Mechanisms by which the surface expression of the glycosyl-phosphatidylinositol-anchored complement regulatory proteins decay-accelerating factor (CD55) and CD59 is lost in human leukaemia cell lines

Michiyo HATANAKA*†¶, Tsukasa SEYA†‡, Misako MATSUMOTO†, Tomoko HARA†, Masaru NONAKA§, Norimitsu INOUE‖, Junji TAKEDA‖ and Akira SHIMIZU*

*Department of Clinical Pathology, Osaka Medical College, Takatsuki 569, Japan, †Department of Immunology, Center for Adult Diseases Osaka, Higashinari-ku, Osaka 537, Japan, ‡PRESTO, JRDC, Kyoto 619-02, Japan, §Department of Biochemistry, Nagoya City University Medical School, Mizuho-ku, Nagoya 467, Japan, and ‖Department of Immunoregulation, Research Institute for Microbial Diseases, Suita, Osaka 565, Japan

We have investigated the mechanisms of defects in the glycosyl-phosphatidylinositol (GPI)-anchored complement regulatory proteins delay-accelerating factor (DAF) and/or CD59 in a panel of human leukaemia cell lines that lack surface expression of these proteins: U937 ($DAF^+/CD59^-$), CEM ($DAF^-/CD59^+$), TALL ($DAF^-/CD59^-$) and a substrain of Ramos [Ramos(−)] ($DAF^-/CD59^-$). Northern blotting and reverse transcription-PCR revealed that the main cause of the DAF and/or CD59 deficiency is the failure of mRNA expression in most of the cell lines, except in Ramos(−) in which sufficient mRNA for DAF and CD59 was produced. U937, CEM and TALL cells were not defective in GPI anchor formation as assessed by the detection of other GPI-anchored proteins. No gene abnormality corresponding to DAF or CD59 was detected by Southern blotting. Thus the cause of the defects of DAF and/or CD59 in these leukaemia cell lines except for Ramos(−) is virtually undetectable steady-state levels of the relevant mRNA, most likely attributable to lack of transcription in these cell lines. On the other hand, Ramos(−) cells failed to generate a GPI anchor, whereas they normally expressed DAF and CD59 transcripts. The transfection of phosphatidylinositol-glycan class A (PIG-A) cDNA into Ramos(−) cells restored DAF and CD59 expression, indicating that the defective mechanism in GPI anchor formation is similar to that in paroxysmal nocturnal haemoglobinuria (PNH) cells, i.e. a deficiency of the PIG-A gene product. Thus the mechanisms of the defects of DAF and/or CD59 in human leukaemia cell lines are not uniform, and in most cases are different from that proposed to cause PNH.

INTRODUCTION

Human tissues and cells are generally resistant to homologous complement [1]. The so-called homologous restriction of complement activation is attributable to the membrane complement regulatory proteins decay-accelerating factor (DAF; also known as CD55), membrane cofactor protein (MCP; CD46), complement receptor type 1 (CR1; CD35) [2] and CD59 [3]. Of these, DAF and CD59 belong to the group of proteins that are linked to cell membranes via a glycosyl-phosphatidylinositol (GPI) anchor [4]. DAF is a 65 kDa protein that inhibits the assembly of or accelerates the dissociation of C3/C5 convertases and thereby regulates the complement cascade at the C3 step [5,6]. CD59, a 20 kDa protein, inhibits the final step of the complement cascade by preventing the formation of the membrane attack complex [7–10]. These proteins are distributed in all haematopoietic cells and in a wide variety of other tissues, but are not present in the blood cells of patients with paroxysmal nocturnal haemoglobinuria (PNH) [11–13]. The deficiency of these proteins leads to the increased susceptibility of erythrocytes to complement-mediated lysis, and thus the patients suffer haemolytic anaemia [14,15]. Other GPI-anchored proteins with different functional entities are simultaneously absent from PNH cells, suggesting that a defect of GPI anchor synthesis is the main cause of the lack of DAF and CD59 in the blood cells of patients with PNH. Miyata et al. [16] have cloned a cDNA encoding a protein termed PIG-A (phosphatidylinositol-glycan class A) which is necessary for the synthesis of the early intermediates in GPI anchor biosynthesis. All patients with PNH that were tested had an abnormality exclusively in the PIG-A gene [17], indicating that a defect in PIG-A is responsible for the pathogenesis of PNH.

DAF and/or CD59 are diminished under some pathological conditions. We have previously reported that DAF is not present in the affected cells of some non-Hodgkin's lymphoma patients [18] and that CD59 is absent from leukaemia cells in some patients [19]. These patients had no history of PNH or a Cromer-related hereditary deficiency of DAF [20]. Together with our findings that DAF and/or CD59 are absent from several human leukaemia cell lines [21,22], we suggested that defects of DAF and/or CD59 are associated, with a high frequency, with haematological malignancies. In the present study we have focused on the mechanisms by which the expression of DAF and/or CD59 is regulated in these malignant cells, in particular whether defects occur through similar mechanisms as those proposed for PNH [17,23,24]. We examined the levels of mRNA, gene abnormalities and GPI-anchor-forming ability in DAF- and/or CD59-deficient leukaemia cell lines.

