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Substratum-dependent stimulation of fibroblast migration by the gelatin-binding domain of fibronectin

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SUMMARY

Nanomolar concentrations of native fibronectin and its RGDS-containing cell-binding domain have previously been reported to stimulate fibroblast migration in the transmembrane (or 'Boyden chamber') assay; in contrast, the gelatin-binding domain (GBD) of fibronectin has consistently been reported to be devoid of migration-stimulating activity in this assay. We have examined the effects of fibronectin and several of its purified functional domains on the migration of human skin fibroblasts in what is presumably a more physiologically relevant assay involving the movement of cells into a 3-D matrix of native type I collagen fibrils. We report that: (a) femtomolar concentrations of GBD stimulate fibroblast migration into such collagen matrices; and (b) fibronectin, as well as peptides containing all other of its functional domains, do not exhibit migration-stimulating activity when tested in the femtomolar to nanomolar concentration range (i.e. 0.1 pg/ml to 1 μg/ml). The correct assignment of migration-stimulating activity to GBD, rather than to a contaminant, was confirmed by: (a) the use of several fibronectin and GBD purification protocols; (b) the neutralization of GBD migration-stimulating activity by monoclonal antibodies directed against epitopes present in this domain; (c) the time-dependent generation of migration-stimulating activity by the proteolytic degradation of native fibronectin; and (d) obtaining an identical dose-response curve with a genetically engineered GBD peptide. The cryptic migration-stimulating activity of GBD was not affected by the presence of serum or native fibronectin, but was inhibited by TGF-β1. Parallel experiments using the transmembrane assay confirmed that GBD was devoid of migration-stimulating activity in this assay when membranes coated with gelatin were used, but revealed that significant stimulation of migration was achieved with membranes coated with native type I collagen. Cells pre-incubated with GBD for 24 hours whilst growing on plastic tissue culture dishes and then plated onto native collagen matrices in the absence of further GBD also displayed an elevated migration compared to controls. Taken together, these observations suggest that: (a) the interaction of GBD with a putative cell surface receptor (and not the collagen substratum) initiates a persistent alteration in cell phenotype which is manifest by an increase in migratory activity when these cells are cultured on a native collagen substratum; and (b) GBD may play a hitherto unrecognised role in the control of cell migration in response to the local release of proteases during pathological processes, such as tumour invasion and wound repair.

Key words: Fibronectin, Cell motility, Collagen matrix, Wound healing

INTRODUCTION

The initiation, directionality and cessation of cell motility are regulated by the complex interplay of various matrix macromolecules and cytokines (Rosen and Goldberg, 1989; Stoker and Gheradi, 1991; Schor, 1994). The temporal and spatial distribution of the adhesive glycoprotein fibronectin within the extracellular matrix during embryonic development (Thiery et al., 1985), wound healing (Clark, 1990; Grinnell et al., 1981) and tumour invasion (Lacovara et al., 1984) is consistent with it contributing to the control of cell migration during these physiological and pathological processes. The effects of fibronectin on cell migration in vitro have commonly been investigated in variations of the transmembrane (or 'Boyden chamber') assay (Zigmond and Hirsch, 1973). Previous studies using this assay have indicated that fibronectin at concentrations of 1-100 μg/ml stimulate the migration of human skin fibroblasts, as well as various other normal and transformed cell types (Postlethwaite et al., 1981; Clark et al., 1988; Aznavoorian et al., 1990). Native fibronectin has similarly been reported to stimulate cell migration in other assays that also involve the assessment of cell motility on plastic tissue culture dishes (Ali and Hynes, 1978).

Fibronectin is a multifunctional molecule capable of interacting with the cell surface, as well as with various other macromolecular constituents of the extracellular matrix.

Limited proteolytic digestion of native fibronectin has revealed that each of its two constitutive polypeptide chains consists of a linear array of discrete protease-resistant globular domains joined together by short stretches of extended protease-sensitive linking sequences. Functional analyses of the protease-resistant domains have indicated that they each express characteristic and discrete affinities. For example, digestion of fibronectin with thermolysin yields several well-characterised functional peptides referred to as Hep-1/Fib-1 (29 kDa), gelatin-binding domain (43 kDa), Cell (110 kDa), Hep-2 (30 kDa) and Fib-2 (20 kDa), in accordance with their respective affinities for heparin (Hep), fibrin (Fib), gelatin and the cell surface (Cell) (Zardi et al., 1985).

The migration-stimulating activity of native fibronectin in the transmembrane assay is shared by several of its purified functional domains. For example, the cell-binding domain (also at concentrations of 1-100 μg/ml) stimulates the migration of a number of cell types, including fibroblasts (Postlethwaite et al., 1981; Seppa et al., 1981); in addition, cells of neural crest origin are responsive to the Hep-2 (Perris and Johansson, 1987) and C-terminal Fib-2 (Fukai et al., 1991) domains. Several of these activities have now been attributed to defined amino acid sequences, such as the RGDS motif located in the cell binding domain (Pierschbacher and Ruoslahti, 1984). Cells of neural crest origin respond to other amino acid sequences as well, including the LDV motif located in the alternatively spliced IIICS region (McCarthy et al., 1986; Humphries, 1990; Humphries et al., 1987). In the context of the present study, it should be noted that: (a) relatively high concentrations of native fibronectin and its biologically active fragments (in the region of 1-100 μg/ml) appear to be required for biological activity to be seen in transmembrane and related assays; and (b) the gelatin-binding domain (GBD) has consistently been reported to be devoid of migration-stimulating activity in these assays (Postlethwaite et al., 1981; Seppa et al., 1981; McCarthy et al., 1986; Clark et al., 1988; Aznavoorian et al., 1990).

In spite of the widespread use of the transmembrane assay, it is not clear whether the results obtained are truly informative regarding cell behaviour in the considerably more complex in vivo milieu (Schor, 1994). With this potential difficulty in mind, we have developed an alternative migration assay system, involving the assessment of cell movement into three-dimensional matrices of type I collagen fibres (Schor, 1980). Using this more physiologically relevant assay system, we have previously reported that native fibronectin at concentrations of 50-100 μg/ml stimulated the migration of various transformed cell lines, but actually inhibited the migration of human skin fibroblasts (Schor et al., 1981a,b), i.e. a result very different from that obtained in the transmembrane assay. We have also reported that transforming growth factor-beta 1 (TGF-β1) inhibits fibroblast migration into such native collagen matrices (Ellis et al., 1992), a result standing in marked contrast to its reported stimulatory activity in the transmembrane assay (Postlethwaite et al., 1987). The apparently assay-dependent nature of these opposing effects of TGF-β is consistent with the view that the biological activity of potentially multifunctional cytokines is modulated by the 'context' of the extracellular matrix (Nathan and Sporn, 1991).

