Fibrinogen-mediated epidermal cell migration: structural correlates for fibrinogen function

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Summary

Previously we showed that epidermal cells are able to use fibrinogen (FGN) as a migration substratum during wound closure. The goal of the present study was to determine the structural features of FGN that allow this migration. Pieces of glass coated with native, fragmented, or other modified forms of FGN were implanted into full-thickness skin wounds of adult newts such that migrating epidermal cells would encounter the implant. In this system, a coating of FGN allowed considerably more migration than a coating of BSA. At high concentrations, heat-denatured FGN supported as much migration as the same amount of intact FGN. Fraction I-9, a circulating form of FGN missing a 20–30K (K = 10^3 Mr) carboxy-terminal segment of the Aa chain, was no less effective than intact FGN. Comparison of the isolated Di and E fragments of FGN showed migration only on Di, but never to the extent seen on intact FGN containing the same amount of D^. Plasmin digestion of D t in the presence of EDTA, a process which produces D 3, a fragment differing from D x by the loss of the carboxy-terminal 109 amino acids of the y chain, caused a significant loss of activity in the D fragment. Migration was good on implants coated with relatively high concentrations of purified Aa chains but y chains were inactive. Migration over intact FGN was almost totally blocked by 230 μM-Arg-Gly-Asp-Ser (RGDS), a peptide known to interact with integrin-type receptors. This same concentration of γ'400-411, a peptide modeled after the carboxy-terminal 12 amino acids comprising the platelet receptor recognition domain in the γ chain, had no effect. These results are consistent with the idea that newt epidermal cell migration over FGN-coated glass involves integrin-type receptors capable of interacting with the Aa and perhaps the γ chains of FGN. The ability of fraction I-9 to support as much migration as intact FGN shows that the RGDS sequence in the carboxy-terminal segment of the Aa chain (\(\alpha_{572-575}\)) is not required for full activity. The contribution of the Arg-Gly-Asp-Phe (RGDF) sequence in the amino-terminal segment of the Aa chain (\(\alpha_{95-98}\)) to I-9 activity remains to be determined. Absence of an effect on migration by the γ'400-411 peptide suggests that epidermal receptors do not recognize this domain of the γ chain. Thus, the ability of FGN to support epidermal cell migration appears to reside primarily in its Aa chain, which may function in concert with a site in the γ chain that does not coincide with the site recognized by platelets.

Key words: epidermal migration, extracellular matrix, fibrinogen.

Introduction

Fibrinogen (FGN), a clottable adhesive protein, is composed of three pairs of non-identical chains (Aa, Bβ, γ) linked by a series of disulfide bonds and arranged into three main structural units: a central E domain flanked by identical D domains. Appropriate digestion with plasmin produces a number of major fragments, which include one E fragment (representing the E domain), two D1 fragments (representing the D domains) and two fragments making up the carboxy-terminal ends of the Aa chains (representing the so-called polar appendages; Doolittle, 1984).

Fibrinogen is a major component of the provisional extracellular matrix in wounds (Clark et al. 1982). The fact that individuals with congenital FGN deficiencies show impaired wound healing (Bloom, 1981) suggests that wound-associated deposition of this protein plays an important part in the repair process beyond its role in restoration of vascular integrity. In addition to the sites involved in self assembly into fibrin, FGN is able to bind to platelets (reviewed by Hawiger, 1987), monocytes
(Altieri et al. 1988), macrophages (Sherman, 1983), fibroblasts (Dejana et al. 1984) and endothelial cells (Dejana et al. 1985; Cheresh, 1987). Previously we have shown that newt epidermal cells will migrate over solid substrata coated with FGN (Donaldson & Mahan, 1983; Donaldson et al. 1987). In the present study we have used various fragments and other modified forms of FGN to map the site(s) in this protein which are responsible for its ability to support migration. To our knowledge this and our previous studies (Donaldson & Mahan, 1983; Donaldson et al. 1987) are the first to present evidence that FGN might play an important role in epidermal wound healing, and the first to document the capacity of normal cells in situ to utilize FGN as a migration substrate.

