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Research Article

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An In Vivo Animal Model of Gene Therapy for Leukocyte Adhesion Deficiency

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Abstract

Leukocyte adhesion deficiency (LAD) is an inherited disorder of leukocyte function that is caused by defects in the CD18 gene and is associated with diminished cell surface expression of CD11/CD18 proteins. We have developed an in vivo model for gene therapy of LAD. Recombinant retroviruses were used to transduce a functional human CD18 gene into murine bone marrow cells which were transplanted into lethally irradiated syngeneic recipients. A reliable flow cytometric assay for human CD18 in transplant recipients was developed based on: (*a*) the availability of human specific CD18 monoclonal antibodies and (*b*) the observation that human CD18 can form chimeric heterodimers with murine CD11a on the cell surface. Human CD18 was detected on leukocytes in a substantial number of transplant recipients for at least 6 mo suggesting that the gene had been transduced into stem cells. Expression was demonstrated in several lineages of a variety of hematopoietic tissues, but was consistently highest and most frequent in granulocytes. Murine granulocytes demonstrated appropriate posttranscriptional regulation of human CD18 in response to activation of protein kinase C. No apparent untoward effects of human CD18 expression were noted in transplant recipients. These studies suggest a specific strategy for LAD gene therapy that may be effective and safe. (*J. Clin. Invest.* 1991. 88:1412–1417.) Key words: CD18 • recombinant retroviruses • bone marrow transplantation • integrins

Introduction

Leukocyte adhesion deficiency (LAD)[1] is an inherited disorder of leukocyte function that is associated with recurrent life-threatening infections (1–3). Leukocytes from affected patients are deficient in three cell surface glycoproteins that mediate important cell–cell interactions (4). These glycoproteins are dimeric molecules that contain a common β subunit, called CD18, and one of three unique α subunits including CD11a (LFA-1), CD11b (Mol), and CD11c (p150,95). The molecular basis of LAD appears to be an abnormality in the expression or function of the shared subunit CD18 (5–7).

The only effective cure for LAD is allogeneic bone marrow transplantation (8). The relative success of this therapeutic modality suggests an alternative approach based on somatic gene therapy. This strategy would involve stably transducing a functional CD18 gene into autologous hematopoietic stem cells ex vivo which would be used to reconstitute a normal hematopoietic system in the setting of an autologous bone marrow transplant. As a first step towards the development of this therapy we described, in a previous study, the use of recombinant retroviruses to transduce a normal CD18 gene into EBV-transformed lymphoblasts of an LAD patient (9). Retroviral-mediated gene transfer into these cells led to reconstitution of cell surface expression of CD11a/CD18 and correction of functional defects in adhesion.

Despite these encouraging results in vitro, many important questions remain about the feasibility of gene therapy for LAD. Is it possible to achieve therapeutic levels of CD18 expression in appropriate lineages following reconstitution with retroviral transduced stem cells? Furthermore, will recombinant derived CD18 be regulated appropriately in differentiated hematopoietic cells of transplant recipients? Finally, will constitutive and/or ectopic expression of CD18 affect hematopoietic reconstitution or lead to disease?

As part of our goal to design a safe and effective genetic treatment for LAD, we describe in this report an in vivo murine model of human CD18 gene transfer. Our strategy was to use recombinant retroviruses to transduce a functional human CD18 gene into bone marrow cells derived from C3H/HeJ mice; lethally irradiated syngeneic recipients were reconstituted with the genetically modified bone marrow cells. A specific and sensitive assay for human CD18 expression in transplant recipients was developed based on previous observations in somatic cell hybrids (10) and retroviral transduced cell lines (Krauss, J. C., L. M. Bond, R. F. Todd III, and J. M. Wilson, unpublished data) that human CD18 will form heterodimers with murine CD11a on the cell surface. Expression of human CD18 in murine leukocytes was readily detected and quantified by flow cytometry using a human specific MAb to a CD18 epitope.

