The Bone Marrow Fibrosis of Hairy-Cell Leukemia Is Caused by the Synthesis and Assembly of a Fibronectin Matrix by the Hairy Cells

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Hairy-cell leukemia (HCL) is a proliferation of clonal B-lymphocytes with features of activation. The disease has a number of distinctive characteristics, prominent among which is the fine reticulin fibrosis invariably present in the bone marrow. However, fibroblast infiltration has never been noted in the marrow and the origin of the fibrosis has not been established. The present studies show that the hairy cells (HCs) of HCL produce an insoluble matrix of fibronectin (FN) in vitro. FN synthesis was shown by the appearance of cellular FN on the surface of cells cultured in serum-free medium and by immunoprecipitation of the metabolically labeled protein from HC aggregates. Moreover, the HCs were shown to assemble FN into disulphide-bonded multimers. This assembly was blocked by a 70-kD amino-terminal fragment of the molecule that blocks FN multimer formation by fibroblasts. HCs expressed abundant VLA-5, an FN receptor not present on normal circulating B lymphocytes, but important in matrix formation. Furthermore, HCs were shown to adhere to an FN fragment containing the VLA-5 binding site. It is therefore suggested that the VLA-5 of HCs is implicated in their assembly of FN matrix. The in vivo relevance of the findings was established by the demonstration of FN in association with infiltrating HCs in bone marrow sections from patients with HCL. It is concluded that the HCs synthesize and assemble an FN matrix and that this is at least partly responsible for the bone marrow fibrosis so characteristic of the disease. © 1994 by The American Society of Hematology.

Hairy-Cell Leukemia (HCL) is a monoclonal proliferation of B lymphocytes with a number of unusual features. One such feature is the characteristic fine fibrosis of the bone marrow (BM) that is rarely seen in other chronic lymphoproliferative disorders. The fibers associate intimately with individual hairy cells (HCs) and in this respect can be readily distinguished from the coarse fibrous bundles typical of myeloproliferative disorders.

The origin of the BM fibrosis of HCL is unclear; in particular, significant infiltration by fibroblastic cells is not seen. This contrasts markedly with myelofibrosis (MF), in which a prominent infiltrate of fibroblasts is present that, in turn, is thought to be the result of the release of stimulatory cytokines from abnormal cells of megakaryocytic origin. This secondary fibrotic process results in typical bundles of extracellular matrix composed predominantly of collagen types I and type III. Although the composition of the fine fibers seen in HCL is not known, the apparent differences in their form and origin as compared with those of MF suggest that a different process is likely to be responsible.

During a study of HC/adhesive protein interactions, we observed that homotypic aggregates of HCs contain large amounts of fibronectin (FN). This led us to postulate that HCs synthesize and assemble an FN matrix and that this is at least partly responsible for the bone marrow fibrosis characteristic of the disease.

MATERIALS AND METHODS

Cell Sources and Cell Culture

Patient Material

Cells from 9 patients with HCL were studied; all had typical disease as determined by clinical presentation, distinctive peripheral blood (PB) and BM morphology, tartrate-resistant acid phosphatase positivity, and immunophenotype (CD5+, CD11c+, CD19+, CD25+, and FMC7+). In 8 cases, material was obtained from PB (from patients in leukemic phase of HCL; HCs >5 × 10^5/L), whereas in 2 of these cases and 1 aleukemic patient, splenic mononuclear cells were obtained. In 5 of the patients, BM trephine material was available for immunohistochemical study. Numbers of patients studied in each phase of the investigation are given in parentheses in the legends. PB samples from 3 cases of chronic lymphocytic leukemia (CLL) and 1 of B-prolymphocytic leukemia (B-PLL) and cells from a patient with a leukemic centroblastic/centrocytic (CB/CC) lymphoma were also examined in certain experiments. In each case, cells were defined on the basis of typical morphology and immunologic markers and, in the case of CB/CC leukemia, on typical node appearances. Informed consent was obtained for all PB samples. Splenectomy and BM biopsy were performed for clinical reasons, but the patients understood that some of the material would be used for research purposes.

Culture Conditions

Short-term cell cultures were performed in RPMI 1640 medium supplemented with 0.5% bovine serum albumin (BSA) at 37°C in 5% CO2 in air unless otherwise stipulated. In experiments in which CLL cells were used these were immediately after purification (viability >90%).
Proteins and Protein Digestion Fragments

The purity of proteins and fragments used in this study was determined to be greater than 90% using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Coomassie blue staining of 8% polyacrylamide gels. Protein concentrations were determined using the BioRad Protein Assay (BioRad, Richmond, CA).

