J. Cell Sci. 63, 287–301 (1983)
Printed in Great Britain © The Company of Biologists Limited 1983

FIBRONECTIN-INDEPENDENT ADHESION OF FIBROBLASTS TO EXTRACELLULAR MATRIX MATERIAL: PARTIAL CHARACTERIZATION OF THE MATRIX COMPONENTS

P. A. HARPER, PATRICIA BROWN AND R. L. JULIANO*
Department of Pharmacology, University of Texas Medical School, Houston, TX 77025, U.S.A.

SUMMARY

Fibroblasts can adhere to extracellular matrix (ECM) material by fibronectin-dependent (type I) and fibronectin-independent (type II) mechanisms. In this report we investigate the biochemical characteristics of ECM that contribute to type II adhesion. ECM capable of mediating type II adhesion is produced primarily by normal diploid fibroblasts, but not by transformed cells or epithelial cells. Treatment of fibroblast ECM under conditions that result in the removal of most of the ECM lipid or most of the ECM glycosaminoglycan does not impair type II adhesion. Likewise, treatment of the ECM with large amounts of purified collagenase does not block type II adhesion. However, treatment of ECM with low doses of trypsin or with an agent that reacts with tyrosine residues, results in complete ablation of the ability of the ECM to support type II adhesion. On the basis of these observations we suggest that the matrix component(s) mediating type II adhesion are non-collagenous proteins or glycoproteins.

INTRODUCTION

Fibroblasts can interact with and adhere to macromolecular components of the extracellular matrix (EMC) in which they are normally situated. The chemical makeup of the ECM is extremely heterogeneous and includes lipids, proteoglycans, collagens and other proteins (Cathcart & Culp, 1979*a*,*b*; Culp, 1978; Grinnell, 1978; Kleinman, Klebe & Martin, 1981; Rollins & Culp, 1979). One mechanism of fibroblast–ECM adhesion is mediated by fibronectin (Fn), a pericellular, multi-domain protein, which appears to be localized at focal cell–substratum contact sites and can bind to macromolecules including collagen, glycosaminoglycans and fibrinogen, as well as to the cell surface (Lindale & Hook, 1978; Mosher, 1980; Pearlstein, Gold & Garcia-Pardo, 1980; Rouslahti & Engvall, 1980; Yamada & Olden, 1978). Recently, however, it has become abundantly clear that cells also exhibit adhesion mechanisms that are not mediated by Fn (Takeichi, 1977; Terranova, Rohrbach & Martin, 1980; Wylie, Damsky & Buck, 1979; Shor & Court, 1979). Thus, we have described a set of Chinese hamster ovary (CHO) cell variants (AD^{v} cells) selected for non-adhesiveness to serum-coated tissue-culture plastic; these cells have totally lost their ability to employ the Fn-mediated adhesion mechanism (Harper

* Author to whom correspondence should be addressed.

& Juliano, 1980). However, AD^v cells readily adhere, via a Fn-independent mechanism, to extracellular matrix (ECM) material produced by normal diploid fibroblasts (Harper & Juliano, 1981*a*). We have also shown that the Fn-independent attachment of AD^v cells to the ECM is mediated, in part, by a high molecular weight glycoprotein (gp 265) expressed on the AD^v cell surface (Harper & Juliano, 1981*b*). The Fn-independent adhesion mechanism (which we now designate type II adhesion) is also expressed in parental cells, but is masked by the predominant Fn-dependent mechanism (designated type I adhesion) (Harper & Juliano, 1981*b*).

We describe here our results on the characterization of the ECM components that help to mediate type II adhesion. We have used three approaches in these studies: (a) examination of the ability of various cell types to produce ECM capable of supporting type II adhesion; (b) attempting the reconstitution of functional matrix by mixing of purified matrix components; (c) selective chemical and enzymic perturbation of the matrix followed by tests of its ability to support type II adhesion. Our data suggest that ECM components known to play a role in Fn-mediated (type I) adhesion, such as collagen and glycosaminoglycans, are not involved in Fn-independent (type II) adhesion. In addition the enzymic and chemical sensitivities of the ECM's capacity to support type II adhesion suggest that the components mediating this process are proteins.

MATERIALS AND METHODS

Cells

The growth and maintenance of parental (WT) and adhesion-variant (Ad^{v}F11) CHO cells has been fully described elsewhere (Harper & Juliano, 1980). Human diploid fibroblasts (HDFs) from skin biopsies (5–15th passage) were cultured in a-MEM plus 10% foetal calf serum and were usually split at a 1:2 ratio after reaching confluence. Swiss mouse 3T3 fibroblasts and hamster BHK fibroblasts were also maintained, usually at subconfluent densities, in the above medium. Confluent cultures of MDCK cells, a dog kidney epithelial line, were obtained from Dr Julia Lever, Department of Biochemistry, University of Texas. Confluent cultures of YN-4 and CL-S1 tumorigenic and non-tumorigenic mouse mammary epithelial lines, were obtained from Dr Dan Medina, Baylor College of Medicine.