Abbreviations used: CR1, complement receptor type 1 (CD35); DAF, decay-accelerating factor (CD55); D-PBS, Dulbecco's PBS; FITC, fluorescein isothiocyanate; GPI, glycosyl-phosphatidylinositol; HUVEC, human umbilical vein endothelial cells; MCP, membrane cofactor protein (CD46); PIG-A, phosphatidylinositol-glycan class A; PI-PLC, phosphatidylinositol-specific phospholipase C; PNH, paroxysmal nocturnal haemoglobinuria; RT, reverse transcription.

¶ To whom correspondence should be addressed, at the Center for Adult Diseases, Osaka.

MATERIALS AND METHODS

Cell lines and antibodies

The human leukaemia cell lines HSB2 and K562 were gifts from Dr. John P. Atkinson (Washington University, St. Louis, MO, U.S.A.). The other cell lines were from the Japanese Cancer Research Resources Bank (JCRB). Data regarding the complement-associated proteins in these cell lines have been reported previously [21]. Substrains of Ramos DAF- and CD59-positive [Ramos(+)] and -negative [Ramos(−)] sublines were established in our laboratory [22]. All cells were maintained in RPMI 1640 supplemented with 10% (v/v) fetal calf serum and antibiotics. No mutagen was used to establish the GPI-anchor-protein-negative cell lines. Cultures were incubated in a 5% CO_2/95% air atmosphere at 37 °C.

Monoclonal antibodies against DAF (1A10) [25] and CD59 (5H8) [26] were gifts from Dr. T. Kinoshita (Osaka University, Osaka, Japan), and Drs. Y. Sugita (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) and M. Tomita (Showa University, Tokyo, Japan) respectively. A monoclonal antibody (J4-57) to CD48 (a GPI-anchored protein) was purchased from Immunotech S.A. A monoclonal antibody against Thy-1.2, named G7, was kindly donated by Dr. T. Tadakuma (Keio University, Tokyo, Japan). Monoclonal antibodies against human Thy-1 (CDw90) and CAMPATH-1 (CD52), both of which are GPI-anchored proteins, were from Chemicon International (Temecula, CA, U.S.A.) and Serotec (Oxford, U.K.) respectively.

Plasmids pME luciferase, pME Thy-1 and pME PIG-A were gifts from Dr. T. Kinoshita [16,17].

Flow cytometric analysis of the surface expression of GPI-anchored proteins

The surface expression of DAF, CD59 and CD48 was assessed by means of flow cytometry as previously described [21]. IA10, 5H8 and J4-57 were used as the primary antibodies for the detection of DAF, CD59 and CD48 respectively. Approx. 1×10^6 cells were incubated with 5–10 μg of the primary antibodies. After two washes, the cells were reacted with 3 μg of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG $F(ab')_2$ as the second antibody. The cells were washed twice and then fixed with paraformaldehyde. The reactivities were evaluated using an Epics Profile II (Coulter Corporation, Hialeah, FL, U.S.A.). To detect Thy-1, 10 μg of biotinylated anti-Thy-1.2 monoclonal antibody G7 was used as the first antibody, which was then reacted with phycoerythrin-conjugated streptavidin (Biomeda, Foster City, CA, U.S.A.).

Northern blotting

Total RNA was extracted from 5×10^7 cells of each cell line with guanidium thiocyanate followed by centrifugation in CsCl [27]. Total RNA (10 μg) from each cell line was denatured with glyoxal and DMSO at 50 °C for 1 h, and then separated on a 1% agarose gel [27]. The RNA was transferred to a nylon membrane (Hybond N^+; Amersham International), irradiated for 5 min with UV light and baked for 2 h at 80 °C. After prehybridization, the membrane was hybridized to [α-^{32}P]dCTP-labelled DNA probes at 65 °C using a Rapid Hybridization System (Amersham). The blot was washed twice with 2 × SSC/0.1% SDS at room temperature. After two washes with 0.1 × SSC/0.1% SDS at 65 °C, RNA blots were visualized by autoradiography.

Southern blotting

Genomic DNA was extracted from each leukaemia cell line using an Iso Quick DNA extraction kit (Micro Probe Corp., Garden Grove, CA, U.S.A.). The DNA (10 μg) was digested with *Kpn*1 or *Pvu*II, and the fragments were Southern-blotted with a DAF or CD59 probe. Briefly, the DNA (10 μg) from each cell line was digested with restriction enzymes overnight at 37 °C. The resulting fragments were separated on 1% agarose gels, alkaline denatured and transferred to nylon membranes (Hybond N^+) according to standard methods [27]. The membranes were prehybridized, hybridized and washed under the same conditions described for the Northern blots.

