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Localisation and cellular origin of hyaluronectin

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ABSTRACT

Hyaluronectin is an extracellular matrix glycoprotein which specifically binds to hyaluronan. Isoforms of hyaluronectin are present in nervous and mesenchymal tissues but, while the nervous tissue isoform has been characterised in some detail, less is known about the mesenchymal isoform. Although its tissue localisation suggests a role in tumour development, neither its cellular origin nor its exact function are known. In this study we demonstrate hyaluronectin synthesis in fibroblasts and smooth muscle cells in vitro. The pattern of immunolocalisation of hyaluronectin in fibroblasts depended on the cell type, length of time spent by the cells in culture and cell density. Immunoreactivity in sparsely plated migratory cells was seen mainly in a patchy distribution at the attached cell surface and in the migration tracks left by the cells on the subtratum. In stationary cells a more uniform distribution associated with the attached cell surface was observed, while in confluent cultures hyaluronectin immunoreactivity was mainly seen as a network of fibrillar material above the cell. The pattern of staining was distinct from that of other hyaluronan-binding proteins. Immunoprecipitation, using antihyaluronectin antibodies, of the substratum-attached material deposited by human fetal fibroblasts revealed a family of proteins ranging from 22 to 90 kDa, the major protein being of approximately 60 kDa. These results lead us to propose that hyaluronectin plays an important role in cell migration, probably by regulation of hyaluronan distribution and binding.

Key words: Extracellular matrix; hyaluronan; hyaluronan-binding proteins; hyaluronectin.

INTRODUCTION

Hyaluronan (HA) is a linear polysaccharide which is present in the extracellular matrix of all connective tissues, wherein it influences cell migration, proliferation and differentiation and plays a role in morphogenesis, angiogenesis, wound healing and immune responses (Laurent & Fraser, 1992). In addition to the physical effects of HA on these events, its hydrodynamic properties, for example, may help to maintain the shape of a developing structure, several specific HA binding proteins, both extracellular and cell surface, have been identified which may mediate its effects on cell behaviour (for reviews see Toole, 1990; Knudson & Knudson, 1993). These include the HA receptor complex which incorporates a receptor for HA-mediated mobility (RHAMM) and has been characterised extensively in cultured fibroblasts and smooth muscle cells although little is known about its tissue distribution (Turley & Torrance, 1985; Turley, 1992; Yang et al. 1994). Another cell surface hyaluronan-binding protein has been described on the basis of immunofluorescence studies on cultured human fetal lung fibroblasts (Gupta et al. 1991). The family of CD44 cell surface receptors also includes isoforms with HA binding ability, which may have a role in cell adhesion and migration (Underhill, 1992). The best known of the extracellular HA binding proteins are the aggregating proteoglycans, sharing some common structural features with other HA binding proteins (Toole, 1990). The interrelationship of the cellular distribution and function of these HA binding proteins has not been established but is likely to lead to a clearer idea of how differential expression of the proteins may contribute to the regulation of HA modulated cell behaviour.

Hyaluronectin (HN) is a glycoprotein found in fetal and adult mammalian tissues and its only known ligand is HA. Isoforms of the protein are present in nervous and mesenchymal tissues. In the fetus the

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nervous tissue isoform is found associated with growing axons and dendrites and in areas containing large extracellular spaces (Delpech et al. 1987), while in the adult it is found predominantly at the nodes of Ranvier and around approximately 10% of neurons (Delpech et al. 1982). Its structure and biochemical properties have been investigated in some detail (Delpech et al. 1987, 1991), and its cellular localisation has also been demonstrated in the cytoplasm and cell processes of cultured oligodendroglial cells (Asou et al. 1983). The mesenchymal form is associated with undifferentiated mesoderm in the fetus but can no longer be detected as the tissue differentiates and the main locations subsequently in the fetus are the upper layer of the dermis and the mucosal connective tissue of the gut (Delpech & Delpech, 1984; Delpech et al. 1986). In the adult the mesenchymal form is found in a limited distribution, associated with the loose connective tissue and in tissues undergoing renewal and proliferation. For example, in normal tissues HN is found in the extracellular matrix (ECM) of hair follicles, the intralobular connective tissue of the mammary gland, the subendothelium of arteries and the lamina propria of the large intestine (Delpech & Delpech, 1984). It also marks the proliferation of connective tissue in tumours, both in fibrosarcomas and the desmoplastic response to some carcinomas (Delpech et al. 1979; Delpech & Delpech, 1984; Bertrand et al. 1992; Ponting & Kumar, 1995). Less is known about the biochemistry of the mesenchymal form of the protein. Its tissue distribution suggests a significant role in fetal development and tumour progression, but neither its cellular origin nor its function have been clearly elucidated. Its localisation to the stroma of tumours suggests production by (myo)fibroblasts (Delpech et al. 1979; Bertrand et al. 1992; Ponting et al. 1993). HN has been localised in the cytoplasm of fibroblasts in vitro using an immunoenzymological procedure, although no details of the staining pattern were given (Delpech et al. 1986). It has also been isolated from human peripheral blood monocyte conditioned medium (Delpech et al. 1992).

The aim of this study was to identify non-nervous tissue cells types producing HN in vitro and to compare the cellular distribution of the protein identified by using HN-antibody with that demonstrated for the other HA binding proteins. In addition, the localisation of HN was compared with that of talin, a cytoplasmic marker for cell-substratum adhesion structures, and fibronectin (FN), a well characterised ECM molecule associated with cell adhesion and migration.

MATERIALS AND METHODS

Materials

Tissue culture grade glutamine, fetal calf serum, Dulbecco's Modified Eagle's Medium, Minimum Essential Medium ($\times 10$), nonessential amino acids ($\times 100$) were purchased from Flow Laboratories Ltd, Rickmansworth, UK. Benzyl penicillin was from Glaxo Laboratories Ltd, Greenford, UK. 4-chloro-1-naphthol, 1-ethyl-3(-3-dimethylamino-propyl)carbodiimide hydrochloride, ethylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic acid (EGTA), HA (human umbilical cord grade I), levamisole, leupeptin, paranitrophenylphosphate, pepstatin, phenylmethylsulphonyl fluoride (PMSF), streptomycin sulphate B.P., monoclonal anti-talin, anti-α-smooth muscle actin and antifibronectin antibodies were obtained from Sigma Chemical Company Ltd, Poole, UK. Streptomyces hyaluronidase (EC 4.2.99.1) was from Calbiochem/Novabiochem, Nottingham, UK. Peroxidase, fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate-conjugated secondary antibodies and monoclonal anti-epithelial membrane antigen were purchased from DAKOPATTS Ltd, High Wycombe, UK. Citifluor mountant was obtained from Agar Scientific, Stansted, UK. Gel electrophoresis reagents and chromatography columns were from Bio Rad Ltd, Hemel Hempstead, UK. Nunc-immuno plate 196 well ELISA plates and prestained molecular weight markers for PAGE were from Gibco BRL, Uxbridge, UK. 6-aminohexyl-agarose gel (AH-Sepharose 4B) was from Pharmacia Ltd, Milton Keynes, UK. DE52 preswollen microgranular anion exchanger was from Whatmann, Maidstone, UK. General laboratory reagents of Analar grade were obtained from BDH, Merck Ltd, Lutterworth, UK. [^{3}H]amino acids were purchased from Amersham International, Aylesbury, UK. Polyclonal anti-HN antibody, raised against human brain HN, and antiserum from which antibodies to HN had been removed were generous gifts from Dr B. Delpech, Rouen, France. Monoclonal antibody to CD31 was a kind gift from Dr P. Dias, St Judes Hospital, Memphis, TN, USA. Monoclonal antibody to cytokeratin 8 was a kind gift from Professor E. B. Lane, University of Dundee, UK.