FN Purification

FN was purified by gelatin-sepharose affinity chromatography according to the method of Engvall and Ruoslahti (fresh frozen human plasma unsuitable for therapeutic use was provided by BTS Liverpool [Liverpool, UK]).

Cathepsin D Digestion

The 70-kD cathepsin D digest fragment of FN was prepared as described by McKeown-Longo and Mosher. Essentially, FN (2 mg/mL) was dialyzed into a 50 mmol/L tris/acetate buffer, pH 3.5, and was digested for 14 hours using 310 μg/mL cathepsin D pretreated with soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (PMSF). The FN digest was stopped with pepstatin and...
Immunoprecipitation of metabolically labelled FN from HC aggregates

- 240kD

Fig 2. Newly synthesised FN is associated with cultured HCs. Highly purified HCs were metabolically labeled (with 35S-methionine) in culture. Immunoprecipitation from cell extracts with polyclonal anti-FN antibody precipitated a protein of approximate molecular weight for FN (220 kD; left-hand lane). Precipitation with an irrelevant polyclonal (antilaminin) antibody produced no precipitate (right-hand lane) (n = 2).

dialyzed into tris-buffered saline (TBS), pH 7.4. The 70-kD fragment was purified from the digestion mixture by its retention on a gelatin-sepharose column.

Tryptic Digestion

The cell-binding domain (CBD)-containing and CS-1-containing tryptic digest fragments of FN were generated as described by Garcia-Pardo et al. FN (in TBS, pH 7.6) was digested using TPCK-treated trypsin (Sigma, St Louis, MO) 1:200 for 60 to 90 minutes at 37°C. The fragments of interest were retained on a heparin-sepharose affinity column. The 80-kD cell binding and 38-kD CS-1-containing fragments were then purified using ion-exchange chromatography.

Radiolabeling of Proteins and Cell Components

Protein Iodination

FN was iodinated with 125I (Amersham, Arlington Heights, IL) using iodobeads (Pierce, Rockford, IL) according to the manufacturer's recommended method. Samples were desalted using a Sephadex G25 column (Pharmacia, Uppsala, Sweden) and specific activity determined.

Cell Surface Iodination

Highly purified HCs (approximately 10⁶ cells) were surface-la beled with 2 mCi 125I by a lactoperoxidase technique using glucose oxidase to generate hydrogen peroxide according to published methods.

Metabolic Labeling of HC Products

Purified HCs (2 x 10⁶) were cultured in methionine-free minimal essential medium (MEM) supplemented with 100 U/mL penicillin/streptomycin, 100 μg/mL L-glutamine, and 10% fetal calf serum (depleted of FN on a gelatin-sepharose affinity column). After 2 hours, this medium was pulsed for 2 hours with 1 mCi 35S-methio-
nine. Culture was continued for a further 4 hours before lysis for immunoprecipitation.

Antibodies and Immunologic Techniques

Rabbit anti-FN polyclonal antibody was purchased from Behring (Marburg, Germany). Mouse monoclonal anti-FN and cellular FN were from Sigma and a second anticalcellular FN antibody was obtained from Cymbus Bioscience (Southampton, UK).

Anti-Integrin Monoclonal Antibodies (MoAbs)

Anti-β1 (4B4) MoAb was obtained from Coulter (Hialeah, FL), anti-α5 (HP1/3 and HP2/4) MoAbs were a kind gift from Dr A. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain), and anti-α1 (SAM-1) MoAb was purchased from Serotec (Oxford, UK). Other MoAbs, including class-specific controls, were purchased from Becton Dickinson (Mountain View, CA), unless otherwise specified.

APAAP reagents (Dakopatts, Glostrup, Denmark) were used according to standard protocols. Immunoperoxidase reagents (Sigma) were used according to manufacturer’s protocols. In the cell adhesion assay, peroxidase-conjugated f(ab²) fragments (rabbit anti-mouse polyclonal antibody from The Binding Site, Birmingham, UK) were used.

Specific Assays and Techniques

FN Assembly by HCs

Cells (10⁴) were cultured in individual cultures under constant agitation on a roller mixer in serum-free medium as described above, with the additional presence of 125I-labeled FN (5 to 20 µg/mL) and unlabeled FN or its fragments when indicated. Triplicate cultures were stopped after appropriate time intervals and samples were analyzed according to the following techniques.

