Enhanced assembly of basement membrane matrix by endodermal cells in response to fibronectin substrata

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Summary

Basement membranes are complex extracellular matrices contributing to the regulation of growth, migration and differentiation of many cell types. However, little is known about the mechanisms regulating the deposition and assembly of basement membrane from its constituents. We have investigated the role of extracellular matrix molecules in the control of basement membrane matrix assembly by cultured endodermal (PFHR-9) cells. In the presence of fibronectin-depleted serum, substrata of fibronectin or laminin induced an increase in deposition of laminin, type IV collagen and proteoglycans by PFHR-9 cells, in comparison to cells adherent to type I collagen-coated, vitronectin-coated or uncoated substrata.

Direct effects of fibronectin or laminin on the degree of cell spreading or rate of proliferation were not responsible for enhanced matrix deposition. The effect did not result from a redirection of basement membrane components to the matrix, since there was no decrease in matrix constituents released to the culture supernatants. Furthermore, the synthesis and release of other molecules that are not basement membrane constituents was unaltered in response to different extracellular matrix substrata. Experiments with fibronectin fragments showed that a 105×10^3 M_r 'cell'-binding domain (containing the cell attachment sequence Arg-Gly-Asp-Ser) was an important contributor to enhanced matrix deposition, while the N-terminal 29×10^3 M_r heparin-binding domain also contributed to the effect, particularly with respect to heparan sulfate proteoglycan deposition. It seems that fibronectin has a dual role of action in promoting basement membrane matrix assembly, through direct cell surface interactions, and through the binding of fibronectin to other matrix components that may nucleate or stabilize the matrix assembly.

Key words: laminin, proteoglycans, cell adhesion.

Introduction

Basement membranes are complex pericellular matrices that separate parenchymal from stromal compartments in tissues. Their structure and composition are increasingly well understood, and from a combination of in vitro experiments with basement membrane components and in vivo observations, it is apparent that they are essential for the maintenance of diverse tissue functions and are integral to differentiation processes (Bissell and Barcellos-Hoff, 1987; Gospodarowicz et al. 1984; Timpl and Dziadek, 1986). They are now appreciated to be markedly heterogeneous in molecular composition and structural organization (Abrahamson et al. 1989; Couchman, 1987; Desjardins and Bendayan, 1989; Hunter et al. 1989; McCarthy et al. 1989; Sanes et al. 1990), depending on the tissue of origin and the developmental status. The major collagenous and non-collagenous components of basement membranes have been isolated and their structures analyzed (Hassell et al. 1986; Paulsson et al. 1986; Timpl and Dziadek, 1986), and some of the potential interactions between components have been discussed (Timpl and Dziadek, 1986; Yurchenco et al. 1986). It is, however, not known how basement membrane synthesis in vivo is controlled. Some growth factors such as transforming growth factor-beta (TGF-β) can promote extracellular matrix synthesis (Bassols and Massagué, 1988; Ignnotz and Massagué, 1986) but the process is clearly complex.

Our starting point has been the observation that both in wound healing (Colvin, 1989; McDonald, 1988) and in embryonic basement membranes, (Gibson et al. 1983; Mayer et al. 1981; Thiery et al. 1989) fibronectins can be abundant. This family of closely related glycoproteins have been shown to promote adhesion and migration of a number of cell types, including many that synthesize basement membranes, such as epithelial, endothelial and muscle cells (for review, see Hynes, 1990). It has been proposed previously that fibronectin-rich extracellular matrices may provide a temporary or provisional matrix for tissues that subsequently differentiate and form basement membranes (Colvin, 1989). We have investigated a possible new role for fibronectins in the promotion of basement membrane assembly in endodermal cells. The PFHR-9 endodermal cell line has the advantage that it does not deposit fibronectins into an extracellular matrix, thus enabling the effects of these glycoproteins to be investigated when supplied exogenously.

We provide evidence that fibronectins, when present as a substratum, will promote the assembly of basement membrane-type matrix containing laminin, type IV collagen and proteoglycans. Qualitative and quantitative changes in assembled matrix were seen, mostly resulting
from an interaction of the 'cell-binding domain of fibronectin with the cells. PFHR-9 cell adherence to fibronectin, which is partly sensitive to arginine-glycine-aspartic-acid (RGD)-containing peptides, leads to the enhanced deposition of basement membrane matrix at the cell–substratum interface. In contrast, type I collagen and fibronectin-depleted, serum-coated substrata were ineffective in enhancing basement membrane matrix assembly.