Methods

Primary cell cultures

Fibroblast and smooth muscle cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) con-

taining 10% (v/v) fetal calf serum (FCS) and PSG (100 μg/ml benzyl penicillin, 100 μg/ml streptomycin sulphate, 300 μg/ml L-glutamine). Endothelial cells were cultured in DMEM supplemented with 10% (v/v) newborn calf serum and PSG.

Placental fibroblasts. Samples of freshly obtained fullterm human placental tissue were washed in phosphate buffered saline (PBS) followed by culture medium and finely diced. The tissue was divided and treated by the following methods. (1) Finely minced tissue was plated directly onto uncoated tissue culture grade plastic dishes with sufficient complete medium to barely cover the tissue. The plates were incubated undisturbed for 24 h, then 1 ml of complete culture medium was added to each well. Colonies of cells were scraped into the medium and replated. (2) Sterile type I collagen extracted from adult rat tails (a gift from Dr A. Sattar) was used to coat 24 well plates for 1 h at 37 °C, then rinsed 3 times with PBS. Medium containing finely minced tissue was plated and the cell cultures expanded as above. (3) Collagen gels were prepared using sterile type I collagen (as above) by mixing 8.5 ml of the cold collagen extract with 1 ml cold ×10 concentrated MEM and 0.5 ml 4.4% $NaHCO_3$, giving a final collagen concentration of approximately 1.5 mg/ml. Finely minced tissue was added to the collagen and aliquoted into 24 well plates. Usually after 2–3 d cells were seen to migrate from the tissue into the collagen, whereupon the gel was digested by incubation with 0.1% (w/v) collagenase in PBS at 37 °C for 45 min. The cells were recovered by centrifugation, plated into 24 well plates, and expanded as above.

Embryonic chick cells. 8-d-old chick embryos were removed from their eggs and decapitated. The skin was minced and the tissue treated as for placental cell cultures.

Bovine aortic endothelial and smooth muscle cells. Freshly obtained bovine aortas were cut open longitudinally and washed with PBS. The lumen was gently scraped twice with a scalpel blade to remove endothelial cells, washed again with PBS and scraped again, more firmly, to collect smooth muscle cells. The endothelial and smooth muscle cells were separately seeded in culture medium in 24 well plates and colonies of cells expanded as detailed for placental cell cultures.

Other cell types. Human adult skin fibroblast cells, human fetal skin fibroblast cells, and human fibrosarcoma cell line were gifts from Ms A. Spreadborough, Mr G. Rushton and Dr T. Wheldon respectively; Swiss 3T3 mouse fibroblast and MRC-5 human embryo lung fibroblast cell lines were obtained from Flow Laboratories Ltd, Rickmansworth; human MCF-7 breast carcinoma cell line was obtained from the European Collection of Animal Cell Cultures, Porton Down. MCF-7 and MRC-5 cells were cultured in DMEM containing 10% (w/v) FCS, PSG and nonessential amino acids.

Indirect immunofluorescent staining of cultured cells

The cells were grown on glass coverslips for 1–2 d and each cell type stained with the antibodies to the following cell lineage specific markers: von Willebrand factor (1:500) and CD31 (1:500) as endothelial cell markers; epithelial membrane antigen (1:50) and cytokeratin 8 (1:50) as epithelial cell markers; α-smooth muscle actin (1:400) as a marker of smooth muscle and myofibroblast cells. Controls were incubated with PBS. Cells were also stained with anti-HN polyclonal antibody (1:30), in which case the negative control was antiserum from which antibodies to HN had been removed. To investigate any variation in staining arising from length of time spent in culture, human fetal skin fibroblasts (HFSF), passage 6, were stained for HN after 2, 6 and 24 h in culture and postconfluent.

To stain the cells the coverslips were gently washed twice in PBS and fixed in ice cold methanol for 5 min. The cells were incubated for 1 h at 37 °C with the primary antibody, washed in PBS and incubated with fluorescein isothiocyanate (FITC) conjugated swine antirabbit or rabbit antimouse secondary antibodies (1:40 in PBS) for 30 min at room temperature. After washing, the coverslips were mounted in Citifluor. For double staining, the primary antibodies (polyclonal anti-HN [1:30] with monoclonal anti-human cellular FN [1:500], or monoclonal anti-talin [1:800]) were mixed and applied to the cells as above, followed, after washing, by incubation as above with a mixture of FITC and tetramethylrhodamine isothiocyanate (TRITC)-labelled secondary antibodies (both 1:30 in PBS). Stained preparations were examined using a Dialux 22EB incident light fluorescent microscope. Alternatively, FITC fluorescence was observed and photographed using a Carl Zeiss Laser Scan microscope, using 488 nm laser excitation and 525 nm emission filters.

Electrophoresis, Western Blotting and dot blotting

Samples containing 10–25 μg protein were processed using 10% sodium dodecyl sulphate (SDS) polyacrylamide gels under reducing conditions. Gels were Western blotted electrophoretically onto nitrocellu-

lose (Burnette, 1981). For immunoperoxidase staining the nitrocellulose was incubated in 5% (w/v) BSA in PBS for 1 h and incubated overnight with anti-HN polyclonal antibody (1:500). Following incubation with swine antirabbit secondary antibody (1:200) for 1 h, bound antibody was visualised using 0.06% (w/v) 4-chloro-1-naphthol, 0.03% (v/v) hydrogen peroxide in 20% (v/v) methanol. Dot blot assays were performed using a 96 well Minifold apparatus (Bio-Rad Labs) and the presence of HN detected as for the Western blots.

Extraction of HN from cultured cells

Immunohistochemistry. To investigate the possibility that the staining pattern was due to antibody exclusion from the adhesion plaques, human fetal skin fibroblasts (HFSF) were extracted prior to fixation using the method of Neyfakh et al. (1983). HFSF cultured on glass coverslips for 24 h were washed with PBS and incubated in 1% (v/v) Triton X-100, 4% (w/v) polyethyleneglycol 20000 (PEG) in buffer (50 mM imidazole, 50 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, pH 6.8) for 3 min. The cells were washed 3 times for 3 min in the buffer, fixed in methanol (−20 °C) for 4 min and double-stained for HN and talin as detailed above.

Affinity chromatography. HA-Sepharose was generated by coupling purified HA to 6-aminohexyl-agarose gel (AH-Sepharose 4B) by the carbodiimide reaction using the method of Tengblad (1979). Supernatants were loaded (25 ml/h) on a 50 ml column and the column washed with PBS followed successively by 1 M NaCl in PBS and 3 M NaCl in PBS. HA binding proteins were eluted using 0.2 M glycine-HCl, pH 2.2 (50 ml/h) and 10 ml fractions collected. The eluted fractions were neutralised with NaOH, dialysed against distilled water, freeze-dried and examined by dot blot assays and by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting techniques.

Presence of HN in fetal calf serum (FCS)

To exclude the possibility that the staining was due to exogenous uptake of HN, the FCS used in the medium was tested by the dot blot technique for the presence of HN. In addition, 25 ml FCS was dialysed against 3 changes of PBS then applied to the HA-Sepharose column and eluted as detailed above. The pooled fractions from the different elutions were dialysed against PBS, freeze-dried and tested by the dot blot technique for the presence of HN. Proteins eluted from the column by the glycine-HCl buffer were examined by SDS–PAGE and Western blotting/immunostaining.

Culture of cells in serum free medium

Staining of cells. To eliminate any contribution made to the staining by the HN present in the serum, HFSF (passage 8) were subcultured in serum free medium. After 2 d in culture, the cells were trypsinised, resuspended in serum-free medium, plated on coverslips in Leighton tubes, cultured for 2–3 d and stained by indirect immunofluorescence staining.

