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Cell surface sialylation and ecto-sialyltransferase activity of human CD34 progenitors from peripheral blood and bone marrow

Reinhard Schwartz-Albiez[1], Anette Merling[1], Simona Martin[2], Rainer Haas[3] and Hans-Jürgen Gross[4]

[1] *German Cancer Research Center, Tumor Immunology Program, Heidelberg, Germany,* [2] *Institute for Transuranium Elements, European Commission Joint Research Centre, Karlsruhe, Germany,* [3] *University of Duesseldorf, Department of Hematology and Clinical Immunology, Duesseldorf, Germany,* [4] *Department of Clinical Chemistry, University of Ulm, Germany*

Surface expressed negatively charged sialoglycans contribute to the regulation of adhesive cellular interactions and are thus involved in the growth and differentiaton of hematopoietic progenitor cells. In particular, the cell surface sialylation state may govern the liberation of CD34+ hematopoietic precursors from bone marrow stroma cells and extracellular matrix. In order to assess the overall surface sialylation of live human CD34+ hematopoietic precursor cells, we applied a previously described flow cytometric enzyme assay. Cells with and without sialidase pretreatment were incubated in the presence of fluorescent CMP-sialic acid and exogenous ST6Gall. Thus sialylation of surface-expressed lactosamine residues was analysed. We demonstrated that surface lactosamines of CD34+ precursors derived from bone marrow and peripheral blood are over 95% sialylated, predominantly in α2-6 linkage. These results are in accordance with flow cytometric analysis of surface lectin staining. Sialic acid specific lectins MAA and SNA were strongly bound whereas SBA, VVA, and PNA became reactive only after sialidase pretreatment. CD34+ leukemia cell lines TF1 and KG1a also showed a high degree of surface sialylation, whereas cell line KG1 expressed to the largest extent free lactosamines. In these cell lines, α2-6 and α2-3 sialylated residues were present in equal amounts. In a variation of the flow cytometric enzyme assay, live cells were incubated without exogenous STGal I to measure the activity of endogenous ecto-sialyltransferase. Ecto sialyltransferase activity was observed in all CD34+ cells which was able to resialylate major surface glycoproteins such as HLA Class I, CD45, CD43, and CD34. The ecto-sialyltransferase may serve to maintain or increase surface sialylation rapidly without *de novo* synthesis.
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Abbreviations: **CMP-5-F-Neu: cytidine-5′-monophospho-5-fluorescein-thioureido-N-acetyl-neuraminic acid, HPC: hematopoietic precursor cells, MAA: Maackia amurensis agglutinin, MAb: monoclonal antibody, NDVN: Newcastle disease virus neuraminidase (EC 3.2.1.18), PBPC: peripheral blood precursor cells, PNA: Peanut agglutinin, SBA: Soy bean agglutinin, SNA: Sambucus nigra agglutinin, ST6Gall: Galβ1,4GlcNAc α2,6-sialyltransferase (EC 2.4.99.1), VCN: *Vibrio cholerae* neuraminidase EC 3.2.1.18), VVA: Vicia villosa agglutinin.**

Introduction

Self-renewal, proliferation, differentiation, extravasation and homing of hematopoietic precursor cells (HPC) are processes which require adhesion contacts to bone marrow stroma cells and their surrounding extracellular matrix (ECM) and subsequent release in coordinated fashion [1]. It became apparent that protein-carbohydrate interactions play a role during these processes of hematopoiesis [2]. In particular, sialylated glycoconjugates and sialic acid-recognizing surface-expressed lectins have been described to be involved in adhesion of HPC [3]. In the complex interplay of surface receptors, sialoglycans may have either repulsive or adhesive effects depending on the quantity of their expression contributing to the overall negative surface charge and the function of their respective counter-receptors.

To whom correspondence should be addressed: Reinhard Schwartz-Albiez, Tumor Immunology Program, German Cancer Resarch Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Tel.: +49-6221-42713; E-mail: r.s-albiez@dkfz.de

The homing of recirculating B cells to murine bone marrow is directed by the α2-6 sialoglycan-specific CD22 lectin on B lymphocytes, belonging to the Siglec family of sialic acid-recognizing lectins [4]. P- and E-selectins recognizing glycans such as sialylated Lewis x seem to direct the homing of HSC to the bone marrow [5]. Further, P-selectin interacts with the sialoglycoprotein CD162 (PSGL-1) on HPC [6]. Other mucin-like sialoglycoproteins, CD34, CD43, and CD164 are expressed on primitive HSC and may serve as adhesive receptors for interactions with bone marrow stroma.

Interestingly, ligation of CD162 on CD34+ HSC results in suppression of HSC proliferation [6]. Also the sialomucin CD164 expressed on HPC may be a signalling molecule which can suppress HPC proliferation [7]. Crosslinking of the sialomucin CD43 induces apoptosis in HPC [8]. Myeloid precursors upregulate α2-6 sialylation of the integrins CD 11b and CD18 during late phases of bone marrow differentiation which in turn may entail reduced binding capacity of the integrins to fibronectin and bone marrow stroma [9]. It was hypthesized that upregulation of α2-6 sialylation regulates release of myeloid cells from the bone marrow into the peripheral blood [9].

