Expression of human liver HSPGs on acute myeloid leukemia

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Abstract
Heperan sulfate proteoglycans (HSPGs) play important biological roles in cell-matrix adhesion processes and are essential regulators of growth actions. The expression of the different HSPGs in itself is tightly regulated providing strict controls on the activities of the bound ligands. Human liver is a target for a number of pathogens, and HSPGs have been demonstrated in several cases to play a pivotal role in infectivity. Despite HSPGs important biological functions, little is known about its cell-specific distribution patterns. Human liver HSPG was isolated, and a specific monoclonal antibody (mAb) 1E4-1C2 was produced. Distribution of HSPG reactive to this mAb was studied in normal blood cells, hematopoietic cell lines and blood cells isolated from patients with various hematologic disorders using indirect immunofluorescence. There was no expression of molecules recognized by this mAb on lymphoid (Daudi, Jurkat, Sup-T1) and mononcytoid (U937) cell lines. Peripheral blood cells, normal bone marrow, together with leukocytes isolated from patients with acute lymphoblastic leukemia, chronic myelocytic leukemia, Hodgkin’s disease or Non-Hodgkin’s lymphoma, were also negative. In contrast, 1E4-1C2 showed significant positive results on human myeloid cell lines HL-60 and K562. Moreover, it is interesting that this mAb also recognized epitopes on leukocytes isolated from acute myeloblastic leukemia. These results suggest that malignancies of cells in myeloid lineage may cause expression of HSPGs that are detected by this specific mAb, making it a potential co-marker for the diagnosis of acute myeloid leukemia.

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Introduction
Acute myeloid leukemia (AML) is a complex disease with considerable phenotypic and genotypic heterogeneity. Most cases of AML are sporadic, characterized by acquisition of somatic mutations in hematopoietic progenitors that confer a proliferative and/or survival advantage, impair hematopoietic differentiation and confer properties of limitless self-renewal [1]. A recent study suggests that the certain leukemia oncogenes such as the MLL-ENL fusion commandeer programs of self-renewal in committed progenitors and enforce phenotypical expression of markers associated with hematopoietic stem cells [2]. These may not only be valid targets for therapeutic intervention, but might also provide insights into approaches to confer properties of self-renewal.

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to adult somatic cells for therapeutic benefit, such as tissue regeneration. AML is usually diagnosed by examination of a patient with a blood smear having a total white count from 1000/mm³ to >200,000/mm³. Ten percent of patients, however, still have normal white blood count without circulating blasts. Bone marrow aspiration and biopsy also need to be performed for cytogenetic and immunophenotypic studies. Bone marrow in a patient with AML typically is hypercellular, with absent or a decreased level of megakaryocytes [3]. Cytogenetic studies are important prognostic indicators and are generally performed at the time of the initial bone marrow aspiration and biopsy [4]. Cytogenetic as well as molecular genetic studies are of major importance in diagnosing AML and defining the major AML subtype [4]. However, these tests require viable and dividing cells and can take up to 2 weeks for a complete analysis.

Classification and diagnosis of AML basically rely on the morphologic and cytochemical system proposed in 1976 [5,6]. The World Health Organization has revised this classification system to include unique clinical and biological subgroups (M0-M7). Immunophenotyping, using monoclonal antibodies (mAbs) to identify myeloid- and lymphoid-associated antigens, has been very helpful in defining such subgroups. The percentage of positive reacting blasts should be greater than 20%, with one or more of myeloid-associated antigen, CD33 or CD13, to establish the presence of AML [7]. Individual antibodies that correlate well with the morphologic classification include CD34 and CD117 in subgroups M0 and M1. The expression of these surface antigens on myeloblasts, however, does not always agree with morphologic or cytogenetic staining features [8,9]. Therefore, the use of additional mAbs that can identify other phenotypic markers might be of clinical importance. A majority of myeloid blast cells express differentiation markers asynchronously, and this unusual coexpression of normal differentiation antigens is common.