Analysis of the conditioned medium. HFSF which had been cultured for 2 d in serum-free medium were seeded into T175 cm^2 flasks and incubated in fresh serum-free medium for 3 d. The medium, approximately 120 ml, was collected and concentrated to ~ 20 ml by placing in dialysis tubing and covering with PEG 20000, for 3–4 h at 4 °C. 100 µl of the concentrated conditioned medium was tested by the dot blot technique for the presence of HN. Fresh medium was similarly treated and used as a control. To check for binding to HA, an ELISA assay was performed. Briefly, wells of an ELISA plate were incubated with 50 µl/well of a 20 µg/ml solution of purified HA overnight at 4 °C, the wells were blocked for non-specific binding and incubated with 50 µl of the conditioned medium for 2 h at room temperature. Duplicate incubations were made on uncoated plates. HN bound to the plate was detected using polyclonal anti-HN antibody (1:500). Alkaline phosphate-conjugated secondary antibody (1:400) was applied for 2 h and antibody binding was detected using 5 mM para-nitrophenylphosphate, 1 mM levamisole, 0.5 mM $MgCl_2$, in 0.01 M diethanolamine. Assays were performed in triplicate. The remainder of the conditioned medium was dialysed against PBS, applied to an HA affinity column and the fractions tested by dot blot and Western blotting techniques for HN.

Detection of HN by immunoprecipitation

Radiolabelling of cells. Subconfluent HFSF cells were metabolically labelled using [^{3}H]-labelled amino acids. The cells were incubated in serum free medium for 24 h followed by 10 µCi/ml of a mixture of [^{3}H]-labelled amino acids in fresh serum free medium for 24 h under standard culture conditions. The conditioned medium and substrate attached material (SAM) were harvested.

Harvesting of medium. The conditioned medium (approximately 250 ml) was collected and concentrated to approximately 5 ml using PEG 20000 as above. 2 ml was subsequently dialysed against buffer (0.15 M NaCl, 0.1 M sodium acetate buffer, pH 5.0) and divided into 2 aliquots. 1 ml was digested with 2 turbidity-reducing units of Streptomyces hyaluronidase and protease inhibitors [0.5 mM phenylmethylsulphonylfluoride (PMSF), 1 μM Pepstatin, 0.1 mM Leupeptin, 1 mM EDTA] at 37 °C for 2 h. 1 ml was used as a control, i.e. the enzyme was omitted. Both aliquots were heated at 100 °C for 3 min and dialysed against immunoprecipitation buffer (see below). 400 μl of each of the digested and control sample was used for immunoprecipitation.

Solubilisation of substrate attached material (SAM). The method of Culp (1976) was used to isolate SAM. The adherent radiolabelled cells were washed 3 times with PBS and incubated in 0.5 mM EGTA and protease inhibitors in PBS for 1 h at 37 °C with agitation. The suspended cells were removed, the substratum was gently washed by pipetting with 0.5 mM EGTA in PBS (EGTA wash) and rinsed in PBS followed by distilled water. The cell suspension was centrifuged for 10 min at 300 *g* and the supernatant added to the EGTA wash. The pooled EGTA-solubilised material was concentrated to 5 ml as above, dialysed against PBS and immunoprecipitated. The SAM was extracted by incubation with 25 ml per flask of 0.2% (w/v) SDS at 37 °C for 1 h with agitation. The pooled extract was concentrated to 5 ml as detailed above, dialysed against PBS and immunoprecipitated. In a parallel experiment unlabelled cells were cultured in Leighton tubes and treated as above. Cultures treated with EGTA only or with EGTA followed by SDS were double stained for talin and HN using the immunofluorescent staining techniques described above.

Immunoprecipitation. The method of Copper et al. (1981) was used to specifically precipitate antigens recognised by the anti-HN polyclonal antibody, from the conditioned medium, EGTA-solubilised material and SAM. To remove any nonspecific binding, 400 μl of the concentrated medium or extract was mixed with 2 ml immunoprecipitation buffer [0.4 M NaCl, 5 mM EDTA, 0.5 M Tris-HCl pH 8.0, 1% (v/v) Nonidet P40] and added to 60 μl of a 50% (v/v) suspension of Protein A Sepharose in immunoprecipitation buffer, in the absence of antibody. The suspension was mixed for 2 h at 4 °C and the supernatant recovered by centrifugation (5500 *g*) for approximately 10 s. The Sepharose pellet was washed with 100 μl of immunoprecipitation buffer and the wash added to the supernatant. The mixture was split into 2 equal aliquots. To one aliquot 5 μl of the affinity purified polyclonal rabbit anti-HN antibody was added, while to the other 5 μl of control solution was added, i.e. serum from which the antibody had been removed by affinity chromatography. Thereafter, both samples were treated identically. The mixture was incubated for 2 h at room temperature, after which 30 μl of fresh 50% (v/v) Protein A Sepharose suspension in immunoprecipitation buffer was added. The suspension was mixed and the supernatant removed as above. The Sepharose pellet was washed twice by mixing with 1 ml of immunoprecipitation buffer and centrifuging as above. The Sepharose was washed twice as before with 1 ml 10 mM Tris-HCl, pH 6.8 and resuspended in 15 μl of double-concentration electrophoresis sample buffer; the suspension was heated at 100 °C for 3 min and centrifuged as above. Each supernatant was applied to an SDS polyacrylamide gel as a single sample, electrophoretically resolved and visualised by fluorography (Pulleyblank & Booth, 1981).

The abbreviations are listed in Table 1.

Table 1. *Abbreviations*

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic acid
FCS	Fetal calf serum
FN	Fibronectin
HA	Hyaluronan
HFSF	Human fetal skin fibroblasts
HN	Hyaluronectin
PBS	Phosphate buffered saline
PEG	Polyethyleneglycol
PMSF	Phenylmethylsulphonyl fluoride
RHAMM	Receptor for hyaluronan-mediated mobility
SDS–PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

RESULTS

Immunocytochemistry

The anti-HN antibody stained all fibroblasts, irrespective of species, the smooth muscle and the fibrosarcoma cells. Some slight staining of bovine aortic endothelial cells was also observed but the breast carcinoma cells were negative. Three patterns of specific staining were observed: one was reminiscent of focal adhesion in that it was a punctate staining associated with the attached surface, the second was a clear staining of the migration tracks, while the third was a strong fibrillar staining of confluent cells (Table

Table 2. *A summary of patterns of HN staining in cultured HFSF and comparison with those of FN*

State of cell in culture	Pattern of stain for: HN	FN
Newly adherent (2 h culture)	Amorphous cytoplasmic staining with some patchy or punctate staining of the cell extensions	Amorphous cytoplasmic staining. Punctate and fibrillar staining of cell extensions and under cell body
Migratory	Strong staining of migration tracks. Fine fibrillar and punctate staining, the latter sometimes in linear arrays, associated with the attached surface	Punctate and fibrillar staining associated with attached surface
Stationary	Almost complete staining of the attached surface. Negative patches correspond to talin-positive areas	Strong extracellular fibrillar staining of substratum
Confluent	Staining associated with attached surface as above, and also strong fibrillar network staining above the cells	Strong fibrillar staining of substratum and of network above cells

2). The extent of these patterns in any one culture varied markedly depending on how long the cells had been allowed to adhere after subculture, on the passage number, and on the cell type (Table 3). In addition, positive cultures displayed some amorphous staining for HN in the cytoplasm of the cells, principally in the perinuclear area.

In HFSF stained after 2 h in culture, FN was distributed in a punctate pattern in approximately 50% of the cells but occasionally a discrete fibrillar pattern was also seen (Fig. 1*A*, *B*). No staining of migration tracks was observed. HN was observed as a nonspecific cytoplasmic localisation and a pattern of punctate or more broad patches (Fig. 1*D*, *E*), associated with the attached surface of cell extensions and reminiscent of adhesion structures. The staining for HN was less extensive, structured and sharp in any one cell than that of FN. Small migration tracks, where present, were also positive for HN. No staining was observed for talin and only approximately 10% of the cells contained α-smooth muscle actin (not shown).