Materials and methods

Materials

Two preparations of intact FGN were used with identical results: (1) Kabi human FGN (Helena Laboratories, Beaumont, TX) that was further purified by gelatin-affinity chromatography to remove contaminating fibronectin and (2) peak 1 FGN that contained no detectable fibronectin, factor XIII or plasminogen, prepared as previously described (Finlayson & Mosesson, 1963; Amrani et al. 1988). FGN purity was verified by SDS–PAGE (Laemmli, 1970) followed by staining with Coomassie blue.

Plasma fraction 1–9 FGN, which differs from intact FGN in that 1–9 is missing a 20–30K piece from the carboxy terminus of the z chain, was prepared and characterized as described previously (Galanakis et al. 1978; Amrani et al. 1988) and was also fibronectin-free.

Denatured FGN was prepared by heating Kabi or peak 1 FGN, diluted in PBS, to the concentrations indicated in Fig. 1, in a water bath at 65–67°C for 30 min.

Fibronectin fragments D1 and E were prepared and characterized as described by Kloczewiak et al. (1987). Fibrinogen fragment D3 was obtained by a 5 h plasmin digestion of D1 at 37°C in the presence of 5 mM-EDTA. Enzymatic action was inhibited by the addition of aprotonin (Sigma). D3 was purified by G25 column chromatography. SDS–PAGE was used to monitor the conversion of D1 to D3.

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Lyophilized, reduced and carboxymethylated FGN chains Aα and γα (the active form of γα rather than γγ), were solubilized in 5% acetic acid and stored at 4°C as stock solutions (2 mg ml⁻¹).

Immuno globulin fractions of goat antisera to the D and E fragments of human FGN were purchased from Miles Laboratories (Naperville, IL, USA). An immunoglobulin fraction of normal goat serum was prepared by ammonium sulphate precipitation of whole goat serum obtained from Miles.

The synthetic peptides, Arg-Gly-Asp-Ser (RGDS), and His-His-Leu-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val (Y404-R), were purchased from Peninsula Laboratories (Belmont, CA).

Animals

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

Coating glass implants and culture dishes

All glass implants (1.2x2.5 mm) were incubated in 10 μl of test protein and allowed to dry overnight. Implants treated with heat-denatured FGN were dried at 65–69°C. All other implants were dried at 23°C. In culture dishes (Falcon 2001) 15 μl aliquots of test proteins were applied in 50 mm² circles (seven circles per dish) and allowed to dry overnight at 23°C. Immediately before use, all glass implants and culture dishes were washed four times with distilled H2O. Intact FGN, fraction 1–9, fragments D1, D3 and E, were all diluted in PBS. The Aα and γα chains were diluted in 5% acetic acid.

Migration on implanted substrata

This assay has been described in detail elsewhere (Donaldson et al. 1987; Donaldson & Mahan, 1983), but briefly, after a full thickness piece of skin was removed from the dorsal surface of each hind limb, the limb was amputated and placed in Holtfreter’s solution (HS). Subsequently, a piece of glass coated with the protein to be tested was inserted partway under the skin so that epidermal cells migrating from the edge of the wound would encounter the protein-coated glass. In most experiments implanted limbs were incubated at 23°C for 9 h in HS and then fixed in 10% formalin. When the effects of synthetic peptides on migration were studied, limbs were incubated in 60% CEM 2000, a serum-free culture medium (Scott Laboratories, Fiskeville, RI, USA) to allow peptide effects on pH to be monitored. In these experiments, the incubation time was extended to 16 h. In all experiments, drawings of the implant were made with the aid of a drawing tube fitted to a dissecting microscope. Distance migrated was determined from the drawings by measuring planimetrically the area of a standardized region of the implant covered by epidermal cells. This value was then divided by the width of the region measured and adjusted for magnification. Differences were tested for significance with Student’s t-test.