HSPGs interact with various extracellular matrix components, adhesion molecules, proteinase inhibitors, growth factors and transcription factors and control spatial distribution and bioavailability of these molecules. HSPGs in liver act as a critical receptor for apoE and are involved in lipid metabolism [10,11] and are likely involved in some of the pathology associated with Alzheimer’s disease [12,13]. Human liver is also a target of a number of pathogens, and heparan sulfate has been demonstrated in several cases to play a pivotal role in infectivity [14-16]. The liver is known to be a rich source of HSPGs. While heparin is intracellular and found in granulated cells [17], HSPGs are extracellular and commonly found on cell membranes, suggesting their importance as a receptor or coreceptor for various heparin-binding proteins [18]. Recently, human liver HSPG with an elevated sulfation level was isolated and purified [19]. Since HSPGs are involved in controlling the growth and differentiation of a variety of cell types, mAbs were prepared against this human HSPG and used as a tool to distinguish between hematopoietic cell types. We report that human liver HSPG is expressed on the other cell types, most interesting on certain malignant cells in myeloid lineage. We propose to apply a specific mAb prepared against this human HSPG as a tool to detect leukemic cells.

### Materials and methods

#### Cells, reagents and antibodies

HSPG D2 fraction, used as an antigen in this study, was isolated and purified from human liver [19]. Normal blood samples were buffy coats isolated from healthy normal humans. Hematopoietic cell lines (Daudi, Sup T-1, Jurkat, U937, HL-60 and K562) used in this study were kindly provided from Dr. Watchara Kasinrerk, Faculty of Associated Medical Sciences, Chiang Mai University. All cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS (Gibco, Grand Island, NY), 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B in a humidified atmosphere of 5% CO₂ at 37°C. The study of bone marrow aspirates was performed using specimens from patients with Hodgkin’s disease (HD, n=2) and Non-Hodgkin’s lymphoma (NHL, n=10). For peripheral blood, the buffy coats tested were prepared from EDTA anticoagulated whole blood of patients with various hematologic malignancies (acute myeloblastic leukemia (AML, n=12), acute lymphoblastic leukemia (ALL, n=5) and chronic myelocytic leukemia (CML, n=1)). All patients were admitted at Maharaj Hospital, Faculty of Medicine, Chiang Mai University. There was no treatment performed at the time of blood collection. Individual cells tested were washed twice with cold 1% bovine serum albumin (BSA)-phosphate buffered saline (PBS) containing sodium azide, at pH 7.2 (1% BSA-PBS azide) and adjusted to 1×10⁷ cells/ml before each assay. In direct immunofluorescence assays, PE-conjugated anti-CD33 and PE-conjugated anti-CD42b (Zymed, San Francisco, CA) were used as markers for myeloblastic cells and as a negative control antibody, respectively. mAb MT99/3, IgG1 specific to all cell lines tested was a kind gift from Dr. Watchara Kasinrerk, Faculty of Associated Medical Sciences, Chiang Mai University and was used as positive control. Anti-HIV p24, IgG1 isotype control for the indirect immunofluorescence assay was kindly provided from Dr. Sakchai Dettrirat, Faculty of Associated Medical Sciences, Chiang Mai University.

#### Hybridoma production

The HSPGs from human liver were isolated and purified as previously described [19]. Antibody against the highly anionic, high molecular weight (D2) fraction (Fig. 1) was generated by immunization of a female BALB/c mouse at 1-week intervals using D2 fraction of 100 μg/μl/dose via the intraperitoneal route. Splenocytes were collected and fused with P3-X63Ag8.653 myeloma cells by standard hybridoma fusion techniques using 50% polyethylene glycol and HAT medium selection. The IgG1 isotype of the mAb was determined using an isotyping ELISA kit (Calbiochem).