Materials and methods

Materials

General chemicals were from Sigma Chemical Co. (St Louis, MO). Sodium $^{35}$Sulfate (specific activity 38.8 mCi/mmol) and $^{35}$Smethionine (specific activity 10.23 mCi/mmol) were from Amersham Corporation (Arlington Heights, IL). DEAE-Sephacel, Sephadex G-50, gelatin–Sepharose and heparin–Sepharose were purchased from Pharmacia (Uppsala, Sweden). Chondroitinase ABC was from ICN Immunobiologicals (Lisle, IL). Vitrogen (type I collagen) was obtained from Collagen Corporation (Falo Alto, CA). Vinyl and laminin were gifts from Dr D. F. Mosher (University of Wisconsin, Madison, WI) and Dr D. R. Abrahamson (University of Alabama at Birmingham), respectively.

Cell culture, attachment, proliferation and spreading assays

PFHR-9 cells, a teratocarcinoma-derived cell line (Chung et al. 1977; Engvall et al. 1984), are a gift from Dr H. P. Bachinger (Shrirer's Hospital, Portland, OR). These were grown in Dulbecco's Minimum Essential Medium (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, VA) 0.02% glutamine, 1 mM sodium pyruvate and 25 mM Heps. All routine and experimental cultures were incubated at 37°C in 5% carbon dioxide in a humidified incubator.

Substrata were prepared by air drying 2–3 μg of protein onto 12 mm diameter glass coverslips (Fisher) or multiwell plates (Linbro; Flow Laboratories) except for Type I collagen where 500 μl of 100 μg/ml–1 vitamin in 0.1% acetic acid were adsorbed for 6 h. They were then washed (10 min each) with phosphate-buffered saline (PBS), treated with 1% heat-denatured bovine serum albumin (BSA) in PBS and washed again with PBS. Trypsinized cells were seeded (5 x 10^5 cells/cm–2) in medium containing fibronectin-depleted serum onto coated substrata. To measure proliferation rates, cells were suspended at 24 h intervals, and the number of cells/well was counted on a Coulter counter (Coulter Electronics Inc., Hialeah, FL). For measurement of spread cell area, a minimum of 50 cells/sample were analyzed using a Microplan II Image Analysis System (Laboratory Computer Systems, Inc., Cambridge, MA) from photomicrographs (final magnification of ×480).

Attachment assays were performed as previously described (Woods et al. 1986). Tetradecamer amino acid peptides of the sequence YAVTGRGDSSPAK or YAVTGRGESSPAK (both prepared by Dr G. Anantharamanah, University of Alabama at Birmingham, AL and sequenced by Dr P. Neame, Shriner's Hospital Clinical Biochemistry Laboratory, Birmingham, AL) was electrophoresed as a standard. Polypeptides corresponding to A and B chains of laminin were excised from the Coomassie blue-stained PAGE gel and analyzed for radioactivity. The remaining matrix containing $^{35}$S-labeled HSPG was subsequently treated with nitrous acid according to the method of Shively and Conrad (1976). Samples of chondroitinase ABC–released material and the material sensitive to nitrous acid were chromatographed on a Sephadex G-50 column and the fractions analyzed for $^{35}$S radioactivity. The amount of laminin secreted into the medium was quantified by immunoprecipitation. Protein A–Sepharose beads were hydrated in immunoprecipitation buffer (0.5 mM sodium chloride, 0.01 M Tris–HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.02% sodium azide, 0.2 mM PMSF and 5 mM NEM), before incubation (90 min, room temperature) with 25 μl of polyclonal anti-laminin serum. The beads were thoroughly washed by centrifugation and resuspension with the same buffer, incubated with radiolabeled culture supernatants for 90 min, and washed 5 times with immunoprecipitation buffer followed by 3 washes with 0.01 M Tris- HCl, pH 8.8. The immunoprecipitates were solubilized in 2% SDS and analyzed for radiolabel content. A non-immune rabbit serum was used as a control.
Antiserum against laminin