Migration from skin explants

This assay has also been described previously (Mahan & Donaldson, 1988), but briefly, pieces of skin were removed from the hind limbs of newts and explanted onto the bottom of tissue culture dishes coated with the protein to be tested. Each dish received seven pieces of skin (one from each of seven animals) and 5 ml of 60% CEM 2000. After incubation for 18 h at 23°C the area covered by epidermal cells that migrated from the explant was determined planimetrically.

Binding of FGN, D1, and E to glass implants (ELISA assays)

Pieces of glass were coated with FGN, fragment D1, and E as in the migration experiments. Glass coated with bovine collagen was used as a negative control. The coated glass was treated with a primary antibody solution (either anti-D or anti-E goat serum gamma fraction) diluted 1:39 in PBS–BSA–Tween for 2 h at room temp (anti-E) or overnight at 4°C (anti-D). Unbound antibody was removed by five washes with NaCl–TWEEN. The pieces of glass were then incubated in affinity-purified anti-goat IgG–peroxidase conjugate (Sigma) diluted in PBS–BSA–TWEEN to 1:39 for 2 h at room temperature. Unbound second antibody was removed by five NaCl–TWEEN washes. After incubating the glass in an O-phenylenediamine–hydrogen peroxide substratum for 15 min, the reaction was stopped with 2.5 M-sulfuric acid and the absorbance was measured at 492 nm.

Results

Implants coated with native FGN supported consider-
Fig. 1. Ability of native FGN, heat-denatured FGN, and the 1-9* fragment of FGN to support epidermal cell migration. Pieces of coverslip glass coated with the indicated proteins were implanted into skin wounds on the amputated hind limbs of adult newts and incubated for 9h in Holtfreter’s solution. The distance migrated by the epidermal cells was then determined (as described in Materials and methods) and these values were used to compare the relative effectiveness of each coating material. Each point shows the mean ± S.E. for at least eight limbs. In this and all other figures where it appears, the shaded area represents the background value (mean ± S.E.) for distance migrated on glass coated with 1 mg ml⁻¹ of BSA. At 10⁻⁶M, the mean for denatured FGN is not statistically different from the mean for native FGN (t-test, P = 0.34); at this same concentration, the 1-9 fragment supports slightly more migration than native FGN (P = 0.02). *1-9 is a circulating form of FGN that is missing a 20-30K piece from the carboxy terminus of the Aα chain. O O, 1-9; • • , FGN; • •, denatured FGN.  

ably more migration than those coated with BSA (Fig. 1). To determine if this ability of FGN to support migration is dependent on its native conformation, we also tested heat-denatured FGN and found that at higher concentrations, denatured FGN was as effective as the native form. At lower concentrations however, native FGN was better (Fig. 1).

Based on visual inspection of coated glass stained with Coomassie Blue, denatured FGN appeared to bind to glass as well as native FGN at all concentrations tested. The coating with denatured FGN, however, was more particulate and not as homogeneous as native FGN. Decreased migration on denatured FGN at lower concentrations may therefore be more a reflection of its particulate nature than any actual loss in its migration-supporting capacity. These data suggest that the native conformation of FGN is not essential for it to support epidermal migration.

Since the Arg-Gly-Asp (RGD) sequence in a number of proteins has been implicated in their ability to bind to various cell types, we were interested in learning if the RGD sequences in FGN were involved in epidermal cell migration. Both RGD sequences in FGN are in the Aα chain, one at residues 95–97, the other at residues 572–574, which occurs in the carboxy-terminal polar appendage (Doolittle, 1984; Henschen et al. 1983). To examine the influence of the RGD sequence in the polar appendage, we tested 1-9 FGN, a circulating form missing 20–30K from the carboxyl end of the Aα chain, including the carboxyl RGD sequence. It is evident from Fig. 1 that 1-9 was as good a substratum as intact FGN. In fact, at high concentrations, 1-9 was significantly better than the intact molecule. The distal RGD sequence in FGN is therefore not required if the proximal part of the Aα chain and the other two chains are also present.