Preparation of substrata

The preparations of Fn-coated, gelatin-coated and ECM-coated substrata have been described in detail elsewhere (Harper & Juliano, 1981*a*,*b*). The preparation of ECM-coated substrata in tissue-culture dishes entails: (a) the growth of human diploid fibroblasts (or other cells) to the desired density (most experiments were done with 1–2 day post-confluent cultures or 5 to 8($\times 10^5$) cells per 35 mm dish); (b) washing the cell layer with phosphate-buffered saline, pH 7·2 (PBS) followed by a 30 min incubation at 37 °C in PBS containing 1 mM-EGTA, 1 mM-phenylmethyl sulphonyl fluoride and 1 mM-benzamidine; (c) removal of the cells by sharply tapping the dishes, followed by gentle washing with PBS. For fibroblasts this results in complete cell removal and the retention on the dish of a layer of ECM capable of supporting type I or type II adhesion. This procedure was applied to other cell types, although the nature of the material retained on the dish under these conditions has not been investigated (in some cases up to 60 min incubation with EGTA-containing solution was required to achieve full removal of cells). The material derived by EGTA treatment of fibroblasts has been extensively analysed by Culp (1978), who has termed this material SAM (substrate-attached material). It is composed of proteoglycans and other ECM components (Rollins & Culp, 1979) as well as fragments of cell membrane and cytoskeleton.

Collagen gels consisting of 2 mg/ml collagen (Vitrogen Corporation) were prepared according to the manufacturer's instructions. In some instances purified glycosaminoglycans (GAGs; obtained from Dr B. Mathews, Department of Pediatrics, University of Chicago) were either incorporated into the matrix prior to gelation or the gels were equilibrated with a solution of the GAGs just before use.

Conditioned growth medium (a-MEM, 10% foetal calf serum plus antibiotics) was collected from confluent monolayers of HDF or WT CHO cells or from exponentially growing suspension cultures of WT and AD^{v}F11 cells. The medium was centrifuged to clear it of cells, and stored frozen until needed. To prepare substrata consisting of adsorbed components from the conditioned medium, plastic tissue-culture dishes (35 cm diameter) were incubated with 2 ml of media for 2–4 h at 37 °C. The medium was then replaced by a sample of adhesion buffer (a-MEM containing 1 mg/ml bovine serum albumin (BSA) and antibiotics).

Radiolabelled ECM substrata were prepared by metabolically labelling the HDFs from which they were derived. Monolayers 50–60% confluent were incubated with 10 μCi/ml [^{35}S]sulphuric acid to label sulphated GAGs, 10 μCi/ml [^{35}S]methionine to label proteins, or 10 μCi/ml [^{3}H]oleic acid to label the phospholipids; labelling was done in regular growth medium. The HDFs were allowed to reach confluency and ECM substrates were prepared as above.

Adhesion assay

Measurement of the adhesion of ^{3}H-labelled WT or AD^{v}F11 cells to the various substrata has been fully described elsewhere (Harper & Juliano, 1980). Adhesion experiments were usually conducted in a-MEM plus 1 mg/ml BSA (adhesion buffer).

Extraction and chemical modification of the ECM

Immediately following the preparation of the ECM, the dishes were incubated for 60 min at 37 °C with one of the following: (a) 0·5 unit/ml hyaluronidase; (b) 0·5 unit/ml chondroitinase AC or ABC; (c) 100 units/ml of protease-free bacterial collagenase (all of the above prepared in adhesion buffer); (d) 4 M-urea; (e) 0·1% Triton X-100 (TX100); (f) PBS control (all of the above in PBS for 60 min at 37 °C), or with various concentrations of trypsin in PBS for 10 min at room temperature. Following incubation the dishes were washed extensively with PBS containing 1 mg/ml BSA and then used as substrata in the adhesion assay. If the dishes were extracted with trypsin, 10 mg/ml soy bean trypsin inhibitor was added to the wash to stop the action of the protease.

Metabolically labelled ECM was extracted with known amounts of the various agents mentioned above. Following incubation the dishes were extensively washed and any material remaining associated with the dishes was solubilized in hot 0·2% sodium dodecyl sulphate (SDS). The radioactivity of known samples of both the extracted material and the SDS-solubilized material was determined in a scintillation counter and the data expressed as the amount of material removed and as a percentage of the total on the dish.

More extensive collagenase treatments of the ECM were done as follows. Collagenase (Biofactures, type III) was prepared in calcium acetate buffer (pH 7·4) and incubated with fibroblast ECM at doses ranging from 0–200 units/ml for 60 min at 37 °C. As a control for the efficacy of the enzyme in degrading collagen, the same doses of enzyme in 1 ml were incubated with 2 ml of solidified Vitrogen (Collagen Corporation) and the liquification of the gel was scored on the basis of 0 (solid) to +4 (liquid).

Chemical modification of the ECM generally followed the procedures described by Grinnell & Minter (1979) for the modification of serum-coated substrata. Thus tyrosine residues were modified by iodination induced by chloramine T as follows. ECM substrata previously washed with PBS were incubated with 0·4 mg/ml chloramine T plus various concentrations of sodium iodide in 0·1 M-sodium phosphate buffer (pH 7·1) for 5 min at 22 °C. The reaction was stopped by the addition of 1 ml of 0·5 mg/ml sodium metabisulphite and the treated ECM substrata were well washed with PBS prior to use. Carboxy groups were modified by treatment with a water-soluble carbodiimide (EDAC) and glycine methyl ester. The PBS-washed ECM substrata were incubated with various concentrations of EDAC and 1·0 M-glycine methyl ester in 0·9% NaCl for 10 min at 22 °C. The treated ECM were then washed twice with 1 M-glycine and twice more with PBS prior to use.