#### Distribution of human liver HSPG epitope on normal human peripheral blood cells by two-step staining

The binding of mAb to cells was analyzed by indirect immunofluorescence followed by direct immunofluorescence using FITC-conjugated rabbit anti-mouse Isg and
PE-conjugated rabbit anti-CD33 (DAKO A/S, Denmark), respectively. Heparinized whole blood was drawn from healthy subjects (n=10). Samples were centrifuged at 250×g;uffy coat was collected, washed and adjusted to 1×10⁷ cells/ml with cold 1% BSA-PBS azide. To block non-specific FcR-mediated binding of mAb, cells were pre-incubated for 30 min on ice with 10% human AB serum before staining. Fifty microliters of cell suspension was then incubated with equal volume of 1E4-1C2 mAb for 30 min on ice. Cells were washed twice with cold 1% BSA-PBS azide and incubated with FITC-conjugated rabbit-anti-mouse Igs for another 30 min. Cells were then washed 3 times with the same buffer and suspended to 50 µl before adding equal volume of PE-conjugated rabbit anti-CD33. The mixture was incubated on ice for another 30 min before washing. Finally, remaining red blood cells were lysed before suspending with 500 µl of 0.5% paraformaldehyde and analyzed on a FACScalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer. Individual populations of blood cells were gated according to their forward and side scatter characteristics. PE-conjugated anti-CD42 mAb (Zymed, San Francisco, CA) was used as negative control in direct immunofluorescence.

Distribution of human liver HSPG epitope on hematopoietic cell lines by indirect immunofluorescence assay

Cell lines (Daudi, Sup T-1, Jurkat, U937, HL-60 and K562) were cultured in RPMI 1640 supplemented with 10% fetal calf serum in 37°C incubator with 5% CO₂ and 95% humidity. Before any assay, cells were collected and washed twice with PBS and adjusted to 1×10⁷ cells/ml with cold 1% BSA-PBS azide. Non-specific FcR-mediated binding of mAb was blocked with 10% AB serum for 30 min on ice. Fifty microliters of each cell line (1×10⁷ cells/ml) was individually incubated with 50 µl of cell culture supernatant of clone 1E4-1C2 or mAbs MT99/3, positive control, for 30 min on ice. RPMI 1640 and washing buffer were used as negative control and conjugate control, respectively. Cells were then washed twice with cold 1% BSA-PBS azide and incubated with FITC-conjugated rabbit-anti-mouse Igs for another 30 min. Cells were washed 3 times with the same buffer. Finally, cells were suspended with 500 µl of 0.5% paraformaldehyde and investigated by flow cytometry.

Distribution of human liver HSPG on blood samples of leukemic patients by two-step staining

Bone marrow samples (10 NHL and 2 HD) were anti-coagulated with EDTA. Blood samples (5 ALL, 1 CML and 12 AML) were collected from patients with a specific diagnosis made by a hematologist (morphology-based diagnosis from bone marrow smear). Samples from both sources were spun down and buffy coat was collected, washed twice with cold 1% BSA-PBS azide and adjusted to 1×10⁷ cells/ml. To block non-specific FcR-mediated binding of mAb, cells were pre-incubated for 30 min on ice with 10% human AB serum before staining. Fifty microliters of cell suspension was then incubated with equal volume of 1E4-1C2 mAb for 30 min on ice. DMEM high glucose, washing buffer and mAb anti-HIV p24 were used as negative, conjugate control and isotype control, respectively. Cells were washed twice with cold 1% BSA-PBS azide and incubated with FITC-conjugated rabbit-anti-mouse Igs for another 30 min. Cells were then washed 3 times with the same buffer and suspended to 50 µl before adding of the equal volume of PE-conjugated rabbit anti-CD33. For the direct immunofluorescence, PE-conjugated rabbit anti-CD42b mAb and PE-conjugated mouse IgG1 mAb

Figure 1  HSPG D2 fraction isolated from human liver. HSPGs were isolated and purified from normal human liver. These were separated into four major peaks by DEAE-Sepharose and Sepharose CL-6B, respectively. All fractions detected the absorbance at 280 nm.