In view of these various observations, the objective of this study has been to examine the effects of native fibronectin and several of its principal functional domains on the migration of human skin fibroblasts into native type I collagen matrices and compare this to the effects of these molecules in the more commonly used transmembrane assay.

MATERIALS AND METHODS

Reagents

Monoclonal antibodies specifically recognizing epitopes in the following domains of fibronectin were used in this study: gelatin-binding domain (A003, Gibco BRL, Paisley, Scotland and MAS 508p, Sera Lab, Crawley Down), cell-binding domain (FN12-8, Pierce and Warriner, Chester) and disulphide bonded C-terminal domain (FN21-1, Pierce and Warriner, Chester). RGDS was obtained from Sigma Chemical Co, Poole, Dorset.

Cells and culture conditions

Fibroblast lines were established in our laboratory by explant culture (Ham, 1980) from foreskin biopsies (cell lines FSF37, FSF44; donor age range 1-2 years) and forearm skin biopsies (SK319, SK526, SK539; donor age range 20-70 years). Stock cultures were maintained in Eagle's MEM supplemented with 15% (v/v) donor calf serum, 1 mM sodium pyruvate, 2 mM glutamine, non-essential amino acids at 37°C in a moist atmosphere containing 5% CO_2. Cells were grown on 90 mm plastic tissue culture Petri dishes and passaged at a split ratio of 1:5 upon reaching confluence 7-10 days after plating. All experiments were performed at passages 10-18. Cultures used in this study were shown to be free of mycoplasmal contamination. The majority of experiments were performed with the SK319 cell line; the generality of these results was confirmed in comparative experiments with the other lines.

Migration assays

Type I collagen was extracted overnight from rat tail tendons in 3% acetic acid, dialysed for 2 days against distilled water, diluted to 2 mg/ml and used to make 2 ml collagen gels in 35 mm plastic tissue culture dishes as previously described (Schor and Court, 1979). Unless otherwise indicated, cell migration was assessed in serum-free growth medium (SF-MEM). In these assays, collagen gels were overlaid with 1 ml of either SF-MEM or SF-MEM containing 4× the final concentration of the putative effector molecule to be tested (e.g. native fibronectin or one of its fragments). Confluent stock cultures of fibroblasts were then trypsinised, pelleted by centrifugation, resuspended in SF-MEM at 2×10^5 cells/ml and 1 ml samples of this inoculum pipetted onto the overlaid gels. Considering the 2 ml volume of the collagen gel, the 1 ml medium overlay and the 1 ml cell inoculum, this procedure gives the correct final concentration of test molecule. The cultures were incubated for 4 days and the percentage of fibroblasts present within the collagen gel matrix at that time ascertained by microscopic observation of 15-20 randomly selected fields, as previously described (Schor, 1980). Replicate gels were counted for each experimental condition. Certain assays were conducted in the presence of 5% serum. In these cases, cells were resuspended at a concentration of 2×10^5 cells/ml in MEM containing either 20% donor calf serum or 20% fibronectin-stripped donor calf serum. 1 ml of this cell suspension was then plated onto the gels overlaid with SF-MEM±effector molecule, thus giving a final concentration of 5% serum in both control and test cultures. Fibronectin-stripped serum was prepared by gelatin-affinity chromatography and levels of fibronectin depletion greater than 99% were confirmed by ELISA using an anti-fibronectin polyclonal antibody purchased from Dako, Ltd (High Wycombe, UK).

Comparative migration data were obtained using the transmembrane assay, as employed in the majority of previous studies. Accordingly, 8.0 μm pore polyvinylpropylene-free polycarbonate Nucleopore

membranes (Costar, UK) were coated with either: (a) gelatin, by overnight immersion at 37°C in a 10 μg/ml aqueous solution, followed by 3× washes with PBS and air drying; or (b) native collagen, by immersion in a cold solution of 2 mg/ml collagen (as used to make the collagen gels), followed by air drying. The gelatin was prepared from the collagen stock solution by heating samples of it at 60°C for 30 minutes, followed by rapid subsequent cooling (to ensure efficient denaturation). The coated membranes were fitted into a 48-well MicroChemotaxis Chamber (Neuroprobe Inc, MD, USA), with the bottom well containing different concentrations of GBD dissolved in SF-MEM BSA and a total of 2×10^4 cells suspended in 50 μl SF-MEM + 0.1% then plated into the upper wells. The chambers were incubated for 4-5 hours at 37°C in a humidified CO_2 incubator and the membranes then removed, fixed in methanol and stained with Mayer's Haemotoxylin. Cells remaining on the top of the membranes were wiped off and the membranes then mounted onto glass slides. These were examined under bright field illumination at a magnification of 250× and the number of cells which had migrated onto the lower surface of the membrane were counted in 15 random fields per well.

A pictorial comparison of the native collagen matrix and transmembrane migration assays is presented in Fig. 1. These assays differ with respect to a number of parameters, including: (a) the nature of the substratum (matrix of native collagen fibres versus coated polycarbonate membrane); (b) duration (4 days versus 4-5 hours); and (c) expression of data (percentage of total cells within 3-D collagen matrix versus cells on undersurface of membrane per high power field). Fibroblasts penetrate the collagen matrix to a maximum depth of approximately 100 μm during the 4-day duration of the collagen matrix migration assay.

Purification and characterisation of fibronectin fragments

Unless otherwise indicated, proteolytic degradation fragments of fibronectin were prepared by digesting 1 mg/ml human serum fibronectin (Bioproducts Laboratory, Elstree, Herts, UK) in 0.1 M NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.0, with 3% (w/w) thermolysin (Boehringer Mannheim, Lewes, E. Sussex, UK) at 30°C for 30 minutes; the reaction was then quenched with 0.2 volumes 0.2 M EDTA. The following fragments were generated and purified from the thermolysin digest by hydroxyapatite chromatography, as described by Zardi et al. (1985): Hep-1/Fib-1 (29 kDa), GBD (43 kDa), Cell (110 kDa), Hep-2 (30 kDa) and Fib-2 (20 kDa). Preparations of the gelatin-binding domain (GBD) were additionally: (a) purified from thermolysin digests of fibronectin by a combination of heparin and gelatin affinity chromatography; and (b) purchased directly from Gibco. The commercially available preparations of fibronectin and GBD (from Bioproducts and Gibco, respectively) were further purified prior to use by SDS-PAGE, followed by cutting out and extracting the region of the acrylamide gel containing protein of the expected molecular mass; these preparations of fibronectin and GBD are referred to as 'electrophoretically re-purified'. The identity and purity of all proteolytic fragments were confirmed by a combination of PAGE and N-terminal amino acid sequence. The *gap 1-3* recombinant construct of truncated rat fibronectin containing the intact gelatin-binding domain, as well as the adjacent I-4 and I-5 repeats (Sottile et al., 1991), was expressed in insect cells under serum-free conditions and isolated by gelatin-agarose affinity chromatography (Mooradian et al., 1989).