Analysis of [^{35}S]sulphated GAGs

Following extraction with chondroitinase, 4 M-urea, 0·1 % TX100 or various concentrations of trypsin as described above, the [^{35}S]sulphated GAG component of the resulting ECM was analysed by agarose gel electrophoresis as described by Mourao & Machado-Santelli (1978).

Gel electrophoresis

The ECM material was extracted with various agents as described above. Any material remaining on the dish was solubilized into an excess of hot SDS, then precipitated with 10 vol. of acetone: ammonia (5·3 : 0·3, v/v). The precipitate was resolubilized directly into gel solubilizer and a sample was analysed by polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). After the gels were stained with Coomassie Brilliant Blue to visualize the proteins, the gels were prepared for PPO-enhanced autoradiography according to the method of Laskey & Mills (1975).

RESULTS

Type II adhesion to various ECM substrata

The ability of various types of ECM, prepared by EGTA extraction, to support type II adhesion was assessed by measuring the attachment of ^{3}H-labelled AD^{v}F11 cells to ECM-coated substrata. As seen in Table 1A there is a progressive, but not necessarily linear, increase in the ability of HDF ECM to support type II adhesion as the cell density of the original fibroblast layer increases. In contrast to the ECM from diploid fibroblasts, ECM derived from a variety of epithelial or fibroblastic cell lines failed to support adhesion of AD^{v}F11 cells (Table 1B). This suggests either that these permanent cell lines fail to make the attachment factor required by F11, or that the material is readily lost during the procedure for ECM preparation.

Attempts at reconstitution of ECM for type II adhesion

We have previously shown that, unlike Fn substrata, the ECM from HDF cells could promote that attachment of the non-adhesive variant AD^{v}F11 cells. The adhesion assay that we have described, however, does not distinguish between matrix constituents that have been deposited by the cells and soluble components subsequently adsorbed to the substratum. To test whether it was possible to reconstitute the adhesive property of the ECM with conditioned media alone, dishes were prepared coated with material adsorbed from WT-, AD^{v}- or HDF-conditioned media. The ability of both WT and AD^{v} cells to adhere to these various substrata was assessed and compared with that of ECM substrata. The results obtained differed markedly between WT and AD^{v} cells (Table 2A). Clearly, conditioned media from all sources promoted the attachment of the WT cells. The AD^{v} cells, however, did not adhere to substrata treated with conditioned media alone. Thus, it would seem unlikely that soluble HDF components are responsible for ECM-mediated adhesion of the AD^{v} cells.

Collagen, a major component of the ECM, has been implicated, both directly and indirectly, in cell–substratum attachment. To determine whether the adhesion of the AD^{v} cells was mediated by collagen, we assessed the ability of both cell lines to attach

Table 1. *Adhesion of AD^{v}F11 cells to ECM-coated substrata*

A. *Effect of cell density – human diploid fibroblast (strain 1580) ECM*

Diploid fibroblast cells per 35 mm dish	% Adhesion of F11
0 (serum only)	4·0
$1{\cdot}4 \times 10^5$	20·2 ± 2·2
$3{\cdot}3 \times 10^5$	20·3 ± 5·6
$3{\cdot}6 \times 10^5$	26·8 ± 3·8
$5{\cdot}5 \times 10^5$	33·8 ± 5·8
$8{\cdot}0 \times 10^5$	49·6 ± 5·7

ECM was prepared from strain 1580 fibroblast cultures at the indicated density. The adhesion of AD^{v}F11 cells was measured during 60 min at 37 °C in adhesion buffer as described in Materials and Methods.

B. *AD^{v}F11 adhesion to ECM from various cell types*

Source of ECM	% Adhesion of F11
Human diploid fibroblasts (confluent)	49·6 ± 5·7
MDCK cells (confluent)	19·3 ± 2·9
Serum-coated dishes	5·2 ± 3·6
Human diploid fibroblasts (confluent)	52·9 ± 4·1
Swiss 3T3 (confluent)	1·3 ± 0·3
Serum-coated dishes	4·1 ± 0·9
Human diploid fibroblasts (confluent)	53·2 ± 3·4
BHK fibroblasts (confluent)	2·9 ± 0·5
Serum-coated dishes	3·4 ± 0·6
Human diploid fibroblasts (confluent)	35·1 ± 0·8
Cl-Sl cells (confluent)	4·0 ± 0·6
YN-4 cells (confluent)	3·4 ± 1·0
Serum-coated dishes	2·7 ± 0·5

All adhesion studies were performed in adhesion buffer for 60 min at 37 °C using a 5 % CO_2 incubator.

to various types of collagen substrata. As seen in Table 2B, all of the collagen substrata were capable of promoting the attachment of WT cells. The AD^{v} cells, however, failed to attach, whether the substrate consisted of native collagen in the form of a gel, collagen dried down onto the substrate as a thin film, or heat-denatured collagen (gelatin). No adhesion was observed for either cell line in the absence of Fn. Recently, much work has been focused on the role of GAGs in substrate adhesion, especially as both collagen and Fn can interact directly with these matrix components (Jilek & Horman, 1979; Lindale & Hook, 1978; Mosher, 1980). We therefore investigated the possibility that the AD^{v} cells could adhere to the substrate via a complex of Fn, collagen and various GAGs; substrata composed of collagen plus GAGs present either during or after gelation were assayed for the adhesion of AD^{v} or WT cells. The results we obtained were similar to those for collagen alone (Table 2C). Although WT