Figure 2  A gel and western blot using mAb 1E4-1C2. (A) Heparinase III/chondroitinase ABC digested D2 fraction was analyzed by SDS-PAGE (non-reducing) at 200 V for 45 min. Gel was stained with silver (Left panel). (B) The samples were blotted onto PVDF membrane and reacted with mAb 1E4-1C2. The reaction was probed with HRP-conjugated rabbit anti-mouse IgGs (DAKO) following DAB substrate (Sigma) (right panel). Molecular weight is indicated in kDa.
were used as negative control and isotype control, respectively. The mixture was incubated on ice for another 30 min before washing. Finally, remaining red blood cells were lysed before suspending with 500 μl of 0.5% paraformaldehyde and analyzed on a FACSCalibur (Becton Dickinson, Sunnyvale, CA) flow cytometer. Individual populations of

**Figure 3** Normal peripheral blood cells do not express molecule(s) recognized by anti-human liver HSPG, 1E4-1C2. Buffy coat isolated from normal healthy donors was stained with mAb specific to human liver HSPG (1E4-1C2) by indirect immunofluorescence technique using anti-HIV p24 as isotype control and DMEM high glucose, cell culture medium, as negative control. The cells were subsequently stained with PE-conjugated anti-CD33 using PE-conjugated mouse IgG as isotype control by direct immunofluorescence. 1% BSA-PBS azide was used as conjugate control in both steps. Contaminated red blood cells were lysed before analysis by flow cytometer. A: cell control, B: negative control, C: conjugate control, D: isotype control and E: 1E4-1C2. Data are representative of 10 independent donors.
blood cells were gated according to their forward and side scatter characteristics.

Results

HSPGs are an important group of molecules involved in many cellular processes especially those of growth and differentiation. A human liver HSPG was isolated, fractionated and purified to better understand its mechanism of action. Specific mAb was prepared against the highly sulfated D2 fraction (Fig. 1) using standard hybridoma technique [19]. Clones were characterized and used as a tool to study the expression of human liver HSPGs. The mAb 1E4-1C2 recognizes an HSPG having a molecular weight >200 kDa. Treatment of this HSPG with the heparinase III/chondroitinase ABC to remove glycosaminoglycan chains gave a prominent band corresponding to a core protein of ~60 kDa (Fig. 2A). Western blot analysis showed that mAb 1E4-1C2 recognized core protein of this HSPG (Fig. 2B). This mAb does not bind to any population of normal peripheral blood cells (PBLs) (Fig. 3) or hematopoietic cells in lymphoid or monocytoid lineages (Fig. 4). In contrast, it shows significant binding to HL-60 and K562, which are of myeloid lineages (Fig. 4).

Since HSPGs play an important role in cell growth and differentiation, we decided to examine whether HSPG was expressed on hematopoietic cells and not on normal blast cells in bone marrow. We performed indirect followed by direct immunofluorescence assays on patients’ bone marrow aspirates and peripheral blood samples. PE-conjugated anti-CD33 and PE-conjugated anti-CD42 were used to verify the myeloid cell population and as a negative control mAb, respectively. The results showed that all normal bone marrow blood samples of 10 NHL and 2 HD were negative using mAb 1E4-1C2 (Fig. 5). It should be noted that staging bone marrow samples from patients with Hodgkin and Non-Hodgkin lymphomas were used as normal bone marrow samples these marrows showed cells with normal maturation patterns. Cells of patients diagnosed with CML (n = 1) and ALL (n = 5) were also negative (Fig. 6). In contrast, mAb 1E4-1C2 showed a significant positive response with 13 of the 18 various subtypes of AML blood samples tested (2 unclassified AML, 3 M1, 3 M2, 3 M3 and 2 M4 subtypes). The populations recognized by mAb 1E4-1C2 were all AML as specified by PE-conjugated anti-CD33, which is a marker for all subtypes of AML. The negative staining with PE-conjugated anti-CD42b, which are specific to human platelet, showed the validity of method and confirmed that AML population was not recognized by non-specific antibodies. The different patterns of expression detected by 1E4-1C2 mAb are shown in Figs. 7-11.