Experiments with D₁ and E fragments
In addition to cleaving the polar appendages from the rest of the FGN molecule, plasmin digestion in the presence of calcium produces two other major fragments, which represent the central and terminal domains. The first is called the E fragment and the second, the D₁ fragment, each of which contains portions of the Aα, Bβ and γ chains. Neither fragment contains an RGD sequence (Thorsen et al. 1986). The D₁ fragment possesses a binding site for platelets near the carboxy terminus of its γ chain (Marguerie et al. 1982). When we tested molar equivalent concentrations of the D₁ and E fragments as migration substrata we found activity only in D₁ (Fig. 2).

In no case however, did D₁ support as much migration as a molar equivalent amount of intact FGN (Fig. 2).

Binding data from ELISA assays using antisera against the D and E fragments showed that glass implants exposed to a given molar concentration of either fragment bound approximately the same amount of that fragment as when a molar equivalent solution of FGN was used. Thus from Fig. 3A, it is clear that the lack of activity on implants coated with fragment E is not because E failed to...
bind. Nor can the inability of the D₁ fragment to produce full activity be explained by any quantitative difference in its binding compared to intact FGN (Fig. 3B).

Assuming that full activity might be due to a cooperative interaction between the D₁ and E fragments, we tested implants coated with a mixture of D₁ and E. Despite successfully producing coatings containing roughly as much of each fragment as in coatings of intact FGN (Fig. 3A,B) migration was no better than when D₁ alone was used as a substratum (Fig. 4).

**Effect of antibodies against D and E epitopes**
When epidermal cells were allowed to migrate over intact FGN in the presence of antibodies against the D and E fragments, both inhibited migration, with anti-D being more effective than anti-E (Fig. 5). The inhibitory effect of anti-D was consistent with the ability of the D₁ fragment to support migration. The inhibition produced by anti-E was somewhat surprising. Since Fig. 3A shows that the anti-E used was quite specific for fragment E, the inhibitory effect of anti-E cannot be explained by cross reactivity with fragment D. It may be that in intact FGN the active site(s) in the D domain lies close enough to the E domain to be sterically blocked by antibodies binding to E epitopes.

**Conversion of D₁ to D₂**
The interaction of platelets with fragment D₁ can be prevented by degradation of this fragment with plasmin in the absence of Ca²⁺, a treatment that causes loss of the
Fig. 6. Ability of the D$_1$ and D$_2$ fragments of FGN to support epidermal cell migration. Protocol same as in Fig. 1. D$_3$ was produced by digesting D$_1$ with plasmin in the presence of EDTA. D$_3$ differs from D$_1$ in that it is missing the carboxy-terminal portion of the γ chain, a region which includes the amino acid residues involved in platelet binding. Each bar shows the mean ± S.E. for ten limbs. At both concentrations shown, migration was significantly better on D$_1$ than on the corresponding amount of D$_3$ (P<0.01 and 0.05, respectively).

Fig. 7. Ability of the Aα and γ chains of FGN to support epidermal cell migration. Protocol same as in Fig. 1. Each point shows the mean ± S.E. for at least nine limbs.

**Experiments with Aα and γ chains**

When we tested isolated Aα and γA chains in our migration assay (purified β chain was not available), we obtained considerable activity on Aα but none on γA (Fig. 7). Again, Coomassie Blue staining showed that the γA chain bound to glass at least as well as the Aα chain. Comparison of Figs 1 and 7 shows that on a molar basis, it took approximately 20 times more Aα chain than intact FGN to produce 0.4 mm of migration, an amount near the maximum produced by any coating. Comparison of Coomassie Blue-stained coatings of the Aα chain and intact FGN showed that the Aα chain was perhaps slightly less efficient in its binding to glass, such that it took approximately twice as much Aα chain protein to achieve the same staining intensity as intact FGN. This difference in binding is not enough to account for the difference in migration on the two substrata at molar equivalent concentrations. Implication of the γ chain by the D$_3$ experiments and the inactivity of the γA chain by itself as a substratum led us to ask if the Aα chain in intact FGN functions in conjunction with the γ chain. To test this, we coated glass with a mixture of Aα and γA chains at a final concentration of 10$^{-7}$ M for each chain, a concentration somewhat below the point where the Aα chain becomes distinctly active (see Fig. 7). This had no enhancing effect on Aα chain activity (data not shown).