Table 2. *Attempts at reconstitution of type II adhesion*

A. *Effect of conditioned media on adhesion*

Media	% Adhesion	
	WT	$AD^{V}F11$
HDF-conditioned	94·0	4·8
WT-conditioned	91·2	5·9
$AD^{V}F11$-conditioned	94·9	ND

Conditioned media from confluent monolayers of HDF and WT cells or from suspension cultures of WT and $AD^{V}F11$ cells were collected as described in Materials and Methods. The adhesion of both WT and $AD^{V}F11$ cells to plastic substrata coated with these various media was assayed during 90 min at 37 °C as described. No difference was observed whether conditioned medium was used to precoat the substratum or was present throughout the assay. The data represent the means of three determinations. ND, not determined.

B. *Collagen as a substrate*

Substrata	% Adhesion	
	WT	$AD^{V}F11$
Native collagen gel	84	4
Dried native collagen	81	2
Dried gelatin	85	4
Tissue-culture plastic	87	8

Various collagen substrata and tissue-culture plastic were coated with FN (human serum CIG) as described in Materials and Methods. These substrata were then assayed for their ability to promote cell attachment during 90 min at 37 °C. The data represents the mean of three determinations.

C. *GAGs as a substrate*

Substrata	% Adhesion	
	WT	$AD^{V}F11$
Collagen + serum	85	1
Collagen + CIG	83	6
Collagen + CIG + hyaluronic acid	88	1
Collagen + CIG + heparan sulphate	82	1
Collagen + CIG + chondroitin sulphate	88	1
Collagen + all the above	88	3

Collagen gels were prepared and in some instances were incubated with various GAGs (10 mg/ml) and serum (10 %) or CIG (100 μg/ml) for 60 min at room temperature. After washing with PBS the ability of both WT and AD^{V} cells to adhere to these substrata was then determined during 90 min at 37 °C. The data represent the mean of three determinations. Essentially the same results were obtained if the GAGs, serum and CIG remained throughout the adhesion.

cells adhered readily to all substrates tested, in no instance, except for ECM-coated dishes, was AD^v cell attachment observed.

Selective extraction and modification of the ECM

WT cells appeared capable of adhering to any of the reconstituted matrices, unlike AD^v cells, which failed to attach. Our results, however, do not rule out the possibility that the various components we tested failed to assemble in the correct orientation or stoichiometry. Therefore, we tried the converse approach; namely, selective extraction. It would be reasonable to assume that components that are extracted without perturbing AD^v adhesion are non-essential for cell attachment via the type II process.

Efficiency of the extracting agents. We have previously reported that extracting the ECM with a variety of agents has little or no effect on the suitability of the matrix to support cell attachment (Harper & Juliano, 1981*b*). To verify whether the conditions of the extraction were appropriate we determined the amount of material removed by the extracting procedures using ECM derived from HDF metabolically labelled with

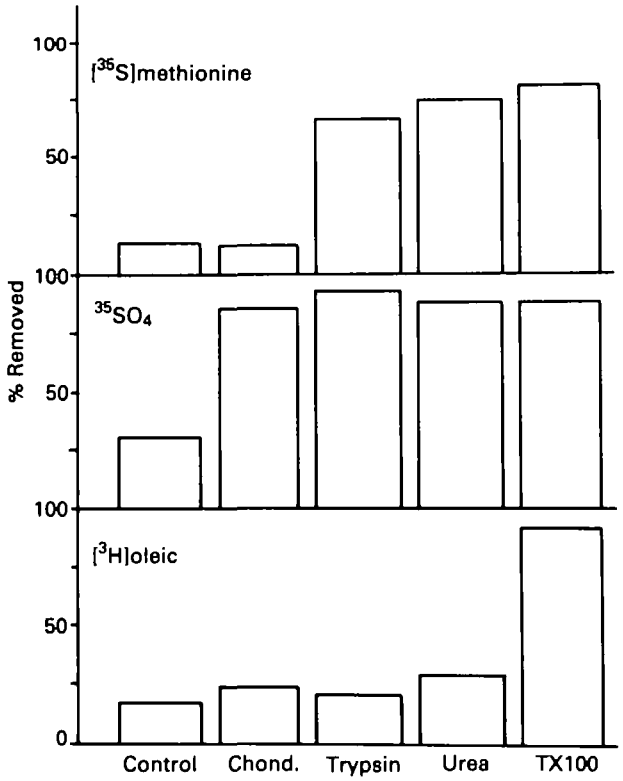

Fig. 1. Efficiency of the extracting agents. ECM substrata derived from HDFs incubated with [^{35}S]methionine to label protein, [^{35}S]sulphate to label sulphated GAGs or [^{3}H]oleic acid to label lipid, were extracted with various agents as described in Materials and Methods. The amount of radioactivity that was released was determined and expressed as a percentage of the total in the ECM. Chond. (chondroitinase), 0·5 ml; trypsin, 5 μg/ml; TX100, 0·1 %; urea, 4 M.

