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Ectodomain shedding of CD200 from the B-CLL cell surface is regulated by ADAM28 expression

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ABSTRACT

CD200, a membrane glycoprotein of the immunoglobulin superfamily, is overexpressed in CLL. Soluble in serum CD200 (sCD200) is correlated with poor prognosis in CLL.

ADAM (a disintegrin and metalloproteinase) enzymes are implicated in membrane protein shedding. ADAM28 mRNA expression in CLL was correlated with plasma sCD200 levels, and release into culture from CLL cells. siRNA for *ADAM28* decreased release of sCD200 from cultures and transfection of a cloned ADAM28 gene into CD200$^+$ cells enhanced release of sCD200.

Our data support the hypothesis that ADAM28 plays a role in the shedding of CD200 from B-cell CLL cells.

1. Introduction

Chronic lymphocytic leukemia (CLL) of B cells (B-CLL) is a tumor of CD5$^+$CD19$^+$ and CD23$^+$ lymphocytes. It is the most common type of leukemia among adults in the western world and is characterized by a profound but poorly understood immunodeficiency state [1].

CD200, a membrane glycoprotein of the immunoglobulin superfamily (IgSF), exerts immunosuppressive function in autoimmune disease, fetal rejection and transplant tolerance [2]. CD200 is expressed on cells in many tissues, including B cells [3], and it exerts its immunoregulatory function following interaction with its receptor (CD200R) [2]. CD200 expression is increased in several hematology malignancies [4–6] and is linked with disease progression/prognosis in lymphoma and leukemia [5,6]. We and others have reported increased expression of CD200 on cells from B-cell CLL patients compared to normal B cells [7–9] and reported that elevated levels of a soluble form of CD200 (sCD200) are associated with poor prognosis [10].

ADAM (A Disintegrin And Metalloprotease) enzymes are a family of transmembrane proteases, composed of several domains. ADAMs are involved in cell adhesion, cell signaling, migration, degradation of extracellular matrix (ECM) and proteolytic ectodomain shedding [11–13]. Ectodomain shedding is a process in which a cell surface protein is cleaved near its transmembrane domain with the released protein ectodomain exerting a function. ADAMs are over expressed in human cancers [12,14] and implicated in cancer metastasis [15–17].

ADAM28 is expressed in human lymphocytes and is upregulated in certain cancer cells [18–20]. In contrast with other ADAMs, where furin-like proprotein convertases are involved in prodomain removal, ADAM28 is activated by autocatalytic removal of the prodomain with the mature transmembrane protein expressed on the cell surface [18]. The active form of ADAM28 cleaves specific sites in protein substrates, including CD23 and insulin-like growth factor binding protein-3 (IGFBP-3) [21]. ADAM 28 is expressed as two alternative forms, a membrane-anchored form (ADAM28m) and a short secreted form (ADAM28s) [22]. Immunoblotting with antiserum against the disintegrin domain of human ADAM28m showed two bands of 87 and 67 kDa in peripheral blood leukocytes. The 67 kDa form may represent a processed form of ADAM28 or a truncated, secreted form, ADAM28s [22].

We have asked whether elevated levels of sCD200 in CLL plasma reflect the action of unique ADAMs, and screened expression of 12 different ADAM species in CLL cells, correlating mRNA expression with soluble CD200 levels in patient serum. Our data show expression of ADAM28 is significantly correlated with sCD200, and siRNA-mediated inhibition of ADAM28 expression attenuates shedding of sCD200.

2. Materials and methods

2.1. Clinical samples

Peripheral blood was collected from consenting B-CLL patients and from healthy volunteers (all protocols were approved by institutional review boards). Cells were

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Table 1
Primer sequences used in real-time PCR.