(1) Detergent extraction. Sequential detergent extraction was performed essentially as described by McDonald et al. The deoxycholate-soluble fraction consisted of labeled FN obtained by extraction successively with 3% Triton X-100 in phosphate-buffered saline (PBS), 0.1 mg/mL DNase I in 1 mol/L NaCl, and 2% deoxycholate. Total radioactivity in the deoxycholate-soluble and -insoluble fractions was determined.

(2) Whole cell lysate. The total cellular material present in each of the cultures was obtained by sequential centrifugation and washing in PBS at 4°C in the presence of PMSF (2 mmol/L final concentration). The total material was then solubilized in 4% SDS at 70°C for 10 minutes either in the presence or absence of 5-mercaptoethanol. Samples were then examined by SDS-PAGE analysis using an 8% polyacrylamide gel.

(3) Fragment inhibition. Triplicate samples were analyzed as described in (1) above after 8 hours of incubation either in the presence or absence of the 70-kD cathepsin D FN-digest fragment (300 µg/mL).

Immunoprecipitation of Metabolically Labeled FN From HCs

Cultured HCs metabolically labeled exactly as described above were washed in 3 cycles of centrifugation/resuspension and then lysed in NP40 lysis buffer at 4°C and then in 4% SDS buffer. At all steps enzyme inhibitors (10 µg/mL aprotinin, 2 mmol/L PMSF, 10 µmol/L EDTA, 8 mmol/L Iodoacetamide) were freshly added. Immunoprecipitation was performed from each of three samples: culture medium, NP40-extractable material, and SDS-extractable material (SDS was taken into mixed micelle form using 1% Triton X 100 according to published methods). FN was specifically immunoprecipitated using a rabbit anti-FN polyclonal antibody. Precipitated complexes were recovered using protein-G sepharose (Pharmacia) and analysis by SDS-PAGE and autoradiography using 8% polyacrylamide gel and reducing conditions.

Immunoprecipitation of Integrins

Surface radio-iodinated HCs prepared as described above were lysed in 1% NP40 lysis buffer in the presence of freshly prepared enzyme inhibitors (as above). Cellular debris was removed by centrifugation and the solubilized material was extensively precleared with protein G sepharose alone (twice) and in the presence of irrelevant IgG1 and IgG2b MoAbs (twice). The precleared samples were then specifically immunoprecipitated using anti-β1, α5, and α1, and control antibodies.

HC Adhesion Assay

A specific HC adhesion assay was developed using 96-well tissue culture plates coated by overnight incubation at 4°C with FN or its fragments. Plates were washed with PBS and 10⁵ HCs suspended in 100 µL of Hanks’ Balanced Salt Solution were allowed to adhere for 15 minutes at 37°C. Plates were washed three times with PBS by inversion and then fixed for 2 minutes in 2% paraformaldehyde.

The number of adherent HCs was specifically determined by an immunocassay. An anti-CD11c MoAb was allowed to bind to the adherent HCs; this antibody was then detected using peroxidase-conjugated F(ab²)₂ fragments of antimouse polyclonal antibody with colorimetric analysis of 0-phenylenediamine (OPD) substrate development.

Fig 4. A 70-kD aminoterminal fragment of FN inhibits matrix assembly by HCs. Cells were cultured for 10 hours in the presence of radiolabeled FN (30 µg/mL) with or without unlabeled FN (300 µg/mL) or its 70-kD cathepsin D digest fragment (300 µg/mL). Both the whole molecule and the 70-kD fragment clearly inhibit incorporation into matrix (the deoxycholate-insoluble fraction).
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RESULTS

HCs Form In Vitro Aggregates Containing Polymeric FN

Circulating HCs had no detectable adhesive proteins bound to or associated with their cell surfaces. In particular, we were unable to show FN, vitronectin, thrombospondin, laminin, or von Willebrand’s factor immunocytochemically in cytospin preparations.

After in vitro culture in serum-free medium for 6 hours, the HCs formed loose, net-like homotypic aggregates. When these aggregates were examined immunocytochemically for the presence of adhesive proteins, abundant FN, but not other serum adhesive proteins, was now found to be present. The FN appeared at cell-cell junctions and in the form of long fibers (Fig 1). Homotypic aggregates did not form under these culture conditions when other B-lymphoproliferative disorders were examined; moreover, no FN was detected immunocytochemically at the surface of these cells.