[^{35}S]methionine to label protein, [^{35}S]sulphuric acid to label sulphated GAGs and [^{3}H]oleic acid to label the lipid components of the cells. The amount of radioactivity that was extracted was determined and expressed as a fraction of the total associated with the ECM on the dish. As seen in Fig. 1, 0·5 unit/ml of chondroitinase ABC removed 85 % of the GAGs but did not extract either protein or phospholipid significantly. Urea extracted both protein and GAGs, whereas 0·1 % TX100 removed all three components. In all instances, no significant reduction in adhesion was observed. It is interesting to note that both urea and TX100 extracted a major portion of the protein of the ECM without reducing the adhesion of the cells. Trypsin, on the other hand, at 1·0 μg/ml reduces AD^{V} adhesion to 25 % but removes only 30 % of the protein component of the matrix. It would appear that the adhesion-promoting factor is resistant to simple solubilization, but requires a protease to release it from the substrate or to inactivate it.

Effects of various extracting agents on ECM-mediated adhesion. Glycosaminoglycans are integral components of the ECM. To extract these components specifically, we incubated the ECM with chondroitinase AC to extract chondroitin 4- and 6-sulphate, hyaluronidase to extract hyaluronic acid or chondroitinase ABC to extract chondroitin 4- and 6-sulphate and dermatan sulphate. Collagenase was also used since

Table 3. *Extraction of fibroblast ECM*

A

Treatment of ECM	% Adhesion	
	WT	AD^{V}F11
ECM (none)	100	100
ECM + chondroitinase AC	ND	93
ECM + hyaluronidase	ND	83
ECM + chondroitinase ABC	99	99
ECM + collagenase	ND	75

ECM substrata were prepared and subsequently incubated with PBS, 0·5 u./ml chondroitinase AC, 0·5 u./ml chondroitinase ABC, 10 u./ml hyaluronidase or 100 u./ml collagenase for 60 min at 37 °C. The resulting substrata were then assayed for their ability to promote cell attachment during 90 min at 37 °C. The results are expressed as the percentage of attachment to the unperturbed control ECM for each cell type and are the means of three determinations. ND, not determined.

B

Treatment of ECM	% Adhesion of F11	Liquefaction of vitrogen
None	50·6 ± 4·3	0
Collagenase 50 u.	32·8 ± 4·5	+
Collagenase 100 u.	49·3 ± 5·0	++
Collagenase 200 u.	51·8 ± 3·5	+++

Collagenase treatment for 1 h at 37 °C in calcium acetate buffer.
Adhesion assay, 1 h at 37 °C in adhesion buffer. 0, solid; +++, liquid.

collagen is a major fibrillar component of the ECM and can interact with the GAGs. As seen in Table 3, none of these extractions resulted in any decrease in AD^v adhesion. It should be noted, however, that the chondroitinases used do not remove heparan sulphate. Of all the GAGs heparan sulphate has been most strongly implicated in substrate adhesion and therefore, if still present on the substratum, could be responsible for the observed attachment. To determine which classes of SO_4-GAGs were removed not only by the enzymes but also by urea and TX100, we analysed the matrix material remaining on the dish after the ECM was extracted. Fig. 2 shows an autoradiogram of the [^{35}S]sulphated GAGs left on the dish after various extractions. Following treatment with chondroitinase ABC, most if not all of the chondroitin sulphate (CS) and dermatan sulphate (DS) were removed. Urea and TX100 appear to remove most of the heparan sulphate (HS), whereas trypsin removes most of the CS and a significant amount of the DS and HS. Although none of the agents simultaneously removes all three of the major classes of sulphated GAGs, there is no consistent pattern for retention of any one particular class. Since chondroitinase ABC, urea and TX100 treatments do not affect AD^v cell attachment, it seems likely that none of the GAG classes removed by these treatments (CS, DS, HS) is directly involved in type II adhesion.

Proteolytic sensitivity of the ECM. We have previously reported that the ECM component that mediates type II adhesion is trypsin-sensitive (Harper & Juliano, 1981*b*). Incubating the substrate with 5 μg/ml of trypsin completely abolishes AD^v

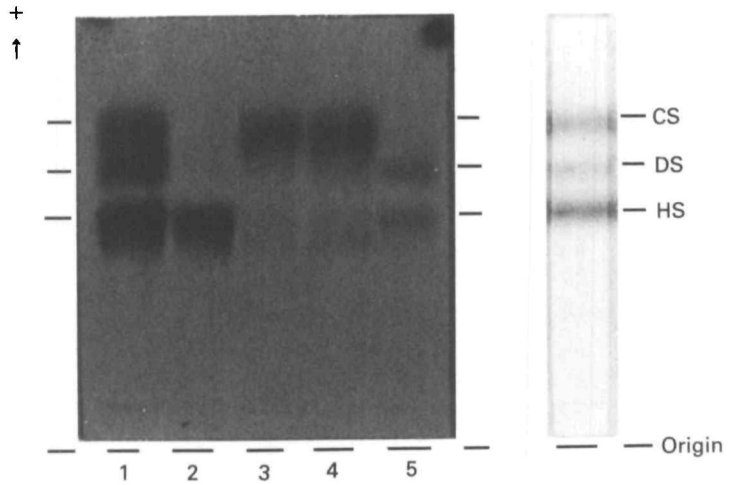

Fig. 2. Identification of sulphated GAGs in the ECM. Sulphated GAGs labelled with [^{35}S]sulphuric acid were isolated from ECM that had been extracted with various agents, and the GAGs were analysed by agarose gel electrophoresis. Equal samples of the total extract were applied to the gel and the resulting autoradiogram is shown and compared with known standards. Lane 1, PBS control; lane 2, extracted with 0·5 u./ml chondroitinase ABC; lane 3, extracted with 4 M-urea; lane 4, extracted with 0·1 % TX100; lane 5, extracted with 1 μg/ml trypsin; CS, chondroitin sulphate; DS, dermatan sulphate; HS, heparan sulphate.