These data point to the intricate regulation of adhesion and de-adhesion by CD34+ HSC-expressed sialoglycoproteins and also underlines the functional role of the overall content of surface sialoglycans.

No data were yet available on the overall sialylation of HPC in bone marrow and peripheral blood. We therefore investigated surface sialylation of CD34+ HPC derived from bone marrow and peripheral blood by a flow cytometric enzyme assay [10,11] and surface lectin staining.

Ecto-sialyltransferase activity has been observed in human B cells [11]. This enzyme is able to sialylate surface glycoproteins and thus may mask lactosamine residues by rapid surface sialylation or to produce sialylated glycan ligands for specific lectins such as the siglec CD22. Ecto-sialyltransferase on B lymphocytes has been determined by the flow cytometric enzyme assay [10,11]. In particular, live cells were incubated in the presence of fluorescent CMP-sialic acid and absence of exogenous sialyltransferase. Fluorescent sialic acid was covalently incorporated into surface glycans by means of a surface-expressed sialyltransferase. We have applied this assay modification to assess ecto-sialyltransferase activity on CD34+ HPC.

An ecto-sialyltransferase on HPC may be involved in the emigration of these cells from distinct compartments and finally of mature hematopoietic cell from the bone marrow into the peripheral blood.

Materials and methods

Materials

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma (St Louis, MO). 2,3-dehydro-NeuAc, Vibrio cholerae neuraminidase (VCN; EC 3.2.1.18), Newcastle Disease virus neuraminidase (NDV; EC 3.2.1.18), and α2,6 sialyltransferase (St6GalI, EC 2.4.99.1, from rat liver) were obtained from Roche (Mannheim, Germany). CMP-NeuAc, CMP-5-F-Neu (cytidine-5′-monophospho-5-fluoresceinthioureido-*N*-acetylneuraminic acid), was prepared enzymatically (CMP contamination in each preparation <5%) as described previously [12].

Cell lines

Cell lines KG1 and its variant KG1a derived from a patient with acute myeloblastic leukemia (AML) and the erythroleukemic cell line TF1 were cultivated in RPMI 1640 medium containing 10% fetal calf serum, 10 mM Hepes, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Additionally cell line TF1 required recombinant IL-3 (5 ng/ml, Reliatech, Braunschweig, Germany) for *in vitro* growth.

Methods

Separation of CD34+ HPC from peripheral blood and aspiration of bone marrow cells

Peripheral blood precursor cells (PBPC) were collected from female patients with primary or metastatic breast cancer who had received cytotoxic chemotherapy and additionally R-metHuG-CSF (filgrastim, Amgen, Thousand Oaks, CA) before PBSC harvest [13]. PBPC collection began when a distinct population of CD34+ cells was measurable in the peripheral blood. CD34+ cells were selected from leukapheresis products using a Baxter Isolex 300 SA Magnetic Cell Separation System (Baxter Immunotherapy, Irvine, CA) as previously described. The enrichment procedure was performed immediately after leukapheresis. The cells were resuspended in PBS containing 1% human serum albumin and 0.2% sodium citrate and 0.5% immunoglobulin (Venimmun, Behringwerke, Marburg, Germany).

Bone marrow aspirates were taken from breast cancer patients as well as from healthy donors and were used in heparinized form for further studies. From analysis of surface markers, no difference between bone marrow-derived CD34+ from either healthy persons or breast cancer patients was observed so all bone marrow biopsies were regarded as containing normal HPC. The patients were treated under the ethical guidelines of the Joint Committee on Clinical Investigations of the University of Heidelberg. Informed consent was obtained from each patient.

Flow cytometric detection of cell surface sialylation

Cytometric detection of surface sialylation by endogenous ecto-sialyltransferase and exogenous sialyltransferase of rat liver was performed as previously described [10,11]. In brief, the cells were washed with PBS and then treated with or without VCN (5 mU/1 $\times$ 10^6 cells/100 μl PBS) or NDVN (2 mU/100 μl PBS) for 30 min at 37°C. Enzymatic reaction was stopped by

addition of ice-cold PBS followed by extensive washing in PBS. Then, cells were incubated for 120 min at 37°C with the addition of CMP-5-F-NeuAc at 25 μmol/L and α2,6 sialyltransferase of rat liver (50 mU/ml) in the presence of 250 μM 2,3 dehydro-2-deoxy-*N*-acetylneuraminic acid as inhibitor for sialidase activity. In some experiments we tested for endogenous, surface-active sialyltransferase. For this, exogenous rat liver sialyltransferase was omitted from the experiment as described [11]. Enyzmatic activity was stopped by addition of ice-cold PBS and 3 times washing in PBS. In control experiments, resialylation was performed in the presence of 12 mM CTP in order to competitively inhibit sialyltransferase activity. Viability of cells did not suffer from enzymatic treatment as monitored by incorporation of propidium iodide in flow cytometry. For cytometric analysis using a FACScan cytometer (Becton Dickenson, Moutain View, CA), cells were washed twice and the cell pellet resuspended in RPMI 1640 medium containing 1 mg/ml propidium iodide. Green fluorescence of 1×10^4 viable cells was analyzed by excluding dead cells identified by red fluorescence (propidium iodide label). Fluorescence intensity was determined as the mean fluorescence of fluorescent NeuAc/1×10^6 cell stained. As background values we used the fluorescence of cells incubated at 0°C with fluorescent CMP-glycosides or incubated at 37°C in the presence of CTP as indicated above.