Methods

Vectors and viruses

A full length cDNA for human CD18 (9) was cloned into the BamHI site of a previously described retroviral vector (11) that expresses the recombinant gene from heterologous sequences spanning the 5′ region of the chicken β-actin gene (called BA-CD18). Sequences 5′ to the immediate early (IE) gene of human cytomegalovirus (from SpeI [at −580 of IE gene] to PstI [site in vector sequence] of CDM8, ref. 12) were subcloned into PUC19 and a portion containing IE enhancer se-

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1. *Abbreviations used in this paper:* IE, immediate early; LAD, leukocyte adhesion deficiency.

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quences was removed on a XhoI (from the polylinker) to NcoI (−220 of the IE gene) fragment (for numbering of IE enhancer see ref. 13). Synthetic linkers were used to convert the NcoI site to a XhoI site and the modified fragment was cloned into the unique XhoI site of BA-CD18 located 5′ to the β-actin promoter. This new vector is called CMV-BA-CD18. Structures of these vectors are presented in Fig. 1 *A*.

Retroviral vectors (10 μg) were mixed with pSV2Neo (1 μg) and transfected into the ecotropic packaging cell line Ψ Cre as described (9). Individual clones of transfected cells (25 clones/producer) were isolated and supernatants were tested for titer of virus by infecting NIH3T3 cells and analyzing the infected cells for the abundance of proviral DNA (9). The single best virus producer from each vector was used in subsequent analyses. DNA blot analysis of infected unselected NIH3T3 cells revealed 0.2 copies of provirus/cell for BA-CD18 and 1 copy of provirus/cell for CMV-BA-CD18. Comparison to viruses that encode a selectable marker yields an approximate titer of 2×10^5 CFU's/ml for BA-CD18 and 1×10^6 CFU's/ml for CMV-BA-CD18 (data not shown).

Flow cytometry

One color analysis of peripheral blood. Indirect immunofluorescence and flow cytometric analysis was performed on peripheral blood as described (14). A monoclonal antibody to human CD18 (IB4, ref. 15) was conjugated to biotin as described (16). The conjugate was incubated with whole blood which was secondarily stained with a streptavidin-fluorescein-isothiocyanate conjugate. The erythrocytes were lysed and the nucleated cells were fixed with a whole blood staining kit (Coulter Electronics Inc., Hialeah, FL) and analyzed by flow cytometry. Each analysis was compared to indirect immunofluorescence staining by an isotype identical biotin-conjugated negative control MAb.

Two color analysis of cell suspensions. Suspensions of cells were prepared from tissues and stained simultaneously with a biotin-human CD18 conjugate (IB4) and the following rat MAbs: antimurine(m)-CD18 (M18/2.a.12.7, ref. 17), antimurine (m)-CD11a (M17/4.4.11.9, ref. 17), antimurine(m)-CD11b (M1/70.15.115, ref. 18), anti-m-GR-1 (19), or anti-m-CD45r (20). Whole blood was secondarily stained with streptavidin fluorescein-isothiocyanate conjugate and goat anti-rat phycoerythrin. The erythrocytes were lysed and the nucleated cells were fixed with a whole blood staining kit (Coulter) and then analyzed by two color flow cytometry. For the analysis of tissues, the animals were euthanized 6 mo after bone marrow transplantation.

Phorbol myristate acetate (PMA)–mediated regulation of human CD18

Whole blood was obtained from two recipient animals as well as two normal human volunteers. The blood was divided in aliquots and either (*a*) held at 4°C or (*b*) incubated at 37°C with PMA at 100 ng/ml final concentration, each for 30 min. The blood was then stained with human CD18 antibody (IB4, Fig. 4 *A;* ref. 15), or an antibody which cross-reacts with both human and murine CD11b (M1/70.15.115 Fig. 4 *B;* ref. 18), or a murine CD18 antibody (M18/2.a.12.7, Fig. 4 *C;* ref. 17), and analyzed by flow cytometry as described in Fig. 1. The relative fluorescence of a given epitope was obtained by determining the linear channel number representing the mean fluorescence of all cells, minus the mean linear fluorescence of cells stained with an isotype identical control antibody. N.D. is not done.