Accession #	Gene	Sequence 5′–3′
NM_001109.3	ADAM8	ACACCCGGCAGGCACCAAAG GTGGGAGCTCGGCTCCTTGC
NM_001005845.1	ADAM9	TTTTCCCGCCACTGCACGAAGT AGAAGAGCTGTCTTGCCACAGA
NM_001110.2	ADAM10	GGTTGGCCAGATTCAACAAAAC TTTGGATCCCCACATGATTCTG
NM_003474.4	ADAM12	TTCCTGCTGCAACTGCTGAACA GGAATTGTCATGGACCATTCAG
NM_207197.1	ADAM15	TTCGAAGAGGCAGCTGCCCATT AACATGGACCACTCCACCAGCA
NM_003183.4	ADAM17	TTCATCCACCCTCGAGTTCCCA TACAAAGGAAGCTGACCTGGTT
NM_033274.2	ADAM19	GAAGGAGGGCCGTGTGGTGC GGATGGGAGGAGGCCCCAGG
NM_003814.4	ADAM20	AGCACTGCAGCTCTGATGG GCATGGCTGACCTTTAGGG
NM_003813.2	ADAM21	TGCATCCATAAGAAGTGTGTCA TCCCCTTCATATTGCAGGTC
NM_014265.4	ADAM28(m)	CCACTGGCACCAGGCCACAC GAGGCTGGGGGCTCATTGCC
NM_021777.3	ADAM28(s)	AATCCTTTCCCCTGTGCATG AATGGTCCTTTGACCATGGT
NM_021794.3	ADAM30	CCTCCATTCTGTGAGGAAGTG CCCAAATTGACGAGGGAAT
NM_025220.2	ADAM33	CTTGAGCTGGAGAAGAACCAC TAGTGGCAATGATCCGTGTG
NM_002046.3	GAPDH	TCATCCATGACAACTTTGGTATCG TGGCAGGTTTTTCTAGACGGC
NM_000194.2	HPRT1	CAAGCTTGCTGGTGAAAAGGA TGAAGTATTCATTATAGTCAAGGGCATATC

isolated by density centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The B-CLL cells were isolated from blood by negative selection (RosetteSep, StemCell Technologies, Vancouver, BC) according to the manufacturer's instructions.

2.2. Isolation of total RNA and synthesis of cDNA

Total RNA was extracted using TRIzol^{R} Reagent (Invitrogen) and treated with RNase-free Dnase I (Promega, Madison, WI) for 30 min at 37 °C. 2 μg RNA was reverse transcribed with SuperScript II (Life Technologies Inc., Rockville, MD) using a random oligonucleotide hexamer (Takara Bio Inc., Shiga, Japan) at 42 °C for 50 min, followed by heating at 70 °C for 15 min. cDNA was stored at −20 °C until PCR analysis (Table 1).

2.3. qPCR

Primers specific for individual ADAM isoforms were designed based on sequences available in the GenBank. PCR was performed on an ABI Prism 7900 Real Time PCR instrument (Applied Biosystems, Foster City, CA) with SYBR Green I as a double-stranded DNA-specific binding dye. Cycling conditions were, 95 °C for 10 min, and after initial denaturation (95 °C, 5 min) 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions used triplicate samples on a 384 plate, amplifying mRNAs for ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28m, ADAM28s, ADAM30 and the two housekeeping genes, HPRT1 and GAPDH.

Results were expressed as relative gene expression in comparison to HPRT1 using the $2^{-\Delta\Delta Ct}$ method [23], where $2^{-\Delta Ct} = 2^{[-\Delta Ct_{(ADAM)} - Ct_{(HPRT)}]_{B-CLL}}$. Applying the same formulation using GAPDH as comparator did not change any of the conclusions discussed below.

2.4. ELISA essay for soluble CD200

High binding 96-well plates (Corning Life Sciences) were coated overnight at 4 °C in Tris–HCl, pH 8.1 with a capture monoclonal anti-CD200 antibody (1B9) at 1.25 μg/ml concentration. All washings used PBS + 0.01% Tween20. Plates were blocked for 1 h at room temperature with blocking buffer (5% FBS in PBS) and different concentrations of CD200Fc (standard curve) or samples (plasma or supernatant) were added in 100 μl buffer. Plates were incubated for 3 h at room temperature, followed by washing and incubation for 2 h with 100 μl detection antibody (rabbit anti-CD200 serum, 1:500 dilution). After further washing, 100 μl goat anti-rabbit IgG-HRP antibody (1:12,500 dilution) was added with incubation at room temperature for 30 min. 100 μl TMB substrate was added with 10 min incubation at room temperature in the dark. The reaction was stopped using 50 μl sulfuric acid per well, and plates read in an ELISA plate reader at 450 nm. The sensitivity of detection of sCD200 is ~50 pg/ml.

