bar represents the mean ± S.E. of at least 10 explants. *Significantly different from its control, P < 0.02.

Epidermal cell migration is, therefore, a specific feature of this particular peptide.

The response of explants in CB3-coated dishes to higher concentrations of RGDS was remarkably similar to the response in collagen-coated dishes (Fig. 1). That is, there seemed to be a small amount of migration in both that was RGDS-insensitive. The effect of higher concentrations of RGES on CB3-mediated migration likewise resembled its effect on collagen (Fig. 2).

Monensin is a monovalent ionophore that has been shown to inhibit secretion and endocytosis in a variety of cell types (Ledger & Tanzer, 1984). When 2 μM-monensin was added to the medium bathing skin explants, there was a small, albeit statistically insignificant, effect in dishes coated with fibronectin or collagen. In CB3-coated dishes, monensin was two to four times more inhibitory than in dishes coated with the other two substrata, an effect that was statistically significant (Fig. 6).

Since mammalian keratinocytes are known to secrete fibronectin after they become culture-adapted and fibronectin secretion is inhibitable by monensin in some cell types (Uchida et al. 1979), we tried to determine if epidermal cells migrating on CB3 were secreting fibronectin. To investigate this possibility, epidermal cells were allowed to migrate on dishes coated with CB3 in the presence of 35S-labelled methionine. At the end of the experiment, the cells and the medium were assayed for labelled proteins using gel electrophoresis and fluorography. Fig. 7 shows the results of a typical experiment. In fluorograms of cell extract, there were numerous labelled bands, including one with the approximate molecular weight of fibronectin. In the medium, however, there was no evidence of labelled proteins.

Using this same approach, Clark et al. (1985) showed that cultured human keratinocytes not only synthesize fibronectin, but secrete most of it into the medium. They found that fibronectin made up approximately 0.7% of the newly synthesized protein that was retained in the cells.
Fig. 7. [35S]methionine-labelled proteins in the cell extract and medium of cells migrating on CB3. Skin explants were placed in plastic dishes coated with CB3 (10 μg ml⁻¹). After 18 h in medium containing [35S]methionine, proteins in the medium were precipitated with trichloroacetic acid. This precipitate and the pooled epidermis from all explants were each solubilized in 100 μl of SDS. Samples were then analysed by electrophoresis and fluorography. Lane 1 represents the cell extract; lane 2, an equal volume of solubilized medium precipitate. The arrow indicates the position in an adjacent well, of human plasma fibronectin, which we have found has the same mobility as newt plasma fibronectin.

much was secreted into the medium as was retained. Our densitometer scans of gels containing cell extract showed that we could detect bands that represented as little as 1-2% of the total protein in the gel. The putative fibronectin band represented 2-4% of the total. Even when the precipitated proteins from the medium were loaded onto gels at a concentration twice that of an optimal amount of cell extract, no fibronectin was found. While this does not rule out the possibility that newt epidermal cells secrete fibronectin when they migrate across CB3, it shows that if they do, the amount is much less than for human keratinocytes in vitro.

Discussion

The capacity of many cell types to adhere to collagen is mediated by fibronectin (see review by Kleinman et al. 1981). Other cell types bind collagen directly (see list, Dedhar et al. 1987). Earlier we found that CB3 and CB8 (fragments of the alpha 1 chain of type I collagen that do not contain the fibronectin binding site) support newt epidermal cell migration about as well as CB7, the fragment containing the fibronectin binding site (Donaldson et al. 1982). This suggested that newt epidermal migration over collagen does not involve fibronectin. That conclusion is supported by data from the present study showing distinctly different relative effects of RGDS and RGES, depending on the substratum. In the presence of RGDS, the fibroblast attachment sequence in fibronectin, epidermal cell migration is more effectively blocked on fibronectin-coated substrata than on those coated with type I collagen. With RGES, the reverse is true. We interpret this to mean that fibronectin and collagen-mediated migration involve different mechanisms and that keratinocyte-derived fibronectin is not involved in migration over collagen. O'Keefe et al. (1985) found similarly that attachment of human keratinocytes to type IV collagen in short-term adhesion assays does not require the participation of fibronectin. One possible explanation for our peptide results would be the existence of two classes of ECM receptors on newt epidermal cells, one for fibronectin, a different one for collagen.

There are precedents for the existence of multiple receptor classes in other cell types. Hepatocytes, for example, have separate receptors for collagen, fibronectin and asialoglycoproteins (Rubin et al. 1978, 1979, 1981, 1984). Recently, distinctly different receptors for fibronectin and collagen have been isolated from human osteosarcoma cells (Dedhar et al. 1987). When incorporated into liposomes, both receptor types on osteosarcoma cells bind to their respective substrata, but not others. Despite this specificity, both receptors appear to recognize the RGD sequence, their specificity presumably residing in their ability to recognize different flanking sequences in each protein (Akiyama et al. 1985; Pytela et al. 1986). The recognition of RGDT but not RGES by the osteosarcoma collagen receptor (Dedhar et al. 1987) makes it clearly different in its specificity from the putative collagen receptors in newt epidermal cells, which do recognize RGES. To our knowledge the only other receptor that recognizes RGES is the receptor in mouse melanoma cells that mediates attachment of these cells to the REDV sequence in the type III connecting segment in fibronectin (Humphries et al. 1986). In addition to RGES, the melanoma receptors recognize RGDS and, of course, REDV. This cross-reactivity with several sequences makes the melanoma receptor less stringent than the fibroblast fibronectin receptor (Humphries et al. 1986).