In the 6 h culture the majority of cells were migrating and in these a patchy distribution of HN was seen at the attached surface of the permeabilised cells, often showing a more or less clear linear arrangement, but not as finely fibrillar as that of FN (Fig. 1*C*, *F*). A prominent characteristic was the localisation of HN in the cell migration tracks (Fig. 2*A*) and areas adjacent to the cell where it had apparently spread and retracted. This staining was patchy, often appearing as a continuation of that directly below the cell. HN was also observed at the initial site of cell adhesion, seen as a strongly staining circle (Fig. 2*B*) at the start of many of the adhesion tracks. Closer examination of the tracks stained for HN revealed a punctate distribution, occasionally seen as a series of semicircular depositions along the track, a shape reminiscent of that of the leading edge of the migratory fibroblast (Fig. 2*C*). The staining visualised the entire trail left by the cell and was comparable to that observed by Halfter et al. (1990) for ECM components. Controls, incubated with immune serum from which the anti-HN antibody had been removed by affinity chromatography, showed negligible staining.

In stationary cells, characterised by their well-spread morphology and mainly seen in the 24 h cultures, HN almost completely covered the attached surface. However, this pattern was interrupted by areas of negative staining taking the form of sharply defined strips of varying length (Fig. 3*A*). Although the patchy distribution predominant in the migrating cells was reminiscent of 'footprint' or adhesion plaque staining, these areas appeared to coalesce in the stationary cells. Double staining showed that the HN-negative strips were talin positive (Fig. 3*B*). HN therefore appears to be excluded from the focal adhesions as defined by the presence of talin, this protein being a cytoplasmic component of the focal adhesion structure. To examine whether this pattern was due only to exclusion of antibody from the adhesion plaques, the coverslips on which fetal fibroblasts had been cultured were stained following relatively mild detergent (Triton X100) extraction, which removes the cells, leaving the ECM. The staining for HN retained the same pattern: although the cell was no longer present, the pattern of migration tracks and footpad material was still present (Fig. 4). No staining was observed for talin.

Table 3. *Semiquantitation of staining of cells with anti-hyaluronectin (HN) polyclonal antibody*

Cell type*	Intensity of staining	
	Track**	Cell surface***
Adult human skin fibroblasts p2	++	+
Adult human skin fibroblasts p12	+	++
Fetal human skin fibroblasts p3	+++	+
Fetal human skin fibroblasts p14	++	++
Human placental fibroblasts p2	+++	+
Human embryo fibroblast cell line (MRC5)	±	+
Swiss 3T3 mouse fibroblast cell line	±	+
Embryonic chick fibroblasts p4	++	+
Bovine aorta smooth muscle cells p3	+++	±
Bovine aorta smooth muscle cells p20	+++	±
Bovine aortic endothelial cell p5	±	−
Human breast carcinoma cell line (MCF7)	−	−
Human fibrosarcoma cell line	++	±

Relative intensity of immunofluorescent staining: − no staining; ± weak; + moderate; ++ strong; +++ very strong. * The 'cell type' of primary cultures was designed on the basis of morphology and immunocytochemical staining using lineage specific markers as described in the text. ** 'Track' denotes the punctate staining of the attached cell surface and migration tracks associated with migrating cells. *** 'Cell surface' denotes the more extensive staining associated with the attached cell surface of stationary cells. p, passage number.

In the older (24 h) cultures the staining for FN was far more extensive than in the newly adherent cells, revealing a more fibrillar arrangement on the attached side of the cell (not shown). There was no obvious colocalisation of FN and HN, HN covering almost all the attached cell surface to some degree and, while FN was clearly organised into distinct fine structures; HN appeared to be somewhat amorphous. Although the proportion of cells staining for α-smooth muscle actin was greatly increased in the 24 h cultures, no colocalisation with HN was observed (not shown).

Fibroblasts and smooth muscle cells when confluent revealed a meshwork of fibrillar material over the cells (Fig. 5), similar to that of FN (not shown). Although FN appeared in such a distribution even when only 2 or 3 cells were in contact with one another, HN formed a network only when the cells were confluent.

The staining for HN of different passage cells and of different cell types had characteristic patterns (summarised in Table 2). The HFSF retained their migratory morphology and hence strong track staining both in the cultures grown for longer periods (i.e. > 24 h) and of high passage. In contrast, the human adult skin fibroblasts generally showed weaker staining at all passages. The pattern of punctate cellular and track staining was strong in the younger cultures (passage 2) but diminished with increasing passage so that the staining in the human adult skin fibroblasts at passage 12 was greatly reduced. In the older passage human adult skin fibroblasts few tracks were observed, the staining being more confined to the attached surface of the predominant well spread stationary cells. The level of staining of the smooth muscle cells was considerably stronger than for the human adult skin fibroblasts, was mainly in the migration tracks and persisted into high passage. The fibrosarcoma cells possessed the pattern of staining characteristic of fetal or low passage adult fibroblasts, i.e. strong staining of extensive migration tracks (not shown). The staining of the fibroblast (3T3) cell line for HN was considerably weaker than the primary cell cultures. Little staining was seen under the cell and no cell tracks were observed. In subconfluent fibroblast cell lines extensive HN-positive fibrils apparently extended between cells (Fig. 6), similar to those seen in confluent primary cell cultures. Endothelial cells possessed very faintly staining migration tracks.

Synthesis of HN

HN was detected by dot blot assay in the FCS and although affinity chromatography removed much of the HN, some HN still remained, presumably tightly bound to HA in the serum. Proteins that bound to the HA column, and were immunodetected using anti-HN antibodies, comprise a major band at 49 kDa and minor bands at approximately 180, 47, 38, 34 and 28 kDa (Fig. 8).

To test whether the pattern of staining was due to uptake of HN from the serum, the HFSF were stained following culture in the absence of serum (not shown). The level of staining for HN was very much reduced, both in intensity and reflecting decreased migration track deposition, but retained the same pattern. That of talin was also much more sparse and diffuse suggesting that the reduction in HN deposition may be correlated, at least partially, with diminished cell-substratum binding in the serum free medium as observed by Bayley & Rees (1982).

Dot blot and ELISA analysis showed the presence and HA binding ability of HN in serum free conditioned medium harvested from human fetal and adult fibroblasts and from bovine smooth muscle cells, but extraction of HN from the HFSF medium by affinity chromatography was unsuccessful. Although a small amount of protein bound to the HA column from the conditioned medium, no HN was detected by immunostaining of a Western blot.