Flow cytometric analysis of lectin staining

For flow cytometric analysis of lectin-stained cells, the following lectins were applied: FITC-conjugated *Vicia villosa* (VVA), soybean agglutinin (SBA); PE-conjugated peanut agglutinin (PNA) obtained from Sigma, StLouis; biotinylated Sambucus nigra agglutinin (SNA); and Maackia amurensis agglutinin (MAA) were obtained from Vector Labs, Burlingame, CA. Lectins were dissolved (1 mg/ml) and diluted for immunofluorescence staining at optimal concentration as determined in pilot experiments (LFA-FITC 1:100, VVA-FITC 1:20, SBA-FITC 1:200, PNA-PE 1:100, MAA-biotin, and SNA-biotin 10 μg/ml each). Cell were stained in several steps each of 30 min at 4°C followed by extensive washing. In the first step, cells were stained with FITC-conjugated-lectins, followed by CD34-PE staining. In case of PNA-PE, cells were subsequently stained with CD34-Cy5 and for biotinlytad-lectins, cells were further treated with streptavidin-FITC (Dianova) diluted 1:50.

CD22Rg contains the first three immunoglobulin-like domains of CD22 linked to the Fc part of IgG1. The cDNA specific for CD22Rg was integrated into the immunglobulin expression vector pIg. CD22Rg was purified from COS transfectant culture supernatants by chromatography on protein A-Sepharose 4B.

CD22Rg was preincubated together with goat anti-human IgG1 (Fc-specific) coupled to biotin in equimolar concentrations (5 μg/ml) in PBS + 1% BSA for 1 h at room temperature. The complex was applied to VCN-/NDVN-pretreated or untreated cells for 20 min at room temperature. Reactivity of CD22Rg was detected by incubation with Streptavidin coupled to FITC. Cells were incubated with goat immunoglobulin (100 μg/ml) for 10 min at room temperature before the specific staining procedure, followed by 2 washing steps with PBS containing 1% BSA. As negative controls we used human IgG1 (5 μg/ml) precomplexed with goat anti-human IgG1 (Fc-specific).

Labeled lymphocytes were suspended in PBS containing 1 mg/ml sodium azide and analyzed immediately. Flow cytometric analysis was performed using a FACScan cytometer (Becton Dickinson, Mountain View, CA) equipped with an argon laser emitting light at 488 nm. At least 10,000 cells were counted from each sample. Viability of cells was tested by adding propidium iodide to lymphocyte suspension. Viable lymphocytes were gated by forward scatter (FSC) versus side scatter (SSC). Three colour fluorescence was measured per single cell. Compensation of the respective fluorescence channels was carried out with single fluorescent cells. The data were recorded by logarithmic mode for FL1, FL2, FL3 by linear mode for FSC and SSC and analyzed by the Lysis II software (Becton Dickinson, Mountain View, CA). All cells with fluorescence intensity higher than that observed in 95% of the control cells stained only with secondary antibody were considered positive.

Immunoprecipitation and Western blotting of fluorescent NeuAc-labeled surface glycoproteins

The capacity of endogenous ecto-sialyltransferase to resialylate mAb-defined surface structures was assessed by labeling KG1 cells, with and without prior VCN treatment, by application of CMP-5-F-Neu without exogenous sialyltransferase as described above. Cells were washed and lysed for 30 min on ice in lysis buffer (TBS containing 1% Triton X-100 and a protease inhibitor cocktail, Roche, Mannheim, Germany). The cell lysate was centrifuged in an Eppendorf centrifuge for 10 min at 0°C, and the supernatant was then taken for unspecific absorption of cellular proteins and specific immunoprecipitation of surface proteins for 1 h using 400 μl lysate together with 10 μl purified mAb (1 mg/ml), 10 μl rabbit anti-mouse antibody, and 20 μl ProteinA Sepharose CL4B as previously described [11]. Antibodies used for immunoprecipitation were anti-CD34 epitope class 2 mAb 0786 (Beckman Coulter, Krefeld, Germany), anti-CD43 mAb WR14 (obtained by Leucocyte Typing Conference IV), anti-CD45 mAb CBL124 (Dianova, Hamburg, Germany), and anti-MHC class I mAb W6/32 (kindly obtained from Dr. G. Moldenhauer, German Cancer Research Center, Heidelberg, Germany).

