Fibromodulin - an Extracellular Matrix Protein: Characterization of its Unique Gene and Protein Expression in B-Cell Chronic Lymphocytic Leukemia (B-CLL) and Mantle Cell Lymphoma

Eva Mikaelsson¹, Amir Hossein Danesh Manesh¹, Alfred Lüppert¹, Mahmood Jeddi-Tehrani¹,², Mohammad-Reza Rezvany¹, Ramazan Ali Sharifian³, Reza Safaie³, Azam Roohi⁴, Anders Österborg¹,⁵, Fazel Shokri⁴,⁶, Håkan Mellstedt¹,⁵, and Hodjattallah Rabbani¹

¹Immune and Gene Therapy Lab, CCK, Karolinska University Hospital, Stockholm, Sweden
²Department of Immunology, Avesina Research Center, Tehran, Iran
³Clinic of Hematology and Oncology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
⁴Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
⁵Departments of Oncology (Radiumhemmet) and Hematology, Karolinska University Hospital, Stockholm, Sweden
⁶National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran

Corresponding author:
Håkan Mellstedt, M.D., Ph.D., Professor
Department of Oncology (Radiumhemmet)
Karolinska University Hospital
SE-171 76 Stockholm, Sweden
Tel: +46-8-5177 4641, Fax: +46-8-31 83 27
e-mail: hakan.mellstedt@karolinska.se

Running head: Fibromodulin - a tumor marker in B-CLL and MCL.
The study was supported by grants from the Swedish Research council/SIDA/SAREC, the Iranian Ministry of Health and Medical Education, the Swedish Cancer Society, the Torsten and Ragnar Söderberg Foundation, the Cancer Society in Stockholm, the King Gustaf Vth Jubilee Fund, the Cancer and Allergy Foundation, the Gunnar Nilsson Cancer Foundation and the Karolinska Institute Foundations.

Word counts: 4501
Word count Abstract: 173
ABSTRACT

Fibromodulin is an extracellular matrix protein normally produced by collagen-rich tissues. By studying the global gene expression profile of B-cell chronic lymphocytic leukemia (B-CLL) cells, fibromodulin was found to be the most over-expressed (>300-2200 times) gene\(^1,2\).

In this study, fibromodulin was found to be expressed at the gene level (RT-PCR) in all B-CLL patients (n=75) and in the majority (5/7) of patients with mantle cell lymphoma (MCL). No mutations in the fibromodulin gene were detected. Fibromodulin was also detected at the protein level in the cytoplasm of the B-CLL cells and in the supernatant following \textit{in vitro} cultivation, but not at the cell surface. Fibromodulin was not found in T-CLL, B-PLL, T-PLL, hairy cell leukemia, follicular lymphoma, lymphoplasmacytic lymphoma, multiple myeloma, ALL, AML, CML and in 36 hematological cell lines. Normal blood mononuclear cells (T and B lymphocytes, monocytes), tonsil B cells and granulocytes did not express fibromodulin. Activation (PMA/ionomycin) of normal T and B lymphocytes induced a weak fibromodulin gene expression but not to the extent seen in freshly isolated B-CLL cells.

The reason for the exclusive ectopic expression of fibromodulin in B-CLL and MCL is not known. However, the unique protein expression makes it likely that fibromodulin is involved in the pathobiology of B-CLL and MCL.
INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) cells originate from the CD5⁺ B-lymphocyte subpopulation and display distinct immunophenotypic features as well as various genetic defects and chromosomal aberrations. There appears to be an indispensable role for the micro-environment non-tumoral cells in the onset and progression of B-CLL. This involves follicular dendritic cells and CD4⁺ T lymphocytes as well as bone marrow derived stromal cells and endothelial cells. Stromal cells have a role in extending leukemic cell survival. Direct physical contact between leukemic cells and stromal cells is essential for inhibition of apoptosis. Similarly, endothelial cells have the capacity to protect B-CLL cells from apoptosis by means of physical interaction. Viability of B-CLL cells cultured on fibronectin is consistently higher than the viability of control cultures. It has been suggested that direct cell-cell contact and/or matrix or membrane-bound cytokines rather than soluble factors may be important for the in vivo survival of B-CLL cells. Bidirectional malignant lymphocyte-microenvironment interactions may lead to the amplification of a micro-environment able to inhibit apoptosis of B-CLL cells.

Over-expression of numerous genes has been shown using microarray technique including a marked increase of fibromodulin (>300-2200 times). Fibromodulin (42 – 80 kD) is a member of the extracellular class II leucin-rich proteoglycan family involved in the regulation of the assembly of collagen in connective tissues. It is a cytosolic protein with a secretory sequence but no transmembrane or extracellular domain. Although fibromodulin has so far been ignored as a molecule, which may be involved in the pathogenesis of cancer, it has become evident that this and other members of the proteoglycan family are not only involved in collagen fibrillogenesis and cell adhesion but also contribute to the modulation of cytokine activity, tumor growth suppression and prevention of apoptosis. Sulfation of tyrosine residues at the N-terminal part of fibromodulin indicates that fibromodulin might be involved in chemokine receptor signaling.

In the present study, we extended our preliminary finding and could now demonstrate that fibromodulin is a rather unique, non-mutated molecule selectively expressed at the gene and protein level in B-CLL and mantle cell lymphoma (MCL) cells as well as in the supernatant of cultured B-CLL cells. The reason for the
exclusive expression of fibromodulin in these two B cell malignancies is not known. It is urgently warranted to understand the biological functions of fibromodulin in B-CLL/MCL as this unique molecule may be utilized as a target for therapeutic intervention.

