FIBONECTIN IS A COMPONENT OF THE SURFACE COAT OF HUMAN NEUTROPHILS

S. T. HOFFSTEIN, G. WEISSMANN AND E. PEARLSTEIN
Departments of Medicine and Pathology, New York University Medical Center, New York, N.Y. 10016, U.S.A.

SUMMARY

Although adherence to surfaces is central to neutrophil function many of the determinants of neutrophil adherence are still unknown. The possible involvement of cell surface material, fibronectin in particular, was therefore studied. Surface coat material was visualized ultrastructurally by the ferrocyanide-reduced osmium technique of Karnovsky (1971). Loosely attached surface coat material was seen distributed uniformly on cells in suspension. Indirect immunofluorescence indicated the presence of fibronectin on the neutrophil surface. Distribution of fibronectin as determined by indirect immunoferritin localization corresponded with the distribution of cell coat material. Some, if not all, of this fibronectin was synthesized by neutrophils themselves since metabolically labelled fibronectin could be obtained by immunoprecipitation after short-term culture with [35S]methionine. Neutrophils also adhere to Sepharose beads to which gelatin is covalently linked (GS) but not to plain Sepharose beads (PS). In the process they transfer surface coat material to GS but not PS. Similar transfer was seen when cells were permitted to adhere to glass or plastic coverslips. Indirect immunofluorescence showed that fibronectin-containing material was transferred from neutrophils to GS but not PS. Parallel studies with antisera to 2 other plasma proteins, factor VIIIIR and alpha-antitrypsin showed that neutrophils did not transfer these to either GS or PS beads. The data suggest that material antigenically and functionally related to fibronectin is associated with the extracellular coat of neutrophils and is transferred with cell surface material to surfaces to which neutrophils adhere.

INTRODUCTION

Human peripheral blood neutrophils adhere readily to glass, nylon or charged plastic surfaces in the absence of serum or other exogenous proteins (Kvarstein, 1969a; Wright et al. 1978; Rasp, Clawson, Hoidal & Repine, 1979), although adherence may be modified by the presence of serum or plasma. Optimum adherence in the presence of serum or plasma is dependent on the presence of divalent cations (Garvin, 1961; Penny, Galton, Scott & Eisen, 1966; Kvarstein, 1969b; Bryant, DesPrez, Van Way & Rogers, 1969), and requires normal cytoskeletal function since it is inhibited by vincristin (Schneier, Gall, Carpe & Boggs, 1977) and colchicine (Penny et al. 1966; Boxer et al. 1978) and enhanced by heavy water (Schneier et al. 1977). Beyond this, little is known about the mechanisms whereby neutrophils adhere to surfaces. Because the surface proteins of cells in general, and fibronectin in particular, have been implicated in adhesion (see review by Pearlstein, Gold & Garcia-Pardo, 1980), we tested the possibility that fibronectin may be associated
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with the neutrophil surface and may mediate polymorphonuclear leukocyte adherence to some substrates.

MATERIALS AND METHODS

Cells

Purified neutrophil populations (95-98%) were obtained from heparinized human peripheral blood sedimented through a Hypaque-Ficoll gradient (Boyum, 1968). The bulk of the contaminating red cells were removed by sedimentation with dextran and the remainder by resuspending the cell pellet in 1 vol. phosphate-buffered saline (PBS) and 3 vol. H₂O for 30 s, then adding 1 vol. 0.6 M-NaCl. The cells pellets were washed 3 more times in 20-ml portions of PBS containing 0.6 mM-Ca²⁺ and 1 mM-Mg²⁺ (PiCM). Cells were centrifuged at 180 g (7 min) for each wash.

Sepharose beads

Gelatin-Sepharose beads were prepared by coupling gelatin to CNBr-activated Sepharose 4B at a ratio of 10 mg of protein/1 g of gel. Fibronectin-Sepharose beads were prepared by coupling fibronectin (derived from plasma) to CNBr-activated beads at a ratio of 3 mg of protein/1 g of gel. Opsonized beads were prepared by incubating washed beads in 4 times their volume of human serum for 30 min at 37 °C (Goldstein, Kaplan, Radin & Frosch, 1976). The serum was either fresh or inactivated by heat at 57 °C for 30 min.