Hybridization probes

A plasmid carrying DAF cDNA sequences was a gift from Dr. D. M. Lublin (Washington University, St. Louis, MO, U.S.A.) [28]. The plasmid was digested with *Eco*RI and the resulting 2.0 kb fragment (covering the whole DAF coding region except for five amino acids in a signal peptide sequence) was purified by agarose-gel electrophoresis. CD59 cDNA was prepared as follows. cDNA transcribed from K562 RNA was amplified by PCR. The primer sequences were similar to those used in reverse transcription (RT)-PCR for RNA detection, but with additional *Eco*RI restriction sites at the 5′ and 3′ ends. The product (429 bp) was purified by agarose-gel electrophoresis and cloned into the *Eco*RI site of pBluescript KS (Stratagene, La Jolla, CA, U.S.A.). The inserted product was then sequenced on an ABI 373A DNA sequencer (Perkin-Elmer, Foster City, CA, U.S.A.). The plasmid was digested with *Eco*RI and the resulting fragment was purified by agarose-gel electrophoresis. The DAF and CD59 cDNA inserts were labelled with [α-^{32}P]dCTP using the random-primed DNA labelling reaction [27] and used for probing Northern and Southern blots.

RNA detection by RT-PCR

Samples of total RNA (1 μg) from each cell line were reverse-transcribed using random primers. cDNA was then amplified by 35 cycles using the following forward and reverse primers: DAF (with an additional *Bam*H I site at the 5′ end of the 5′ primer, and an *Eco*RI site at the 5′ end of the 3′ primer), 5′-ATG GAT CCG ACT GTG GCC TTC CCC CAG AT-3′ and 5′-ATG AAT TCG TTA CAT GAG AAG GAG ATG GT-3′; CD59, 5′-CTG TGG ACA ATC ACA ATG GGA ATC CAA GGA-3′ and 5′-GGT GTT GAC TTA GGG ATG AAG-3′. A two-cycle amplification method was employed, i.e. each cycle consisted of incubation for 1 min at 95 °C and 1 min at 60 °C. The amplification products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

The nucleotide sequences of the resulting products were all confirmed on a DNA sequencer.

Transfection

pME was used as an expression vector [29]. Ramos cells (1×10^7) were mixed with the pME Thy-1 plasmid (40 μg) or pME PIG-A plasmid (60 μg) in the transfection buffer (Hepes-buffered saline, consisting of 20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM dextrose [30]). The total volume was 0.8 ml for pME Thy-1 and 0.4 ml for pME PIG-A. The optimal conditions (240 V, 950 μF) were determined

by electroporating pME luciferase followed by measuring the level of luciferase activity expressed in the cytosol [31]. Ramos cells resisted transfection, since even under the optimal conditions the luciferase activity was 2% of that in control K562 cells (results not shown). When pME Thy-1 was transfected into K562 cells, the positive population was 48.3% of the total (results not shown).

Ramos cells containing DNA were electroporated in 0.4 cm cuvettes at 350 V, 500 μF (for pME Thy-1) or 240 V, 950 μF (for pME PIG-A) with a Gene Pulser (Bio-Rad, Richmond, CA, U.S.A.). After culture for 2 days, the levels of Thy-1, DAF and CD59 expression were evaluated by flow cytometry. Surface expression of DAF and CD59 was also assessed by a Protein A rosette assay [21].

Transfection was also performed with Lipofectamine (Life Technologies, Grand Island, NY, U.S.A.) as described in the manufacturer's booklet.

Phospholipase C digestion of cells

Cells (4×10^6) were suspended in 200 μl of Dulbecco's PBS (D-PBS) containing 0.5% BSA and digested with phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma, St. Louis, MO, U.S.A.) at a concentration range of 0.5–1.0 unit/ml [32,33]. After an incubation for 1 h at 37 °C, the reaction was stopped by the addition of cold D-PBS containing 0.5% BSA. After two washes in the same cold D-PBS buffer, the levels of GPI-anchored proteins were assessed by flow cytometry.

RESULTS

Surface expression of DAF and/or CD59 on human leukaemia cell lines

We assessed the levels of the proteins MCP, CR1, DAF and CD59 on human leukaemia cell lines by flow cytometry, and found that three [TALL, CEM and Ramos(−)] of the 32 cell lines tested were DAF-negative (Figure 1). TALL, Ramos(−) and U937 cells were CD59-negative. CEM, a DAF-negative cell line, expressed as much CD59 as K562 cells. All cell lines expressed MCP but virtually no CR1, consistent with previous reports [21,22]. Thus we have a panel of cell lines with double-positive DAF and CD59 [represented by K562, HSB2 and Ramos(+)], single-negative DAF (CEM), single-negative CD59 (U937) and double-negative DAF and CD59 [TALL and Ramos(−)].