A polyclonal antiserum was raised in rabbits by subcutaneous injection at two-week intervals (Westgate et al. 1984) of 0.5 mg laminin (essentially free of entactin) purified from the murine Engelbreth-Holm-Swarm tumor. Serum was collected biweekly and subjected to ELISA and immunoblotting analysis, to confirm monospecific reactivity to laminin. No reactivity against murine entactin, type IV collagen or other matrix components was detected. The antiserum was subsequently shown to react with all mouse, rat and human tissue basement membranes when examined by indirect immunofluorescence microscopy. A full characterization of this antiserum will be described elsewhere.

Analysis of SPARC (secreted protein acidic and rich in cysteine) and tissue plasminogen activator

Cultures were radiolabeled with 10 μCi ml⁻¹ [³⁵S]methionine in fibronectin-depleted medium and grown for 3 days. Supernatants, together with a PBS wash, were immunoprecipitated with antibodies to SPARC (a kind gift from Dr. B. L. M. Hogan, Vanderbilt University, TN). Each sample was preclearced with 10 μl of Protein G-Sepharose (Pharmacia) for 90 min at room temperature. Conjugates of Protein G-Sepharose with SPARC antiserum or non-immune serum were prepared by incubation for 90 min at room temperature with 10 μl serum in immunoprecipitation buffer. These were sedimented, extensively washed in immunoprecipitation buffer, and incubated for 45 min in 1% BSA in PBS at room temperature. Conjugates were sedimented, washed 5 times with immunoprecipitation buffer, then incubated with the precleared, radiolabeled culture supernatant for 90 min at room temperature. Immunoprecipitates were sedimented and resuspended repeatedly for 1 h in immunoprecipitation buffer, until no further radiolabel was detected in the washes. They were then washed 3 times in 0.01 M Tris–HCl, pH 6.8, before suspension in a 5% SDS solution to release the radiolabel, which was quantitated by scintillation spectroscopy.

Further supernatants were analyzed for tissue plasminogen activator content (tPA) using the Biopool solid-phase assay kit (Biopool AB, Umeå, Sweden). External standards were supplied with the assay and used to obtain a standard curve from which the results were calculated.

Results

Indirect immunofluorescence studies

PFHR-9 cultures seeded on fibronectin, laminin and native type I collagen substrata for 72 h showed both qualitative and quantitative differences in the deposition of the major basement membrane components. Qualitatively, the staining for laminin, type IV collagen and HSPG was most extensive in cultures grown on fibronectin. The basement membrane matrix produced by these cells on control (no precoating, Fig. 1A–C) and native type I collagen substrata (Fig. 1D–F) were markedly reduced in comparison to cells on fibronectin substrata (Fig. 1G–I).

Immunofluorescent staining of laminin deposited by cells grown on fibronectin substrata showed a very pronounced 'chicken-wire' pattern (Fig. 1G). In contrast, cells on uncoated substrata (Fig. 1A) and type I collagen substrata (Fig. 1D) showed much-reduced matrix deposition with only sparse and spotty laminin deposits, although intracellular staining was extensive.

When cultures were stained for type IV collagen or HSPG, cells grown on fibronectin substrata (Fig. 1H and I) again elaborated a more extensive network of matrix compared to cells grown in either control conditions (Fig. 1B and C) or on native type I collagen substrata (Fig. 1E and F). Fibronectin was not the only matrix component capable of increasing matrix deposition by PFHR-9. Laminin substrata also promoted deposition of type IV collagen (Fig. 1Q) and HSPG (Fig. 1R). In contrast, substrata coated with vitronectin (which probably adsorbs from serum onto control (uncoated) substrata) did not appear to induce differences in the amount or form of laminin, type IV collagen or HSPG when compared with serum-coated substrata (not shown).