Electron microscopy; ferrocyanide-reduced osmium fixation (FRO)

Cells were fixed in 1-2.5% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4) containing 0.025% CaCl₂. They were then washed overnight in buffer and post-fixed for 2 h at 4 °C in 2% OsO₄ and 1.5% K₄Fe(CN)₆ in phosphate buffer (pH 6) (Karnovsky, 1971; Dvorak, Hammond, Dvorak & Karnovsky, 1972). They were dehydrated in a graded series of alcohols without a buffer wash after the osmium post-fixation.

Tannic acid mordanting

Other cell preparations were fixed in glutaraldehyde, post-fixed in 2% osmium and treated with 1% tannic acid (A.R. no. 1764, Mallinckrodt, St Louis, MO) as previously described (Simionescu & Simionescu, 1976).

Immunofluorescence and immunoferritin microscopy

Neutrophils suspended in PiCM were permitted to adhere to glass coverslips or gelatin-Sepharose beads for 30 min at 37 °C. They were then washed again in buffer and incubated in the cold with 10% human immunoglobulin G (Ig G) (Miles, Elkhard, IN) in PiCM. The purpose of this IgG was to occupy Fc receptor sites on the plasma membrane and so minimize the non-specific binding of the rabbit IgG. The immune serum did not cross-react with the IgG. The excess was poured off and rabbit antihuman plasma fibronectin, previously shown to be monospecific (Pearlstein & Gold, 1978), was diluted 1:5 before use. As controls, anti-human factor VIIIR (Behring Diagnostics, Somerville, N.J.) and anti-alpha-antitrypsin antisera (Cappel Laboratories, Downington, PA) were used at dilutions of 1:5 and 1:10. The cells were incubated with the antisera for 60 min at 4 °C and then washed extensively with cold PiCM. The second antibody used for immunofluorescence was fluorescein-conjugated goat anti-rabbit immunoglobulins (Behring Diagnostics) diluted 1:40 in 10% human IgG, and for immunoferritin electron microscopy ferritin-conjugated goat anti-rabbit (Cappel) was diluted 1:10 as above. Incubation with the second antibody was carried out at 4 °C for 45 min and the coverslips were then washed extensively with PiCM and fixed in the cold. In some experiments cells were prefixed with 1% formaldehyde (freshly generated from paraformaldehyde) and 0.1 M-cacodylate buffer plus 10% sucrose for 30 min at 0-4 °C. The
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Fixative for electron microscopy was a combined aldehyde and osmium fixative, which we used previously (Zurier, Hoffstein & Weissmann, 1973), and the fixation for light microscopy was cold, 2% formaldehyde (freshly generated as above) in 0.2 M-cacodylate buffer. After extensive and overnight washing in PiCM containing 0.5% bovine serum albumin, the latter cells were viewed in a Zeiss universal microscope equipped with an epifluorescence illuminator.

Immunoprecipitation

Neutrophil cultures (2 x 10⁶ cells with < 2% monocytic cell contamination) were labelled for 18 h with 75 μCi/ml [³⁵S]methionine in Hams F-10 medium (Grand Island Biological Co.) and proteins precipitated from the medium and cell extracts by a double-antibody technique. Cell pellets were dissolved in PBS (140 mM-NaCl, 5 mM-PO₄, pH 7.4) containing 0.5% NP-40 (Particle Data Labs, IL), 2 mM-PMSF and 25 U/ml Trasylol (Sigma). The cell extract and conditioned medium were clarified by centrifugation at 10000 g for 60 min. Anti-fibronectin or normal rabbit sera were added, incubated for 2 h at 37 °C and immune complexes precipitated using sheep anti-rabbit antiserum (Meloy) for 24 h at 4 °C. The immunoprecipitates were washed by centrifugation and dissolved in 2% sodium dodecyl sulphate (SDS) for electrophoresis.