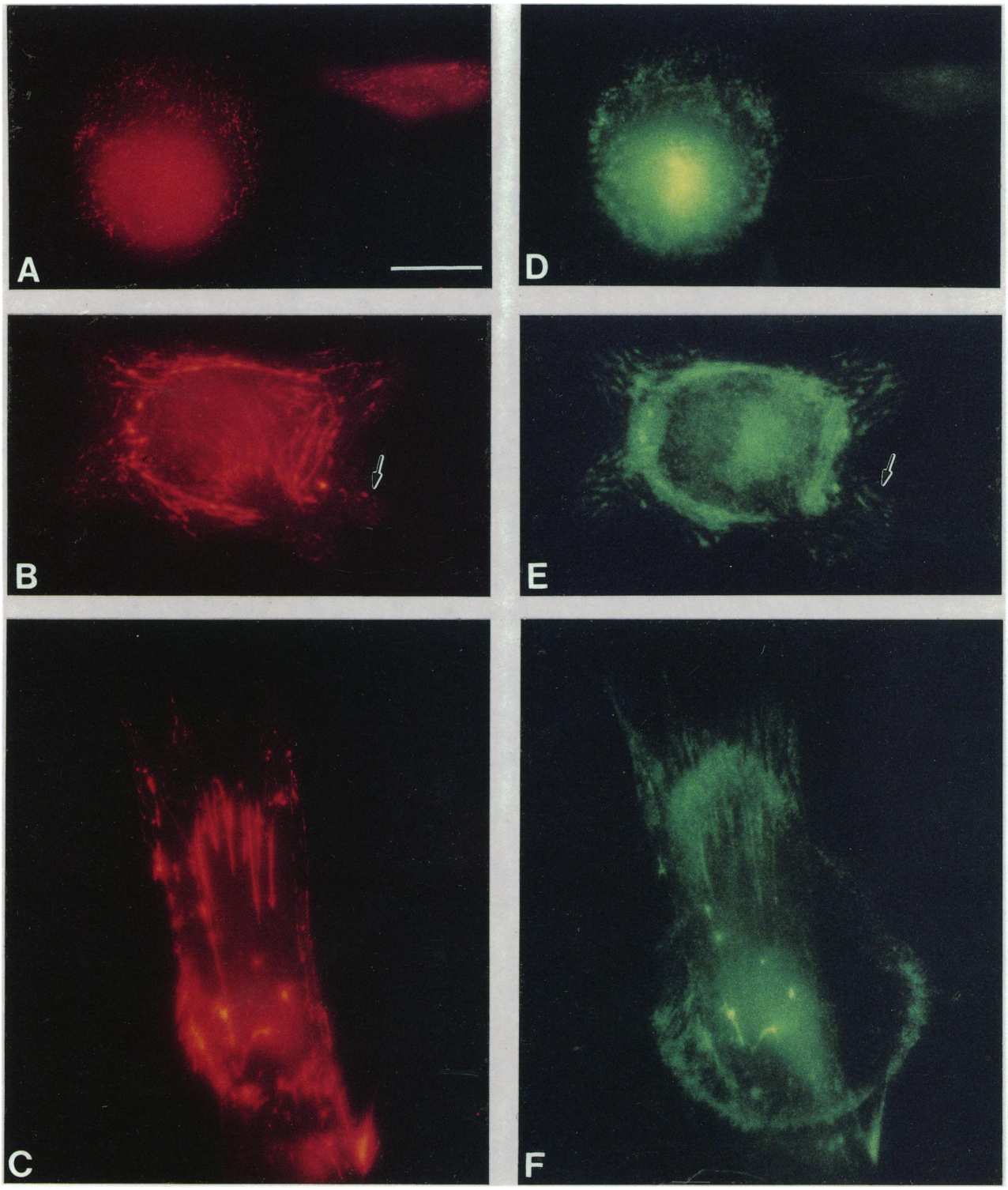

Fig. 1. Immunofluorescence staining for FN and HN in human fetal skin fibroblasts fixed with cold methanol (*A*, *B*, *C*) stained with monoclonal anti-cellular FN antibody. (*D*, *E*, *F*) stained with polyclonal anti-HN antibody. For all cells the staining for both HN and FN was localised with or under the attached surface. (*A*) and (*D*) show the same rounded fibroblast, 2 h after plating, not yet displaying any clear cell extensions. (*B*) and (*E*) show a slightly more spread fibroblast, also 2 h after plating. (*C*) and (*F*) show a migrating fibroblast fixed 6 h after plating. Some degree of colocalisatioin can be seen in the fibrillar staining of both antigens. The semicircular staining seen using the anti-HN antibody is probably material deposited by the leading edge of the cell as it moved. Bar for all figures, 20 μm.

Immunofluorescence studies of cultures following treatment with EGTA revealed that, while all the cells had been removed, and talin was no longer detected, the pattern of staining for HN persisted (Fig. 7). Further treatment with SDS, however, to remove the tightly bound SAM, eliminated all the staining.

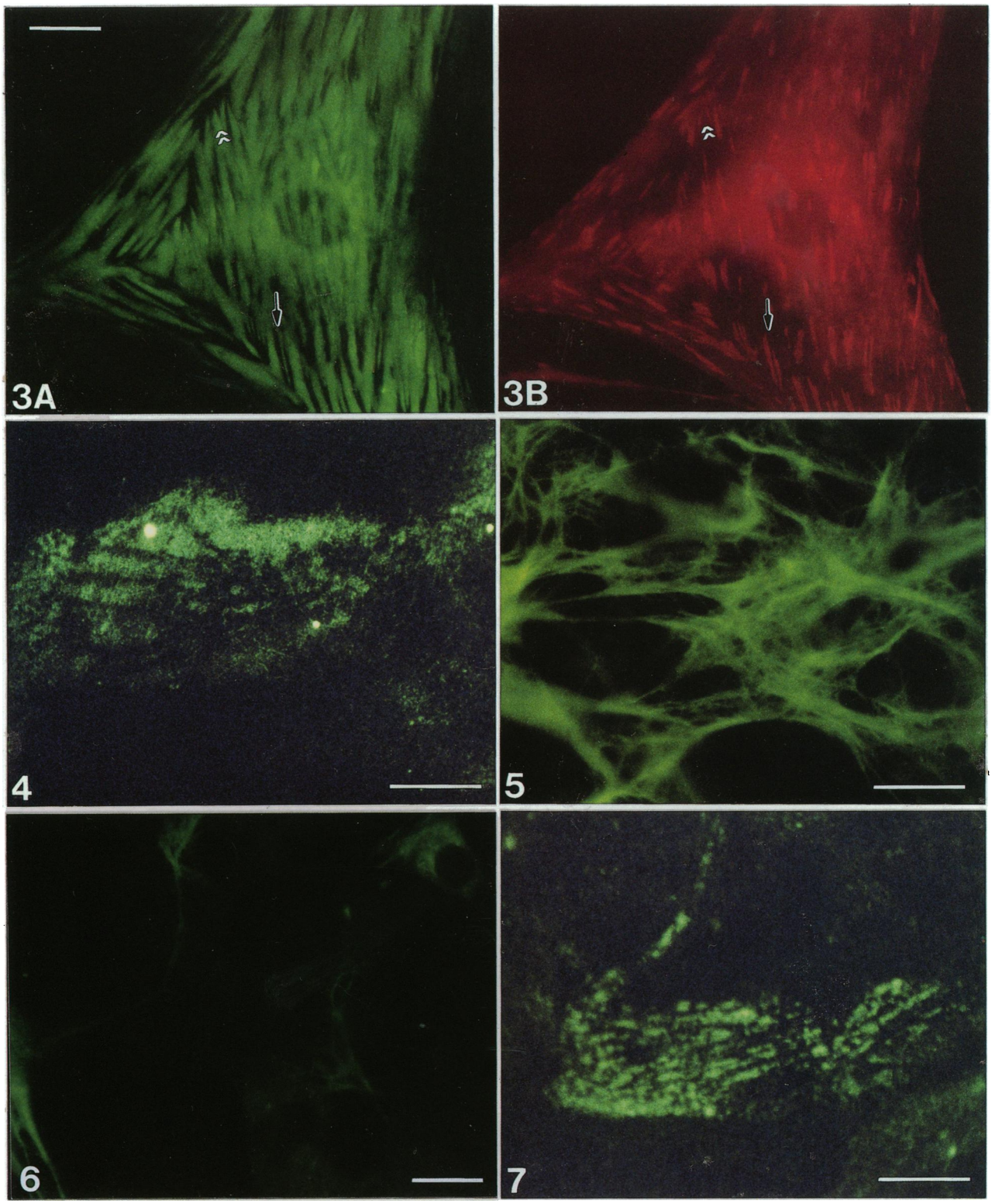

Fig. 3. Immunofluorescence staining of a human adult skin fibroblast fixed with cold methanol after 24 h in culture and double stained with (*A*) polyclonal anti-HN antibody and (*B*) monoclonal anti-talin antibody. The double staining seems to show a consistent inverse or mutual exclusion (→, «). The staining for talin extends closer to the edge of the cell than does that for HN. Bar, 20 μm.