In order to assess which domains of fibronectin promoted the enhanced deposition of extracellular matrix by PFHR-9 cells, purified fibronectin fragments were used as substrata. Incubation of PFHR-9 cells on the collagen-binding (40 x 10³ M*) fragment of fibronectin did not lead to appreciable matrix deposition, and the staining for laminin, type IV collagen and HSPG resembled that of cells grown on control or type I collagen substrata (not shown). In contrast, when the cell-binding (105 x 10³ M*; Fig. 1J–L) or the N-terminal heparin-binding (29 x 10³ Mr) fragment of fibronectin did not lead to more extensive network of matrix deposition than when cells were incubated on either control or type I collagen substrata. The C-terminal heparin-binding (31 x 10³ M*) domain gave similar results to those where the 29 x 10³ M* fragment was used as a substratum (not shown). The amount of matrix production by any of these fragments appeared, however, to be less than that of cells grown on intact fibronectin substrata. When a combination of 105 x 10³ M* and 29 x 10³ M* fragments was used as substrata (Fig. 1P), matrix deposition also appeared to increase above that seen with the individual fragments, although it was noted that the pattern of matrix deposition on the combined substrata of 105 x 10³ M* and 29 x 10³ M* fragments was different from the chicken wire pattern seen on substrata of intact fibronectin. Matrices examined by immunofluorescence microscopy after 24 h incubation were quite sparse in comparison to those seen after 48 and 72 h culture periods.

Table 1. Laminin synthesis by PFHR-9 cells on different substrata (control set as 100%; n=3)

<table>
<thead>
<tr>
<th>Substratum</th>
<th>% cts min⁻¹</th>
<th>% cts min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 (1104.7)</td>
<td>100 (581.0)</td>
</tr>
<tr>
<td>Collagen I</td>
<td>101.8 (1124.6)</td>
<td>109.4 (417.0)</td>
</tr>
<tr>
<td>Laminin</td>
<td>102.0 (1789.0)</td>
<td>118.2 (450.3)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>103.8 (3699.0)</td>
<td>136.0 (518.2)</td>
</tr>
<tr>
<td>105 x 10³ M*</td>
<td>144.9 (1501.4)</td>
<td>110.0 (419.1)</td>
</tr>
<tr>
<td>29 x 10³ M*</td>
<td>127.6 (1410.3)</td>
<td>129.0 (457.2)</td>
</tr>
<tr>
<td>105 + 29 x 10³ M*</td>
<td>165.7 (2001.4)</td>
<td>157.0 (521.8)</td>
</tr>
</tbody>
</table>

Immunoprecipitates of supernatant laminin are corrected for radiolabel precipitated with non-immune serum. (Counts are average per well in a 24-well plate.)
composed of heparin-binding fragments also appeared to result in increased laminin deposition over that detected in matrices on uncoated or type I collagen-coated substrata, with an increase of approximately 25%. When the 105×10^3 M_r and 29×10^3 M_r fragments were combined as a substratum, the PFHR-9 cells responded by increasing laminin deposition by approximately 86%.

Fibronectin substrata promoted the incorporation of three times the amount of chondroitin/dermatan sulfate into the matrix compared with control substrata. The cell-
Fig. 1. Immunofluorescence staining of PFHR-9 extracellular matrix for laminin (1st column), type IV collagen (middle column) and HSPG (3rd column). Cells were seeded for 72 h on uncoated substrata (A–C), type I collagen (D–F), fibronectin (G–I), 105×10^3 M, cell-binding domain of fibronectin (J–L), 29×10^3 M, N-terminal heparin-binding domain of fibronectin (M–O), and laminin (Q–R). The substratum was 105×10^3 M+ 29×10^3 M, fragments of fibronectin in P. A substantial matrix is present where cells have been seeded on fibronectin, its cell-binding domain or laminin, but is markedly reduced in amount on the other substrata. Small arrows indicate areas of matrix deposition, while the long arrow shows an intracellular accumulation of matrix component. The extent of matrix deposition is inversely correlated with the ability to discern individual cells (e.g. E and Q). Since the matrix is a fine substratum cost, it does not always stain intensely for type IV or HSPG. Bar, 50 μm.