Immunoprecipitates were subjected to SDS-PAGE and subsequent Western blotting. Western blots were incubated overnight at 4°C in PBS containing 5% bovine serum albumin, then in PBS containing 5% bovine serum albumin and 0,2% Tween 20, sheep anti FITC IgG Fab fragments coupled to peroxidase (Roche), diluted 1:3000 for 1 h a 4°C. Immunoprecipitates

Table 1. Percentage of free and sialylated lactosaminyl residues on the surface of CD34+ hematopoietic precursor cells

Biopsy	*Origin of sample*	*Free Lac*	*Sialylated Lac*	*α2,6NeuAc*	*α2,3NeuAc*
Bm1	Bone marrow	5	95	66,8	33,2
Bm2	Bone marrow	3	97	11,0	89,0
Bm3	Bone marrow	3	97	71,0	29,0
Bm4	Bone marrow	5	95	72,0	28,0
Bm5	Bone marrow	4	96	57,0	43,0
Pb1	CD34+ PBMC	3	97	71,3	28,7
Pb2	CD34+ PBMC	2	98	17,9	82,1
Pb3	CD34+ PBMC	5	95	62,0	38,0
KG1	AML	78	22	42,0	58,0
KG1a	AML	6	94	37,3	62,7
TF1	erythroleukemia	12	88	50,9	49,1

were washed 4 times in PBS + 0,2% Tween 20 and then 3 times in PBS; ECL staining was performed using Super Signal West Dura extended duration substrate according to the manufacturer's protocols (Perbio, Bonn, Germany).

For the detection of CD34 molecules in various cell lines, cells were lysed, the lysates subjected to SDS-PAGE and Western blots as described above, and then stained with anti-CD34 mAb 0786 of the class 2 epitope (Immunotech), diluted 1:1000, followed by incubation with goat anti-mouse peroxidase conjugated antibody, diluted 1:3000 (Dianova). Immune complexed were visualized by the ECL methods as described.

Results

Cell surface sialylation of CD34+ hematopoietic precursors derived from bone marrow and peripheral blood

In order to determine the overall surface sialylation of live CD34+ HPC derived from bone marrow or peripheral blood, we applied the flow cytometric enzyme assay for fluorescently labelling surface glycans as previously described [10,11,14]. For this, cells were incubated in the presence of CMP-5-F-Neu and exogenous ST6Gal I with and without sialidase pretreatment. Sialylation was measured over a period of 120 min at 37°C in a standardized fashion. Incorporation of fluorescent neuraminic acid into surface glycans was monitored by flow cytometry. This method enables us to estimate (a) free lactosaminyl residues on untreated cells, (b) totally available lactosaminyl residues including those which were naturally sialylated on VCN pretreated cells, (c) α2-6 sialylated lactosaminyl residues by subtracting results of NDVN-treated cells from those of VCN-treated cells, and (d) α2-3 sialylated lactosaminyl residues by subtracting results of untreated cells from those of NDVN-treated cells.

Figure 1 shows a typical experiment with bone marrow-derived cells (Bm1 of Table 1) where incorporation of fluorescent neuraminic acid in the presence of ST6GalI with and without sialidase pretreatment is depicted at the *x*-axis and anti-CD34 reactivity at the *y*-axis. Thus incorporation of exogenous neuraminic acid can be differentiated between CD34+ hematopoietic precursors on one side (upper right panel) and CD34− stroma cells and differentiated hematopoietic cells which already lost their CD34 surface antigen on the other side (lower right panel). In this representative biopsy CD34+ cells consisted of about 5% of all cells harvested which could be moderately cell surface sialylated by exogenous ST6GalI (arbitrary units of mean fluorescence 147) indicating that most

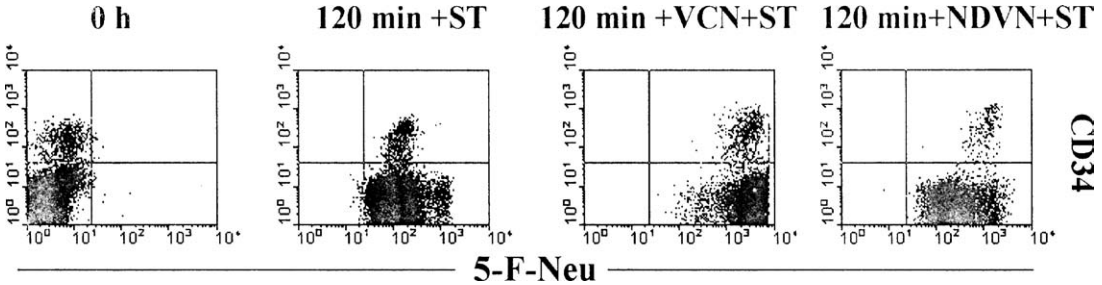

Figure 1. Flow cytometic analysis of fluorescein-labeled surface neuraminic acid of CD34+/CD34− cells derived from bone marrow (biopsy Bm1). Cells were labeled in the presence of CMP-5-F-Neu and ST6GalI for times indicated, without or with VCN or NDVN pretreatment. Immunofluorescence of 5-F-Neun is given at the *x*-axis while CD34 reactivity is indicated at the *y*-axis. 5-F-Neu Ac-labeled CD34+ cells are located in the upper right panel while 5-F-Neu-labeled CD34− cells are in the lower right panel.

lactosaminyl sites were masked by sialylation. In contrast this population shows markedly increased incorporation of fluorescent neuraminic acid after cellular pre-treatment with VCN (mean fluorescence 3200; representing now the majority of available lactosaminyl sites on the cell surface). Only 50% of the CD34+ cells could be resialylated after NDVN pretreatment cleaving specifically α2-3-linkage of sialic acids (mean fluorescence 1070). Calculating from the fluorescent mean values, approximately one third of the available lactosaminyl sites were present in α2-3 sialylated linkage in this CD34+ subpopulation. In the fraction of CD34-cells of this biopsy, distribution of sialylated and non-sialylated surface-expressed lactosamines was almost similar with the exception of a subpopulation with slightly higher proportion of free lactosamines.