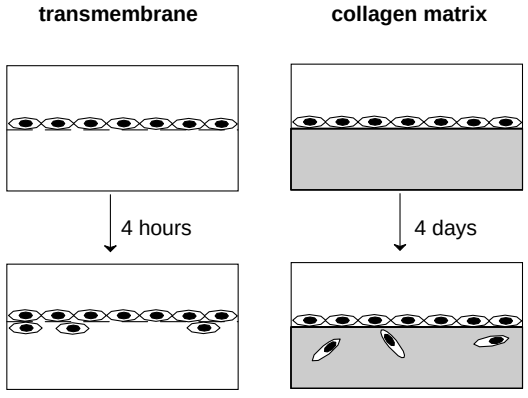

Fig. 1. Schematic comparison of the transmembrane and native collagen matrix migration assays. Details of the protocols for each assay are given in Materials and Methods. These assays differ with respect to a number of parameters, including: (a) the nature of the substratum (matrix of native collagen fibres versus coated polycarbonate membrane); (b) duration (4 days versus 4-5 hours); and (c) expression of data (percentage of total cells within 3-D collagen matrix versus cells on undersurface of membrane per high power field). Fibroblasts penetrate the collagen matrix to a maximum depth of approximately 100 μm during the 4-day duration of the collagen matrix migration assay.

Where indicated, samples of purchased fibronectin and GBD were purified by anion exchange (Mono Q column) or reverse phase (ProRPC HR5/10 column) chromatography using an FPLC system fitted with an LCC-500 controller (Pharmacia Biotech, St Albans, Herts). For anion exchange chromatography, buffer A consisted of 10 mM sodium orthophosphate, pH 7, and buffer B was the same with 1 M NaCl. The mono Q column was equilibrated with 90% buffer A + 10% buffer B prior to sample injection. After sample injection, the column was washed with this same buffer mix for 4 minutes at a flow rate of 1 ml/minute and bound sample eluted with a 20-minute 10%-50% buffer B gradient. The column was washed by increasing buffer B to 100% and held for 4 minutes before returning to the start conditions. For the reverse phase chromatography, solvent A was 18 M-Ohm water + 0.1% trifluoroacetic acid and solvent B was 100% acetonitrile (BDH, hypersolvent grade). The column was equilibrated at a flow rate of 0.3 ml/minute with solvent A prior to sample injection. The bound sample was eluted by a 60-minute 0%-50% gradient of solvent B, and then washed by increasing solvent B to 100% for 12 minutes before returning to start conditions.

In order to determine the number and approximate molecular mass of biologically active degradation peptides, fibronectin digests were resolved in replicate tracks by SDS-PAGE using a Hoefer cooled vertical slab system (SE 600 series), according to the method of Laemmli (1970). Electrophoresis was performed under reducing conditions. Pre-stained molecular mass markers were obtained from Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK, as follows: phosphorylase B (139.9 kDa); bovine serum albumin (86.8 kDa); ovalbumin (47.8 kDa); carbonic anhydrase (33.3 kDa); soybean trypsin inhibitor (28.6 kDa); lysozyme (20.7 kDa). Gels containing one set of replicate tracks were stained with 0.25% Coomassie brilliant blue dissolved in 50% methanol and 10% acetic acid. The remaining set of replicate tracks were sliced and eluted for subsequent determination of migration-stimulating activity, according to the method described by Gherardi et al. (1989). In this procedure, the unstained replicate tracks were sliced into 5 mm width pieces; each slice was then extracted by placing it into 1 ml of 0.1 M ammonium hydrogen carbonate containing 1 mM SDS and incubating at 37°C overnight whilst mixing end over end. Samples (2-5 μl) of this extract were diluted to 1 ml with SF-MEM and assessed for migration-stimulating activity.

RESULTS

Effects of fibronectin and its purified functional domains on fibroblast migration into native collagen matrices

In these experiments, stock fibroblasts were trypsinised, resus-

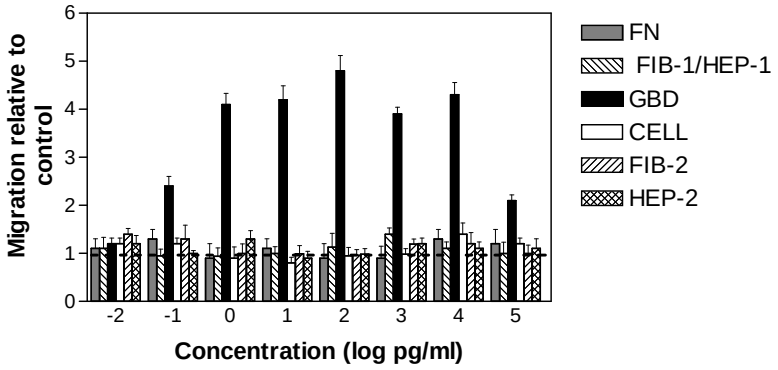

Fig. 2. The effects of fibronectin and its proteolytic degradation fragments on the migration of human skin fibroblasts into native collagen matrices. The cell migration assays were performed as described in Materials and Methods. This involved overlaying pre-formed collagen gels with 1 ml of serum-free MEM (controls) or serum-free MEM containing the requisite concentration of native fibronectin (FN) or one of its proteolytic fragments. Trypsinised SK319 fibroblasts were suspended in serum-free MEM to give an inoculum containing 2×10^5 cells/ml and 1ml of this was plated onto replicate control and fibronectin (fragment)-overlaid gels. After a 4-day incubation period at 37°C, the cells on the surface and within the 3-D matrix of the gel were counted in 15 randomly selected fields by microscopic observation and these data were used to calculate the percentage of total cells present within the gel matrix. Results from five experiments are presented. In order to facilitate comparison of results obtained in different experiments, data are normalised by expressing them as 'relative stimulation' of migration, this being calculated by dividing the percentage of cells in the gel matrix for each experimental point by the control value obtained in that particular experiment. Dotted line indicates control level of migration. Values are +s.e.m.

pended in serum-free MEM (SF-MEM), plated onto the surface of collagen gels and incubated for 4 days in the absence (control) and presence of the various fibronectin functional domains at concentrations ranging from 0.01-100,000 pg/ml. The number of cells within the collagen gel matrix were then determined and these data expressed as a percentage of total cells (as described in Materials and Methods). In contrast to previously published results obtained with the transmembrane and related migration assays, we found that concentrations of the 43 kDa gelatin-binding domain (GBD) as low as 0.1 pg/ml significantly stimulated cell migration. This biological activity exhibited a bell-shaped dose-response curve, with maximal stimulation of migration achieved at concentrations of GBD between 1 pg/ml and 10 ng/ml (Fig. 2). Native fibronectin, as well as all other of its tested fragments (i.e. Fib-1/Hep-1, Cell, Fib-2, and Hep-2), were completely devoid of migration-stimulating activity within this concentration range. The appearance of cells on the surface of control gels and within the 3-D collagen matrix of GBD-stimulated cultures is presented in Fig. 3. Cells migrating in response to GBD were morphologically indistinguishable from the (considerably smaller number of) cells within the gel matrix of control cultures (data not shown).