2.5. Knockdown of ADAM 10, 17 and 28 expression with Stealth RNA interference (RNAi)

Three independent Stealth RNAi samples, complementary to ADAMs 10, 17 or 28 were purchased from Invitrogen and tested for specific suppression of the respective ADAM mRNA expression in CD200-stably transfected human breast cancer cells (MDA-MB-231^{CD200}). Transfection was performed using 2 × 106 adherent MDA-MB-231 cells and 3 μg siRNA (or control siRNA) using lipofectamine as transfection agent. qPCR was used to assess mRNA expression for the different ADAMs as well as a control housekeeping gene (GAPDH). Culture supernatants were also assessed for sCD200 release from the (CD200-transfected) MDA-MB-231 cells. When fresh CLL cells were treated with 3 μg siRNA, 10^7 fresh B-cell CLL cells were used, and transfection used Amaxa^{TM} Nucleofector^{TM} Technology, applying Solution-V with a U-013 running program. After transfection, cells were resuspended with 900 μl pre-warmed AIM-V media and incubated for 48 h, 72 h or 96 h at 37 °C until harvest. Culture supernatants were stored at −20 °C for sCD200 ELISA assay.

2.6. Transfection of Ly5CD200^{+} cells with a cloned ADAM28 cDNA

A glycerol stock of the human full-length cDNA clone of Adam 28 was obtained from Origene (Rockville, MD), containing 1.5 kb full-length cDNA of the human Adam 28 inserted into the pCMV6-XL5 mammalian expression vector. Plasmid DNA was purified using an EndoFree Plasmid Maxi Kit from Qiagen (Valencia, CA) and verified by DNA sequencing. A pmaxGFP vector was provided by Amaxa Inc. (Gaithersburg, MD) and used as a positive control for transfection. 1×10^6 Ly5 B cell lymphoma cells were centrifuged, resuspended in 100 μl of Nucleofector Solution V (Amaxa Inc.), and mixed with 2 μg of plasmid DNA. Electroporation was performed as above and cells cultured in triplicate in serum free medium with supernatants collected at 48 h post-transfection to measure sCD200.

2.7. Immunoblotting of CLL extract with anti-ADAM28 antibody

Cells were lysed on ice in 400 μl of 50 mM Tris–HCl buffer (pH 7.5), 150 mM NaCl, 1 mM PMSF, SDS 0.1% and Na. Deoxycholate 0.5% containing a cocktail of proteinase inhibitors (Roche Diagnostics, Mannheim, Germany). SDS-PAGE (7.5% total acrylamide) was performed under reducing conditions and resolved proteins were transferred to polyvinylidene difluoride membranes with a semidry blotter. Membranes were probed with a commercial Rabbit polyclonal antibody against the cytoplasmic domain of ADAM 28 (ab39875; Abcam, Cambridge, UK), at a dilution of 1:1000 using Rabbit anti-beta actin (ab8227; Abcam), at a dilution of 1:5000, as positive control. Blots were performed with 2% milk in TBST overnight at 4 °C. Goat anti Rabbit HRP was added as developer with protein bands detected with ECL Western blotting reagents (Amersham Pharmacia Biotech).

2.8. Statistical analysis

Data were analyzed using *JUMP5* software. Differences in mRNA expression of ADAM28 between paired groups was determined by Student's *t*-test. Correlations between the mRNA expression levels of the ADAMs species and sCD200 levels used a Pearson correlation coefficient. *p*-Values <0.05 were considered significant.

3. Results

3.1. mRNA expression of different ADAM isoforms in B-CLL cells

mRNA expression of ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28, ADAM30 and ADAM33 was screened by qPCR in CLL cells from 18 different patients (% CD5^{+} cells varied from 65% to 92% in these different samples), and CD19^{+} B cells from six normal donors. Fig. 1 shows the relative expression level of ADAMs 9, 10, 17, 28, and 30 was significantly higher ($p < 0.0001$) in CLL cells compared to normal CD19^{+} B cells (nominally set as one). mRNA expression for ADAM 8 and 12 was significantly lower ($p < 0.0001$) in the same cells compared to control cells. While data are shown in Fig. 1 using CD19^{+} cells from all donors (CLL and normal) no significant differences were seen when comparing relative mRNA expression in