Native CD34+ cells from all bone marrow biopsies and blood samples were highly sialylated at their surface-expressed lactosaminyl sequences; free lactosamines accessible for the exogenous ST6GalI represented only approximately 5% (Table 1). Glycans on CD34+ cells from healthy donors were to the largest extent sialylated in α2-6 linkage. As an exception glycans of bone marrow CD34+ cells from a patient with a Hodgkin's lymphoma (Bm2) were more α2-3 than α2-6 sialylated. CD34+ cells from sample Pb2 derived from healthy donor had an exceptionally high proportion of α2-3 linked lactosamines. This deviation from the otherwise observed sialylation pattern cannot be explained at the moment. It should be noted however that, for example, inflammatory processes may also influence the sialylation pattern.

CD34− cells from these bone marrow biopsies also expressed highly sialylated glycans on their surface; however most of these were α2-6 sialylated over 95% (Table 2).

In addition we determined cell surface sialylation of three established CD34+ cell lines: KG1, derived from a patient suffering from undifferentiated acute myeloblastic leukaemia, its variant KG1a; and the erythroleukemia-derived cell line TF1.

Determination of surface sialylation by the fluorometric method revealed that, of the parental cell line KG1, only 22% of cell surface-expressed lactosaminyl glycans were sialylated in an almost equal ratio between α2-6 and α2-3 sialylation (Table 1). In contrast, 94% of lactosamines were sialylated in KG1a cells with a preponderance of α2-3 sialylation (63%). Surface lactosamines of cell line TF1 were covered by sialic acid to a large extent (88.4%) with an almost equal ration between α2-6 and α2-3 sialylation.

Determination of surface sialylation and unmasked oligosaccharide sites by lectin staining confirmed these results although one has to consider that lectin staining covers a slightly different spectrum of oligosaccharide sequences than lactosamine (re)sialylation by ST6Gal I. The high degree of surface sialylation on native CD34+ cells was observed also by strong staining with SNA (specific for α2-6 linked sialic acid), somewhat weaker with MAA (specific for α2-3 linked sialic acid), and almost negative staining with VVA, PNA, and SBA recognizing terminal Gal and GalNAc residues (Figure 2, shown for blood sample Pb2, summarized data not shown). Incubation with lectins VVA, PNA, and SBA yielded only significant staining after VCN or NDVN treatment of cells. Since VVA staining (preferentially recognizing terminal GalNAc residues) was for most biopsies and blood samples stronger than PNA and SBA, one may conclude that CD34+ cells express to a larger extent in addition to lactosamine sequences also α2-6 and α2-3 sialylated oligosaccharides with subterminal GalNac.

Staining of KG1 cells with SNA was very weak (data not shown). Staining with MAA was almost negative which may be due to the weak overall sialylation of KG1. In contrast KG1a showed strong staining both with SNA and MAA with an estimated ratio between α2-6 and α2-3 sialylation of 38 to 62% which is identical to the result obtained by resialylation of lactosaminyl sequences. Terminal Gal and GalNAc sites as assessed by staining with VVA, SBA, and PNA were more accessible on KG1 than on KG1a cells. On KG1a cells staining with VVA, SBA, and PNA became effective after VCN pretreatment of the cells. Together these results demonstrate that KG1 and KG1a differ largely in their content of surface sialylation. It seems that in KG1a cells a great proportion of GalNAc sites are sialylated. Possible candidates for this type of sialylation are sialylated Thomsen-Friedenreich (TF) and T nouvelle (Tn) carbohydrate structures present on these acute myeloblastic cell lines (unpublished data). In erythroblastic leukaemia TF1, cells most accessible surface glycan sites were sialylated as apparent by SNA and MAA staining and increased VVA, SBA, and PNA staining after sialidase treatment.

CD34+ cells of some samples were also strongly stained with the CD22Rg lectin construct recognizing α2-6 sialylated lactosamine sequences which decreased after VCN, but increased

Table 2. Percentage of free and sialylated lactosaminyl residues on the surface of CD34-bone marrow cells

Biopsy	*Origin of sample*	*Free Lac*	*Sialylated Lac*	*α2,6NeuAc*	*α2,3NeuAc*
Bm1	Bone marrow	5	95	66,8	33,2
Bm2	Bone marrow	5	95	93,0	7,0
Bm3	Bone marrow	7	93	93,0	7,0
Bm4	Bone marrow	18	82	98,0	2,0
Bm5	Bone marrow	4	96	57,0	43,0