Results and Discussion

Two retroviral vectors containing human CD18 cDNA were constructed and used to generate cell lines that stably produce high titer stocks of ecotropic virus. In both vectors, human CD18 is expressed from a transcript initiated at sequences from the chicken β-actin gene that are located internal to the viral

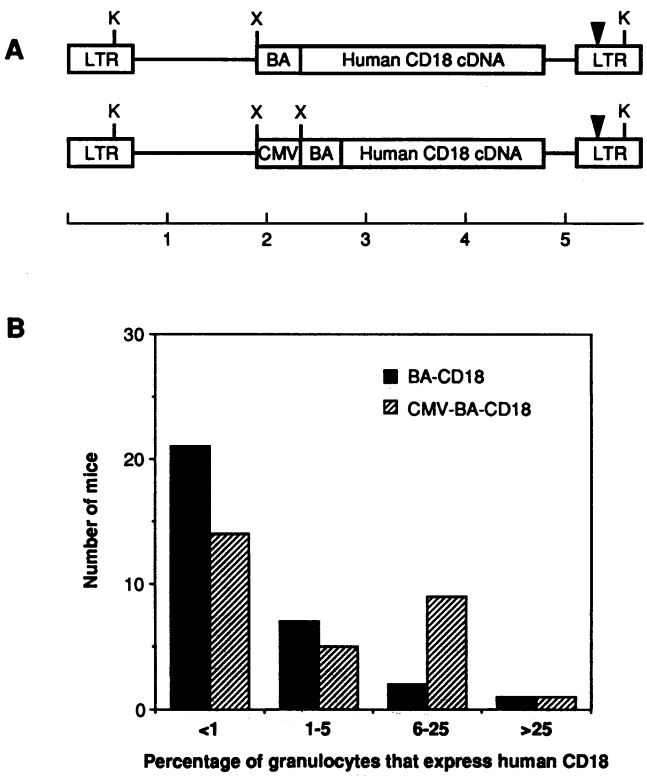

Figure 1. (*A*) Structures of retroviral vectors that express human CD18. The top vector is called BA-CD18 while the bottom vector is called CMV-BA-CD18. *LTR,* long terminal repeat sequences; *BA,* promoter sequences from the chicken β-actin; *CMV,* enhancer sequences from the immediate early gene of cytomegalovirus; *K,* KpnI; and *X,* XhoI. The scale indicates vector length in kilobases. (*B*) Expression of human CD18 in peripheral blood of transplant recipients. Blood was harvested 6 wk after transplantation and analyzed for cell surface expression of human CD18 as described in Methods. The analyses were performed on a subpopulation of nucleated cells that were enriched for granulocytes based on forward and ninety degree light scatter. This panel summarizes the expression of CD18, in terms of percentage of total granulocytes that specifically stained with the human CD18 MAb (i.e., < 1%, 1–5%, 6–25%, and > 25%), in animals transplanted with BA-CD18 transduced (*shaded bars*) or CMV-BA-CD18 transduced (*stippled bars*) bone marrow.

transcriptional unit (11); the vectors differ by the presence (CMV-BA-CD18) or absence (BA-CD18) of enhancer sequences derived from the immediate early gene of cytomegalovirus. Fig. 1 *A* presents the structures of these vectors.

Bone marrow was isolated from C3H/HeJ mice, transduced with either CMV-BA-CD18 or BA-CD18 ecotropic virus, and transplanted into syngeneic lethally irradiated recipients (40 recipient animals for each virus) as described (11). Approximately 75% of the animals survived the transplant. This rate of survival is similar to that achieved with retroviruses expressing other genes (for example, ref. 11) suggesting that CD18 gene transfer had no apparent effect on hematopoietic reconstitution. Animals that survived the transplant (60/80) were subsequently analyzed for expression of human CD18 in peripheral blood leukocytes by flow cytometry using a human CD18 MAb. In these analyses, nucleated cells were separated into lymphocyte or granulocyte enriched fractions based on light scatter properties; Fig. 1 *B* summarizes the analysis of the