The migration-stimulating activity of GBD was also apparent in the presence of 5% whole calf serum, 5% fibronectin-stripped serum and 1 μg/ml fibronectin in SF-MEM (Fig. 4). Identical results were obtained with GBD purified from fibronectin digests by a combination of heparin and gelatin affinity chromatography or purchased commercially, either with or without subsequent electrophoretic re-purifica-

Table 1. The effects of GBD on fibroblast migration into native collagen matrices

Cell line	% Migration		Migration relative to control	*P* value
	Control	1 ng/ml GBD		
FSF37	2.0±0.5	7.1±0.4	3.55	0.0078
FSF44	2.3±0.1	7.5±0.3	3.26	0.0018
SK319	1.9±0.3	8.2±0.3	4.32	0.0023
SK526	1.2±0.1	4.5±0.7	3.75	0.0218
SK539	2.1±0.2	7.4±0.8	3.52	0.0119
Mean	1.9±0.7	6.9±0.8	3.52	0.0119

Data summarising the effect of GBD on the migration of five fibroblast lines.

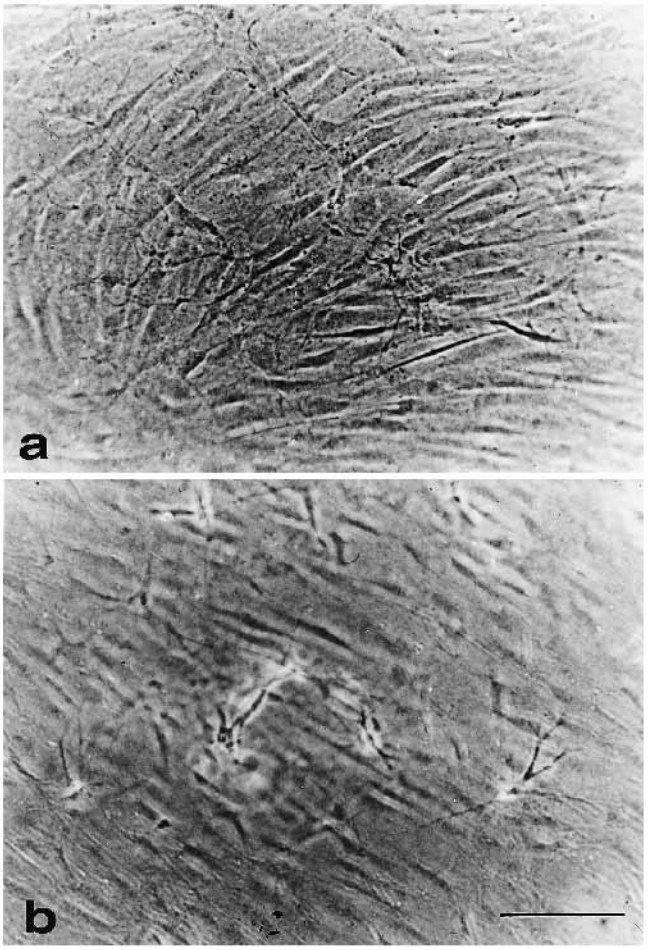

Fig. 3. The appearance of fibroblasts in the collagen matrix assay. (a) Cells on the surface of control gel cultures after 4 days of incubation. (b) Cells within the 3-D collagen matrix after 4 days of incubation in the presence of 1 ng/ml GBD. The monolayer of cells remaining on the surface is out of focus. Bar, 50 μm.

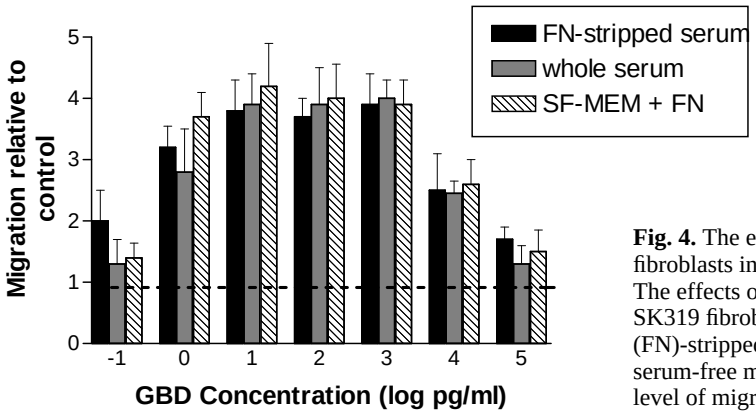

Fig. 4. The effects of GBD on the migration of human skin fibroblasts in the presence of serum and exogenous fibronectin. The effects of different concentrations of GBD on the migration of SK319 fibroblasts were assessed in the presence of 5% fibronectin (FN)-stripped serum, 5% whole serum and 1 μg/ml fibronectin in serum-free medium (SF-MEM+FN). Dotted line indicates control level of migration. Values are +s.e.m.

tion (data not shown). We have previously reported that greater than 95% of fibroblasts attach to the surface of the collagen matrices within 2 hours of plating (Schor et al., 1981a). Fibroblast attachment was not affected by any concentration of GBD in serum-free or serum-containing medium (data not shown).

Data presented in Table 1 indicate that GBD stimulated the migration of all five fibroblast lines examined in this study.

Several experiments were performed in order to confirm that the observed migration-stimulating activity of GBD was not due to a possible contaminant. In the first instance, both native fibronectin and GBD were subjected to anion exchange and reverse-phase chromatography and the collected column fractions tested for migration-stimulating activity. Data presented in Fig. 5 indicate that a single peak of migration-stimulating activity was collected in fractions obtained from each of the two GBD separations; in both cases, migration-stimulating activity co-eluted with the A_{280} protein peak. No biological activity was recovered in either of the two fibronectin chromatographic separations.

The native fibronectin used to generate GBD and the other peptide fragments used in the above experiments was purified from human plasma. Since blood contains a number of proteins known to stimulate cell migration (and certain of these may conceivably co-purify with fibronectin), we next compared the activities of proteolytically produced GBD with the Gap 1-3 genetically engineered construct of truncated rat fibronectin containing the intact gelatin-binding domain, as well as the adjacent I-4 and I-5 repeats (Sottile et al., 1991). This construct was expressed in insect cells grown under serum-free conditions. Data presented in Fig. 6 indicate that GBD (generated by the degradation of human plasma fibronectin) and Gap 1-3 displayed identical dose-response curves. In addition, the migration-stimulating activity of GBD and the Gap 1-3 recombinant construct were both completely neutralised by monoclonal antibodies (A003 and MAS 508p) recognising epitopes within the gelatin-binding domain, but were unaffected by monoclonal antibodies (FN12-8 and FN21-1) directed against epitopes present in other domains of fibronectin (Table 2).