Table 2. [35S]sulfate incorporation into matrix glycosaminoglycans by PFHR-9 cells on different substrata (control set as 100%; n=3)

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Chondroitin/dermatan sulfate</th>
<th>Heparan sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (unc)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Collagen I</td>
<td>86.3</td>
<td>89.5</td>
</tr>
<tr>
<td>Laminin</td>
<td>210.2</td>
<td>128.7</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>301.7</td>
<td>109.4</td>
</tr>
<tr>
<td>105×10^3 M</td>
<td>225.3</td>
<td>109.9</td>
</tr>
<tr>
<td>29×10^3 M</td>
<td>81.4</td>
<td>136.7</td>
</tr>
<tr>
<td>105+29×10^3 M</td>
<td>78.7</td>
<td>63.3</td>
</tr>
</tbody>
</table>

*A significant portion of the radiolabel remained after chondroitinase ABC and nitrous acid treatments. Since the matrix on these substrata is markedly clumped, it is likely that the values shown here underrepresent the actual matrix glycosaminoglycans, due to inaccessibility.*

binding domain of fibronectin was mostly responsible for this effect, while substrata of laminin also led to increased amounts of these glycosaminoglycans in the matrix compared with controls. Type I collagen substrata had comparable levels of matrix chondroitin/dermatan sulfate (CS/DS) to controls. Only laminin substrata and those of the 29×10^3 M heparin-binding domain of fibronectin led to increased heparan sulfate deposition into the matrix of PFHR-9 cells (Table 2). The amount of nitric acid-susceptible material in the combined 105×10^3 M, 29×10^3 M, fragment-containing substrata were surprisingly low, at 63.3% of control levels. However, a considerable amount of radiolabel remained as an SDS-soluble residue after chondroitinase ABC and nitrous acid treatment. It is unlikely that all this material was sulfated glycoprotein, but may have represented proteoglycan resistant to chondroitinase ABC and nitrous acid treatment in the aggregated matrix.

Secretion of basement membrane components and other products into PFHR-9 culture supernatants

In order to ascertain whether the effect of fibronectin substrata was to redirect specifically the export of basement membrane constituents to the ventral cell-substratum interface or merely to increase cellular export of matrix components, supernatants from radiolabeled cultures were also analyzed for laminin and proteoglycan content. As shown in Table 1, the amounts of laminin released into the culture medium by PFHR-9 cells varied with substratum conditions in a similar way to that seen with matrix deposition. Highest amounts were seen in supernatants from cultures on fibronectin substrata and those composed of 105+29×10^3 M, fibronectin fragment mixtures. Similarly, when [35S]sulfate-labeled proteoglycans from the supernatants of cultures on fibronectin substrata were compared with those of controls on uncoated substrata, an increase of approximately 25% in secretion of both HSPG and CS/DSPG was noted, although the proportion of these two proteoglycan types was unaltered (not shown).

These results show that there is no decrease in supernatant laminin or proteoglycan concomitant with an increased matrix pool of these components. Therefore, it appears that the fibronectin substrata promoted an overall increase in extracellular matrix export. Moreover, there did not seem to be a strong specific vectorial component in promotion of matrix assembly by PFHR-9 cells in response to either fibronectin or laminin substrata.

In order to examine whether the effect of substratum-adsorbed fibronectin was to elevate levels of synthesis of all exported macromolecules or only those of the extracellular matrix, we estimated the synthesis of SPARC and tPA. The former, although synthesized by PFHR-9 and other basement membrane-synthesizing cell types (Dziadek et al. 1986; Mason et al. 1986), appears not to be a basement membrane component in uovo (Sage et al. 1989). The latter (Saikusa and Rifkin 1988) may be involved in the regulation of matrix turnover. SPARC was immunoprecipitated from radiolabeled culture supernatants, while tPA synthesis was estimated by a solid-phase immunoassay. The results (Table 3) showed that their synthesis and release by cultured endodermal cells was largely unaffected by the substratum on which the cells were grown. It can be inferred, therefore, that the effects of fibronectin may be to elevate specifically the synthesis and export of basement membrane matrix molecules.