Table I. Cell Surface Expression of Human CD18 and Various Murine Epitopes on Nucleated Cells from the Tissues of Mouse 15-6, Six Months after Bone Marrow Transplantation

			Blood		Spleen		Bone marrow			
Exp	MAb-1	MAb-2	Lymphocyte	Granulocyte	Lymphocyte	Granulocyte	Lymphocyte	Granulocyte	Thymus	Lymph node
A	h-CD18	con	14	75	11	55	16	74	24	7
B	con	m-CD18	65	97	61	68	32	85	96	91
C	con	m-CD11a	91	97	75	61	49	91	98	98
D	con	m-CD11b	11	97	9	56	6	88	2	7
E	con	m-GR-1	4	96	5	37	8	83	7	11
F	con	m-CD45r	39	4	33	23	60	7	15	16
G	h-CD18	m-CD18	12	75	10	40	10	68	19	5
H	h-CD18	m-CD11a	11	77	10	37	11	73	23	6
I	h-CD18	m-CD11b	7	75	6	36	6	72	0	2
J	h-CD18	m-GR-1	1	50	3	30	2	68	2	2
K	h-CD18	m-CD45r	3	1	8	12	8	5	4	3

Cells were gated into lymphocyte and granulocyte populations (blood, spleen, and bone marrow) or a single population (thymus, lymph node) and stained using the monoclonal antibodies listed under column MAb-1 (control or h-CD18) and MAb-2 (control, m-CD18, m-CD11a, m-CD11b, m-GR-1, or m-CD45r). Cells were stained and analyzed by two color immunofluorescence as in Fig. 2. Data are presented as the percentage of cells in a specific quadrant of the histogram. The percentage of cells positive for a particular epitope was determined by setting a cursor at the channel where the negative control antibody demonstrated no fluorescence, and the percentage of cells that were more fluorescent than the cursor was recorded as the percent of cells expressing the epitope. The cursors remained at the same channels between the various hematopoietic tissues. Human CD18 was stained with fluorescein isothiocyante (green) and the murine epitopes were stained with phycoerythrin (red). The table summarizes the positive fluorescence in the following quadrants: green positive, red negative—exp. A; green negative, red positive—exp. B, C, D, E, F; and green positive, red positive—exp. G, H, I, J, K.

granulocyte enriched fractions. Analysis of blood harvested 6 wk after transplantation revealed significant human CD18 expression in granulocytes from 25 of 60 surviving mice. The number of cells expressing human CD18 ranged from undetectable to 70% of total granulocytes and did not qualitatively change when analyzed at 6 mo (22 of 60 animals assayed) following transplantation. The lymphocyte gated fraction consistently demonstrated 5- to 10-fold fewer CD18 expressing cells than the granulocyte gated fraction. Analyses of peripheral blood from the highest expressing animals for the presence of replication competent virus were consistently negative (11). These studies suggest that a substantial number of animals have been partially reconstituted with retroviral transduced hematopoietic stem cells.

Two animals that expressed human CD18 in > 25% of peripheral blood granulocytes 6 mo after transplantation were characterized further. Leukocytes were separated into granulocyte and lymphocyte enriched fractions and analyzed by two color flow cytometry for the coexpression of human CD18 with several murine leukocyte antigens including murine CD18, CD11a, CD11b (a granulocyte/monocyte marker), GR-1 (a granulocyte marker), and murine CD45r (a murine B lymphocyte marker). Data from the animal that expressed human CD18 in 14% of the lymphocyte fraction and 75% of the granulocyte fraction (animal 15-6, reconstituted with CMV-BA-CD18 transduced bone marrow) are summarized in Table I and selected histograms are presented in Fig. 2. Direct evidence to support the expression of human CD18 in granulocytes was provided in experiments that detected human CD18 in 50–75% of cells expressing the lineage specific markers, GR-1 and CD11b. Human CD18 was detected in 3% of CD45r positive cells indicating that the recombinant gene was active in a small subpopulation of lymphocytes. Finally, expression of human CD18 in granulocytes had no apparent effect on the expression of endogenous CD11a, CD11b, and CD18 relative to bone marrow transplant recipients in which human CD18 was not expressed (data not shown).