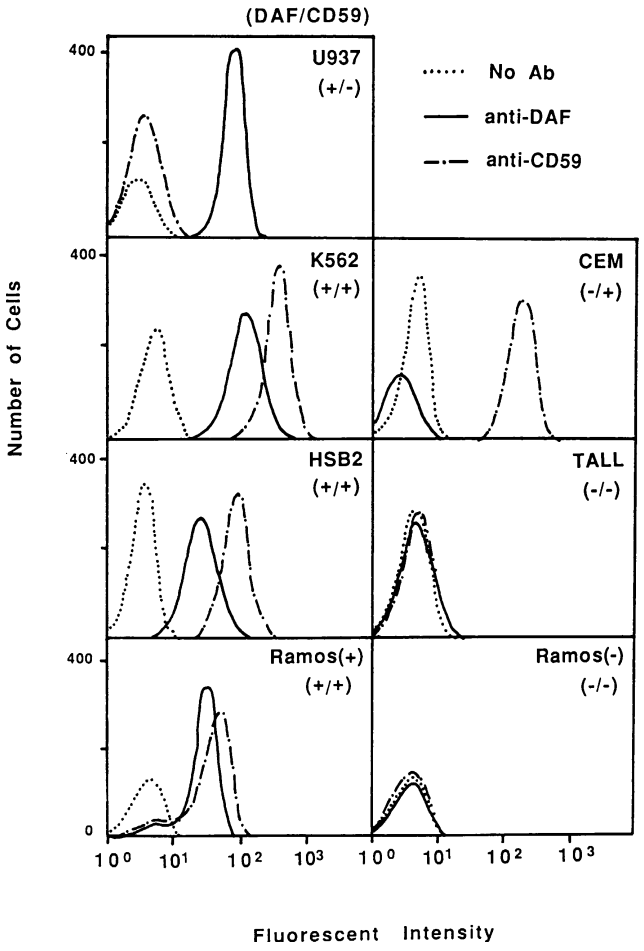

Figure 1 Levels of DAF and/or CD59 expression on human leukaemia cell lines

Cells (1×10^6) were incubated with antibodies against DAF (IA10) and CD59 (5H8). Non-immune mouse IgG was used as a control (No Ab). The cells were stained with FITC-labelled goat anti-mouse IgG $F(ab')_2$ as the second antibody and analysed by flow cytometry.

DAF and CD59 mRNAs in human leukaemia cell lines

We next examined the mRNA levels of DAF and CD59 in the cell lines by Northern hybridization. The DAF probe developed two major bands of 2.7 and 2.0 kb in the DAF-positive cell lines U937, K562, HSB2 and Ramos(+) (Figure 2). Among the DAF-negative cell lines, Ramos(−) cells expressed mRNA similar in size and amount to that in the positive counterpart. CEM and TALL cells showed no DAF transcripts (Figure 2).

The CD59 probe hybridized to three distinct bands corresponding to 2.3, 1.6 and 1.1 kb in the CD59-positive cell lines K562, CEM, HSB2 and Ramos(+). The 1.6 and 1.1 kb bands were rather faint compared with the 2.3 kb band. Among the CD59-protein-negative cell lines, Ramos(−) expressed as much CD59 transcript as Ramos(+), whereas it was undetectable in U937 and TALL cells. The blot was also hybridized to a β-actin probe to confirm that comparable amounts of total RNA were applied to each track.

The presence of mRNA was further assessed by RT-PCR. When amplified for 35 cycles using primer pairs specific for DAF, the product (487 bp) was detected in the DAF-positive cell lines U937, K562, HSB2 and Ramos(+), but not in the DAF-negative cell lines CEM and TALL (Figure 3, top). Similar to the Northern blots, comparable amounts of amplified DAF cDNA were detected in Ramos(−) cells as in Ramos(+) cells.

CD59 mRNA was also analysed by RT-PCR using primer pairs specific for CD59 (Figure 3, bottom). After 35 cycles of amplification, the predicted product (411 bp) was detected in the CD59-positive cell lines K562, CEM, HSB2 and Ramos(+), but not in the CD59-negative cell lines U937 and TALL. Again, comparable amounts of amplified CD59 cDNA were detected in Ramos(−) and Ramos(+) cells.

Sensitivity of DAF and/or CD59 to PI-PLC

We examined whether the DAF- and/or CD59-deficient cell lines can synthesize the GPI anchor moiety. In the DAF- or CD59-single-positive cell lines, DAF and CD59 are actually linked on the membranes via a GPI anchor, since PI-PLC (0.1 μg/ml) decreased the CD59 level in CEM cells and the DAF level in U937 cells (Figure 4). These results confirmed that the expressed DAF and CD59 are present as GPI-anchored forms and thus