TGF-β1 has been reported to bind to fibronectin (Fava and McClure, 1987; Mooradian et al., 1989) and may therefore be responsible for certain of the reported biological activities of allegedly purified fibronectin. In this regard, we have previously demonstrated that TGF-β1 in the concentration range 0.1-10,000 pg/ml has no effect on the migration of confluent adult fibroblasts into native collagen gels (Ellis et al., 1992). Data presented in Fig. 7 confirm this finding and further

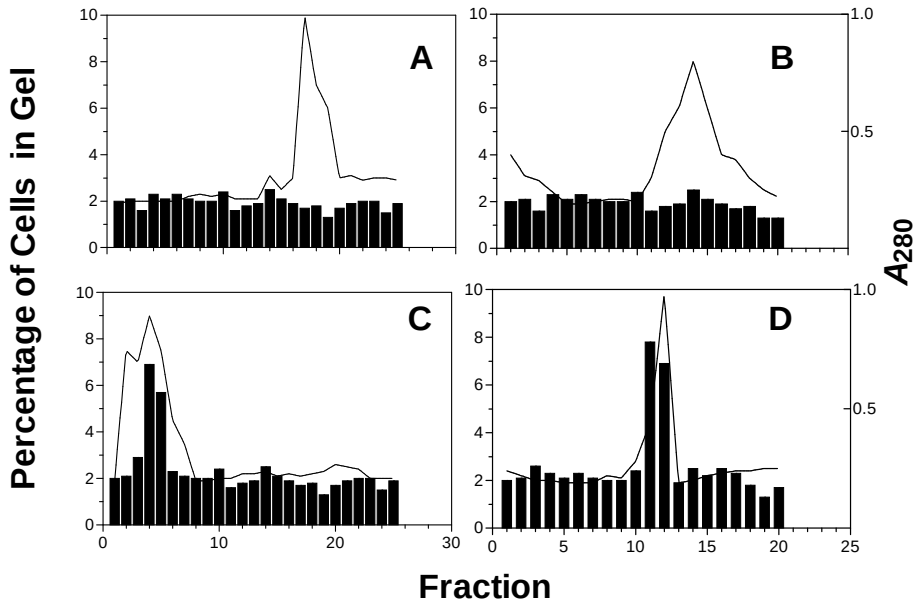

Fig. 5. Chromatographic purification of fibronectin and GBD. Fibronectin (A,B) and GBD (C,D) were purified by anion exchange Mono Q (A,C) and reverse phase ProRPC (B,D) FPLC chromatography, as described in Materials and Methods. The collected fractions were assayed for migration-stimulating activity (bars) and these were co-plotted with the A_{280} values (lines) indicating total eluted protein.

Table 2. The effects of antibodies against different domains of fibronectin and RGDS peptide on the stimulation of fibroblast migration by GBD

Antibody	GBD (pg/ml)	% Cells in gel + antibody	% Cells in gel (−antibody)	Inhibition
Anti-GBD (A003)	Proteolytic			
0.1 μg/ml	0	1.7±0.2	(*1.8±0.3*)	-
	10	1.9±0.4	(7.8±0.4)	**98.3**
	100	1.2±0.9	(7.5±0.1)	**110.5**
1.0 μg/ml	0	2.0±0.1	(*1.8±0.3*)	-
	10	1.8±0.1	(7.8±0.4)	**100.0**
	100	1.8±0.6	(7.5±0.1)	**100.0**
Anti-GBD (A003)	Recombinant			
0.2 μg/ml	0	1.8±0.3	(*2.2±0.1*)	-
	10	1.8±0.3	(8.4±0.4)	**106.4**
	100	7.7±0.4	(8.5±0.3)	12.6
1.0 μg/ml	0	1.7±0.0	(*2.2±0.1*)	-
	10	1.8±0.4	(8.4±0.4)	**106.5**
	100	4.8±0.3	(8.5±0.3)	**59.7**
Anti-GBD (MAS 508p)	Proteolytic			
0.1 μg/ml	0	2.4±0.1	(*2.9±0.3*)	-
	10	3.0±0.2	(8.9±1.1)	**98.3**
	100	2.1±0.1	(9.1±0.4)	**112.9**
1.0 μg/ml	0	2.6±0.2	(*2.9±0.3*)	-
	10	2.8±0.2	(8.9±1.1)	**101.7**
	100	0.9±0.8	(9.1±0.4)	**136.7**
Anti-GBD (MAS 508p)	Recombinant			
0.2 μg/ml	0	1.7±0.3	(*2.2±0.3*)	-
	10	1.7±0.2	(8.4±0.5)	**92.5**
	100	1.9±0.2	(8.5±0.4)	**104.8**
1.0 μg/ml	0	1.9±0.2	(*2.2±0.3*)	-
	10	1.9±0.1	(8.4±0.5)	**104.8**
	100	2.1±0.0	(8.5±0.4)	**101.6**
Anti-cell binding	Proteolytic			
1:1,000	0	1.9±0.2	(*1.7±0.2*)	-
	100	12.3±0.4	(11.2±0.3)	–11.5
Anti-C terminus	Proteolytic			
1:1,000	0	3.0±0.3	(*1.7±0.2*)	-
	100	11.6±0.5	(11.2±0.3)	–4.2

The effects of different concentrations (0-100 pg/ml) of both proteolytically derived GBD and (Gap 1-3) recombinant GBD on fibroblast migration were determined in the presence or absence of the indicated monoclonal antibodies. The following antibodies were used: anti-gelatin binding domain (A003), anti-cell binding domain (FN12-8) and anti-disulphide bonded C-terminal domain (FN21-1). Data are presented concerning the percentage of cells within the collagen matrix in the presence and absence (in parentheses) of antibody. The percentage inhibition was calculated relative to the control level of migration (italics) obtained in the absence of both GBD and blocking agent. Values of relative inhibition greater than 50% are indicated in bold.

indicate that TGF-β1 *inhibits* the migration-stimulating activity of GBD in a dose-dependent fashion. Related data indicated that neutralising antibodies to TGF-β1 did not inhibit the effects of GBD on cell migration (data not shown).