Selected animals were euthanized and various tissues were harvested and analyzed for human CD18 expression by two color flow cytometry. Table I summarizes the flow cytometric analysis of tissues from animal 15-6. Cells derived from spleen and bone marrow were separated into lymphocyte and granulocyte gated fractions based on light scatter while thymic and lymph node derived cells demonstrated a single population of cells comprised primarily of lymphocytes/thymocytes. Spleen and bone marrow cells demonstrated a pattern of human CD18 expression that closely resembled the results obtained in peripheral blood; expression of human CD18 was consistently more frequent in granulocytes than in lymphocytes. Human CD18 was detected in a small subpopulation of lymph node derived cells (7% in the lymphocyte-gated fraction and 3% of CD45r positive cells) and in a greater number of thymocytes (24% of cells in the lymphocyte-gated fraction). Identical results were obtained with tissues harvested from a second animal that expressed human CD18 in > 25% of granulocytes.

The flow cytometric analysis of hematopoietic tissues demonstrated consistent lineage-specific expression of human CD18 with substantially greater expression in granulocytes than lymphocytes. This could be caused by selective reconstitution of the myeloid compartment with transduced cells or diminished transgene expression in the lymphoid compartment of an animal fully reconstituted with transduced cells. In

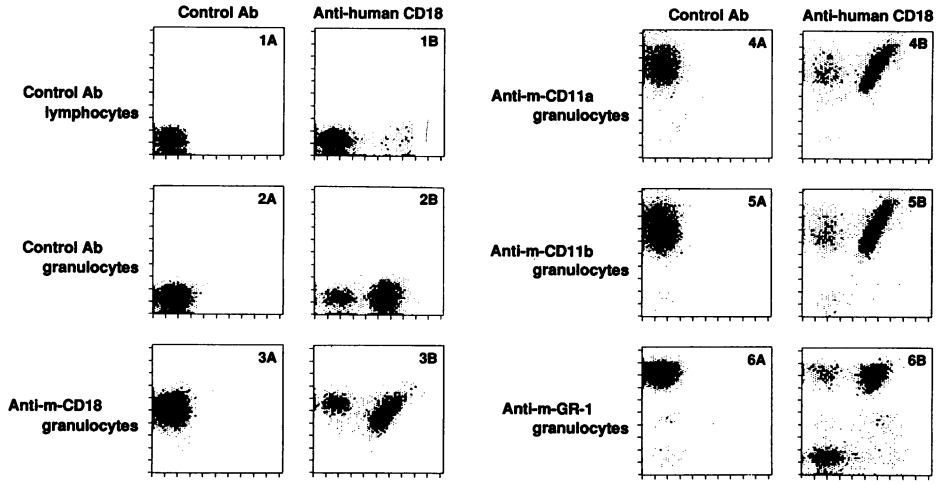

Figure 2. Two color flow cytometric analysis of peripheral blood. Whole blood from animal 15-6 was collected and analyzed by two color indirect immunofluorescence, as described in Methods. Red fluorescence is measured on the *y* axis and green fluorescence is measured on the *x* axis. Indirect immunofluorescent staining with the human CD18 and murine antigen reagents was compared to indirect immunofluorescence staining by isotype-identical negative controlled MAb. Cells (lymphocyte or granulocyte enriched fractions) were stained with MAb to murine antigens (1, control MAb, lymphocytes; 2, control MAb, granulocytes; 3, anti-m-CD18, granulocytes; 4, anti-m-CD11a, granulocytes; 5, anti-m-CD11b, granulocytes; and 6, anti-m-GR-1, granulocytes) and either control MAb (*A*) or anti-human CD18 (*B*).