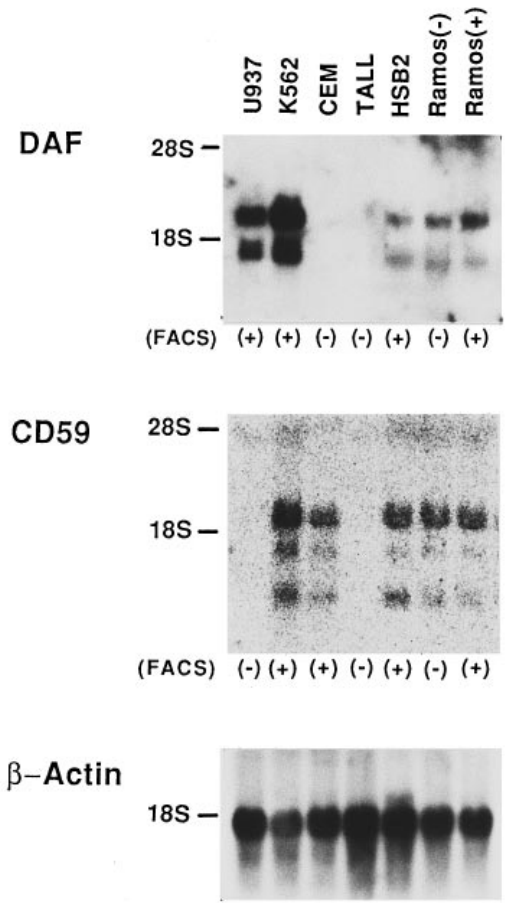

Figure 2 Northern blots of DAF and CD59 mRNAs in human leukaemia cell lines

Samples of total RNA (10 μg) prepared from human leukaemia cell lines were Northern blotted. The blots were hybridized with DAF (top panel) and CD59 (middle panel) probes. To evaluate the amounts of RNA applied to each track, the blots were hybridized with a β-actin probe (bottom panel). The ethidium bromide-stained 18 S and 28 S total RNA standards are indicated. Similar results were obtained in three additional experiments using other samples of RNA. (+) indicates protein expression by flow cytometry (FACS) criteria.

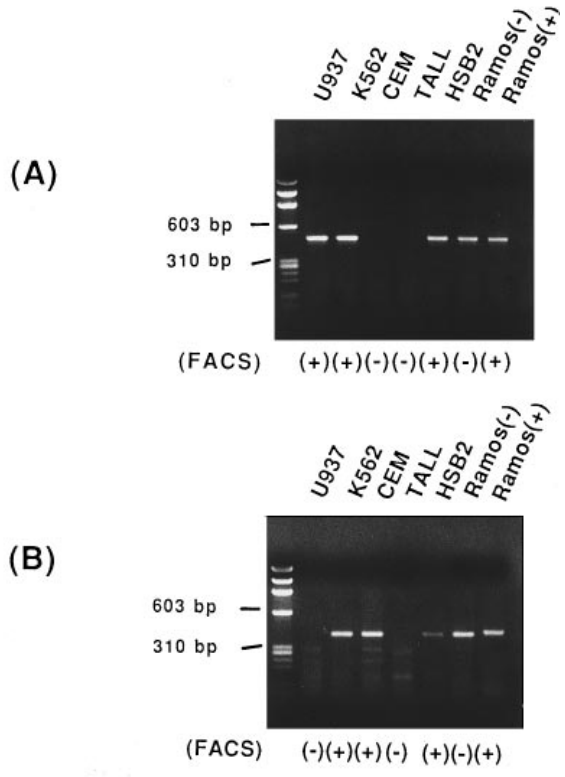

Figure 3 RT-PCR products amplified from human leukaemia cell RNA with primers for DAF or CD59

Samples of total RNA (1 μg) prepared from leukaemia cell lines were reverse-transcribed to cDNA, then amplified for 35 cycles by PCR using primers specific for DAF (top) or CD59 (bottom). Four experiments were performed and a representative one is shown. (+) indicates protein expression by flow cytometry (FACS) criteria.

that GPI anchor biosynthesis is essentially normal in these DAF/CD59-single-positive cell lines.

Levels of GPI-anchored proteins on human leukaemia cell lines

In the DAF/CD59-double-negative cell lines [TALL and Ramos(−)], we measured the expression levels of another GPI-anchored protein, CD48, to assess their GPI anchor biosynthetic ability (Figure 5). CD48 is distributed on leucocytes exclusively as a GPI-anchored protein and no transmembrane forms have been reported. TALL but not Ramos(−) cells are CD48-positive. Although PI-PLC at a concentration of 0.1 unit/ml barely decreased the CD48 level, it did so slightly and reproducibly at a higher concentration (1.0 unit/ml). Similar results were obtained by flow cytometry using anti-(Thy-1) and anti-CAMPATH-1, although their expression levels were far lower than those of CD48 (results not shown). Hence TALL cells possess GPI anchor biosynthesis activity, whereas the Ramos(−) subline does not. The GPI anchors synthesized by TALL cells are only partly sensitive to PI-PLC, like those in human erythrocytes [32,33].

Transfection of Ramos cells with Thy-1 or PIG-A cDNA

Ramos(−) cells do not express any of the three GPI-anchored proteins (DAF, CD59, and CD48), although they express DAF and CD59 transcripts, suggesting a defect in GPI anchor biosynthesis. To confirm this speculation, we transfected Ramos(−) cells with mouse Thy-1 (a mouse GPI-anchored protein which is not expressed on human cells) cDNA by electroporation and examined its presence on the membranes by flow cytometry (Figure 6A). Even after the Thy-1 cDNA transfection, Ramos(−) cells remained Thy-1-negative, while the positive control Ramos(+) cells became Thy-1-positive, although the positive populations only represented 2% of the total cells. Since the Thy-1-positive population was so small, we determined the optimal conditions for gene transfection using pME luciferase and a luciferase assay kit. Under the optimal conditions, Ramos(−) cells were transfected with PIG-A, a gene responsible for PNH pathogenesis, and surface-expressed DAF and CD59 were tested (Figure 6B). About 6% of the Ramos(−) cells became DAF- and/or CD59-positive; thus the expression of the GPI-anchored proteins was not restored in Ramos(−) cells until the cells were provided with PIG-A.