Effect of substrata on spreading of PFHR-9 cells

Since fibronectin and laminin promote adhesion and cytoskeletal organization of a number of cell types, we investigated whether the degree of cell spreading on each substratum correlated with extracellular matrix assembly. Spread cell areas were monitored through 3 days of incubation on different substrata (Table 4). Although fibronectin and its cell-binding fragment, as well as laminin, appeared to increase the degree of cell spreading at 24 h, this did not persist at 48 h. Type I collagen, in contrast, appeared to induce comparatively reduced spreading at 48 h but not at the other time points. After

Table 3. Release of SPARC and tPA by PFHR-9 cells into culture supernatants during growth on different substrata

<table>
<thead>
<tr>
<th>Substratum</th>
<th>SPARC (n=3)</th>
<th>tPA (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cts min⁻¹ ± s.d.</td>
<td>% control</td>
</tr>
<tr>
<td>Uncotted</td>
<td>460±116</td>
<td>100</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>386±96</td>
<td>77</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>461±170</td>
<td>100</td>
</tr>
<tr>
<td>Laminin</td>
<td>433±122</td>
<td>94</td>
</tr>
<tr>
<td>105×10^3 M</td>
<td>ND</td>
<td>7.25</td>
</tr>
<tr>
<td>29×10^3 M</td>
<td>ND</td>
<td>7.1</td>
</tr>
<tr>
<td>105+29×10^3 M</td>
<td>ND</td>
<td>6.95</td>
</tr>
</tbody>
</table>

SPARC results are corrected for cts min⁻¹ precipitated by non-immune serum. ND, not determined.

Basement membrane assembly
These results indicate that cell spreading is probably not
PFHR-9 proliferation on extracellular matrix substrata
Substratum 24h (±S.E.M.) 48h (±S.B.M.) 72h(±B.E.M.)
Collagen I
Laminin
Fibronectin
Uncoated

The interaction of the 105×10^{3} Mr region of fibronectin
Interactions of the cell-binding domain with PFHR-9
with several cell types occurs through members of the
role in the enhanced basement membrane matrix depo-
sition, since, at the 48 and 72 h time points, when a
majority of the matrix is deposited, there was no
correlation between the degree of spreading that a
substratum induced and the deposition of matrix.

Interference reflection microscopy of cells grown for 24,
48 or 72 h on uncoated substrata or those coated with type I
collagen, laminin, fibronectin or the cell-binding or
heparin-binding fibronectin fragments showed no appar-
ent difference in cell-substratum adhesion character-
stics. Under no circumstances were focal adhesions
formed; close-contact areas predominated (not shown).

PFHR-9 proliferation on extracellular matrix substrata
There was slight variability in the proliferation of PFHR-9
cells cultured on the different substrata (Table 5), with
more cells being present on type I collagen or control
substrata at 24 h. By 48 h, however, the number of cells on
diff the different substrata were closely similar and this
was maintained through the 72 h time point. The results
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sition on fibronectin and laminin substrata.

Interactions of the cell-binding domain with PFHR-9
cells
The interaction of the 105×10^{3} Mr, region of fibronectin
with several cell types occurs through members of the
integrin family of receptors, some of which are sensitive to
competitive RGD-containing peptides (Hynes, 1990; Ruos-
lahi et al., 1984; Parry 1987), we have investigated
some determining factors in the

Table 4. Spread cell area of PFHR-9 cells on different
substrata

<table>
<thead>
<tr>
<th>Substratum</th>
<th>24h (±S.E.M.)</th>
<th>48h (±S.B.M.)</th>
<th>72h(±B.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated</td>
<td>760.7 (40.5)</td>
<td>806.7 (37.0)</td>
<td>713.4 (29.8)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1068.2 (40.3)*</td>
<td>764.3 (39.7)</td>
<td>650.8 (37.3)</td>
</tr>
<tr>
<td>105×10^{3} Mr</td>
<td>1046.4 (66.3)</td>
<td>738.3 (31.9)</td>
<td>626.5 (35.8)</td>
</tr>
<tr>
<td>Laminin</td>
<td>1017.2 (29.8)*</td>
<td>736.7 (34.3)</td>
<td>501.5 (29.8)*</td>
</tr>
<tr>
<td>Collagen I</td>
<td>720.3 (29.5)</td>
<td>594.6 (21.7)*</td>
<td>621.2 (36.0)</td>
</tr>
</tbody>
</table>

*Significant difference from controls at P=0.01.