HCs Synthesise Cellular FN

FN is synthesised in different forms. The cellular form differs from plasma FN and the two forms may be distinguished using specific MoAbs. The synthesis of the cellular form of the molecule appears to be a constant feature of matrix-forming cells.11,12

Using two different antibodies that specifically recognize cellular FN, we showed immunocytochemically that cellular FN appears at the surface of HCs after short-term culture in serum-free medium.

To show directly FN synthesis by HCs, metabolic labeling experiments were undertaken. As shown in Fig 2, labeled FN was precipitable from cellular aggregates in the detergent-extractable (cell-associated) fraction. No labeled FN was detected in the culture medium.

HCs Can Assemble FN Into Matrix Form and This Assembly Is Inhibited by a 70-kD Fragment Known to Inhibit Matrix Assembly by Fibroblasts

Tissue FN consists of both cellular and plasma forms assembled together into multimeric (matrix) form.11 FN does not self-assemble into multimers under physiologic conditions; instead, newly synthesized cellular FN is assembled together with serum FN into disulphide-bonded multimers by the active participation of matrix-forming cells (normally fibroblasts). It was therefore important to establish whether the FN in HCs was disulphide-bonded, and therefore in a multimeric form. Formation of disulphide-bonded matrix FN was measured using the differential solubility of matrix, and nonmatrix (but surface-bound) FN, in nonionic detergent (deoxygen). When HCs were incubated in the presence of small amounts of 125I-labelled plasma FN, radioactivity became increasingly associated with the cellular fraction. The deoxygen nonextractable (matrix) FN increased progressively with time, whereas the extractable (nonpolymerized) FN remained relatively constant (Fig 3). This increase in insoluble labeled FN incorporation was inhibited by an excess (1 mg/mL) of unlabeled FN (not shown). Cells from 3 cases of CLL did not progressively accumulate labeled FN, although low levels of initial FN binding were seen in two of these cases.

When similarly cultured HCs were examined at different time points by SDS-PAGE analysis of whole cell lysates, increasing amounts of radioactivity were observed in the disulphide-bonded, multimeric form (Fig 3).

Formation of an FN matrix from native FN requires cellular interaction with a number of structural sites on the FN molecule. The best characterized site lies on the amino terminal portion of FN and is contained within a 70-kD amino-terminal fragment generated by cathepsin D digestion. This fragment is a specific inhibitor of FN matrix assembly.7,13

The 125I-FN incorporation experiments that were used to show FN matrix assembly by the HCs were performed in the presence of the 70-kD fragment. The fragment inhibited incorporation of labeled FN into the HC cellular aggregates (Fig 4). An irrelevant protein of approximately 70-kD had no effect on incorporation, whereas whole unlabeled FN solely inhibited the process.

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Fig 5. HCs express large amounts of VLA-5 together with VLA-4. FACS analysis (A) using MoAbs against the α chains of the integrins VLA-4 and VLA-5 and appropriate isotypic controls showed the presence of both integrins (n = 7). VLA-5 was consistently expressed at higher density. Immunoprecipitation analysis (B) showed that both α4 and α5 integrins could be precipitated with associated β1 chain (non-reduced SDS-PAGE and autoradiography). Scanning densitometry confirmed that the cell expressed approximately twice as much α5 as α4; α5 was demonstrable in two of its isoforms (α5/160 and the 160-kD form).

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Flow Cytometric Analysis of Integrin Expression on Hairy Cells

Immunoprecipitation Analysis of Hairy Cell Integrin Receptors
HCs Possess Abundant VLA-5 and This Integrin May Be Involved in Matrix Assembly

The best characterized cellular structure involved in matrix assembly is the integrin receptor VLA-5 (the classical FN receptor). This receptor codistributes with and appears to organize newly formed FN fibrils; it also appears to have a role in the initiation of matrix assembly. The binding of VLA-5 is mediated by the RGD-containing CBD on the FN molecule.

A second important FN-binding integrin is VLA-4 and, in contrast to VLA-5, this integrin is highly expressed by circulating B lymphocytes. Binding of VLA-4 to FN is mediated by an alternative site located in the CS-1 region of FN. This site is structurally separate from the CBD and does not appear to be involved in matrix assembly.