Taken together, these data suggest that the proteolytic cleavage of fibronectin enables a *cryptic* migration-stimulating activity latent within its gelatin-binding domain. In order to test this hypothesis, electrophoretically re-purified fibronectin was digested with thermolysin; the reaction was stopped after various times ranging from 5 minutes to 24 hours and the resulting digests tested for migration-stimulating activity. An initial assessment indicated that migration-stimulating activity

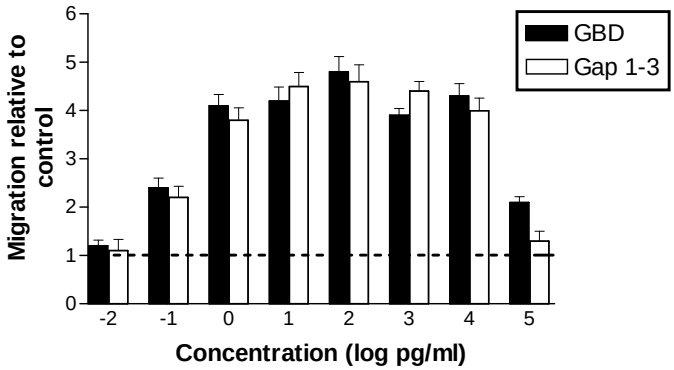

Fig. 6. Comparison of migration-stimulating activity of GBD and the Gap 1-3 genetically engineered construct containing the intact gelatin-binding domain. The data compare the effects of GBD and the Gap 1-3 genetically engineered construct on the migration of SK319 fibroblasts. Dotted line indicates control level of migration. Values are +s.e.m.

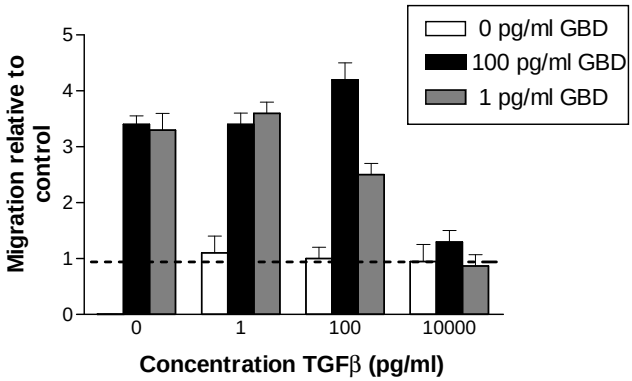

Fig. 7. The effect of TGF-β1 on GBD-stimulated cell migration. SK319 fibroblasts were plated onto the surface of 3-D native collagen matrices in medium containing different concentrations of TGF-β1 (0-10,000 pg/ml) in the absence or presence of either 1 or 100 pg/ml GBD. Migration assays were performed as indicated in Materials and Methods. Dotted line indicates level of migration in control cultures (i.e. in the absence of both GBD and TGF-β1). Values are +s.e.m.

was detectable at concentrations of total protein as low as 100 pg/ml after only 5 minutes digestion with thermolysin; the minimum concentration of total digest required for migration-stimulating activity subsequently decreased to an apparent plateau of between 1 and 10 pg/ml following 30 minutes digestion. For a more detailed analysis, native fibronectin and its 5, 30 and 60 minute digests were next separated by SDS-PAGE in order to identify the biologically active peptides generated by thermolysin digestion. The acrylamide gels contained replicate tracks of each sample. Following electrophoresis, one set of tracks was visualised by staining with Coomassie Blue, while tracks containing the replicate sample set were sliced and extracted for assay of migration-stimulating activity. Data presented in Fig. 8 indicate that native fibronectin produced a major high molecular mass band at the top of the gel and that none of the replicate track slices contained detectable migration-stimulating activity. Migration stimulating activity was detected in two track slices after only

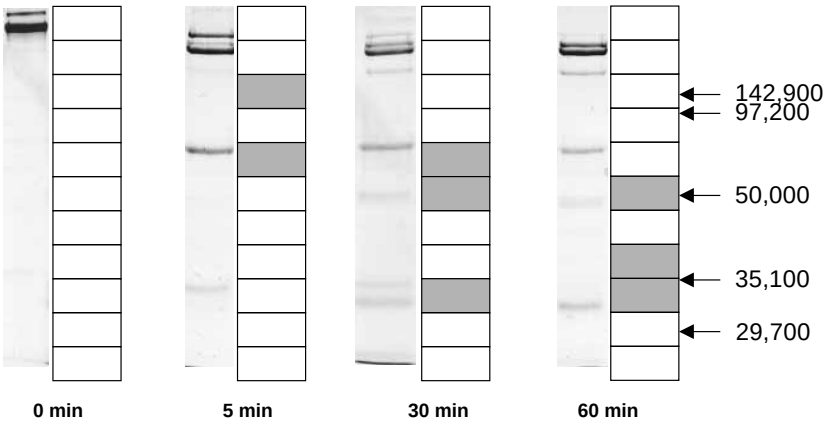

Fig. 8. SDS-PAGE of native and digested fibronectin: correlation with recovered migration-stimulating activity. Samples of the indicated fibronectin digests were analysed by SDS-PAGE using a 10% acrylamide resolving gel. Replicate tracks of each sample were loaded onto the same acrylamide gel. Following electrophoresis, one set of tracks was stained with Coomassie Blue. The corresponding replicate tracks were sliced and eluted for determination of migration-stimulating activity as described in Materials and Methods using SK319 fibroblasts; gel slices with detectable levels of migration-stimulating activity (defined as at least twice the control values) are indicated by shading. Data are presented for undigested fibronectin, as well as for 5-, 30- and 60-minute digestion periods.

5 minutes digestion; the larger biologically active peptide was estimated to have a molecular mass in the region of 100-140 kDa (corresponding Coomassie-stained band or bands not apparent), while the smaller corresponded to a single stained band with an apparent molecular mass of approximately 70 kDa. After 60 minutes digestion, two biologically active tracks were again obtained, these now corresponding to estimated molecular masses of 40-50 and 30-40 kDa, respectively. The migration-stimulating activities of all the biologically active peptides were neutralised by two monoclonal antibodies directed against sites contained within the gelatin-binding domain (antibodies A003 and MAS 508p) and were unaffected by two other antibodies (FN12-8 and FN21-1) directed against epitopes present in the cell-binding and disulphide-bonded C-terminal region of fibronectin, respectively (data not shown).

The substratum- and assay-dependent nature of GBD migration-stimulating activity

GBD has previously been reported to be devoid of migration-stimulating activity when tested in the transmembrane assay. The following experiment was performed: (a) in order to confirm this observation with our preparations of GBD; and (b) to ascertain whether coating the membrane with a film of native collagen modulated the cellular response to GBD. Filters were accordingly coated with a film of gelatin (as used in the majority of previous studies) or native collagen (as used in our collagen matrix assay). As shown in Fig. 9, GBD stimulated fibroblast migration in the collagen gel assay at the lowest concentration tested (1 pg/ml). In agreement with previously published observations, GBD (at concentrations ranging from 1 pg/ml to 100 μg/ml) was totally devoid of migration-stimulating activity when assessed in the transmembrane assay with gelatin-coated filters. Interestingly, GBD did exhibit limited migration-stimulating activity in the transmembrane assay when the filters were coated with native collagen; in this case, migration-stimulating activity was apparent in a relatively narrow range of concentrations (i.e. 1-100 ng/ml) and resulted in a lower maximal stimulation relative to control values (approximately twofold) compared to that achieved in the native collagen assay (approximately four- to fivefold).