Discussion

In the current study, we isolated, fractionated and purified liver HSPG from normal healthy donor. The most highly sulfated HSPG fraction, D2, was selected since it contained an intact core protein and isolated amounts sufficient for immunization and screening. We prepared a set of mAbs reactive with the D2 HSPG fraction. Next, these mAbs were used to screen for epitopes on hematopoietic cells. The 1E4-1C2 mAb was found to be of interest since it was able to detect an epitope on cell lines in myeloid lineage. This characteristic epitope was not observed on normal peripheral blood cells or cell lines in lymphoid or even monocytoid lineage. We propose that malignancies of cells of the myeloid lineage cause the expression of new membrane HSPG that are recognized by this specific mAb. These newly expressed HSPGs may be involved in cell growth and differentiation. However, our preliminary studies used only cell lines. To address whether the newly expressed HSPG is also found in hematopoietic malignancies, we performed the indirect immunofluorescence followed with direct immunofluorescence on bone marrow and blood samples collected from patients with various diagnoses. These morphology-based diagnoses were performed by a hematologist on aspirated bone marrow smears using Wright-Giemsa staining. Normal bone marrow aspirates from patients with HD and NHL were also investigated to address whether normal blast cells could be stained with 1E4-1C2 mAb in an effort to distinguish between normal and malignant blast cells. The results showed that normal blast cells in bone marrow samples failed to react with mAb 1E4-1C2. In addition, malignancies