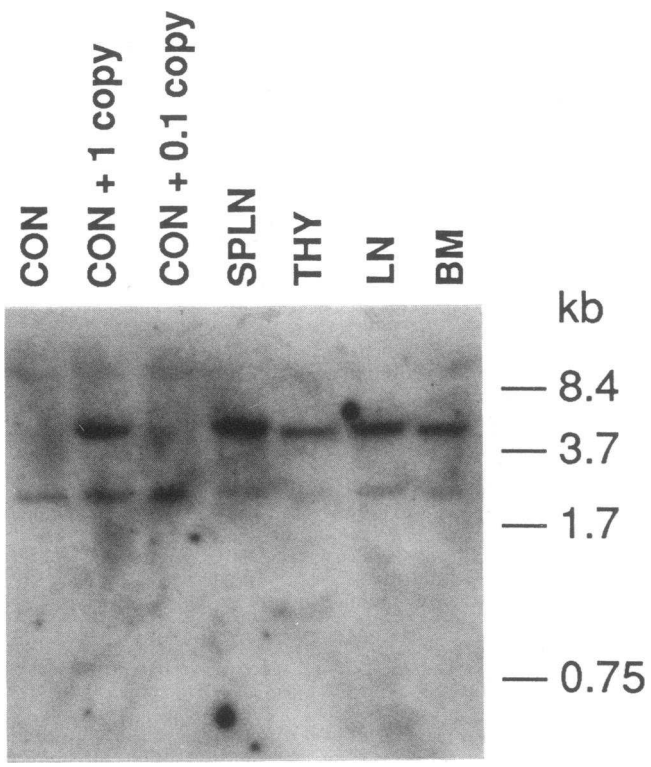

Figure 3. Analysis of hematopoietic tissues for proviral DNA. Tissues (spleen, thymus, lymph node, and bone marrow) were harvested from animal 15-6 and single cell suspensions were prepared and divided into aliquots for flow cytometric analysis (Table I) and DNA blot analysis. DNA was prepared from each tissue, restricted with Kpn1, fractionated in an agarose gel, and subjected to DNA blot analysis using human CD18 cDNA as a probe (9). DNA (10 μg) was obtained from a control mouse (transplanted with untransduced bone marrow [*CON*]), a control mouse supplemented with 7.5 pg of plasmid DNA (*CON + 1 copy*), or 0.75 pg of plasmid DNA (*CON + 0.1 copy*), and from the spleen (*SPLN*), thymus (*THY*), lymph nodes (*LN*), and bone marrow (*BM*), of mouse 15-6. Molecular weight markers in kilobases are shown along the right border.

an attempt to elucidate the basis for this apparent lineage restriction, hematopoietic tissues were analyzed for proviral DNA (Fig. 3). Total cellular DNA was prepared from each tissue, restricted with Kpnl, fractionated in an agarose gel, and subjected to DNA blot analysis using human CD18 cDNA as a probe (9). DNA blot analysis of total cellular DNA from animal 15-6 demonstrated equal and abundant quantities of unrearranged proviral DNA in spleen, thymus, lymph node, and bone marrow (approximately one proviral copy/cell). These results argue against selective myeloid reconstitution and suggest that there may be a lymphoid specific block to expression of proviral derived human CD18 at the transcriptional or posttranscriptional level.

Leukocytes demonstrate important posttranscriptional regulation of CD11/CD18 proteins during the acute inflammatory response in vitro or in vivo (1–3, 21–22). An example of this regulation is the recruitment of CD11b/CD18 to the cell surface of granulocytes and monocytes in response to a variety of stimuli such as lymphokines, phorbol esters, and complement (23–24). To develop an effective gene replacement therapy of LAD, it may be necessary to reconstitute both the absolute level and the regulation of CD11/CD18. We studied the effect of protein kinase C activation by PMA on the cell surface expression of human CD18, murine CD18, and murine CD11b in granulocytes from two reconstituted mice that expressed human CD18 on 70% (15–6) and 30% (15–15) of their granulocytes. Cell surface expression of endogenous CD18 (Fig. 4 *C*) and CD11b (Fig. 4 *B*) increased threefold and four- to sixfold, respectively, following incubation with PMA, while human CD18 increased four- to fivefold (Fig. 4 *A*). Human granulocytes demonstrated similar increases in CD18 (Fig. 4 *A*) and CD11b (Fig. 4 *B*) in response to PMA. Murine CD18 and CD11b increased a similar amount in the granulocytes of a control animal (data not shown). The level of human CD18 on murine granulocytes relative to that on human granulocytes was 6–53% at baseline and 4–38% after incubation with PMA in the two animals studied.