MATERIALS AND METHODS
PATIENTS AND CONTROLS
The WHO Classifications of Neoplasms of the Hematopoetic and Lymphoid Tissues were applied. The diagnosis of CLL was based on immunophenotyping (CD5+/CD19+/CD23+/IgM+) and the presence of >5.0x10⁹/l lymphocytes in peripheral blood. Clinical characteristics of the B-CLL patients are shown in Table 1. The phenotype of T cell leukemic cells was in three patients CD3+/CD4+/CD8- and in two patients CD3+/CD4-/CD8+. AML, ALL and CML diagnosis were based mainly on cytomorphology. Five of the CML patients were karyotyped and were all Philadelphia chromosome positive.

Table 1. Clinical characteristics of the B-CLL patient (n=75) material at testing time

<table>
<thead>
<tr>
<th>Gender</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>68 %</td>
</tr>
<tr>
<td>Female</td>
<td>32 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>50-59 years</td>
<td>15 %</td>
</tr>
<tr>
<td>60-69 years</td>
<td>46 %</td>
</tr>
<tr>
<td>70-79 years</td>
<td>36 %</td>
</tr>
<tr>
<td>80-89 years</td>
<td>3 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rai Stage</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24 %</td>
</tr>
<tr>
<td>I</td>
<td>30 %</td>
</tr>
<tr>
<td>II</td>
<td>13 %</td>
</tr>
<tr>
<td>III</td>
<td>16 %</td>
</tr>
<tr>
<td>IV</td>
<td>17 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Previously untreated</td>
<td>32 %</td>
</tr>
<tr>
<td>Previously treated *</td>
<td>68 %</td>
</tr>
</tbody>
</table>

* The majority of the patients had received chlorambucil or fludarabine therapy. At least one month had elapsed since last treatment.
Heparinized peripheral blood was collected from the patients with B-CLL (n=75) as well as from MCL (n=7), T cell chronic lymphocytic leukemia (T-CLL) (n=5), hairy cell leukemia (HCL) (n=10), B-cell prolymphocytic leukemia (B-PLL) (n=1), T cell prolymphocytic leukemia (T-PLL) (n=1), chronic myelogenous leukemia (CML) (n=15), acute myelogenous leukemia (AML) (n=5), acute lymphoblastic leukemia (ALL) (n=14). Bone marrow tumor cells were obtained from patients with B-CLL (n=2), multiple myeloma (n=4), follicular lymphoma (n=2), and lymphoplasmacytic lymphoma (n=4).

Control blood was drawn from normal healthy donors (n=70) with a mean age of 38 years (range 19-75). Fresh human tonsils were obtained from two children after routine tonsilectomy. This study was approved by the Ethics Committee of each institution and informed consent was obtained from all patients in accordance with the Helsinki declaration.

HEMATOLOGICAL AND FIBROBLAST CELL LINES
Thirty-six cell lines derived from a variety of hematological tumors were also included (see Table 3). Characteristics of the Burkitt’s lymphoma cell lines have been described previously19. One B-CLL cell line (EHEB)20 was obtained from the German collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and another B-CLL cell line (I83-E95)21 was a kind gift from Prof Kenneth Nilsson, Uppsala, Sweden. The natural killer lymphoma cell line (YT) was obtained from DSMZ. The remaining hematological cell lines were provided by the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran, Iran). A human fetal foreskin fibroblast cell line (HFFF-PI 6) expressing fibromodulin was obtained from NCBI. All cell lines were adapted to grow in RPMI-1640 medium (no. 42401) (Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS) (Gibco), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco) (complete medium).

ISOLATION OF PERIPHERAL BLOOD CELLS
Normal peripheral blood mononuclear cells (PBMC) (lymphocytes and monocytes), blood tumor cells and bone marrow tumor cells were isolated from blood or bone marrow respectively using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density
gradient centrifugation as earlier described. Granulocytes were recovered from the top of the erythrocyte layer after Ficoll density gradient centrifugation. Erythrocytes were lysed by hypo-osmosis in cold water. More than 98% of the nucleated cells were granulocytes. Tonsil tissue was cut and passed through a metal grid and suspension of tonsil mononuclear cells was prepared by Ficoll-Hypaque density gradient centrifugation as earlier described.

**ISOLATION OF B AND T CELLS**

PBMC from healthy donors were isolated as described above. T and B lymphocytes were purified by negative selection using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer’s instruction. The purity of the isolated populations was checked by direct immunofluorescence using conjugated monoclonal antibodies (MAb) against CD3, CD19, CD14 (Becton-Dickinson, (B-D) San Jose, CA, USA).

Leukemic B cells and tonsil mononuclear cells were also enriched using nylon wool purification.

**ACTIVATION OF NORMAL B AND T LYMPHOCYTES, TONSIL B CELLS AND B-CLL CELLS**

Stable CD40L transfected mouse fibroblast cells (NIH-3T3) were irradiated (100 Gy) and transferred to 6-well plates (TPP, Trasadingen, Switzerland) (150 000 cells/well) in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and L-Glutamine (2 mM). After overnight incubation at 37°C in humified air with 5% CO₂ fibroblasts were washed in PBS. Isolated normal B-lymphocytes, tonsil B cells and B-CLL cells were added (4 x 10⁶ cells/well) and cultured in a total volume of 2 ml of DMEM medium (no. 41965) (Gibco) supplemented with 10% FBS, penicillin, streptomycin and L-Glutamine at 37°C in humified air with 5% CO₂ for 48 h. Cells cultured in medium alone were used as a control.