Fig. 4. Immunofluorescence staining of a human fetal skin fibroblast extracted with Triton X100, fixed with cold methanol and stained with anti-HN polyclonal antibody. The staining pattern retains a similar motif of negative patches as seen under intact cells. Bar, 30 μm.

Fig. 5. Immunofluorescence staining of confluent human fetal skin fibroblasts fixed in cold methanol and stained with anti-HN polyclonal antibody. The staining reveals a fibrillar network above the cells. Bar, 30 μm.

Fig. 6. Immunofluorescence staining of 3T3 fibroblast cell line fixed with cold methanol and stained with anti-HN polyclonal antibody. Bar, 30 μm.

Fig. 7. Immunofluorescence staining of human fetal skin fibroblast treated with 0.5 mM EDTA, fixed with cold methanol and stained with anti-HN polyclonal antibody. Although the cell was removed by the treatment, the staining pattern on the substratum was retained, indicating the localisation of HN in the substrate attached material. Bar, 30 μm.

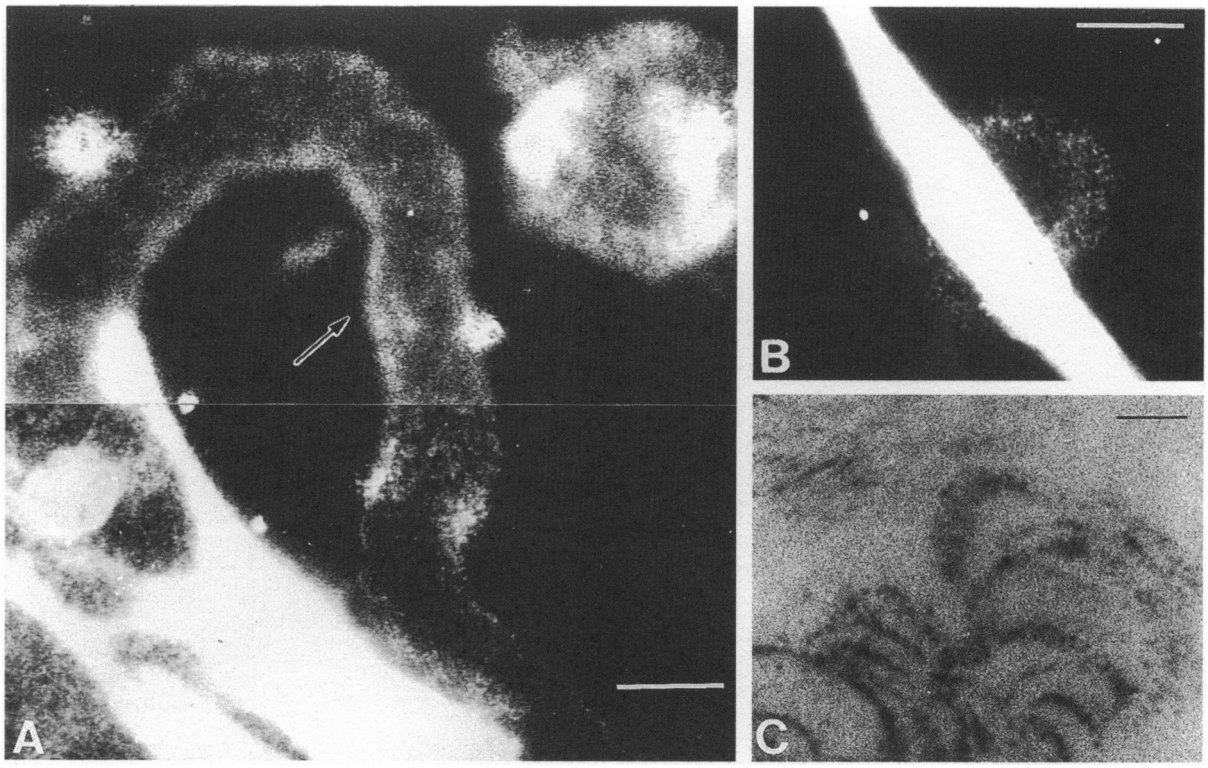

Fig. 2. Immunofluorescence staining of human fetal skin fibroblasts fixed with cold methanol and stained with anti-HN polyclonal antibody. In (*A*) and (*B*) staining was visualised using a laser scan microscope. (*A*) Cell stained after 24 h in culture, displaying a migration track of extracellular material (arrow) deposited during its migration on the substratum. Bar, 30 μm. (*B*) Cell stained after 6 h in culture. The staining of the circular deposition under the cell indicates the initial site of cell–substratum adhesion. Bar, 25 μm. (*C*) Staining was visualised using a fluorescence microscope and shows the track of a migrating fibroblast. The staining reveals a series of semicircular deposits probably indicating the progression of the leading edge of the cell as it migrated by traction via adhesion to the substratum. The staining may give an indication of the size of the successive 'steps' taken by the cell. Bar, 30 μm.

An antibody which specifically recognises HN (raised against human brain HN; Delpech et al. 1979) was used to immunopreciptate HN produced by cultured cell. Radiolabelling and immunoprecipitation of the conditioned medium gave a band above 200 kDa and a weaker band at a slightly lower molecular mass (Fig. 9). The control, using antiserum from which the anti-HN antibodies had been removed, was negative. Incubation of the medium with hyaluronidase prior to immunoprecipitation had no effect on the apparent molecular mass.

The presence of EGTA in the solution used to remove the cells from the substratum would also have removed any peripheral plasma membrane proteins, i.e. proteins associated with but not integrated in the plasma membrane. Immunoprecipitation of the EGTA-solubilised material revealed the presence, albeit weak, of 4 proteins, of M_r approximately 180, 68 and 48 kDa (Fig. 9). This is a different pattern to the SAM and may represent proteins held at the cell membrane and solubilised by the EGTA, or protein(s) selectively solubilised from the SAM. Immunoprecipitation of the SAM, harvested from the substratum by SDS treatment, gave several bands, the most prominent being at approximately 60 kDa, with minor bands of molecular mass ranging between 28 and 90 kDa (Fig. 9). The band seen at approximately 20 kDa is equally strong in the control lane and is therefore likely to be due to nonspecific binding during the process of immunoprecipitation.

DISCUSSION

This study has demonstrated the distribution of HN in cultured mesenchymal cells, i.e. fibroblasts and smooth muscle cells and, to a much lesser extent, in endothelial cells. HN was seen in the ECM, in the form of tracks deposited by the migrating cells, and in the matrix directly under and/or associated with the attached cell surface. The staining under the cell was variable, being patchy in migratory cells but more uniform on the attached surface of the apparently stationary cells. The expression of HN in specific structures appeared within 2 h of subculture. The intensity of staining for HN was markedly influenced by passage number, length of time in culture since

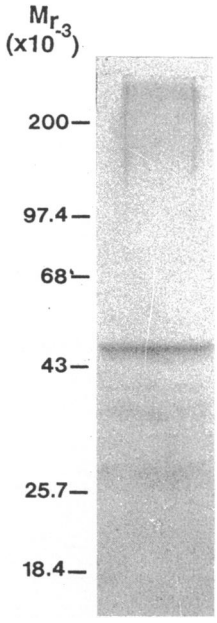

Fig. 8. Immunoblot detection of HN in fetal calf serum. The proteins were affinity purified using HA-substituted Sepharose, resolved by SDS–PAGE using a 10% gel, transferred onto nitrocellulose and the blot stained with anti-HN polyclonal antibody using the immunoperoxidase technique.

plating, and the source of the cell, i.e. cell line or primary culture, fetal or adult.