SDS/polyacrylamide gel electrophoresis

Electrophoresis was performed as described previously (Pearlstein & Waterfield, 1974). Samples were reduced with 0.1 M-dithiothreitol and boiled for 3 min prior to application to the gel. Approximately equal amounts of radioactivity were applied for each sample.

RESULTS

Neutrophil surface coat, ultrastructural studies with ferrocyanide-reduced osmium

Post-fixation with ferrocyanide-reduced osmium (FRO) revealed cell surface material on neutrophils that was not well visualized with either ruthenium red or tannic acid. As reported previously (Dvorak et al. 1972), glycogen and intracellular membranes were well contrasted by this method but the most striking feature made visible by this stain was an electron-opaque material on the outer surface of neutrophils fixed in suspension. At low magnification the cells appeared as if they had been outlined with ink (Fig. 1). Whereas the enhanced contrast of glycogen and intracellular membranes was dependent on the use of alkaline lead as a section stain, the electron opacity of surface coat material was not noticeably increased by lead staining. At higher magnification, the surface coat material was seen to consist of a fine, linear electron-opaque material interspersed with loosely attached, globular deposits of variable size (Fig. 2). Examination of adherent cells showed the globular deposits to be visible on free surfaces but only linear staining could be seen on adherent surfaces (Fig. 3).

Transfer of cell coat material to substrates

Cell coat material was transferred from neutrophils to substrates upon which they crawled. This was evident in experiments in which coverslips were coated with agarose and neutrophils placed in wells cut in the agarose gel. When neutrophils were permitted to migrate on the coverslip, under the agarose for 1-3 h, material
Fig. 1. A cross-section through a neutrophil in suspension. The cell was fixed and stained by the FRO method and section-stained with alkaline lead. Intracellular membranes including the nuclear envelope are well contrasted as is the glycogen. The outer cell membrane is also intensely stained. ×11000.

Fig. 2. A higher magnification view of the outer membrane of a neutrophil in suspension fixed and stained by the FRO method but not section-stained with lead. The intensely stained material on the membrane is seen to consist for the most part of a series of droplets of variable size. ×48000.
Fig. 4. An en face section of the trailing edge of a neutrophil that had been permitted to adhere to and migrate on glass. The direction of movement was from the upper left to the lower right of this micrograph. FRO staining droplets are present on the surface of the retraction fibres and also appear to have been left behind by the cell as it migrated (arrow). × 48,000.

Fig. 5. A, B. Light micrographs of Sepharose beads incubated with neutrophils. Cells did not adhere to the plain bead (A) but did adhere to beads to which gelatin had been covalently linked (B). × 250.

Fig. 3. A transverse section through a neutrophil adherent to and migrating on a glass coverslip. Less stainable coat material is seen on these cells than on neutrophils fixed in suspension suggesting that some of the material has been transferred to the glass surface. Droplets are numerous on the ruffle at the right and on the dorsal surface, but not on the left-hand portion of the cell, which is close to the trailing edge. Only fine linear staining is seen at the adherent surface of this cell and some of this material is seen on the glass. × 15,000.
similar to neutrophil surface coat material was present on the glass substrate, in the well and under the agarose behind the leading front of neutrophil migration but not in areas to which the cells had not yet migrated. Cells that had migrated out of the well appeared to be depleted of coat material when compared to cells within wells or aliquots of the same cells fixed in suspension. Surface coat material was conspicuous on the trailing edge of migrating cells, but patches of membrane on the main cell body were free of stainable material (Fig. 4).

**Interaction with Sepharose beads, ultrastructural studies**

The possibility that neutrophil surface coat material might contain fibronectin was investigated by studying the interaction of neutrophils with Sepharose beads to which gelatin had been covalently linked. Neutrophils do not adhere to plain Sepharose beads but do adhere to gelatin–Sepharose (Fig. 5). Adherence to gelatin beads was not as strong as adherence to glass or to opsonized beads. By light microscopic examination the cells were not as well spread on gelatin as on glass and were easily displaced by vortexing from the gelatin–Sepharose but not from opsonized beads. The distribution of surface coat material on cells adhering to gelatin–Sepharose was similar to that of neutrophil migration on glass (Fig. 6), but less material was
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present on the surface of cells incubated with beads than on control cells. This material was present in quantity on bead surfaces between cells and remained there after the cells were removed by vortexing.