72 h incubation, only cells on laminin substrate showed
any significant difference in spread cell area (a reduction of
20–30 % compared to control and fibronectin substrata).
These results indicate that cell spreading is probably not the
key factor in modulating basement membrane matrix
deposition, since, at the 48 and 72 h time points, when a
majority of the matrix is deposited, there was no
correlation between the degree of spreading that a
substratum induced and the deposition of matrix.

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some determining factors in the

Table 5. Growth and proliferation on various substrata

<table>
<thead>
<tr>
<th>Substratum</th>
<th>24h (±S.E.M.)</th>
<th>48h (±S.B.M.)</th>
<th>72h(±B.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.47±0.43</td>
<td>23.46±2.28</td>
<td>22.79±2.28</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>9.5±2.1.09</td>
<td>24.16±2.91</td>
<td>37.74±1.33</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>11.78±0.59</td>
<td>23.13±0.68</td>
<td>36.94±1.07</td>
</tr>
<tr>
<td>Laminin</td>
<td>7.66±0.41</td>
<td>24.42±0.99</td>
<td>36.42±1.80</td>
</tr>
</tbody>
</table>

Discussion
In the present study, some determining factors in the
deposition of basement membrane matrix components
were investigated in the PFHR-9 cell line. Previous
experiments have shown that these cells produce an
extracellular matrix in vitro, which is rich in laminin,
entactin, type IV collagen and proteoglycans (Chung et al.
1977; Engvall et al. 1982; Keller and Furthmayr, 1986). An
important feature of PFHR-9 cells is that they do not
incorporate fibronectins into their matrices and so any role
of this component in normal basement membrane as-
sembly can be studied by addition of exogenous plasma
fibronectin or its fragments. Fibronectins are a much
studied family of glycoproteins involved in many cellular
functions such as adhesion and migration, and in vivo
these functions are probably important in development and
wound healing (Colvin, 1989; Hynes, 1990; Ruoslahti,
1986a; Thiery et al. 1989; Yamada, 1989). For these
reasons, and since earlier observations had indicated that
fibronectin substrata may increase the capacity of PFHR-9
extracellular matrix to sustain other cell lines (Gospodar-
owicz et al. 1984; Parry et al. 1987), we have investigated
whether fibronectin directly and specifically influences the assembly and composition of basement membrane matrices.

The matrices produced by PFHR-9 cells appear to differ both qualitatively and quantitatively, depending on the composition of the substratum. Immunofluorescence showed enhanced deposition of laminin, type IV collagen and HSPG by PFHR-9 cells grown on fibronectin substrata compared with type I collagen, control (uncoated) or vitronectin-coated substrata. Quantification of radiolabeled matrices indicated a specificity of response of cells to fibronectin. Laminin was deposited into the matrix at 50% more than control values. It is worth noting that fibronectin was not the only glycoprotein that influenced basement membrane assembly, since laminin itself also promoted an effect similar to that of fibronectin. Qualitative examination of matrices assembled on collagen I and vitronectin also gave similar results to controls. Elevated matrix deposition was not merely a preferential deposition of matrix on the substrata, i.e. a redirection of export, since amounts of laminin and proteoglycans released to the medium by cells grown on different substrata also increased in cultures grown on fibronectin. Fibronectin-induced laminin synthesis has also recently been reported for arterial smooth muscle cell cultures (Hedin et al. 1988). The effect of fibronectin and laminin on matrix assembly seemed relatively specific, since release of SPARC and TPA into culture supernatants was not modulated by the substratum on which the cells were plated. Other effects on cell–matrix interactions, regulated through occupation of fibronectin receptors, have also been recently documented. Ligation of fibronectin receptors in synovial fibroblasts, for example, can modulate the gene expression of matrix-degrading enzymes (Werb et al. 1989).