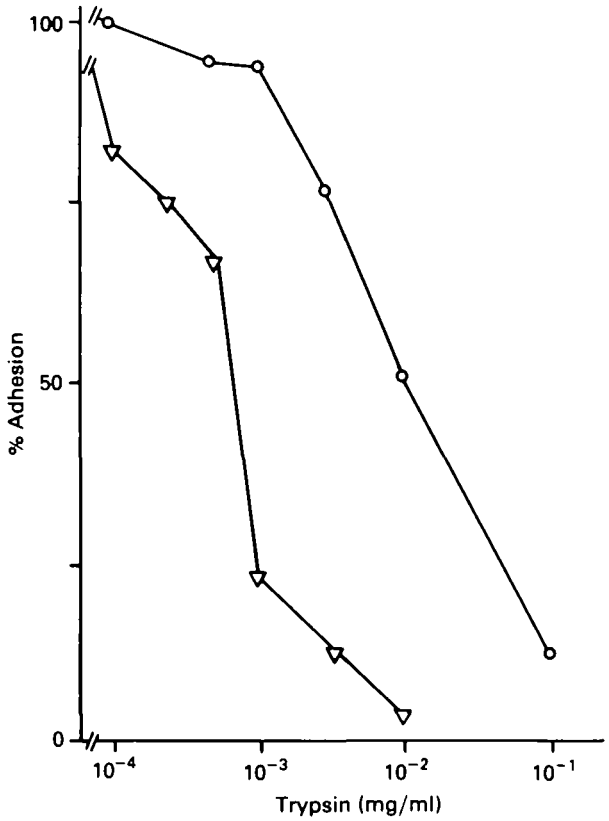

Fig. 3. Adhesion of WT and AD^{v} cells to trypsinized substrata. ECM- and Fn-coated substrata were incubated with various concentrations of trypsin for 10 min at room temperature. Following extensive washing with PBS containing either 1 mg/ml BSA or 1 mg/ml soy bean trypsin inhibitor, the ability of these substrata to support cell attachment was determined as described in Materials and Methods. The data represent the mean of three separate experiments and are expressed as % of untreated control. (○——○) WT on trypsinized Fn substrata; (▽——▽) AD^{v} on trypsinized ECM substrata.

cell attachment. To explore this phenomenon further both ECM and Fn substrata were incubated with various concentrations of trypsin and the ability of both WT and AD^{v} cells to attach to the resulting substrata was determined. The data shown in Fig. 3 clearly indicate that both Fn and ECM substrates are sensitive to trypsin. A reduction of 50% in WT adhesion occurs if Fn substrates are incubated with 6 μg/ml of trypsin, whereas only 0·6 μg/ml is required to reduce AD^{v} adhesion by a similar amount. It is not clear if this implies that the ECM components mediating type II adhesion are more sensitive to proteolysis than Fn or if there is simply more adhesive material available on the Fn substratum.

Chemical modification of the ECM. As described in Materials and Methods, amino acid residues of the ECM were modified using various reagents in a manner similar to that employed by Grinnell & Minter (1979) to modify fibronectin-coated substrata. As seen in Table 4 modification of ECM carboxyl groups with the water-soluble

Table 4. *Chemical modification of the ECM*

A. *Carbodiimide treatment*

Substratum	Treatment	% Adhesion of AD^{V}F11
Diploid fibroblast ECM	None	61·0 ± 2·1
Diploid fibroblast ECM	0·00025 M-EDAC +1 M-glycine methyl ester	51·5 ± 3·7
Diploid fibroblast ECM	0·0025 M-EDAC +1 M-glycine methyl ester	58·9 ± 7·1
Diploid fibroblast ECM	0·025 M-EDAC +1 M-glycine methyl ester	57·4 ± 4·7
Diploid fibroblast ECM	1 M-glycine methyl ester	51·1 ± 3·0
Serum	None	2·8 ± 1·2
Serum	0·025 M-EDAC +1 M-glycine methyl ester	7·9 ± 1·4

B. *Chloramine T treatment*

Substratum	Treatment	% Adhesion of AD^{V}F11
Diploid fibroblast ECM	None	64·1 ± 3·2
Diploid fibroblast ECM	Chloramine T +0·001 mM-NaI	12·6 ± 1·4
Diploid fibroblast ECM	Chloramine T +0·01 mM-NaI	4·4 ± 0·6
Serum	None	3·6 ± 0·4
Serum	Chloramine T +1 mM-NaI	8·8 ± 2·9

Chemical modifications were performed as described in Materials and Methods. Adhesion of F11 cells was measured during 60 min at 37 °C in adhesion buffer.

carbodiimide EDAC plus glycine methyl ester had no effect on the ability of the ECM to support type II adhesion. By contrast, modification of ECM tyrosine residues by chloramine T plus NaI resulted in a marked decrease in the adhesive capacity of the ECM. This differs markedly from the case of Fn-coated substrata, where modification of either carboxyl group or tyrosine residues abolished the ability to support adhesion (Grinnell & Minter, 1979).