The cross-reactivity of fibronectin receptors on mouse melanoma cells with several different peptide sequences makes it clear that one cannot determine, simply from peptide inhibition studies, the amino acid sequence serving as the attachment site in a given substrate. It also raises the possibility that there is only one class of ECM receptor in newt epidermal cells, a receptor that interacts with different sequences in
fibronectin and collagen and does so with different affinities. The more potent effect of RGES on collagen-mediated migration than on fibronectin may be a reflection of a relatively weaker affinity of the collagen active site(s) for the same ECM receptor that mediates migration over fibronectin. This same receptor might be responsible for migration over fibronogen and vitronectin as well, each of which is also sensitive to soluble RGDS (Donaldson et al. 1987). Should newt epidermal cells prove to have a single receptor or receptor complex that mediates migration over a variety of ECM proteins they would resemble platelets, where the IIb/IIIa membrane complex mediates the RGDS-sensitive attachment to fibronogen, fibronectin and vitronectin (Pytela et al. 1986).

At present we are unable to pinpoint the exact location of the active site(s) in either fibronectin or collagen. In fibronectin, migration-promoting activity is limited to domain III and/or IV (Donaldson et al. 1985). In collagen, there seem to be multiple active sites scattered along the molecule (Donaldson et al. 1982). In this respect, newt epidermal cells resemble dissociated hepatocytes, which bind efficiently to all CB peptides of alpha 1(I) (Rubin et al. 1981). Despite the fact that fibronectin, collagen, fibronogen and vitronectin, the four ECM proteins that support epidermal migration, all contain the RGD sequence (Pierschbacher et al. 1985; Fietzek & Kuhn, 1976; Doolittle et al. 1979; Suzuki et al. 1985), it is becoming clear that migration can occur even when this amino acid combination is not available in provided substrata. For example, migration readily occurs on fibronectin even when the RGDS attachment site is blocked by a monoclonal antibody that inhibits fibroblast attachment to fibronectin (Donaldson et al. 1985). The ability of newt epidermal sheets to spread as well over the RGD-deficient CB3 fragment of alpha 1(I) as over intact collagen is further support for this point.

We do not know how this is accomplished. Some epidermal receptors may simply be able to recognize certain amino acid sequences in collagen other than RGD. Alternatively, migrating newt keratinocytes may be able to modify RGD-deficient substrata by secreting an RGD-containing protein such as fibronectin. Secretion of fibronectin has often been reported in mammalian keratinocytes that have spent some time in culture (Alitalo et al. 1982; Clark et al. 1985; Gibson et al. 1983a; O’Keefe et al. 1984, 1985; Kubo et al. 1987). It is not yet clear though that newt keratinocytes migrating from explants have this capacity. In this regard, we found that migration over CB3 was more sensitive to monensin than was migration over either collagen or fibronectin. Since monensin has been shown to inhibit secretion of fibronectin (as well as other proteins) in some cell types (Ledger & Tanzer, 1984; Uchida et al. 1979; Virtanen et al. 1982) these data might seem to support the notion that migration on CB3 requires fibronectin secretion by the migrating cells. However, when we assayed the medium for secreted fibronectin, using the same approach as Clark et al. (1985) used when they demonstrated fibronectin secretion in human keratinocytes, we found none.

Since it is conceivable that an exquisitely small amount of fibronectin might be sufficient to promote migration if it were deposited in precisely the right locations, the possibility that keratinocyte-derived fibronectin might be involved in CB3-mediated migration cannot be excluded by these data alone. The unequivocal demonstration of such a mechanism would have extremely interesting implications, since there are many proteins that do not support migration (Donaldson & Mahan, 1983, 1984a,b; Atnip et al. 1987). Thus, if epidermal cells migrate on CB3 through the secretion of fibronectin, this collagen fragment may provide the migrating cell with some signal that is missing from proteins that do not permit migration.

Alternatively, our inability to detect fibronectin secretion from keratinocytes migrating on CB3 might simply mean that there is none there. This position is supported by our peptide experiments in which cells migrating on CB3 responded to test peptides in a manner more like the response in collagen-coated dishes than in those coated with fibronectin. If, as we are suggesting, migration on CB3 and collagen is independent of keratinocyte-derived fibronectin, why is CB3-mediated migration more sensitive to monensin than migration on collagen? The answer may lie in the ability of monensin to inhibit receptor recycling (Niven & Aplin, 1985). Thus, our monensin data may reflect a difference in the kinetics of receptor uptake and reutilization on the two substrata.

Taking all the above considerations into account, we believe that the dissimilar behaviour of cells migrating on fibronectin and collagen in the presence of RGDS and RGES indicates that there is a fundamental difference in the migration mechanisms on these two proteins. Whether the different behaviour on fibronectin and collagen is caused by different receptors or active sites with different affinities for one class of ECM receptor remains to be determined.

This work was supported by NIH grant AR 27940 awarded to D. J. D.

References


(Received 25 August 1987 – Accepted, in revised form, 15 February 1988)