Isolated T-lymphocytes were stimulated with anti-CD3 antibody (OKT3) (30 ng/ml) (Mabtech, Stockholm, Sweden) using the same culture condition as above.
In addition, normal B- and T-lymphocytes, tonsil B cells and B-CLL cells were also stimulated with 20 ng/ml PMA (phorbol – 12 – myristate – 13 acetate) + 1 µg/ml ionomycin (Sigma, St. Louis, MO, USA).

After 48 h of culture cells were harvested. Activation was confirmed by an increased expression of CD25 and CD69 using monoclonal antibodies (B-D) and flow cytometry. RNA was isolated, cDNA prepared and the relative increase in fibromodulin expression compared to unstimulated cells (time 0 h) was determined by real-time quantitative PCR (see below).

**ACTIVATION OF B-CLL CELL LINES**

Under standard condition the two B-CLL cell lines EHEB and I83-E95 were continuously cultured in RPMI-1640 (Gibco) supplemented with penicillin, streptomycin, L-Glutamine and 10% FBS. Cells in RPMI-1640 were stimulated by co-culture with irradiated CD40L transfected fibroblasts (see above). EHEB cells were also stimulated with 25 ng/ml PMA + 1 µg/ml ionomycin.

Both B-CLL cell lines were also cultured in DMEM medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-Glutamine (2 mM) and 10% FBS (unstimulated) as well as stimulated with 20 ng/ml PMA + 1 µg/ml ionomycin for 48 h. After 48 h of culture cells were harvested. Activation was tested by measuring the expression of CD25 and CD69 using monoclonal antibodies (B-D) and flow cytometry. RNA was isolated, cDNA was prepared and the relative increase in fibromodulin expression compared to unstimulated time 0 h was determined by real-time PCR (see below).

**TRANSFORMATION OF NORMAL B-CELLS WITH EPSTEIN-BARR VIRUS (EBV)**

EBV transformation was performed as previously described. Briefly, purified B lymphocytes (2x10^7 cells) from healthy individuals (n=16) were incubated with cell free supernatant of B95-8 cells at 37 °C for 1h. Cells were washed and incubated in RPMI-1640 supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml).
RT-PCR AMPLIFICATION OF FIBROMODULIN

Total RNA was extracted from tumor cells and normal PBMC using RNAzol B reagent (BioSite, Täby, Sweden) according to manufacturer’s instruction. First strand cDNA was synthesized using 5 µg of total RNA in 20 µl reaction mixture consisting of 5x reaction buffer 4 µl; 10 mM dNTPs 1 µl; 100 uM dithiotheritol (DTT) 1.5 µl; 10 pmol/ml random hexamer (N6) 1 µl, and M-MLV reverse transcriptase 200 units (Gibco). The mixture was incubated at 42 °C for 45 min. PCR amplification was performed using fibromodulin specific primers ACCGTCCCCGATAGCTACTT as sense and CATCCTGGACCTTCCAGCAAA as antisense (GenBank accession No. XM_001782). Briefly, 25 µl reaction mixture of PCR was prepared using 2.5 µl of 10x buffer, 1 µl of 25 mM MgCl₂, 1.5 µl dNTPs (10 mM), 5 pmol of each primer and 1 unit of Ampli-Taq Gold DNA polymerase (Perkin-Elmer/Applied Biosystems, Boston, MA, USA). PCR was performed in 35 cycles, initiated by 1 cycle at 95 °C for 10 min. to activate the Taq Gold DNA polymerase followed by 92 °C; 30 sec, 60 °C; 30 sec, and 72 °C; 30 sec leading to a 448 bp amplicon. PCR product was finally visualized by running agarose gel electrophoresis containing ethidium bromide. To assure the specificity of primers, some of the PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and subjected to sequencing.

REAL-TIME QUANTITATIVE PCR (RT-QPCR)

cDNA samples were used as template and β-actin (endogenous housekeeping gene) was quantified as a positive control against which the different template values were normalized. For β-actin the sense primer was 5’-CGACAGGATGCAAGAGGAGA-3’, antisence primer was 5’-CGTACATCTCGGTTCGCTG-3’ and the probe was 5’-FAM-TCCAACACCTTCAATTCCAGCAGG-TAMRA-3’. Primers for FMOD were the same primers as used for screening. FMOD probe was 5’-FAM-TCCAACACCTTCAATTCCAGCAGC-TAMRA-3’. Ampli-Taq Gold polymerase (Perkin Elmer, Applied Biosystems, New Jersey, USA) was used to catalyze the amplification reaction. PCR conditions were optimized for primers, probes, and MgCl₂. PCR amplification was carried out in 25 µl reaction volume in a 96 well optical PCR plate (N 801-0560, Perkin Elmer). Each reaction volume contained 1xTaqMan Buffer (Perkin Elmer), 5 pmol sense primer (Cybergene AB, Stockholm, Sweden), 5 pmol antisense primer (Cybergene), 2.5 pmol probe (Cybergene), 0.5 units Ampli-Taq Gold and 0.1 units Uracil-N-Glycosilase (UNG) (Perkin Elmer). 1 µl of
the template cDNA was included in each reaction volume except for the non-template control (NTC). Aliquots were then amplified by 1 cycle at 50°C (2 min) followed by 40 cycles of denaturation at 95°C (15 sec.) with annealing and extension at 60°C (1 min.). ABI PRISM 7700 Sequence Detection System (Perkin Elmer) was used for online quantification. For relative quantification of fibromodulin mRNA the following arithmetic formula was used: Relative increase (fold increase) = $2^{-\Delta \Delta CT}$ according to the Perkin-Elmer Instruction Manual (1997) where $\Delta \Delta CT = (CT \text{ of target} - CT \text{ of } \beta\text{-actin})$ at any time point (stimulated samples) – (CT of target – CT of $\beta$-actin) unstimulated samples (at time 0). CT is the point (cycle) at which the amplification plot crosses the threshold. mRNA gene expression is presented as fold increase calculated in relation to unstimulated cells after normalization against $\beta$-actin.