Fig. 7. Glass-adherent neutrophils stained for indirect immunofluorescence with either preimmune serum (A) or anti-fibronectin antiserum (B). The neutrophils have taken up some of the rabbit IgG non-specificity via their Fc receptors and therefore show some patchy fluorescence with the preimmune serum. On the other hand neutrophils treated the same way with anti-fibronectin antiserum had a diffuse fluorescence over their surface in addition to the non-specific patches. × 800.

Identification of fibronectin as a component of neutrophil cell coats

Having established by ultrastructural studies the fact that neutrophils have a loosely attached cell coat, which is transferred to surfaces, particularly gelatin-coated surfaces to which the cells adhere, the next step was to determine whether fibronectin was a component of that cell coat and was transferred with it. Fibronectin was first identified by indirect immunofluorescence. Neutrophils, incubated with anti-fibronectin antiserum showed a diffuse staining pattern in addition to some patches of bright fluorescence (Fig. 7). The bright patches presumably represented aggregates of rabbit or goat IgG bound non-specifically via Fc receptors, since they were also present on control neutrophils incubated with preimmune serum. Material between cells on the glass substrate also fluoresced with immune but not preimmune serum. The immunofluorescent zone on the coverslip was evenly distributed within
the area covered by the original drop of cell suspension, suggesting that material from the suspension had been deposited on the glass and subsequently had bound the anti-fibronectin antibody. The results obtained using a ferritin-labelled second antibody were similar to those obtained at the light microscopic level with a fluoresceinated second antibody. Both experimental cells and cells treated with preimmune serum had scattered aggregates of ferritin-containing immune precipitates on their surface. Cells treated with immune serum displayed, in addition to these clumps, numerous small clusters of ferritin (Fig. 8). The distribution of these small clusters was similar to the distribution of surface coat material revealed by FRO staining.

Transfer of fibronectin with neutrophil cell coats

The ultrastructural studies had shown that material, similar in appearance to neutrophil cell coat material, was transferred to gelatin-Sepharose but not plain Sepharose. Examination of cross-sections of over 50 beads from which cells had been removed by vortexing showed no traces of adherent cell membranes. Thus, we were able to use the beads as a method of collecting neutrophil coat material, and to do indirect immunofluorescence studies of coat material without concern for
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Fig. 9. Indirect immunofluorescence of gelatin–Sepharose beads exposed either: to cells and anti-fibronectin antiserum (A), cells and preimmune serum (B), or heat-inactivated human serum and anti-fibronectin antiserum (C). Gelatin–Sepharose beads incubated with neutrophils and then with anti-fibronectin antiserum fluoresce brightly as do the adherent neutrophils (A, centre bead). An aliquot of the same preparation with preimmune serum (B) shows only a small amount of non-specific fluorescence on the cells. The beads do not fluoresce at all. C is a positive control showing the fluorescence pattern of gelatin beads incubated with serum and then exposed to anti-fibronectin antiserum. × 250.

Table 1. *Indirect immunofluorescence studies*

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<th>FRO-stainable surface coat material</th>
<th>Anti-fibronectin</th>
<th>Factor VIII</th>
<th>Anti-α1-antitrypsin</th>
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<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
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<td>Both negative</td>
<td>Both negative</td>
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<tr>
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<tr>
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<tr>
<td>S beads + serum</td>
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GS, gelatin–Sepharose; S, Sepharose.

non-specific absorption of antibody by Fc receptors. Gelatin–Sepharose beads that had been incubated at 37 °C for 30 min with either neutrophils or serum fluoresced brightly when stained with the immune serum as did the neutrophils themselves (Fig. 9). Beads from aliquots of the same preparations incubated with pre-immune serum did not fluoresce. Control beads without gelatin did not acquire immuno-reactive material when incubated with either serum or cells. Beads incubated with
either cells or serum were also tested with anti-factor VIIIIR and anti-alpha\textsubscript{1}-antitrypsin to see if the neutrophils were non-specifically transferring passively adsorbed plasma proteins. Aliquots from suspensions of gelatin-Sepharose incubated with cells did not fluoresce when incubated with either of the 2 control antisera to plasma protein but suspensions of beads incubated with serum did (Table 1).