Pre-incubation of cells with GBD

In this experiment, stock cultures of fibroblasts growing on plastic tissue culture dishes were washed 3× with SF-MEM and then incubated for 24 hours in SF-MEM, either with or without 1 μg/ml GBD. Both cultures were then trypsinised and washed extensively by repeated (5×) cycles of centrifugation and resuspension in SF-MEM. The cells pre-incubated in the absence of GBD were then plated onto the surface of collagen gels as usual, in the presence and absence of GBD (1 ng/ml). The cells pre-incubated with GBD were plated in SF-MEM in the absence of GBD in the migration assay. Anti-GBD antibody was added to replicate cultures. The results presented in Fig. 10 confirm that GBD present in the assay stimulated the migration of control cells and that this is inhibited by the presence of neutralising antibody. Data presented in the right panel further reveal that: (a) the migration of cells pre-incubated with GBD whilst growing on plastic dishes was also stimulated during the 4-day period of the migration assay in the absence of GBD; and (b) the presence of anti-GBD antibody did not inhibit the migration of these cells.

DISCUSSION

Data presented in this communication indicate that: (a) the gelatin-binding domain (GBD) of fibronectin stimulates fibro-

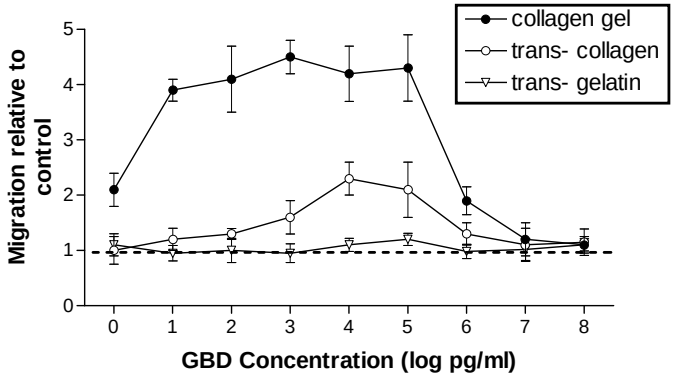

Fig. 9. Comparison of the migration-stimulating activity of GBD as assessed in the native collagen matrix and transmembrane assays. Data are presented comparing the effects of GBD on the migration of SK319, as assessed in the native collagen gel assay and the transmembrane assay (trans-) using membranes coated with either native collagen or gelatin. Dotted line indicates control level of migration.

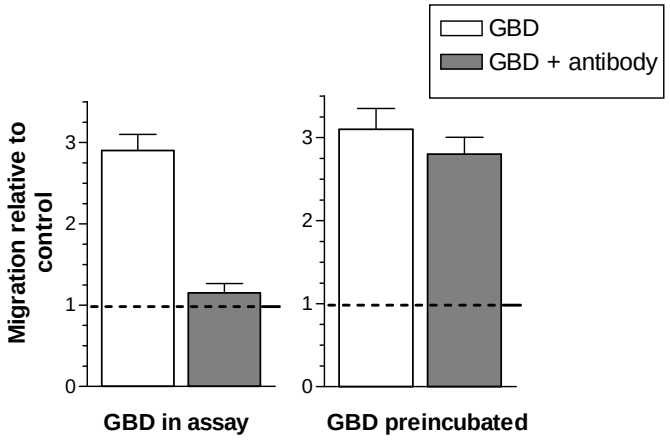

Fig. 10. The effect of pre-incubation with GBD on fibroblast migration. Stock cultures of SK319 fibroblasts growing in 90 mm plastic tissue culture dishes were washed 3× with SF-MEM and then incubated for 24 hours in SF-MEM, either with or without 1 μg/ml GBD. Both cultures were then trypsinised and washed extensively by repeated (5×) cycles of centrifugation and resuspension in SF-MEM. The control cells pre-incubated in the absence of GBD were then plated onto the surface of collagen gels as usual, in the presence and absence of GBD (1 ng/ml). The cells pre-incubated with GBD were plated in SF-MEM in the absence of GBD in the migration assay. Anti-GBD antibody was added to replicate cultures. Data are expressed relative to the migration of control cells in the absence of GBD in the migration assay (dotted line).

blast migration into matrices of native type I collagen fibres; (b) under the same assay conditions, native fibronectin and peptides containing all other of its functional domains are devoid of such activity; (c) the migration-stimulating activity of GBD in the native collagen matrix assay is not affected by the presence of serum and native fibronectin; and (d) GBD exhibits limited migration-stimulating activity when tested in the transmembrane assay using filters coated with native collagen, but is devoid of activity when tested in identical assays using filters coated with denatured collagen (i.e. gelatin).

Various lines of evidence support the conclusion that the observed migration-stimulating activity of GBD in the collagen gel assay is not due to a contaminant. In this regard: (a) no migration-stimulating activity was detected in fibronectin purified by a variety of protocols (i.e. anion exchange chromatography, reverse phase chromatography and SDS-PAGE); (b) the migration-stimulating activity of GBD always co-purified with the main protein peak using all of the above-cited purification protocols; (c) a genetically engineered construct containing the GBD region displayed the same migration-stimulating activity as proteolytically generated GBD; and, finally (d) the migration-stimulating activity of both GBD and its genetically engineered counterpart were inhibited by monoclonal antibodies directed against epitopes present within the gelatin-binding domain. It should also be noted that the chromatographic and SDS-PAGE electrophoresis protocols used to purify GBD should be sufficient to resolve all but covalently bound contaminants. In addition, the extremely low concentration of GBD (0.1-1.0 pg/ml) at which significant migration activity was seen makes it highly unlikely that even lower quantities of a putative contaminant maybe capable of affecting cell migration. Various cytokines, including TGF-β1, have been reported to bind to fibronectin and may be responsible for certain of the previously reported biological activities of fibronectin (Fava and McClure, 1987; Mooradian et al., 1989); our results regarding the effects of exogenous TGF-β1 and neutralising TGF-β1 antibody indicate that this cytokine is not responsible for the observed effects of GBD.

As proteolytic degradation fragments of fibronectin have been detected in wound fluid (Wysocki and Grinnell, 1990), our data suggest that concentrations of GBD previously thought too low to be of biological significance may actually play an important role in the regulation of fibroblast migratory activity during wound healing. Proteases released by inflammatory cells may play a central role in the transient and localised generation of GBD (as well as other degradation peptides exhibiting cryptic biological activities). Similarly, the production of proteases by both tumour and associated stromal cells may also generate GBD and thereby influence local tumour invasion; in this regard, we have recently demonstrated that the migration of melanoma cells is also stimulated by GBD (Banyard et al., manuscript in preparation).