SURFACE AND CYTOPLASMIC STAINING AND FLOW CYTOMETRY

Blood leukemic cells of B-CLL patients and PBMC of normal control donors were washed twice with cold phosphate buffered saline (PBS) and fixed with 1% paraformaldehyde in PBS for 10 min. Following washing with PBS, pelleted cells were treated with 0.5 ml FACS permeabilizing solution (B-D) for 10 min. Permeabilized cells were then washed with washing solution (PBS, 0.2% BSA, 0.1% NaN3, 0.5 mM EDTA and 1% saponin) and incubated for 60 min with an appropriate concentration (5 µg/ml) of affinity purified rabbit antibody specific for a C-terminal peptide of human native fibromodulin. The antibody was a kind gift from Dr. P. Roughley, Genetics Unit, Shriners Hospital for Crippled Children, Montreal, Canada. Non-immune polyclonal rabbit IgG (5 µg/ml) served as negative control. Following three washings, F(ab’)2-fragments of FITC-conjugated goat anti-rabbit IgG (Biosource, Camarillo, CA, USA) was added and the cells were incubated for 45 min. Cells were finally washed twice with washing solution and once with PBS, fixed with 1% paraformaldehyde in PBS and kept at 4 °C until analysis by flow-cytometry (FACS Calibur, BD). For direct surface staining, cells were incubated with FITC-conjugated MAb specific for CD19 or CD5 (BD) for 30 min, washed with washing solution without saponin and fixed as above. FITC-labeled irrelevant isotype-matched MAb served as control.
Surface-staining for membrane-bound fibromodulin was performed in indirect immunofluorescence using the same primary and secondary antibodies as above.

**WESTERN BLOT**

PBMC were lysed in buffer containing 2% Triton X-100, 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 mM Na4P2O7, 1% Glycerol, 0.1% SDS and 1% protease inhibitor cocktail. Cell lysate of $5 \times 10^5$ cells was run on a 10% SDS-PAGE gel at 200 V for 1 h under non-reducing conditions. After electrophoresis, resolved proteins were transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) in a mini Transblot cell (Invitrogen, Carlsbad, CA, USA). The membranes were blocked over night with 5% non-fat milk in PBS plus 0.05% Tween 20 (PBS-T). All additional immunostaining steps, as well as washing steps, were performed in PBS-T supplemented with 5% non-fat milk (Semper, Stockholm, Sweden). Filters were incubated with a rabbit polyclonal antibody against native human fibromodulin (a kind gift from Prof D Heinegård, Dept. of Cell and Molecular Biology, Lund University, Lund, Sweden) over night and a mouse anti-HLA class I Mab (Harlan Sera-Lab Ltd., Hillcrest, UK) for 2 h as a control. Following extensive washings, filters were incubated with the secondary antibody, an HRP conjugated goat anti-rabbit antibody or a goat anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) respectively for 1 h. The filters were developed using enhanced chemiluminescence ECL system (Amersham Pharmacia) according to the manufacturer’s instruction.

**IN VITRO SECRETION OF FIBROMODULIN**

PBMC were isolated from peripheral blood of CLL patients and healthy donors. T and B lymphocytes from healthy donors were also enriched by nylon wool purification (see above). Isolated cells including the fibromodulin positive fibroblast cell line (HFFF-PI 6) were cultured for 40h in 6-well plates ($4 \times 10^6$ cells/well) (TPP, Trasadingen, Switzerland) in 2 ml of AIM-V medium (Gibco) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM) at 37°C in humidified air with 5% CO₂. After culturing, supernatants were harvested and analysed by western blot using the same antibodies and conditions as above. Purified fibromodulin control protein was a kind gift from Prof D. Heinegård.
MUTATION ANALYSIS OF THE FIBROMODULIN GENE
PBMC from healthy individuals and B-CLL patients were isolated and subjected to RNA and genomic DNA preparation as previously described. Fibromodulin specific primers were designed to amplify exon 2 and 3 coding regions from genomic DNA of healthy donors and full-length coding region of cDNA and genomic DNA of CLL patients (S: CACAGGCACGCACACTCTCA, AS: GGTTCTCCAGGTTGGTTG (1082 bp DNA); S: GAAAACATCTGCCCTCCATC, AS: AGCCAAACCAAACCATCAAG (350 bp DNA); S: CACAGGCACGCACACTCTCA, AS: AGCCAAACCAAACCATCAAG (1328 bp cDNA). S= Sense, AS= Antisense. The PCR product was cloned into pGEM-T easy vector and subjected to sequences using ABI310 genetic analyzer (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA).