With regard to what can be said about the identity of HN on the basis of this work, its cellular distribution is markedly similar to that of the chondroitin sulphate proteoglycan, versican (Yamagata et al. 1993). The brain isoform of HN has been shown to have homology with versican, a chondroitin sulphate proteoglycan produced by human fibroblasts and it has been proposed that HN may be a fragment of this proteoglycan (Zimmerman & Ruoshlahti, 1989; Delpach et al. 1991; our own unpublished work). The deposition of HN in the ECM differentiates it from receptors such as the isolated and characterised CD44 molecule. The use of the antibody raised by Gupta et al. (1991) against an HA binding protein also described as HN showed that this protein has a different cellular localisation to HN (our unpublished results). The HA binding protein, RHAMM, isolated by Turley and coworkers from cultured fibroblasts also has a different distribution, being localised to the processes, ruffles and perinuclear region of migrating cells (Turley & Torrance, 1985). Immunoelectron microscopy showed it to be localised at the cell surface (Turley et al. 1990) and the protein has been shown to be involved in the modulation of HA induced rapid migration of transformed or stimulated cells (Turley, 1992). With regard to cell type, the distribution of HN is similar to that of RHAMM in that both were observed in cultured smooth muscle cells and, at a much reduced level, in cultured vascular endothelial cells. The two proteins appear to be distinct however on the basis of cellular distribution.

The pattern of staining suggests a role for HN in cell-environment contact. By immunoelectron and interference reflection microscopy techniques the contacts made by fibroblasts with their environment in culture have been partially defined on the basis of the distance between the cell's attached surface and the substratum or ECM. These contacts are focal adhesions, close contacts and ECM contact sites (Izzard & Lochner, 1980; Kolega et al. 1982). The latter are predominant in late cultures, accounting for up to 80% of the cell contacts and have been further subdivided into two forms by immunoelectron microscopy studies (Chen & Singer, 1982); ECM-I, which may be structurally equivalent to the focal adhesion in that it too comprises end-on associations between actin microfilaments and ECM fibrils; and ECM-II which may be the equivalent of the close contact made with the substratum and appears to comprise areas of association of actin and ECM fibrils apposed to each other in parallel either side of the plasma membrane. One of the cytoplasmic components of the focal adhesion structure is talin which is thought to accumulate with bundles of actin filaments where they terminate end-on at the plasma membrane (Burridge & Connell, 1983; DePasquale & Izzard, 1991). As such, the staining of the fibroblasts for talin serves to locate the termini of the stress fibres and hence the focal adhesions and possibly the ECM-I contacts. In the stationary cells these sites were clearly negative for HN. The staining for HN may therefore correspond to the close contact or ECM-II structures although in general the staining showed no specific pattern, but rather appeared to represent a nonspecific ECM deposition excluded from the areas under the attached surface which are concerned with forming contacts. Although it is difficult to distinguish by light microscopy, there appears to be a small area around some of the patches of talin which is also negative for HN, possibly indicating a lack of HN in the close contacts. Similarly, at the periphery of the cell, although the staining for talin is still strong, there is decreased staining for HN, suggesting that the HN distribution is limited to areas where the cell is at a greater distance from the substratum.

The retention of HN in the migration track is a novel observation. The retention of the protein on the substratum after the cells had been removed suggests a strong intercalation of HN into the SAM. This

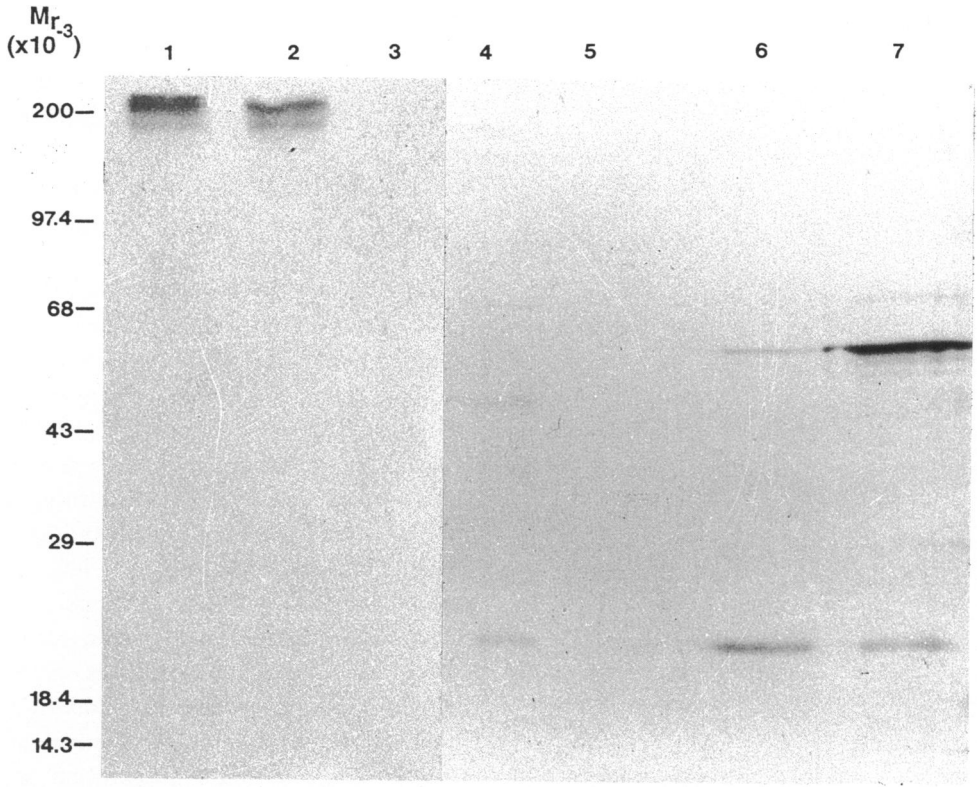

Fig. 9. Fluorograms showing the immunoprecipitation of [^{3}H]-labelled proteins from cultured human fetal skin fibroblasts by anti-HN polyclonal antibody. Lanes 1–3, proteins immunoprecipitated from conditioned medium: 1, using anti-HN polyclonal antibody; 2, protein immunoprecipitated using anti-HN polyclonal antibody following hyaluronidase digestion of the sample; 3, control using antiserum from which the anti-HN reactivity had been removed. Lanes 4, 5, proteins immunoprecipitated from the EGTA-solubilised material: 4, using anti-HN polyclonal antibody; 5, control using antiserum from which the anti-HN antibodies had been removed. Lanes 6, 7, proteins immunoprecipitated from the substrate attached material: 6, control using antiserum from which the anti-HN antibodies had been removed; 7, using anti-HN polyclonal antibody.

material is that left on the substratum when the cell detaches during normal in vitro migration, and has been shown to contain FN, HA, proteoglycans and cytoskeletal proteins (Culp, 1976; Rollins & Culp, 1979). Immunoprecipitation of the SAM in the present study, using anti-HN antibody, revealed a range of M_r species of HN present in this material, possibly degradation products or with different glycosylation patterns. Several mouse fibroblast SAM proteins have previously been identified as co-electrophoresing with, and being tightly bound to HA (Culp, 1976). These are of high molecular mass and are likely to be proteoglycan species. Several other proteins of a range of lower M_r were also observed, however Rollins & Culp (1979) reported that 'little, if any, protein was detected in association with hyaluronic acid' in the SAM.