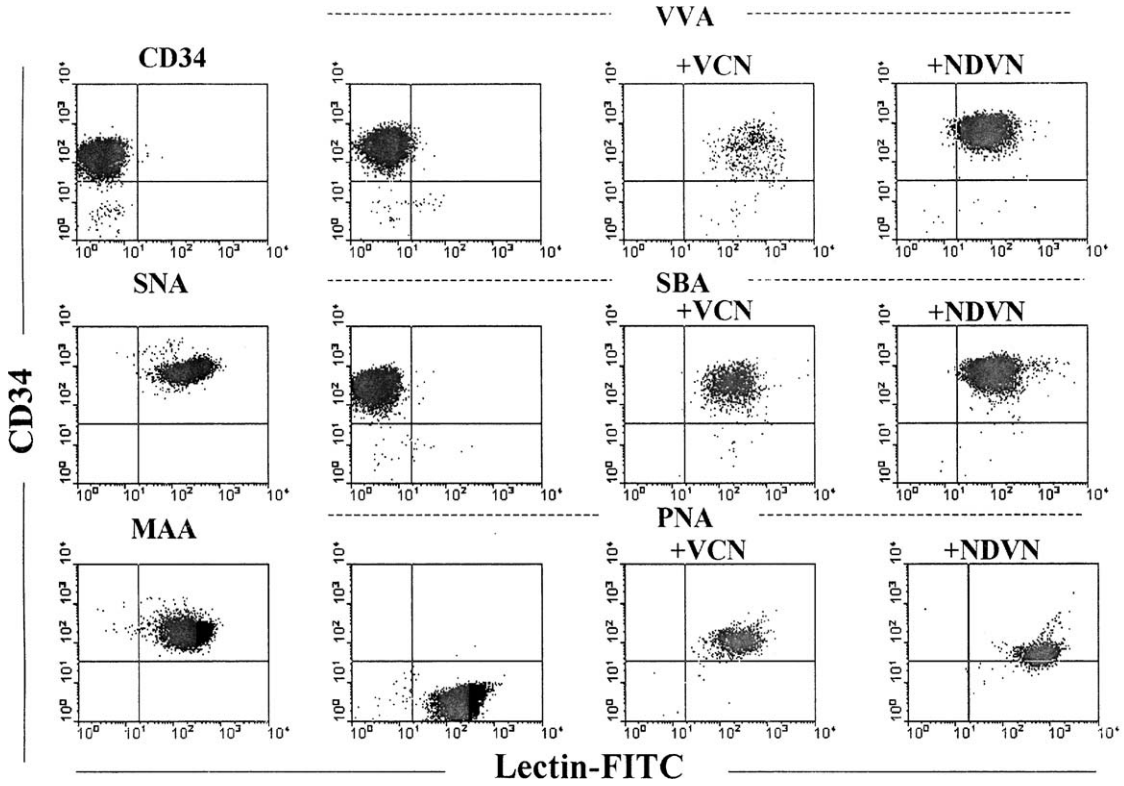

Figure 2. Cell surface lectin staining of selected CD34+ derived from peripheral blood (sample Pb1). Cells were either treated without or with sialidase before staining. Minor contaminations of stained CD34− cells are located in the lower panels.

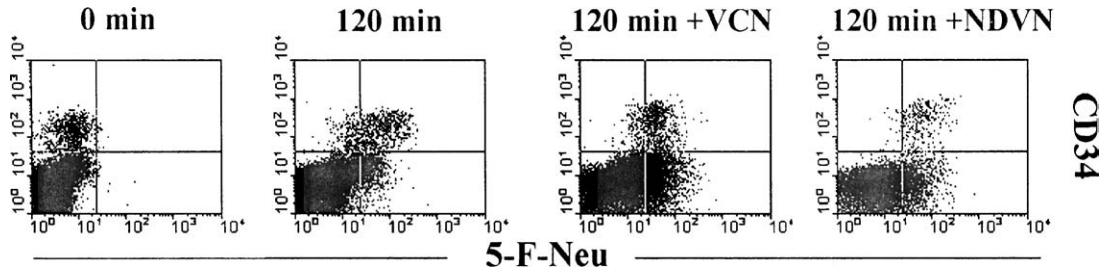

Figure 3. Evidence for ecto-sialyltransferase activity. Incubation of bone marrow cells in the presence of CMP-5-F-Neu without and with sialidase pretreatment and without addition of exogenous ST6Gal I.

after NDVN pretreatment. Possibly α2-3 sialylated oligosaccharide branches sterically inhibit binding of CD22Rg on untreated cells (data no shown).

Activity of ecto-sialyltransferase on CD34+ hematopoietic precursor cells

Since in a previous study we have described an ecto-α2,6 sialyltransferase activity on human B lymphocytes, we were interested whether hematopoietic CD34+ precursor cells also express ecto sialyltransferases. To this end we performed the enzymatic fluorometric surface labeling with CMP-5-F-Neu, however without the addition of ST6GalI. Sialylation without addition of exogenous sialyltransferase could be completely inhibited by CTP.