PAGE of the ^{35}S-labelled ECM. The extreme sensitivity of protease treatment suggested that it might be possible to identify the ECM component by this criterion. Thus ECM extracted with trypsin, urea or TX100 were solubilized and concentrated for analysis by polyacrylamide gel electrophoresis (PAGE). As has been previously reported by Cathcart & Culp (1979*a*,*b*), Coomassie Brilliant Blue staining of the gels for protein resulted in a pattern very similar to that of foetal calf serum. To distinguish ECM components laid down by the cells, [^{35}S]methionine-labelled fibroblasts were

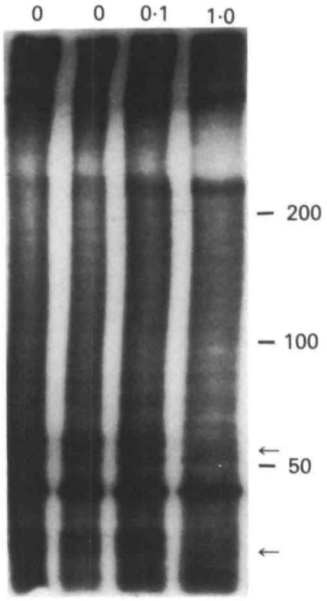

Fig. 4. PAGE of ECM, from [^{35}S]methionine-labelled fibroblasts, treated with various doses of trypsin for 10 min at room temperature. The reaction was stopped with trypsin inhibitor and the ECM analysed by PAGE (7·5% gel) as described in Materials and Methods. 0, no treatment; 0·1 μg/ml trypsin, 1·0 μg/ml trypsin. Equal amounts of CPM were loaded onto each lane. Molecular weight markers ($\times 10^{-3}$) were myosin, albumin, ovalbumin and chymotrypsinogen (as shown on the right). The bands most affected by trypsin treatment are indicated by arrows.

used. As seen in the autoradiograms of Fig. 4, treatment of the ECM with doses of trypsin sufficient to block type II adhesion caused alteration in a complex of bands migrating at between 50 and 60($\times 10^3$)M_r and another complex at $35 \times 10^3 M_r$, but did not reduce either a major high molecular weight (220×10^3) band, which presumably corresponds to cellular fibronectin, or a major band of molecular weight 42×10^3, which probably corresponds to actin. This agrees with the work of others (Murray & Culp, 1980) indicating that Fn and actin are in cryptic locations in ECM material. It is not clear at this point if the proteins of the 50 to 60($\times 10^3$) and $35 \times 10^3 M_r$ complexes, which are altered by trypsin treatment, are actually involved in type II adhesion or if they simply share the same trypsin sensitivity with an unidentified component that mediates this process.

DISCUSSION

In previous publications (Harper & Juliano, 1981*a*,*b*) we have shown that fibroblastic cells have at least two distinct mechanisms for substratum adhesion. One mechanism (which we call type I adhesion) is mediated by the pericellular protein Fn and by unidentified intrinsic membrane and cytoskeletal components. Another mechanism (termed type II adhesion), which is independent of Fn, involves a high molecular

weight glycoprotein on the cell surface, and utilizes components in the ECM. In this paper we provide a preliminary characterization of the nature of the ECM components mediating type II adhesion. Ad^{v}F11 cells, which are defective in type I adhesion, provide a convenient means for studying the type II mechanism in isolation.

Our studies indicate that the components mediating type II adhesion are produced by normal diploid fibroblasts but not by a variety of fibroblastic or epithelial cell lines (Table 1). These components are part of the ECM produced by the fibroblasts and are not shed into the culture medium in free form (Table 2A). Attempts to reconstitute an ECM capable of mediating type II adhesion by mixing together collagen, glycosaminoglycans and fibronectin, three well-known ECM components, were uniformly unsuccessful (Table 2B, C). Either these components do not play a role in type II adhesion or they cannot assume their functional configuration or proper stoichiometry during these reconstitution experiments.

Treatment and extraction of the ECM and correlation of biochemical and adhesion data provide an indication of the nature of the components mediating type II adhesion. First, it is unlikely that these components are lipid in nature; treatment of the ECM with the non-ionic detergent TX100 removes 95 % of the material labelled by [^{3}H]oleic acid (Fig. 1), but does not affect this mode of adhesion. Secondly, a series of experiments suggests that the components mediating type II adhesion are not glycosaminoglycans. Thus extraction of the ECM with chondroitinase ABC or with urea removes 90 % of the $^{35}SO_4$-labelled material (Fig. 1); the enzyme degrades or removes most of the chondroitin sulphate and dermatan sulphate, while the urea removes most of the heparan sulphate (Fig. 2); however, neither treatment with chondroitinase ABC nor urea affects the ability of the ECM to mediate type II adhesion (Table 3A). These results strongly suggest that GAGs are not immediately involved in the type II adhesion process. Thirdly, it seems clear from the data of Table 3A that collagen types I and III are probably not among the components that mediated type II adhesion; collagenase treatment sufficient to liquefy a collagen gel had no effect on type II adhesion. Finally, the component(s) mediating type II adhesion are very sensitive to agents known to degrade or modify proteins. Thus treatment with very low doses of trypsin (Fig. 3) or modification of tyrosine residues by chloramine T iodination (Table 4) abolish the ability of fibroblast ECM to support type II adhesion. These data, taken with the lack of effect of collagenase, suggest that the components mediating type II adhesion are non-collagenous proteins or glycoproteins.