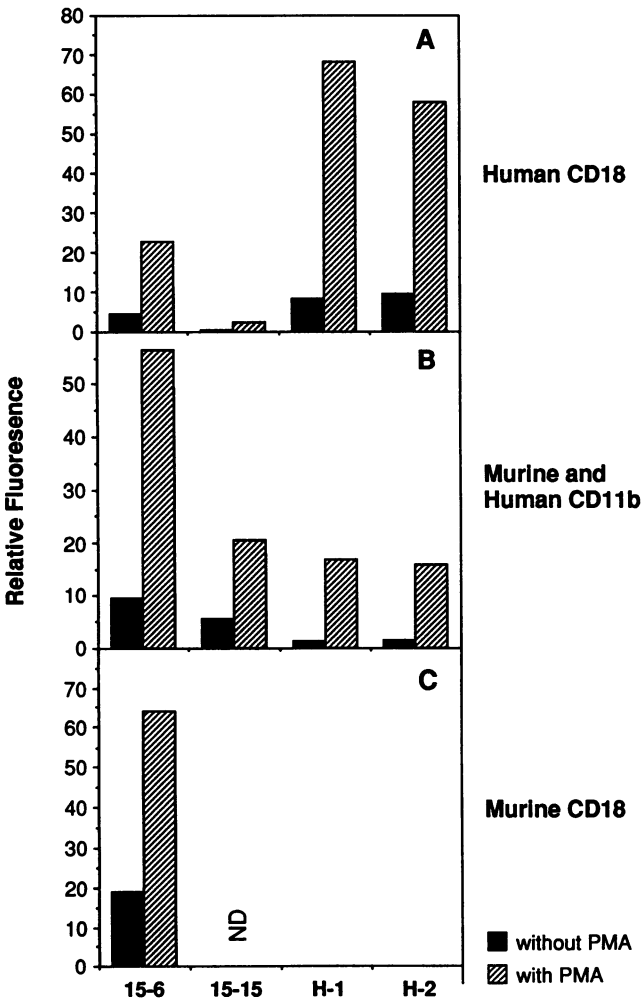

Figure 4. PMA-mediated regulation of human CD18 in murine and human lymphocytes. Whole blood from two mouse recipients (15-6 and 15-15) and two normal human volunteers (H1 and H2) was incubated with (*hatched boxes*) and without (*solid boxes*) PMA and analyzed for cell surface expression of human CD18 (*A*), murine and human CD11b (*B*), and murine CD18 (*C*). The relative linear fluorescence is indicated as histograms.

Selected animals were necropsied after 6 mo to look for pathological consequences of human CD18 expression. Routine analyses of peripheral blood were consistently within the normal range. In addition, there was no apparent gross or histological pathology in tissues of the heart, lung, liver, spleen, kidney, lymph node, or brain (data not shown).

This in vivo model of human CD18 expression addresses several important questions that relate to gene therapy of LAD. We were able to reproducibly introduce a functional human CD18 gene into murine hematopoietic stem cells and detect expression of human CD18 in a substantial proportion of transplant recipients for at least 6 mo. Cell surface expression of human CD18 was found in various hematopoietic lineages with the most abundant expression noted in granulocytes. This is fortunate because granulocyte dysfunction appears to be the most important cellular abnormality in LAD (1–3). Human CD18 was expressed in murine granulocytes at levels that should be therapeutic in LAD derived granulocytes.[2] Furthermore, the proviral derived CD18 subunit undergoes appropriate posttranscriptional regulation in response to a relevant physiological stimulus, activation of protein kinase C. Finally, transduction of a human CD18 cDNA containing heterologous transcriptional elements into stem cells had no apparent effect on hematopoietic reconstitution and was not associated with obvious disease.

These studies suggest a strategy for treating LAD and support its feasibility. Human models must await the demonstration of CD18 transduction into human hematopoietic stem cells.

Acknowledgments

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