APAAP staining showed that HCs consistently express VLA-4 and VLA-5 (n = 9). Figure 5 shows fluorescence-activated cell sorter (FACS) and immunoprecipitation analysis of the expression of VLA-4 and VLA-5 on HCs. Both techniques show that VLA-5 is highly expressed on HCs, whereas VLA-4 is present in smaller amounts.

The relative binding activities of the two integrin receptors were then examined using tryptic digest fragments of FN containing the VLA-4 binding site or the VLA-5 binding site. In an HC-specific adhesion assay, we showed that fragments containing the CBD were far more effective in promoting HC adhesion than those containing the CS-1 (VLA-4 binding) region (Fig. 6).

Taken together, these findings suggest that the VLA-5 of HCs is implicated in the assembly of FN by the cells.

**Discussion**

The present study clearly shows that HCs synthesize and assemble an insoluble matrix of FN.

The synthetic ability of HCs was shown by the appearance of FN on the surface of cells cultured in serum-free medium and by immuno-isolation of the metabolically labeled protein from HC aggregates. Furthermore, the synthesized product was, as expected, the cellular form of the molecule. FN synthesis by cells of B lineage has not been previously shown, but, among hemic cells, is a well-recognized feature of monocytes/macrophages and recently of natural killer cells and some T lymphocytes.

In addition to synthesizing FN, HCs were shown to assemble the molecule into multimers. Newly synthesized product was found exclusively in cell aggregates, and not in the medium. In this regard, the behavior of HCs clearly differs from that of monocyte/macrophages, which produce FN primarily in the soluble form. Furthermore, the HCs clearly differed from related B-cell disorders (CLL, PLL, and CB/CC leukemia) in which no synthesis/assembly was shown under identical conditions. The capacity to assemble FN shown by the HCs is a feature previously only shown in matrix-forming cells.

Matrix formation has been extensively studied in fibroblasts and the mechanisms involved have been largely established. FN does not self-assemble under physiologic conditions, and formation of insoluble FN matrix requires active interaction between the fibroblast and specific binding domains on the FN molecule. The two best characterized sites on FN are the CBD and an area contained within a 70-kD amino-terminal region of the molecule. The CBD is an RGD-containing region that interacts with the VLA-5 integrin at the cell surface. In contrast, the amino-terminal sequence binds to a different, but ill-defined, cell-surface re-
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... the characterization of which is the subject of active investigation. The mechanism involved in matrix assembly by HCs was studied in the light of these concepts and similar mechanisms were shown to be involved. Thus, the 70-kD amino-terminal fragment of FN blocked the assembly of exogenous labeled FN into matrix. Also, HCs were shown to have large amounts of VLA-5 as compared with the VLA-4 generally considered to be the FN-binding receptor of mature B lymphocytes. Furthermore, HCs bound strongly to FN fragments containing the CBD (the VLA-5 binding site). Therefore, it is suggested that the VLA-5 of HCs is involved in matrix assembly. Overall then, HCs under these in vitro conditions resembled professional matrix-forming cells.

We have recently found that HCs possess two other RGD-binding integrins that could potentially interact with the CBD-containing fragment. However, we have also shown that the different HC integrins mediate distinct functional responses and that VLA-5 is the integrin principally involved in the initial spreading response. Because spreading is often associated with FN assembly, these observations are fully in accord with our suggestion that the VLA-5 of HCs is involved in matrix assembly.

Our immunohistochemical studies showed large amounts of FN in HCL BM. When infiltration was patchy, the FN was specifically associated with areas of disease. In contrast, collagen III (a prominent component of fibroblastic fibrosis) was not present. This confirms that fibroblasts are unlikely to be involved in the fibrosis of HCL and points to HCs as the source of the characteristic fibrosis of the disease.

Routine reticulin stains do not detect any specific matrix protein, but the fine reticulin fibrosis of the type observed in HCL is frequently associated with the presence of FN. Most extracellular matrices are complex structures and it is likely that the FN will prove to be bound with other proteins.

Many of the properties of HCs have proved to be shared with normal activated B lymphocytes. In this context, it should be noted that VLA-5 is absent from unstimulated mature B lymphocytes, but appears after cell activation. Furthermore, it has very recently been shown that T cells after in vitro activation can synthesise and bind FN. It is tempting, therefore, to speculate that FN production and/or assembly will prove to have a physiologic role in the function/adhesion of certain other activated B cells; these possibilities are under active investigation in this laboratory.

REFERENCES


The bone marrow fibrosis of hairy-cell leukemia is caused by the synthesis and assembly of a fibronectin matrix by the hairy cells

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