Southern blotting of DAF and CD59 genes

Southern blots were performed of DAF and CD59 genes digested with several restriction enzymes (results not shown). DNA from

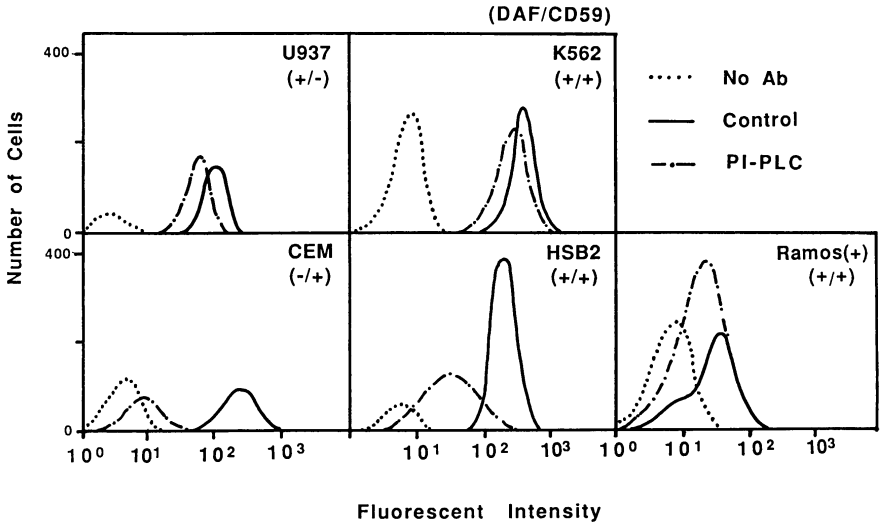

Figure 4 Sensitivity of DAF or CD59 on DAF/CD59-single-positive cell lines to PI-PLC treatment

Cells (4×10^6) were incubated with or without (Control) PI-PLC (0.1 unit/ml) for 1 h at 37 °C. After washing, the levels of DAF on U937 cells and of CD59 on CEM cells were measured by flow cytometry (see Figure 1). Non-immune mouse IgG was used as a control (No Ab). For comparison, the sensitivity to PI-PLC of CD59 on the double-positive cell lines K562, HSB2 and Ramos(+) was also assessed. The procedure for PI-PLC treatment has been described previously [33]. The GPI-anchored proteins on K562 are relatively resistant to PI-PLC, like those on human erythrocytes [32].

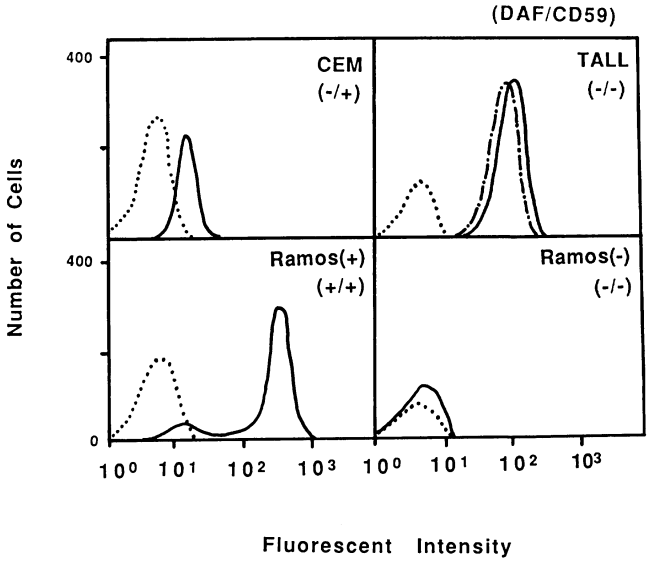

Figure 5 Levels of CD48 (a GPI-anchored protein) on DAF/CD59-negative cell lines

Cells (1×10^6) were incubated with antibodies against CD48. Non-immune mouse IgG was used as a control. The cells were subsequently stained with FITC-labelled goat anti-mouse IgG $F(ab')_2$ as the second antibody and analysed by flow cytometry. Cells were stained with non-immune mouse IgG (dotted line) or anti-CD48 (solid line). For comparison, the levels of CD48 on CEM and Ramos(+) cells were also assessed. A high concentration of PI-PLC (1.0 unit/ml) decreased the level of CD48 on TALL cells (dashed line). The relative resistance of the GPI-anchored proteins on TALL cells to PI-PLC was reproducibly observed, as in the case of K562 cells and erythrocytes.

the defective cell lines showed restriction fragmentation profiles identical to those of a non-defective control (K562) for most of the enzymes studied. Although minor variations in the *Kpn*I fragments of the DAF gene and the *Pvu*II fragments of the CD59 gene were found (results not shown), these variations were not correlated with the defects. Hence no apparent gene abnormality relevant to the absence of mRNA was detected by Southern blot analysis in the DAF/CD59-deficient cell lines.