Do neutrophils synthesize fibronectin?

In order to determine whether fibronectin was synthesized by neutrophils or was taken up exclusively from the medium, purified neutrophils (< 2% monocytic cell contamination) were incubated overnight with \[^{35}\text{S}]\text{methionine}. The cells were homogenized and an immunoprecipitate obtained with anti-fibronectin antiserum. SDS/polyacrylamide gel electrophoresis of this material showed a metabolically labelled band that comigrated with a fibronectin standard (Fig. 10).
DISCUSSION

Although adherence to surfaces or particles is central to neutrophil function in host defense, many of the determinants of neutrophil adherence remain unknown. Studies have been made of ionic requirements, alteration of surface charge and enhancement of adherence induced by chemotactic factors but no consistent hypothesis or theory has emerged (Garvin, 1961; Bryant et al. 1966; Kvarstein, 1969a, b; Hoover, Briggs & Karnovsky, 1978; O'Flaherty, Kreutzer & Ward, 1978; Fehr & Dahinden, 1979). The data presented here, in addition to those in the literature, suggest that for this as for many other biological functions there is redundancy. Neutrophils probably adhere to surfaces by more than one mechanism including fibronectin-mediated adherence. Different combinations of mechanisms available in the neutrophil repertoire may be elicited by different surfaces.

The results obtained in this study indicate that fibronectin is a normal constituent of neutrophil surface coat and suggest that fibronectin, borne by these cells, may mediate their adherence to some surfaces including collagenous substrates. The data also indicate that cell coat material, including fibronectin, is transferred from neutrophils to surfaces to which they adhere and upon which they migrate and may thereby provide signals or modify surfaces for other cells to follow.

The data do not yet enable us to make a definitive statement about the source of the fibronectin observed on the neutrophil surface. Although our studies indicate that neutrophils do synthesize fibronectin, exposure of cells to fluorescein isothiocyanate-labelled fibronectin (unpublished results) indicated that neutrophils like other fibronectin-synthesizing cells also take up exogenous fibronectin. The experiments do however, strongly suggest that the association of fibronectin with the neutrophil surface is specific and functional. Thus, the surface material persists through numerous washes and can be transferred to gelatin-Sepharose but not plain Sepharose under conditions in which other serum proteins are not passively transferred.

As to the role of neutrophil cell coat material and its content of fibronectin, one can only speculate. It may be important in the evolution of the normal inflammatory response. Neutrophils are the first cells to arrive in an inflammatory exudate. Upon exposure to chemoattractants from inflammatory loci, they adhere to the luminal surface of adjacent blood vessels, penetrate the vessel walls and then migrate up the chemotactic gradient through collagen-rich connective tissue.

As the inflammation evolves, neutrophils are followed into the lesion by monocytes, which enter as neutrophil emigration declines. There they attach and develop into tissue macrophages or histiocytes. Healing also requires migration into the lesion of fibroblasts. Attachment of both monocytes and fibroblasts in vitro is enhanced by the presence of fibronectin-containing cellular microexudates, as is fibroblast motility (Ackerman & Douglas, 1978; Pearlstein et al. 1980).

Fibronectin may also serve as a non-antibody non-complement opsonin (Saba, Blumenstock, Weber & Kaplan, 1978). Serum levels are depleted in trauma and sepsis, and uptake of Staphylococcus aureus was shown to be enhanced by fibronectin.
Fibrin clots also contain fibronectin (Pearlstein et al. 1980) and neutrophils interact with and lyse such clots (Plow & Edgington, 1975). Although plasma contains a large quantity of this protein, that already associated with the neutrophil may be cross-linked in position and thus provide a more stable or effective attachment site (Grinnell, Feld & Minter, 1980).

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