The mechanism by which fibronectin promoted basement membrane matrix assembly in PFHR-9 cells was also investigated. Two hypotheses were considered. First, fibronectin may enhance cell adhesion and spreading, which may then lead to early and enhanced matrix deposition. Alternately, fibronectins, through their multiple ligand-binding capabilities may ‘stabilize’ the matrix as it is synthesized and deposited at the cell–substratum interface. To approach these questions we first established whether differences in cell adhesion, spreading or number were seen over the 72 h experimental period. The data showed that those components promoting matrix deposition did induce slightly increased spreading at 24 h but this difference did not persist. Immunofluorescence studies on 24 h cultures showed that matrix deposition on substrata was sparse, the majority being deposited in the following 48 h. By 72 h, when matrices were quantified, no differences were discernable in cell spreading. Furthermore, examination of the nature of cell–substratum adhesion by interference reflection microscopy also showed no differences between cells plated on any of the substrata. Growth rates on all substrata were also essentially the same over the 72 h period. Therefore, we conclude that the effects of fibronectin are not the result of enhanced cell adhesion, spreading or survival. Plating efficiencies of these cells on all substrata were very high. We therefore do not believe that the data stem from selection of PFHR-9 subpopulations, some having enhanced matrix synthesizing capability.

Our results showed that the 105 × 10^6 M cell-binding domain (containing the RGD sequence) was a major influence on PFHR-9 matrix deposition. In addition, the attachment of these endodermal cells to fibronectin substrate was sensitive to the addition of a tetradecamer peptide containing the RGD sequence, whereas a control peptide was much less effective. PFHR-9 cells express the β1-integrin subunit on their surfaces, detectable by indirect immunofluorescence microscopy with a specific antibody. It has been proposed that the αvβ3-integrin binds to fibronectin through the RGD-containing region of the molecule (Takada et al. 1987). Taken together, it seems as though a major signal from fibronectin that leads to enhanced matrix deposition is mediated through the cell surface integrins. We are currently characterizing these receptors in detail, but it is interesting that PFHR-9 cells possess receptors for fibronectin even though synthesis of the ligand is minimal.

The other mechanism that may underlie the effect of fibronectin on matrix assembly is that the substratum serves as a ligand to which secreted basement membrane components may bind. The matrix coat may then ‘nucleate’ and/or ‘stabilize’ the forming matrix. Consistent with this is the known ability of glycosaminoglycans and proteoglycans to bind fibronectin (reviewed by Yamada, 1989; Hynes, 1990). The N-terminal heparin-binding domain as a substratum was seen in these experiments to contribute to matrix deposition, notably of HSPGs, supporting this hypothesis. The cell-binding domain is reported to have a cryptic dermatan sulfate-binding region (Lewandowska et al. 1987), also highly consistent with our data. A type three repeat structure to the N-terminal end of the cell-binding domain of fibronectin is also reported to bind the core protein of basement membrane HSPG (Heremans et al. 1990). These multiple interactions may be instrumental in initiating matrix assembly through the proteoglycans. Proteoglycans are known to occur in virtually all basement membranes (Couchman and Höök, 1988; Roussalki, 1988a; McCarthy et al. 1989), and have the capacity to interact with laminin and type IV collagen (Timpl and Dziedzak, 1986) and, therefore, may in our system form a bridge between the substratum and these major basement membrane components. This may also explain why laminin itself, as a substratum molecule, also led to enhanced basement membrane assembly by PFHR-9 cells.

Proteoglycans also occur on the cell surface, however (Saunders and Bernfield, 1988), and in some cases have transmembrane protein cores. Interactions with fibronectin can lead to striking changes in cytoskeletal organization (Woods et al. 1986). Our latest data for fibroblasts indicate that protein kinase C may be involved in this process (Woods et al. 1990). Therefore, in the present studies, interactions of fibronectin with cell surface proteoglycans may lead to signalling events, in turn affecting basement membrane macromolecule export, either directly, or through the agency of a change in adhesion characteristics. All this is consistent with the idea of continuous exchange of information between cytoskeleton, plasma membrane and extracellular matrix that regulates growth and differentiation (Bissel and Barcellos-Hoff, 1987; Li et al. 1987).

In conclusion, our results with an in vitro system have shown that fibronectin can promote basement membrane formation, in a similar manner to that proposed previously, in tissue recombination experiments (Brownell et al. 1981). This unrecognized function of fibronectin may be important for tissue maturation and wound repair, sites at which this family of glycoproteins are often enriched. The effect on ECM synthesis is probably mediated through a transmembrane signalling event, in addition fibronectin—
glycosaminoglycan interactions may stabilize or 'nucleate' matrix assembly.

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