RESULTS

FIBROMODULIN GENE EXPRESSION IN TUMOR CELLS FROM PATIENTS WITH HEMATOLOGICAL MALIGNANCIES AND HEMATOLOGICAL CELL LINES
PBMC of all B-CLL patients (n=75) expressed fibromodulin at the mRNA level as did the leukemic cells from 5 out of 7 MCL patients. Bone marrow mononuclear cells from B-CLL patients (n=2) also expressed fibromodulin (Table 2, Fig. 1). Fibromodulin was not expressed in tumor cells of patients with T-CLL, B-PLL, T-PLL, hairy cell leukemia, follicular lymphoma, lymphoplasmocytic lymphoma, multiple myeloma, AML, ALL and CML. Neither was fibromodulin expressed in any of 36 different hematological cell lines including 2 B-CLL cell lines kept under standard cell culture conditions (RPMI-1640 medium) (Table 3, Fig. 2). Fibromodulin was not expressed in fresh PBMC (lymphocytes and monocytes) of healthy donors (n=70), isolated normal B cells (purity >90%) (n=6), isolated tonsil B cells (purity >90%) (n=2), isolated T cells (purity >70%) (n=4) and isolated blood granulocytes (purity >98%) (n=10).
Table 2. Fibromodulin gene expression (RT-PCR) in freshly isolated tumor cells of patients with various hematological malignancies and blood nucleated cells of healthy control donors.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>No. of pos. cases/total no. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-CLL (peripheral blood)</td>
<td>75/75</td>
</tr>
<tr>
<td>B-CLL (bone marrow)</td>
<td>2/2</td>
</tr>
<tr>
<td>Mantle cell lymphoma (peripheral blood)</td>
<td>5/7</td>
</tr>
<tr>
<td>B-PLL (peripheral blood)</td>
<td>0/1</td>
</tr>
<tr>
<td>T-CLL (peripheral blood)</td>
<td>0/5</td>
</tr>
<tr>
<td>T-PLL (peripheral blood)</td>
<td>0/1</td>
</tr>
<tr>
<td>Hairy cell leukemia (peripheral blood)</td>
<td>0/10</td>
</tr>
<tr>
<td>Follicular lymphoma (bone marrow)</td>
<td>0/2</td>
</tr>
<tr>
<td>Lymphoplasmocytic lymphoma (bone marrow)</td>
<td>0/4</td>
</tr>
<tr>
<td>Multiple myeloma (bone marrow)</td>
<td>0/4</td>
</tr>
<tr>
<td>CML (peripheral blood)</td>
<td>0/15</td>
</tr>
<tr>
<td>AML (peripheral blood)</td>
<td>0/5</td>
</tr>
<tr>
<td>ALL (peripheral blood)</td>
<td>0/14</td>
</tr>
<tr>
<td>Normal PBMC (lymphocytes and monocytes)</td>
<td>0/70</td>
</tr>
<tr>
<td>Normal blood B lymphocytes *</td>
<td>0/6</td>
</tr>
<tr>
<td>Normal tonsil B lymphocytes*</td>
<td>0/2</td>
</tr>
<tr>
<td>Normal blood granulocytes **</td>
<td>0/10</td>
</tr>
<tr>
<td>Normal blood T lymphocytes ***</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* purity >90%, ** purity >98% *** purity >70%
Table 3. Fibromodulin expression (RT-PCR) in hematological cell lines*

<table>
<thead>
<tr>
<th>Cell lines (total no)</th>
<th>Fibromodulin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkitt’s lymphoma (n=20)</td>
<td>neg</td>
</tr>
<tr>
<td>T cell leukemia (n=3)</td>
<td>neg</td>
</tr>
<tr>
<td>Multiple myeloma (n=4)</td>
<td>neg</td>
</tr>
<tr>
<td>ALL (n=3)</td>
<td>neg</td>
</tr>
<tr>
<td>Promyelocytic leukemia (n=1)</td>
<td>neg</td>
</tr>
<tr>
<td>Myelocytic leukemia (n=1)</td>
<td>neg</td>
</tr>
<tr>
<td>Histiocytic leukemia (n=1)</td>
<td>neg</td>
</tr>
<tr>
<td>NK cell lymphoma (n=1)</td>
<td>neg</td>
</tr>
<tr>
<td>B-CLL (n=2)</td>
<td>neg</td>
</tr>
</tbody>
</table>

* The cell lines were under standard conditions kept in continuous culture in RPMI-1640 with penicillin, streptomycin, glutamin and 10% FBS.
Figure 1. Expression profile of fibromodulin mRNA in leukemic cells of B-CLL (n = 5), ALL (n = 2) and CML (n = 2) patients respectively as well as a healthy control donor (NI 1). Positive (+ve) controls represent the PCR product cloned into pGEM-T easy vector (right panel), B-CLL pooled cDNA (left panel). Negative (-ve) control is the reaction mix without template. Marker is a 100 bp DNA ladder.

Figure 2. Expression profile of fibromodulin mRNA of hematological cell lines. Positive (+ve) controls represent PCR product cloned into pGEM-T easy vector and negative (-ve) control is the reaction mix without template. Marker is a 100 bp DNA ladder.
FIBROMODULIN PROTEIN EXPRESSION IN B-CLL CELLS

Cytoplasmic and surface staining for fibromodulin was done using indirect immunofluorescence. Leukemic B-cells from CLL patients (n=2) were specifically stained in the cytoplasm, but not B cells of control donors. A representative experiment is shown in Fig. 3. No surface staining for fibromodulin could be detected (data not shown). Western blot analysis of cell lysates from 12 CLL patients demonstrated in all patients specific fibromodulin bands around expected size. Representative immunoblots are depicted in Fig. 4.

Supernatants of B-CLL cells cultured for 40h showed the presence of a 60 kD protein reactive with a polyclonal anti-fibromodulin antibody. This was also seen in the culture medium from a fibromodulin positive fibroblast cell line (HFFF-PI 6). No band could be seen in cultures of PBMC or enriched T and B cells of healthy donors (Fig. 5).