As shown in Figure 3 for biopsy Bm1, ecto-sialyltransferase was measured by increase in mean fluorescent intensity and as compared to those value obtained by sialylation with exo-ST6GalI. Ecto-sialyltransferase activity values were low and did not increase after sialidase treatment with regard to mean intensity of fluorescence, a shift in percentage of positive cells was however observed after NDVN treatment. It seems that predominantly free lactosaminyl residues were sialylated. For CD34+ cells of two other bone marrow biopsies, incorporation of 5-F-Neu was enhanced after sialidase treatment. The ecto sialyltransferase activity was able to resialylate both previously α2,6 and α2,3 sialylated glycans (Table 3). CD34+ cells derived from peripheral blood expressed low ecto-sialyltransferase activity with no significant increase after sialidase treatment.

Table 3. Resialylation of free and sialylated glycan residues on the surface of CD34+ hematopoietic precursor cells by ecto-sialyltransferase activity (percentage values)

Biopsy	*Origin of sample*	*Free Lac*	*Sialylated Lac*	*α2,6NeuAc*	*α2,3NeuAc*
Bm1	Bone marrow	100	–	–	–
Bm2	Bone marrow	33	67	57	43
Bm3	Bone marrow	58	42	–	100
KG1	AML	85	15	65	35
KG1a	AML	13	87	–	100*
TF1	erythroleukemia	16	84	9	91

*Sialidase cleavage was exclusively for α2,3 linkage as deduced from comparison of VCN and NDVN treatment.

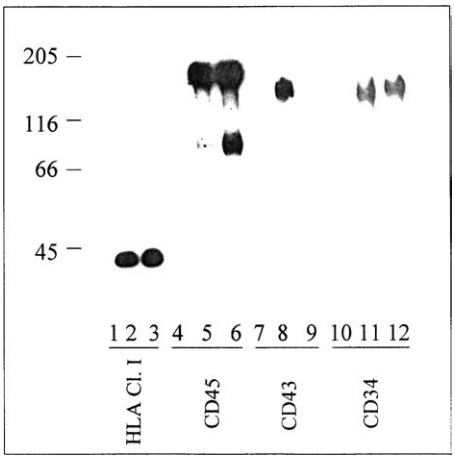

Figure 4. Sialylation of defined surface antigens by endogenous ecto-sialyltransferase. KG1 cells were with or without prior VCN treatment labeled with 5-F-Neu in the absence of exogenous ST6GalI, lysed and antigens precipitated from the lysates as described in Materials and Methods. 5-F-Neu-labeled antigens were visualized on Western blots by staining with an anti-FITC antibody. Lane 1: 0 minute incubation with CMP-5-F-Neu (labeled activated neuraminic acid was added to the cells and immediately removed by 3 times washing), lane 2: 120 min labeling with CMP-5-F-Neu, lane 3: 120 min labeling with CMP-5-F-Neu with VCN pretreatment of cells. The 116 kDa sialylated glycoprotein band in CD45 precipitates has been described earlier as a coprecipitate of CD45 [15].

For CD34+ leukemic cell lines, sialyltransferase activity was also low as indicated by small mean fluorescence values. In these cells more of the previously α2,3 than of the α2,6 sialylated glycans were resialylated by the ecto-sialyltransferase.

Another evidence for the presence of ecto-sialyltransferase activity on CD34+ leukemic cells was obtained by ecto-surface sialylation of defined glycoprotein structures. As shown for cell line KG1, major surface antigens like MHC class I, CD45, CD43, and CD34 were ecto-resialylated (Figure 4). Again VCN pretreatment of the cells did not result in enhanced intensity of the respective bands. With the exception of MHC Class l. I the other resialylated surface antigens are mucin-like structures containing a large proportion of O-glycosylated oligosaccharides. The resialylation of only NDVN-pretreated residues on KG1a cells as shown in the cytometric assay may be correlated to the high proportion of α2-3-linked sialoglycans on mucin-type glycoprotein present on these cells.

CD34 expression in the three CD34+ cell lines studied is different in terms of glycosylation. Three classes of epitopes have been recognized on the CD34 molecule: class I and II epitopes are most likely related to glycosylation; class I epitopes are additionally sensitive to sialidase treatment whereas class III epitope are situated within the protein moiety of the molecule [16]. As observed by flow cytometric analysis of antibody staining, CD34 class I epitopes are weakly expressed on TF1 and KG1 cells and strongly on KG1a; class II epitopes are also much more strongly expressed on KG1a whereas class III epitopes can be recognized on all three cell lines with almost equal staining intensity (data not shown). Although cell line KG1a is derived from KG1, they differ not only in their surface sialylation but also in their protein expression, as shown for surface expressed CD34 (Figure 4).

Discussion

Sialylated surface glycoproteins such as CD34, CD43, CD45, PSGL-1, and CD164 are expressed on hematopoietic precursor cells and mediate interactions with stroma cells and extracellular matrix [1,7,8,17,18]. There are strong indications that the terminal sialic acid on N- and O-linked oligosaccharide chains of these glycoproteins plays a major role in the recognition process. Selectin reactivity depends on the presence of sialic acid in the respective ligand as for example CD34 and PSGL-1 [19]. Also members of the sialic acid-recognizing Siglec-family are expressed on several cell types of the hematopoietic compartment [20]. Surface sialylation of hematopoietic cells is apart from adhesive processes also involved in decisive cellular regulation such as apoptosis [21,22].