**Effects of synthetic peptides**

Migration on intact FGN was blocked almost completely by RGDS, a synthetic peptide known to interact with integrin-type receptors on a variety of cell types (Fig. 8). The IC$_{50}$ for RGDS (the concentration at which migration is inhibited by 50%) was approximately 60 μM. In contrast, γ$_{400-411}$, a peptide modeled after the platelet binding site located in the carboxy-terminal 12 amino acids of the γ chain, had no effect even when used at approximately four times the IC$_{50}$ for RGDS (Fig. 8).

**Discussion**

Evidence presented here indicates that newt epidermal cells interact with FGN primarily through its Aα chains. Thus, purified Aα chains were able to support as much migration as intact FGN if the chains were coated at higher concentrations than intact FGN. This requirement for higher concentrations of the active site(s) in isolated Aα chains may mean that some favorable conformation of the Aα chain has been lost during its preparation. Alternatively, normal function of the Aα chain may require the cooperation of a site in one of the other two chains. The decrease in activity upon conversion of the D$_1$ fragment to D$_3$, a change which involves loss of 109 amino acids from the carboxy-terminal segment of the γ chain, could mean that this putative other site is in the γ chain. However, when the γ chain alone was tested as a substratum it was found to be inactive. This
observation is consistent with the notion that the γ site may not be able to support migration by itself, but acts to maximize the efficiency of the primary site in the ζ chain. When we tried to test this hypothesis by simply adding γ chain to a suboptimal amount of ζ chain prior to coating implants, migration on the resulting coating was similar to that on implants coated with ζ chain alone. Since the conformation of γ chains bound to glass may not be suitable for reactivity with epidermal cells, we do not consider this negative result to be conclusive.

Recently, a great deal of evidence has emerged indicating that many cell types interact with various components of the extracellular matrix through receptors capable of recognizing the amino acid sequence Arg-Gly-Asp (RGD), a sequence present in nearly all extracellular matrix proteins (reviewed by Ruoslahti & Pierschbacher, 1987). Based on the nearly total blockage of FGN-mediated migration, we observed in the presence of a synthetic RGDS peptide, it might appear that the most likely locations for migration-supporting sites in the ζ chain of FGN would be its two RGD sequences, one of which encompasses residues 95–97, and the other, residues 572–574 (Henschens et al. 1983).

The ability of I-9, a catabolic fragment of FGN missing the distal RGD sequence, to support as much epidermal migration as a molar equivalent amount of intact FGN clearly shows that this sequence (α572–574) is not necessary for full activity. The role of the proximal RGD sequence in epidermal migration remains to be established since this sequence (α95–97) lies between the residues comprising the D1 and E fragments (Thorson et al. 1986) and therefore was not tested in the D and E experiments. Speculation on the possible role of RGD sequences in the ζ chain is complicated by the fact that the affinity of RGD-sensitive receptors for RGD sequences in substratum proteins is greatly influenced by the amino acids flanking the RGD sequence, to the point that some RGD sequences may be completely unattractive to a given class of RGD-sensitive receptors (Akiyama et al. 1985; Pytela et al. 1986). Moreover, receptors capable of recognizing the RGD sequence can recognize other sequences as well. Thus, soluble RGDS can also inhibit migration of newt epidermal cells on CB3, a fragment of collagen which contains no RGD sequences (Donaldson et al. 1988).