Figure 3. Intracytoplasmic staining for fibromodulin and surface-membrane staining for CD5 and CD19 of leukemic cells from a patient with B-CLL (A) and PBMC from a healthy control donor (B) (a representative experiment). The shaded area represents background staining with a control antibody (see Materials and Methods). Figures in brackets indicate percent positive cells.
**Figure 4.** Immunoblotting of leukemic cell lysates of two B-CLL patients (lanes 1, 2) and PBMC of a normal healthy control donor (lane 3) incubated with anti-fibromodulin antibody (top) and anti-MHC class I antibody (bottom) (see Materials and Methods).

**Figure 5.** Immunoblotting (Western blot) of supernatants from cells cultured in AIM-V medium for 40h. Culture medium alone (lane 1), fibromodulin positive (PCR) cell line (HFFF-PI 6) (lane 2), PBMC of healthy donor (lane 3), B-CLL cells (lane 4), T cells of a healthy donor (lane 5), B cells of a healthy donor (lane 6). Molecular weight marker to the left.
ACTIVATION OF NORMAL B AND T LYMPHOCYTES, TONSIL B-CALLS, CLL B-CALLS AND B-CLL CELL LINES

In unstimulated normal B- and T-lymphocytes, tonsil B-cells and in B-CLL cell lines cultured under standard conditions (RPMI-1640 medium) no fibromodulin expression at the gene level could be detected.

Culturing of normal B- and T-lymphocytes as well as tonsil B-cells in DMEM medium alone for 48 h induced a weak expression of fibromodulin (12, 4 and 3 fold increase, respectively). A slight increase in fibromodulin was also noted in freshly isolated B-CLL cells (5 fold) (Table 4).

Co-culturing of normal B-lymphocytes, tonsil B-cells and B-CLL cells in DMEM medium with CD40L transfected fibroblasts did not induce fibromodulin expression. Stimulation of T-lymphocytes with anti-CD3 monoclonal antibodies induced a weak fibromodulin expression (fold increase 2.5) (Table 4).

The polyclonal activator, PMA/ionomycin, induced a stronger fibromodulin gene expression in normal B- and T-lymphocytes (24 and 14 fold increase respectively). However, neither B-CLL cells nor tonsil B-cells showed an increased expression upon this kind of stimulation (Table 4, Fig. 6).

The CLL cell lines EHEB and I83-E95 cultured in RPMI-1640 did not express fibromodulin, not even after stimulation with CD40L or PMA/ionomycin. However, when cultured in DMEM, both EHEB and I83-E95 cells showed induction of fibromodulin expression, which was further increased by PMA/ionomycin stimulation (Table 4, Fig. 7).

EBV transformed normal B cells from healthy donors showed a weak fibromodulin gene expression in two out of 16 tested donors (data not shown).
Table 4. Fibromodulin expression (RT-QPCR) in activated cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Stimulator</th>
<th>CD25+ cells (%)</th>
<th>CD69+ cells (%)</th>
<th>Fold increase of FMOD expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched normal B cells (purity&gt;90%)</td>
<td>Unstimulated (0 h)</td>
<td>24</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DMEM medium alone (48h)</td>
<td>32</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + CD40L (48 h)</td>
<td>74</td>
<td>88</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + PMA/ionomycin (48 h)</td>
<td>95</td>
<td>62</td>
<td>24</td>
</tr>
<tr>
<td>Enriched normal T cells (purity&gt;70%)</td>
<td>Unstimulated (0 h)</td>
<td>22</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DMEM medium alone (48 h)</td>
<td>8</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + anti-CD3 (48 h)</td>
<td>73</td>
<td>65</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + PMA/ionomycin (48 h)</td>
<td>ND</td>
<td>ND</td>
<td>14</td>
</tr>
<tr>
<td>Enriched tonsil B cells (purity&gt;90%)</td>
<td>Unstimulated (0 h)</td>
<td>4</td>
<td>54</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DMEM medium alone (48h)</td>
<td>36</td>
<td>51</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + CD40L (48h)</td>
<td>75</td>
<td>65</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + PMA/ionomycin (48h)</td>
<td>76</td>
<td>77</td>
<td>1</td>
</tr>
<tr>
<td>B-CLL</td>
<td>Unstimulated (0 h)</td>
<td>48</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DMEM medium alone (48h)</td>
<td>57</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + CD40L (48h)</td>
<td>96</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + PMA/ionomycin (48h)</td>
<td>100</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>EHEB</td>
<td>RPMI medium alone (0 h)</td>
<td>11</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RPMI medium alone (48h)</td>
<td>17</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RPMI medium + CD40L (48h)</td>
<td>21</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RPMI medium + PMA/ionomycin (48h)</td>
<td>27</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DMEM medium alone (48h)</td>
<td>13</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + PMA/ionomycin (48h)</td>
<td>ND</td>
<td>ND</td>
<td>114</td>
</tr>
<tr>
<td>I83</td>
<td>RPMI medium alone (0 h)</td>
<td>3</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RPMI medium alone (48h)</td>
<td>7</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RPMI medium + CD40L (48h)</td>
<td>11</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>RPMI medium + PMA/ionomycin (48h)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DMEM medium alone (48h)</td>
<td>3</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>DMEM medium + PMA/ionomycin (48h)</td>
<td>33</td>
<td>8</td>
<td>21</td>
</tr>
</tbody>
</table>

* Results are expressed as fold increase in comparison to unstimulated cells time 0 h.
Mean values of two experiments.
Figure 6. Fibromodulin expression in activated B- and T-lymphocytes, tonsil B-cells and B-CLL cells after 48 h of culture. Increase was compared to time 0 h measured by real-time quantitative PCR (RT-QPCR).
Figure 7. Fibromodulin expression in activated B-CLL cell lines after 48 h of culture. Increase was compared to time 0 h measured by real-time quantitative PCR (RT-QPCR).