DISCUSSION

This study demonstrates that the protein deficiencies of DAF and/or CD59 in U937, CEM and TALL cells are caused by the lack of steady-state levels of DAF and/or CD59 mRNAs, whereas that in Ramos(−) cells reflects a defect of the PIG-A gene, the product of which is essential at an early stage of biosynthesis of the GPI anchor [34]. Thus the mechanisms of DAF and/or CD59 deficiency in human leukaemia cell lines are not uniform and are mostly different from that causing PNH. The results are summarized in Table 1.

We believe that the above results are applicable to most intact leukaemia cells. Two findings support this notion. First, we have studied the levels of complement regulatory proteins in a variety of haematologically malignant cells from patients [18,19,35]. In these clinical studies either DAF- or CD59-negative cases are present to similar extents as those in cultured cell lines, the frequency of completely DAF- or CD59-negative cases being 10–20 % [21,35]. Secondly, intact leukaemia cells expressing no DAF and/or CD59 also lacked the corresponding mRNA (M. Hatanaka and T. Seya, unpublished work). Accumulation of data on leukaemia cells from patients should lead to the confirmation of our hypothesis.

The up-regulation of DAF has been found under various pathophysiological conditions. DAF expression on human umbilical vein endothelial cells (HUVEC) is increased by phorbol esters [36], certain lectins [37] and histamine [38]. Complement activation also induces DAF expression on human mesangial cells [39]. Analysis of the 5′-flanking regions of the DAF gene has revealed that the enhancer region contains phorbol 12-myristate

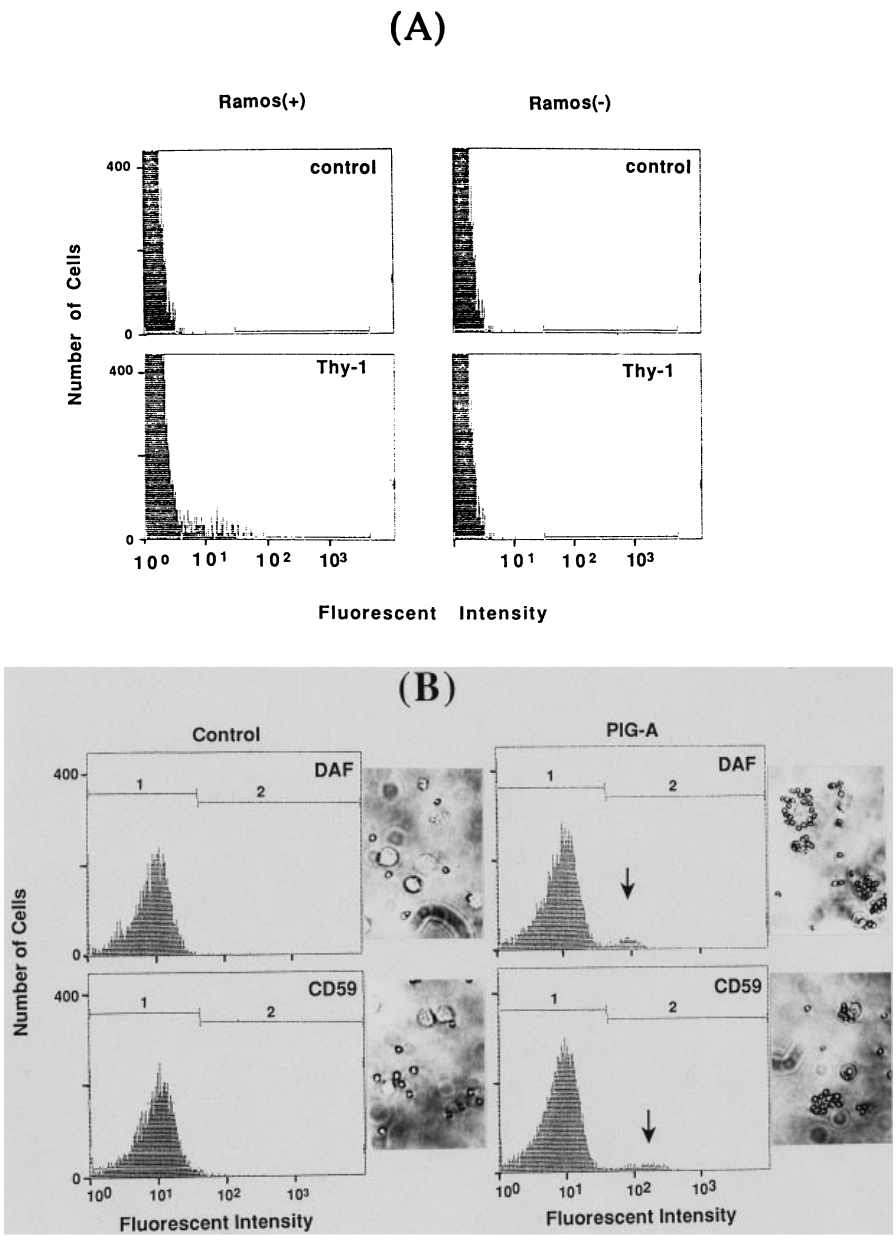

Figure 6 Transfection of (A) Ramos cells with Thy-1 cDNA and (B) Ramos(−) cells with PIG-A cDNA