The ζ and γ chains have also been implicated in binding of FGN to platelets (Hawiger et al. 1982). In platelets, the relative importance of the two chains is the reverse of what we have found for epidermal cells. Not only could isolated multimers of γ chains cause platelet aggregation, but they were approximately five times more effective on a molar basis than isolated ζ chain multimers (Hawiger et al. 1982). Our demonstration that isolated ζ chains will support migration is direct evidence for the existence of epidermal active sites in this part of the FGN molecule. Our evidence for an active site in the γ chain, however, is indirect and consists only of the loss of migration-supporting activity when a carboxy-terminal segment of the γ chain in D1 is removed by plasmin to form the fragment known as D3. Loss of the ability of D1 to inhibit FGN binding to platelets upon conversion to D3 was among the first pieces of evidence indicating that there was a platelet binding site somewhere in the carboxy end of the FGN γ chain (Marguerie et al. 1982). The existence of such a site was firmly established when it was shown that a 27 residue carboxy-terminal cyanogen bromide fragment of FGN could inhibit FGN binding to platelets (Kloczewiak et al. 1982). Through the use of synthetic peptides modeled after various regions of the carboxy-terminal end of the γ chain as inhibitors of FGN binding, the minimal completely active sequence was subsequently localized to γ400–411, the carboxy-terminal 12 amino acids (Kloczewiak et al. 1984). In platelets, γ400–411 and RGDS inhibit FGN binding with a similar IC50 (Plow et al. 1985, 1987; Kloczewiak et al. 1984). When we added soluble γ400–411 to the medium of limbs bearing FGN-coated implants, we found that it had no effect on migration, even at a molar concentration nearly four times the IC50 for RGDS. This suggests that migration over intact FGN does not require the participation of receptors capable of recognizing the platelet binding site located at the carboxy end of the γ chain. If the γ chain is truly involved when epidermal cells migrate over FGN, this in turn could mean that the epidermal active site in the γ chain does not coincide with the platelet binding site.

In studies where the interaction of the D and E fragments of FGN with platelets has been examined, the D fragment is usually more effective than E (Marguerie et al. 1982; Thorson et al. 1986). In the present study, we found a similar relationship for epidermal cells in which migration occurred on the D1, but not the E fragment. In
addition to platelets, FGN binding to monocytes (Altieri et al. 1988), macrophages (Sherman, 1983), fibroblasts (Dejana et al. 1984) and endothelial cells (Dejana et al. 1985) has also been reported. Unlike the situation for platelets and newt epidermal cells, there seems to be a major binding site for fibroblasts (Dejana et al. 1984) and endothelial cells (Dejana et al. 1985) in the E domain with little or no affinity of the D domain for these cells (but see also Dang et al. 1985). Thus, different cell types recognize different regions of the FGN molecule.

The FGN receptors on both platelets (Pytela et al. 1986) and endothelial cells (Cheresh, 1987) can be isolated by affinity chromatography on an RGD-containing matrix. These receptors, therefore, belong to the integrin superfamily (Ruoslahti & Pierschbacher, 1987; Hynes, 1987). Similarly, FGN receptor function on monocytes resides in the integrin receptor Mac-1 (Altieri et al. 1988), which probably also serves as the FGN receptor in macrophages. In the absence of evidence to the contrary, cell surface-associated FN has often been suspected as the agent mediating binding of proteins to cells when the bound protein possesses FN binding sites. While the FGN binding site in fibroblasts has not been fully characterized, the ability of FGN fragment E, which lacks the FN binding site, to inhibit FGN binding (Dejana et al. 1984) argues for the existence of a binding mechanism in these cells that does not involve cell surface-associated FN. Moreover, the interaction with FGN has many features typical of ligand–receptor recognition (Dejana et al. 1984). Our observation that I-9, another FGN fragment lacking FN binding sites, is fully active as an epidermal migration substratum suggests that here too, FGN recognition is FN-independent. From this and the other data we have presented, it appears that soluble RGD S inhibits FGN-mediated epidermal migration by blocking the interaction of an integrin-type receptor on epidermal cells with specific sites in the Aa and perhaps the y chains of the FGN molecule. The exact location of the active sites in FGN and characterization of the receptors involved are topics for further investigation.

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References