MUTATION ANALYSIS OF THE FIBROMODULIN GENE IN HEALTHY INDIVIDUALS AND B-CLL PATIENTS

To determine mutations in the coding region of the fibromodulin gene giving rise to ectopic expression or truncation of fibromodulin, B-CLL cells (n=10) and PBMC of healthy individuals (n=10) were analyzed both at the DNA and cDNA level. No mutations were found in the fibromodulin gene of B-CLL patients compared to the fibromodulin gene submitted to Genbank (gb; XM_001782).
DISCUSSION

Freshly isolated leukemic cells from all B-CLL patients and the majority of patients with MCL expressed the ECM (ExtraCellularMatrix) protein fibromodulin at the gene and protein levels. No mutations in the fibromodulin gene were noted. The fibromodulin protein was detected in the cytoplasm but not on the cell surface. Short-term culture of CLL cells showed the presence of the fibromodulin protein in the cell culture medium. Expression of fibromodulin in hematological malignancies seemed to be exclusive for B-CLL and MCL as fibromodulin was not detected in T-CLL, B-PLL, T-PLL, hairy cell leukemia, follicular lymphoma, lymphoplasmocytic lymphoma, multiple myeloma, AML, CML, ALL as well as in a panel of hematological cell lines. Fibromodulin was not expressed in freshly isolated normal PBMC (lymphocytes and monocytes) including enriched T and B lymphocytes, tonsil B cells as well as purified granulocytes. However, 48h in vitro culture with a strong polyclonal activator (PMA/ionomycin) induced fibromodulin gene expression in normal B and T lymphocytes but no or marginally increase in B-CLL cells and tonsil B-cells.

The reason for this unexpected exclusive and specific expression of fibromodulin in B-CLL and MCL is not known. Gene expression profiling of B-CLL has shown over-expression of many other genes e.g. CD5 (a T cell surface glycoprotein), ZAP-70 (TCR associated signaling molecule) including fibromodulin, which are not B cell specific. Abnormalities of chromosome 1, where the gene for fibromodulin is located (1q32) are rarely seen in B-CLL and no mutations in the gene were found. Recent published data indicate abnormalities of chromosome 1p34-36 28, where the gene for receptor tyrosine kinase Ror1 is located. Over-expression of this gene 29 as well as other genes including CD30, CD21, and interleukin 10, all located on chromosome 1, has been reported in CLL1,30,31. Own unpublished data also indicate that there are several other genes on chromosome 1 which may be exclusively over-expressed in B-CLL cells. Such a gene expression signature involving several chromosomes raises the question of domain or cluster specific regulation involved in the tumorigenesis in B-CLL. This expression pattern including T cell markers implies a possible disruption in regulatory element(s), possibly from a lymphoid progenitor cell i.e. before differentiation into B and T cells. The mechanism behind such a differential expression with no major alteration in the genome might be alteration in DNA methylation covering e.g. fibromodulin locus.
Fibromodulin is a cytosolic secreted protein with a restricted expression pattern mainly to cartilage, bone, connective tissues and tissues rich in collagen. Fibromodulin is involved in fibrillogenesis, cell adhesion and modulation of cytokine activity. This raises the idea of involvement of fibromodulin in tumor suppression and prevention of apoptosis as for other proteoglycans like decorin. In normal tissues fibromodulin seems to be developmentally regulated with higher levels of expression early in life and decreased expression during aging. Fibromodulin knock-out mice demonstrated collagen malformation and symptoms resembling osteoarthritis but no life-threatening structural or physiological defects were observed.

Over-expression of ECM proteins has been observed in malignant cells but the underlying mechanisms are poorly understood. ECM may promote angiogenesis. ECM might interact with cell surface associated heparan sulfate proteoglycans and regulate proliferation and angiogenesis. Myeloma cells express the heparan sulfate proteoglycan, syndecan-1, which is a receptor for the hepatocyte growth factor, which may act as a growth factor for myeloma cells. Tumor stromal cells also produce and secrete increased amount of ECM. This network of cells, ECM and soluble growth factors may support growth of tumor cells and angiogenesis as well as promote tumor progression and spread.

Tyrosine sulfation is a common post-translational modification of proteins and seems to be of importance for protein-protein interactions. Tyrosine sulfation has been noted of ECM proteins including tyrosine residues at the N-terminal of fibromodulin. Analyses of the functional consequences of tyrosine sulfation and/or phosphorylation of the N-terminal of fibromodulin in B-CLL might contribute to a better understanding of the function of this ectopic expressed protein in B-CLL. Sulfation may not exclude phosphorylation of tyrosine residues within the same domain as serine phosphorylation of ECM sialoproteins has been reported.