A role for HA in detachment is endorsed by several observations: for example, variants of a CHO cell line showed that increased ease of detachment was associated with elevated HA synthesis (Atherley et al. 1977; Barnhart et al. 1979). Abatangelo et al. (1982) showed that fibroblasts grown in the presence of HA were more susceptible to mechanical detachment. A migration stimulating factor has been shown to exert its influence by affecting HA synthesis (Grey et al. 1989) and oligosaccharide (3–10 disaccharides), but not macromolecular, HA has been shown to be chemotactic for vascular endothelial cells (Sattar et al. 1994). That HA plays a role in regulating migration has been given credence since it has been identified in the 'footprint' material retained on the substratum in vitro after fibroblast migration (Terry & Culp, 1974; Lark & Culp, 1982) which is believed to comprise the remnants of cell–substratum adhesion structures. New adhesion sites are thought to have little HA, over 80% of the GAG content being heparan sulphate, which is capable of binding FN, but as the structures mature, the ratio of HA and chondroitin sulphate to heparan sulphate increases as HA accumulates (Rollins & Culp, 1979). It has been suggested that HA/chondroitin sulphate complexes may compete with heparan sulphate for cellular FN binding, thereby aiding the detachment of the cells by weakening the cell-substratum linkage (Rollins & Culp, 1979; Laterra & Culp, 1982) or that the increase

in HA deposition pushes apart the cell and substrate, physically loosening the adhesion to the point of release (Toole, 1982).

It is interesting that the increased expression of HN at the attached surface is temporally concomitant with the expression of the tight adhesion structures in stationary cells, but that spatially the two features appear to be mutually exclusive. Assuming the staining for HN in culture to represent, at least in part, the localisation of HA, the observations may be consistent with those of Bayley & Rees (1982) that HA forms a nonspecific layer associated with the attached cell surface. A possibility is that the HN is deposited just after the formation of the adhesion structures so that its deposition is physically excluded from the area under the cell occupied by these structures. The implied presence of HA may help explain the distance maintained between the cell and the substratum, the swelling pressure of the matrix being due to the hydrodynamic properties of the HA molecules. The expression of strong adhesive structures may be the cells' response to such pressure. Conversely, the increased expression of HN may be in order to restrict the swelling capacity of HA.

HN may, in its own right, be an 'anti-adhesion' molecule. While much attention has been focused on the adhesion-promoting molecules such as FN, the existence of anti-adhesion molecules has been recognised (Chiquet-Ehrismann, 1991). A similarity with FN and its receptor is in the redistribution of the proteins in the different phases of cell migration and adhesion (Couchman et al. 1982; Singer et al. 1988). The role of FN and its receptor is thought to be in the transient adhesion made by the migrating cell with the substratum, i.e. the close contacts (Couchman & Rees, 1979), while having a different role in the stationary cells (Couchman et al. 1982). Such a 'dual role' may be possible for HN. It should also be borne in mind, as suggested by Halfter et al. (1990), that the deposition of material in migration tracks is not merely a result of the attachment and subsequent detachment from the substratum in the process of migration of the fibroblast, but may also serve the specific purpose of guiding neighbouring cells. The observations that HN production is increased in vivo when adjacent to 'activated' epithelial cells (Bertrand et al. 1992; Ponting et al. 1993) may therefore be related to a role of HN in promoting epithelial cell migration.

Just as the presence of FN in the ECM is thought to regulate the positioning of its receptors in the plasma membrane of adherent cells, thereby modulating the cytoskeleton and hence the cell shape (Chen et al. 1986; Singer et al. 1988; Roman et al. 1989), the same could be true for HA. It is not known whether there is an integrin-type plasma membrane receptor for HN, but the fibroblast receptor complex for HA has been isolated: Turley and coworkers examined the effect of HA on the migration of fibroblasts and concluded that the organisation of HA on the cell surface by specific binding proteins played a crucial role in the regulation of cell adhesion and hence migration (Turley & Torrance, 1985; Turley et al. 1985; Turley, 1992). This complex has been shown to mediate the role of HA in migration and comprises components which have the ability to bind to actin and to be capable of autophosphorylation. A model analogous to that proposed for FN may therefore be envisaged in which HA plays a similar role to FN, providing the cell with information as to the state of its environment. The role of HN is unclear but the presence in the ECM of a specific HA binding protein may modulate the presentation of HA to the cell, affecting its recognition by receptors. CD44, for example, is known to recognise immobolised HA in preference to soluble HA (Lesley et al. 1990), and the importance of the form of presentation of HA in events such as proliferation and differentiation has been considered (Kujawa et al. 1986*a*, *b*; Elstad & Hosick, 1987). HN may also act as a reservoir for HA, sequestering it as it is produced by the cell, possibly preventing its immediate degradation and regulating the quantity of HA available to the cell, thereby controlling its effect on cell behaviour. This may explain how such a structurally simple molecule as HA can influence fibroblasts in the variety of often contradictory ways observed in vitro.

Conclusive functional studies on HA binding protein have yet to be undertaken. Adhesion studies performed by Gupta & Datta (1991) indicated a role for HA binding protein in the adhesion of rat histiocytoma cells. Turley and coworkers have used purified fibroblast HA binding protein to demonstrate in vitro the role played by the protein in HA-stimulated cell migration by addition of exogenous protein to fibroblasts and smooth muscle cells (Turley et al. 1985; Bourdreau et al. 1991).

Yoneda et al. (1988*a*) have reported that addition of HA to primary cultures of sparsely distributed fibroblasts inhibited proliferation, but caused stimulation in confluent cultures. The minimum size of exogenously added HA capable of incorporating into the matrix was found to be a decasaccharide and it was proposed that a likely candidate for the mediation of HA incorporation into the matrix would be a chondroitin sulphate proteoglycan (Yoneda et al.

1988*b*). This role could be fulfilled by HN. Incorporation of HN into the matrix increased dramatically in confluent cells, forming a strongly staining fibrillar network over the cells and possibly signifying an increased ability of the cell layer to bind HA. The present study shows that some HN was not incorporated into the ECM but could be immunoprecipitated from freshly obtained fibroblast-conditioned medium and was of a minimum of approximately 200 kDa, consistent with that reported for the versican core protein (Zimmermann & Ruoslahti, 1989). From the intensity and extent of staining for HN in stationary cells it might seem surprising that the distribution of HN in normal adult tissues is so limited. Some of the HN immunoreactivity seen in vitro is associated with migration. Since the migration of fibroblasts in vivo is limited to such events as tissue regeneration, this may also explain why, despite the staining of motile fibroblasts in culture, there is no general staining of the ECM in most normal tissues for HN. The strong staining of the fibrosarcoma cells also suggests an increase in HN expression after fibroblast transformation. The increased HN staining observed in breast carcinoma stroma (Bertrand et al. 1992; Ponting et al. 1993) may therefore be a consequence of the fibroblasts being activated in response to, or simultaneously with, the epithelial transformation.

The correlation between the appearance of stress fibres and the increased staining for HN in stationary cells may indicate that this HN expression is largely an in vitro phenomenon. However it is consistent with the limited localisation of HN in vivo to areas of myofibroblasts, for example its increased deposition in stromal tissue in breast carcinoma (Bertrand et al. 1992; Ponting et al. 1993). Furthermore, the staining of the smooth muscle cells accords with the distribution of HN in the intima of arteries and the submucosa of the intestine (Delpech et al. 1979; Lévesque et al. 1994).

The presence of HN in fetal bovine serum poses several questions. The difference in molecular mass between the HN in the bovine serum and that of the bovine brain (present study and our unpublished data) may reflect differences in the roles of nervous and mesenchymal forms of HN. Peripheral blood monocytes may be one of the sources of serum HN (Delpech et al. 1992). Any possible role of HN in the blood is presumably linked to the presence of HA, or possibly prevents its binding to other plasma proteins such as fibrinogen which could cause clotting problems.

In conclusion, this study has localised HN and partially characterised HN in cells in vitro. The specific immunoprecipitation by anti-HN antibodies of metabolically radiolabelled proteins proves conclusively the de novo synthesis of HN by fibroblasts, albeit in low concentration. The present results also show that there are distinct differences in the expression of HN depending on the cell type, length of time spent in culture and the cell density. Caution should therefore be exercised when comparing studies on the effects of HA performed on cells from different sources, the expression of HN by the cell lines being different to that of the primary cultures. This may account for the reported differences in the ability of the cells to respond to exogenously added HA.

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