Direct PAGE analysis of [^{35}S]methionine-labelled fibroblast ECM demonstrates that there are changes in the gel pattern at low doses of trypsin just sufficient to ablate type II adhesion. The proteins affected are in the 50 to 60 and 35×10^3 molecular weight ranges, while other major matrix components such as fibronectin (220×10^3) and actin (43×10^3) are unaffected; however, at this juncture it is not clear if the trypsin-labile components are indeed the components that mediate type II adhesion.

In summary, we have investigated the biochemical characteristics of fibroblast ECM that contribute to type II adhesion. We have shown that treatments that extract or degrade lipids, collagen or glycosaminoglycans have little effect on type II adhesion.

However, treatments that degrade or modify non-collagenous proteins can abolish the ability of the ECM to support this mode of adhesion, indicating that the matrix component mediating type II adhesion is probably a protein or glycoprotein.

This work was supported by NIH grant GM26165 to R.L.J.

REFERENCES

CATHCART, M. K. & CULP, L. A. (1979*a*). Phospholipid composition of substrate adhesion sites of normal, virus-transformed and revertant murine cells. *Biochemistry* **18**, 1167–1176.

CATHCART, M. K. & CULP, L. A. (1979*b*). Initial studies of the molecular organization of the substrate adhesion site. *Biochim. biophys. Acta* **556**, 331–343.

CULP, L. A. (1978). Biochemical determinants of adhesion. *Curr. Top. membr. Transp.* **11**, 327–396.

GRINNELL, F. (1978). Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* **53**, 65–144.

GRINNELL, F. & MINTER, D. (1979). *Biochim. biophys. Acta* **550**, 92–99.

HARPER, P. A. & JULIANO, R. L. (1980). Isolation and characterization of Chinese hamster ovary cell variants defective in adhesion to fibronectin coated collagen. *J. Cell Biol.* **87**, 755–763.

HARPER, P. A. & JULIANO, R. L. (1981*a*). Two distinct mechanisms of fibroblast adhesion. *Nature, Lond.* **290**, 136–139.

HARPER, P. A. & JULIANO, R. L. (1981*b*). Fibronectin independent adhesion of fibroblasts to the extracellular matrix: mediation by a high m w membrane glycoprotein. *J. Cell Biol.* **91**, 647–653.

JILEK, F. & HORMAN, H. (1979). Cold insoluble globulin (fibronectin). VI. Influence of heparin and hyaluronic acid on the binding of native collagen. *Hoppe-Seyler's Z. physiol. Chem.* **360**, 597–603.

KLEINMAN, H. K., KLEBE, R. J. & MARTIN, G. R. (1981). Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* **88**, 473–485.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **227**, 680–685.

LASKEY, R. A. & MILLS, A. D. (1975). Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**, 335–341.

LINDALE, U. & HOOK, M. (1978). Glycosaminoglycans and their binding to biological macromolecules. *A. Rev. Biochem.* **47**, 385–417.

MURRAY, B. A. & CULP, L. A. (1980). Multiple and masked pools of fibronectin in murine fibroblasts cell substrate adhesion sites. *Expl Cell Res.* 237–250.

MOSHER, D. F. (1980). Fibronectin. *Prog. Haemostasis Thromb.* **5**, 111–151.

MOURAO, P. P. S. & MACHADO-SANTELLI, G. M. (1978). Sulphated glycosaminoglycans of cells grown in culture. Dermatan sulphate disappearance in successive fibroblast subcultures. *Cell Differ.* **7**, 367–374.

PEARLSTEIN, E., GOLD, L. I. & GARCIO-PARDO, A. (1980). Fibronectin: a review of its structure and biological activity. *Molec. Cell Biochem.* **29**, 103–128.

ROLLINS, B. J. & CULP, L. A. (1979). Glycosaminoglycans in the substrate adhesion sites of normal and virus-transformed murine cells. *Biochemistry* **18**, 141–148.

RUOSLAHTI, E. & ENGVALL, E. (1980). Complexing of fibronectin, glycosaminoglycans and collagen. *Biochim. biophys. Acta* **631**, 350–358.

SCHOR, S. L. & COURT, J. (1979). Different mechanisms in the attachment of cells to native and denatured collagen. *J. Cell Sci.* **38**, 267–281.

TAKEICHI, M. (1977). Functional correlation between cell adhesion properties and some cell surface glycoproteins. *J. Cell Biol.* **75**, 464–479.

TERRANOVA, V. P., ROHRBACH, D. H. & MARTIN, G. R. (1980). Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. *Cell* **22**, 719–726.

WYLIE, D. D., DAMSKY, E. C. H. & BUCK, C. A. (1979). Studies on the function of cell surface glycoproteins. I. Use of antisera to surface membranes in the identification of membrane components relevant to cell–substrata adhesion. *J. Cell Biol.* **80**, 385–402.

YAMADA, K. M. & OLDEN, K. (1978). Fibronectins–adhesive proteins of cell surfaces and blood. *Nature, Lond.* **275**, 179–184.

(*Received 23 November 1982 – Accepted 21 April 1983*)