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Figure 4  Expression of the antigen recognized by the 1E4-1C2 mAb on various cell lines. The indicated cells were stained with 1E4-1C2 mAb (solid) by indirect immunofluorescence using anti-HIV p24 mAb as isotype control and DMEM high glucose (cell culture medium) as negative control (open). Data are representative of 3 independent experiments.
Figure 5  No expression of the antigen recognized by the 1E4-1C2 mAb on cells in bone marrow. Buffy coat isolated from bone marrow aspirate of patient with NHL or HD was stained with mAb specific to human liver HSPG (1E4-1C2) by indirect immunofluorescence using anti-HIV p24 as isotype control and DMEM high glucose (cell culture medium) as negative control. 1% BSA-PBS azide was used as conjugate control. Contaminated red blood cells were lysed before analysis by flow cytometer. A: cell control, B: negative control, C: conjugate control, D: isotype control and E: 1E4-1C2. Data are representative of 10 NHLs and 2 HDs.
Figure 6  No expression of antigen recognized by the 1E4-1C2 mAb on ALL and CML. Buffy coat isolated from whole blood of patients with CML and ALL was stained with mAb specific to human liver HSPG (1E4-1C2) by indirect immunofluorescence using anti-HIV p24 as isotype control and DMEM high glucose (cell culture medium) as negative control. 1% BSA-PBS azide was used as conjugate control. Contaminated red blood cells were lysed before analysis by flow cytometer. A: cell control, B: negative control, C: conjugate control, D: isotype control and E: 1E4-1C2. Data are representative of 1 CML and 5 ALLs.
Figure 7  Expression of antigen recognized by 1E4-1C2 mAb on cells isolated from AML (unclassified). Buffy coat isolated from whole blood of patient with AML was stained with mAb specific to human liver HSPG (1E4-1C2) by indirect immunofluorescence using anti-HIV p24 as isotype control and DMEM high glucose (cell culture medium) as negative control. The cells were subsequently stained with PE-conjugated anti-CD33, using PE-conjugated mouse IgG as isotype control by direct immunofluorescence. 1% BSA-PBS azide was used as conjugate control in both steps. Contaminated red blood cells were lysed before analysis by flow cytometer. A: cell control, B: negative control, C: conjugate control, D: isotype control and E: 1E4-1C2. Data are representative of 2 samples of unclassified AML.
Expression of antigen recognized by 1E4-1C2 mAb on cells isolated from AML (M4). Buffy coat isolated from whole blood of patient with AML was stained with mAb specific to human liver HSPG (1E4-1C2) by indirect immunofluorescence using anti-HIV p24 as isotype control and DMEM high glucose (cell culture medium) as negative control. The cells were subsequently stained with PE-conjugated anti-CD33, using PE-conjugated mouse IgG as isotype control by direct immunofluorescence. 1% BSA-PBS azide was used as conjugate control in both steps. Contaminated red blood cells were lysed before analysis by flow cytometer. A: cell control, B: negative control, C: conjugate control, D: isotype control and E: 1E4-1C2. Data are representative of 2 samples of M4 subtype.
Figure 9  Expression of antigen recognized by 1E4-1C2 mAb on cells isolated from AML (M3). Buffy coat isolated from whole blood of patient with AML was stained with mAb specific to human liver HSPG (1E4-1C2) by indirect immunofluorescence using anti-HIV p24 as isotype control and DMEM high glucose (cell culture medium) as negative control. The cells were subsequently stained with PE-conjugated anti-CD33, using PE-conjugated mouse IgG as isotype control by direct immunofluorescence. 1% BSA-PBS azide was used as conjugate control in both steps. Contaminated red blood cells were lysed before analysis by flow cytometer. A: cell control, B: negative control, C: conjugate control, D: isotype control and E: 1E4-1C2. Data are representative of 3 samples from M3 subtype.
Figure 10  Expression of antigen recognized by 1E4-1C2 mAb on cells isolated from AML (M2). Buffy coat isolated from whole blood of patient with AML was stained with mAb specific to human liver HSPG (1E4-1C2) by indirect immunofluorescence using anti-HIV p24 as isotype control and DMEM high glucose (cell culture medium) as negative control. The cells were subsequently stained with PE-conjugated anti-CD33, using PE-conjugated mouse IgG as isotype control by direct immunofluorescence. 1% BSA-PBS azide was used as conjugate control in both steps. Contaminated red blood cells were lysed before analysis by flow cytometer. A: cell control, B: negative control, C: conjugate control, D: isotype control and E: 1E4-1C2. Data are representative of 3 samples from M2 subtype.
Expression of antigen recognized by 1E4-1C2 mAb on cells isolated from AML (M1). Buffy coat isolated from whole blood of patient with AML was stained with mAb specific to human liver HSPG (1E4-1C2) by indirect immunofluorescence using anti-HIV p24 as isotype control and DMEM high glucose (cell culture medium) as negative control. The cells were subsequently stained with PE-conjugated anti-CD33, using PE-conjugated mouse IgG as isotype control and PE-conjugated anti-CD42b as negative control for myeloblast cells by direct immunofluorescence. 1% BSA-PBS azide was used as conjugate control in both steps. Contaminated red blood cells were lysed before analysis by flow cytometer. A: cell control, B: negative control, C: conjugate control, D: isotype control, E: 1E4-1C2 and F: PE-conjugated anti-CD42b. Data are representative of 3 samples from M1 subtype.
of cells of lymphoid series and chronic myeloid leukemia failed to react. Only blast cells of hematologic malignancies expressed these HSPGs. Moreover, the blood samples of patients with AML showed positive staining with mAb 1E4-1C2. Only 13 cases of the 18 tested AMLs, however, demonstrated a positive reaction. Many reasons can be used to explain our failure to detect epitope in all samples. First, AML is a malignancy of hematopoietic progenitor cells that can be differentiated into many different cell patterns with unique cytochemistry and immunophenotype [8,9]. According to these differences, AML can be classified into 8 types (M0-M7) where there is a common antigen, such as CD33 [7]. Second, while peripheral blood leucocytes from patients with various kinds of leukemia were collected by venepuncture for testing, morphology-based diagnosis was done by using bone marrow aspirate. It is possible that malignant cells are observed earlier in bone marrow than in the blood [3]. Third, negative staining might result from the difference in HSPG expression of different stages in AML as has been observed for other epitopes associated with M0-M7 subtypes [20-24].

In present study, we have demonstrated that HSPGs are expressed on membrane of myeloid leukemic lineages. The degree of expression probably depends on the stage of cell development, resulting in differential expression. The leukemia cell surface HSPG has a common structure to that of human liver HSPG. We propose to apply indirect immunofluorescence with this mAb specific as an alternative marker to distinguish leukemic cells. Moreover, further characterization of this HSPG and its role in cell proliferation may lead to a better understanding of its regulation of malignant cells.

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