(**A**) Cells (1×10^7) were mixed with pME Thy-1 plasmid (40 μg) in the transfection buffer (total volume 0.8 ml) and electroporated (350 V, 500 μF). After culture for 2 days the levels of Thy-1 on the cells were measured by flow cytometry. Ramos(+) cells expressed Thy-1, although positive populations only represented 2% of the total cells, whereas Ramos(−) cells did not. Ramos cells were electroporated with (lower panels) or without (upper panels) pME Thy-1. (**B**) Cells (1×10^7) were mixed with pME PIG-A plasmid (60 μg) (right panels) or vector only (60 μg) (left panels) in the transfection buffer (total volume 0.4 ml) and electroporated under optimal conditions (see the Materials and methods section). After culture for 2 days, the levels of DAF and CD59 on the cells were measured by flow cytometry. Upper panels, stained with anti-DAF (7% positive compared with control); lower panels, stained with anti-CD59 (5% positive compared with control). Arrows indicate the peak positions of DAF- or CD59-stained Ramos(+) cells. The results of a rosette assay (see [21]) using anti-DAF (upper panels) or anti-CD59 (lower panels) are shown to the side of the graphs (left, vector control cells; right, pME PIG-A transfected cells).

13-acetate/cAMP-responsive elements (AP-1 binding site) [40]. These observations suggest that DAF expression is controlled by transcriptional regulation. Although the precise mechanisms are not identified in the present study, the lack of DAF mRNA in CEM and TALL cells is likely to be the result of transcriptional regulation, probably including altered expression of transcription factor(s).

The regulatory mechanism of CD59 expression appears to be distinct from that of DAF or Ly-6, a murine structural homologue of CD59. The down-regulation of CD59 has only been found in PNH [15,23] and in our case study of acute promyelocytic leukaemia [19]. In contrast to DAF, CD59 expression is not enhanced by histamine or phorbol 12-myristate 13-acetate in HUVEC [38]. Likewise Ly-6, but not CD59, is induced by cytokines in HUVEC [41]. In addition, partial hypomethylation in the 5′ region of CD59 coding sequences reportedly induces the expression of the protein in CD59-negative Burkitt's lymphoma cell lines [42]. The panel of CD59-defective cell lines established

Table 1 Expression of DAF and/or CD59 proteins and their transcripts, as well as GPI anchor formation, in the defective cell lines

Cell line	Protein		mRNA		GPI-anchor-forming activity	Cause of defect of DAF and/or CD59 expression
	DAF	CD59	DAF	CD59		
U937	+	−	+	−	+	No CD59 mRNA
CEM	−	+	−	+	+	No DAF mRNA
TALL	−	−	−	−	+	No DAF or CD59 mRNAs
Ramos(−)	−	−	+	+	−	Failure of GPI anchor formation (defect in PIG-A gene)

for the present study would be a good model for the further investigation of the regulatory mechanisms of CD59 expression.

The lack of DAF/CD59 in these cell lines or in leukaemia cells from patients may be caused by either the leukaemic change or arrested maturation. Which of the two possibilities is the crucial factor remains to be settled. An answer could be obtained by comparing the appearance of DAF/CD59 with that of some differentiation markers. Moreover, another cause, such as a defect in processing mechanisms of primary transcripts or instability of mRNA, cannot necessarily be excluded.

In contrast, the Ramos(−) subline is defective in GPI anchor biosynthesis. This is supported by the following evidence: (1) although Ramos(−) cells normally generate DAF and CD59 transcripts, no proteins are surface-expressed; and (2) transfection of PIG-A cDNA into Ramos(−) cells restored the expression of both DAF and CD59. The possibility that the Ramos(−) subline is derived from PNH cells, however, is excluded, since this subline was established from a patient with Burkitt's lymphoma without PNH [22]. The PIG-A gene may be able to mutate in conjunction with malignant, as well as PNH, transformation.

Transfection efficacy ($\sim 6\%$) was not increased by another method using Lipofectamine (results not shown). The reason for the inefficient restoration of DAF and CD59 (Figure 6) by the transfected PIG-A in the Ramos(−) cell line is thus far unknown; a partial defect in other genes responsible for GPI biosynthesis may not be ruled out.

Occasionally, patients with PNH progress to acute myelocytic leukaemia [43]. It was thought that blasts arose from the injured marrow as a second haematopoietic disorder. Devine et al. [44], however, have shown that blast cells of an acute myelocytic leukaemia patient that progressed from PNH lacked both DAF and alkaline phosphatase, but those from patients without PNH did not. Therefore they suggested that the leukaemic blast cells of PNH patients were derived from the same clone as the abnormal erythrocytes. As our present results show, the lack of both DAF and CD59 in leukaemia cell lines is due to either undetectable steady-state levels of the relevant mRNAs or defective GPI anchor biosynthesis secondary to a PIG-A gene abnormality. In the study by Devine et al. [44] the mRNA levels of GPI-anchored proteins were not evaluated. Therefore the final conclusion as to the relationship between PNH and its leukaemic shift must wait until the analysis of the mRNAs is accomplished.

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