Although an effect of GBD on cell migration has not previously been reported, this domain of fibronectin has been shown to possess a number of other biological activities, including the enhancement of morphological transformation in virally transformed cells (De Petro et al., 1981), the expression of collagenase activity (Lambert-Vidmar et al., 1991a,b; Homandberg et al., 1992) and the promotion of odontoblast differentiation (Lesot et al., 1992). In addition, GBD and its adjacent type III modules have been reported to play a role in fibronectin-fibronectin interactions (Ehrismann et al., 1981; Chernousov et al., 1991; Morla and Ruoslahti, 1992). The possible relationship between these other activities of GBD and its presently reported stimulation of fibroblast migration into native collagen matrices remains to be determined.

The apparent difference in biological activity exhibited by native fibronectin and its purified gelatin-binding domain are intriguing. This observation is consistent with previous reports describing similarly cryptic biological activities of other proteolytic fragments of fibronectin with respect to their stimulation of monocyte migration in the transmembrane assay (Clark et al., 1988), inhibition of cell proliferation (Muir and Manthorpe, 1992), the induction of protease gene expression by adherent rabbit synovial fibroblasts (Werb et al., 1989), adipocyte differentiation (Fukai et al., 1993) and an RGDS-independent mediation of cell migration (Fukai et al., 1991). Degradation of other macromolecules has also been reported to generate peptides displaying cryptic biological activities not shared by the native molecule (Dang et al., 1985; O'Reilly et al., 1994).

The alterations in native fibronectin required for the expression of cryptic biological activity in its various functional domains have recently been investigated by Fukai et al. (1995). In their excellent study, these workers demonstrated that: (a) fibronectin isolated from plasma by conventional purification protocols involving exposure to high concentrations of the urea (a potent denaturing agent) mediated an RGDS-dependent stimulation of cell migration, whilst fibronectin isolated by a gentler ion exchange chromatography protocol did not; and (b) fibronectin isolated by ion exchange was induced to display this bioactivity by subsequent exposure to urea. Hydrodynamic and light scattering data confirmed that

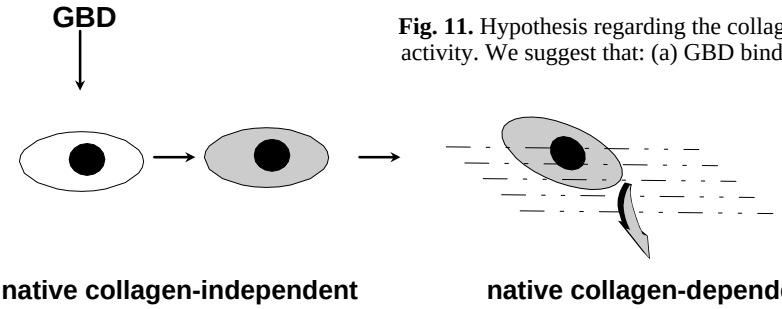

Fig. 11. Hypothesis regarding the collagen-independent and collagen-dependent modes of GBD activity. We suggest that: (a) GBD binds to a cell surface receptor, the ligation of which initiates a persistent alteration in cell phenotype (indicated by shading); and (b) the expression of this altered cell phenotype is manifest by a stimulation of migration on a native type I collagen substratum. According to this hypothesis, the initial interaction of GBD with its putative cell surface receptor and the resultant alteration in cell phenotype are not dependent upon a collagen substratum, whilst the expression of elevated migration is substratum-dependent.

exposure of non-denatured fibronectin to urea induced significant conformational changes in the molecule, which correlated with the enabling of RGDS-dependent migration-stimulating activity. In contrast, the cryptic biological activities of the N-terminal Fib-1 domain (induction of adipocyte differentiation) and C-terminal Fib-2 domain (RGDS-independent stimulation of migration) were not expressed by denatured fibronectin and required the generation of isolated functional domains by proteolytic cleavage. On the basis of these and related observations, they suggested that the spontaneous folding of the urea-denatured molecule results in the inaccessibility of active sites within the Fib-1 and Fib-2 domains, which therefore require proteolytic cleavage from the intact molecule for expression of cryptic activity. Our data regarding the generation of cryptic migration-stimulating activity by the proteolytic generation of GBD is consistent with this interpretation.

In the native collagen migration assay, the putative effector molecule is added to the cultures in a 1 ml SF-MEM overlay 2 hours prior to plating the cells. In a previous study dealing with the effects of TGF-β1 on fibroblast migration, we reported that an equilibrium distribution of TGF-β1 is rapidly established amongst the following three assay compartments, namely: (a) the 2 ml of medium overlaying the collagen gel; (b) the 2 ml of interstitial medium surrounding the collagen fibres; and (c) molecules bound to the insoluble collagen fibres (Ellis et al., 1992). In the case of GBD, this equilibrium is established within 6 hours (data not shown). As the cells are therefore not exposed to a concentration gradient of GBD for the majority of the 72-hour assay period, the observed effect of GBD on cell migration is likely to reflect a chemokinetic response. Initial studies using ^{125}I-labelled GBD indicated that approximately 35% of it is bound to the collagen fibres at equilibrium, with the remaining 65% present in the liquid phase. GBD has a multi-modular structure (Litvinovich et al., 1991) and interacts with collagen via several subsites (Owens and Baralle, 1986; Ingham et al., 1989).

The mechanism by which GBD exerts its effect on cell migration remains to be elucidated. GBD does not contain an RGDS motif and the identity of the minimum amino acid motif required for biological activity is currently under study. The femtomolar concentration range at which it manifests migration-stimulating activity raises the possibility that it may act in a 'cytokine-like' fashion, involving binding to an as yet unknown, presumably high affinity, cell surface receptor. This possibility is consistent with our finding that cells pre-incubated with GBD whilst growing on plastic tissue culture dishes express an elevated level of migration when subsequently trypsinised and assessed in the collagen matrix assay in the absence of further GBD; these results additionally indicate that: (a) the presence of a native collagen substratum is not required during the period of cell exposure to GBD (and hence GBD binding to collagen is not required for expression of its biological activity); and (b) ligation of GBD to its putative cell surface receptor initiates a *persistent* alteration in cell phenotype which is no longer inhibitable by anti-GBD antibody. In this regard, previous studies have revealed persistent effects of a number of cytokines on the synthesis of hyaluronan, which appear to be directly responsible for the observed effects of these cytokines on cell migration in the native collagen assay (Schor, 1994; Ellis and Schor, 1995). Our data further indicate that the stimulation of migration resulting from the hypothesised GBD-induced alteration in cell phenotype requires a native collagen substratum to occur. These various observations are put together in Fig. 11, which summarises our current working hypothesis regarding the collagen-independent and collagen-dependent aspects of GBD's migration-stimulating activity. This substratum-dependence appears to account for the previous failure to note the potent migration-stimulating activity of GBD in the transmembrane assay in which denatured collagen (gelatin) was used to coat the polycarbonate membranes. Further studies are required to identify the putative receptor for GBD and the result of its ligation on cell behaviour.

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