The micro-environment has been suggested to be of importance for the survival of tumor cells of low-grade B cell neoplasms including B-CLL. Cell-cell as well as cell-matrix interactions have been shown to be critical events in preventing apoptosis of malignant lymphocytes. In B-CLL, direct contact between leukemic and bone marrow stromal cells have been found to be essential for tumor cell survival. Bone marrow...
stromal cells express various adhesion molecules involved in hematopoiesis. Leukemic cells exhibit several types of receptors that can bind to the adhesion molecules. This interaction may provide survival signals for the leukemic cells. Fibromodulin may play a role in the physical interaction and signaling between B-CLL cells and the micro-environment. Fresh B-CLL cells died rapidly in vitro when cultured in medium alone. However, when cultured on fibronectin survival was prolonged.

Resistance of B-CLL cells to pro-apoptotic and anti-proliferative effects of transforming growth factor beta (TGF-β) has earlier been reported. TGF-β is secreted by B-CLL cells, without a significant inhibitory effect on the leukemic B cells which is not the case for normal B-cells where autocrine produced TGF-β inhibits differentiation and proliferation. These results may suggest an adaptation of the anti-apoptotic mechanisms of TGF-β in B-CLL cells. Resistance to the inhibitory effects of TGF-β has been suggested to be associated with loss of TGF-β receptors, which has not been confirmed by others, indicating other mechanisms as well. Fibromodulin has been shown to modulate and suppress TGF-β functions in vivo and in vitro. Fibromodulin has binding sites for TGF-β and binding of TGF-β to fibromodulin in B-CLL may inhibit the apoptotic activity of TGF-β.

The two B-CLL cell lines did not express fibromodulin when cultured in standard RPMI medium and no effect of CD40L or PMA/ ionomycin activation was noted. However, when cultured in DMEM medium, fibromodulin expression was induced which was further enhanced by PMA/ionomycin activation. DMEM medium has a higher glucose concentration than RPMI-1640 and contains Fe(NO₃). Glucose and iron have been reported to induce TGF-β production. Iron is of importance for the expression of cell-cycle and apoptosis related genes and may inhibit proliferation of Burkitt lymphoma cells. Iron is also required for PMA-induced expression of certain genes and necessary for protein kinase C activity. Fibromodulin as well as the closely related proteoglycan decorin binds TGF-β, which binding inhibits the function of TGF-β. In myocytes, iron overload increased the expression of decorin and decreased that of TGF-β. Reduction of TGF-β might be due to binding to decorin. Maybe fibromodulin was induced secondary to a glucose/iron-induced TGF-β
expression. As there is no genomic aberration of the fibromodulin locus, the expression might be considered to be the effect of epigenetic factors. Whether fibromodulin can be induced in the other hematological cell lines using those culture conditions is not known at present and requires further studies.

In summary, we have shown that the ECM protein fibromodulin is exclusively expressed and secreted by B-CLL cells and in the majority of MCL cases but not in other hematological malignancies. The gene showed no mutations and the molecular weight was as expected. In normal B- and T-lymphocytes a strong activation signal (PMA/ionomycin) induced a weak expression of fibromodulin. PMA induces cell differentiation signals through the protein kinase C pathways but also apoptosis. Fibromodulin may be a survival factor constitutively expressed in B-CLL cells but upon stimulation/stress also in normal B and T-lymphocytes. Induction of fibromodulin was not seen in tonsil B-cells and the activation signal did not increase fibromodulin expression in B-CLL cells. Tonsil B-cells similar to CLL cells do not have the propensity to undergo apoptosis. Tonsil B-cells may exhibit other survival factors making fibromodulin redundant. The functional role of fibromodulin in the pathogenesis of B-CLL and MCL remains to be established. The unique expression of fibromodulin in B-CLL makes it also an interesting structure for targeted therapy. Spontaneous expansion of CD8+ T-cells recognizing fibromodulin peptides was recently shown in B-CLL.

**ACKNOWLEDGEMENTS**

We thank Ms. Ingrid Eriksson, Ms. Barbro Näsman-Glaser, Dr. R. Payam, K. Esmailzadeh, J. Ghasemi and Ahmadi for their invaluable technical assistance. For excellent secretarial help we thank Ms. Gunilla Burén and Ms. Gerd Ståhlberg.
REFERENCES


12. Hildebrand A, Romaris M, Rasmussen LM, Heinegard D, Twardzik DR, 
Border WA, Ruoslahti E. Interaction of the small interstitial proteoglycans 
biglycan, decorin and fibromodulin with transforming growth factor beta. 

13. Yamaguchi Y, Mann DM, Ruoslahti E. Negative regulation of transforming 

Decorin suppresses tumor cell growth by activating the epidermal growth 

endothelial cells by Akt-dependent and -independent pathways. Ann N Y Acad 
Sci 2002;973:149-152.

in extracellular leucine-rich repeat proteins using mass spectrometry. J Biol 

17. Mikaelsson E, Jeddi-Tehrani M, Osterborg A, Shokri F, Mellstedt H, Rabbani 
H. Fibromodulin - a novel tumor associated antigen exclusively expressed in 
tumor B-cells from patients with chronic lymphocytic leukemia and mantle 

18. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman 
J, Lister TA, Bloomfield CD. The World Health Organization classification of 
neoplasms of the hematopoietic and lymphoid tissues: report of the Clinical 

Biased utilization of immunoglobulin variable region heavy- and light-chain 
genes by the malignant CD5- B lymphocytes from patients with Burkitt's 

den Berghe G, Bontemps F. Resistance to 2-chloro-2'-deoxyadenosine of the 

adhension of activated B-chronic lymphocytic leukaemia (B-CLL) cells via a 


Fibromodulin - an extracellular matrix protein: characterization of its unique gene and protein expression in B-cell chronic lymphocytic leukemia (B-CLL) and mantle cell lymphoma