Surface sialylation does not only mediate specific molecular interactions but is largely responsible for the overall negative charge of a cell which also contributes to cellular repulsive or adhesion processes. In an effort to analyse the overall surface sialylation of human CD34+ HPC, we applied a flow cytometric enzyme assay which is able to quantitativly assess the extent

of free and sialylated lactosamine groups on intact live cells. Further, when cells were treated with sialidases, in particular with specificities α2,3-linked sialic acids prior to labeling, the proportion of both linkages can be calculated from the cytometric data.

From our results it became evident that normal HPC derived from bone marrow or after cytokine stimulation from peripheral blood are highly sialylated. Surface lactosamines are to over 95% masked by terminal sialic acid present to the largest extent in α2-6 linkage. It has to be noted that, by the method applied for this study, naturally α2-3 sialylated lactosamines were after NDVN treatment resialylated by ST6GalI in α2-6 linkage. It may be that not all oligosaccharides desialylated by NDVN were accessible for resialylation by ST6GalI so that the percentage of α2-3-linked sialic acids could be slightly underestimated. In comparison, surface staining by lectins PNA and SBA with and without prior sialidase treatment revealed a similar staining pattern in terms of intensity. Interestingly, staining with VVA, having a propensity for terminal GalNAc, was also significantly increased after sialidase treatment. Therefore it can be assumed that a considerable part of O-glycosylated oligosaccharides are sialylated both in α2-6- and α2-3 linkage. This observation is confirmed by the fact that HPC carry a considerable amount of various sialomucin-type glycoproteins.

Difference both in the extent of sialylation and kind of linkage in samples derived from a patients with leukemia and in established leukemia cell lines in comparison to normal HPC may speak in favour of a different sialylation process in leukemia.

As an example, the CD34 molecule appears in different conformations due to different glycosylation as shown by different bands in SDS-PAGE of CD34 (Figure 5). CD34 of KG1 has a much larger electrophoretic mobility than that of KG1a and TF1. It has been described that, after sialidase treatment of CD34, a band corresponding to a 150 kDa was obtained [16] which points to an effect of sialylation on protein conformation. Since CD34 sialylation of KG1 is much lower than of KG1a cells, we may observe a similar effect to protein folding in that the under-sialylated CD34 of KG1 has a larger electrophoretic mobility. This affords further investigation to see whether low and highly-sialylated CD34 molecules confer different functions at the cell surface.

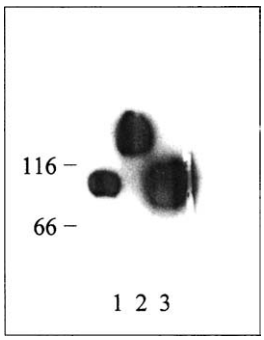

Figure 5. Western Blotting of the CD34 glycoprotein from three different CD34+ cell lines. KG1a (lane 1), KG1 (lane 2) and TF1 (lane 3) cells were lysed, the lysates Western blotted and stained with an anti-CD34 mAb.

We could further demonstrate the existence of an ecto-sialyltransferase by labeling surface glycans with fluorescent neuraminic acid in the absence of exogenous sialyltransferase as shown in flow cytometry and sialylation of distinct surface glycoproteins such as the Major Histocompatibility Complex Class antigen (MHC class I), CD34, CD43, and CD45. In contrast to sialylation by exogenous sialtransferase, sialidase treatment did not increase the extent of surface sialylation by ecto-sialyltransferase. It seems therefore that only unmasked, free lactosamines were accessible for the ectosialyltransferase and those unmasked by exogenous sialidase treatment are perhaps sterically inaccessible for the endogenous enzyme.

Lymphocytes are known to release sialyltransferase activity into the medium [11], however surface sialylation by ecto-sialyltranferase as demonstrated here is not influenced by the soluble form. In an earlier study we reported that conditioned media did not significantly enhance sialylation as compared to unconditioned medium [11]. Since fetal calf serum contains low sialyltransferase activity we performed our assays in serum-free media. Further, in our cell cultures used for the sialylation assays >95% of the cells were viable so that exogenous activity released from damaged or dead cells is rather unlikely. Ecto-sialyltransferase seems to be a membrane-integrated enzyme because treatment to release noncovalently attached molecules from the cell surface did not influence the enzymatic activity [11].

Although this enzyme is able to sialylate major surface receptors, and thereby potentially altering their functions, its role under physiological conditions is not entirely clear. For instance, the existence of CMP-NeuAc in the outer milieu could not directly be demonstrated. It may be that ecto-sialyltransferase is physiologically active during an endocytose salvage pathway at which glycoproteins are resialylated by the ecto-sialyltransferase using intracellular CMP-NeuAc and then recirculate to the surface. Even this hypothetic pathway would result in a much faster sialylation process as compared to *de novo* synthesis. Thereby ectosialyltransferase may function as an enzyme for rapid remodulation of surface glycans and be able to increase sialylation or reconstitute sialylation on a given surface sialoglycan.

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