Glanzmann thrombasthenia is an inherited bleeding disorder characterized by qualitative or quantitative defects of the platelet-specific integrin, αIIbβ3. As a result, αIIbβ3 cannot be activated and cannot bind to fibrinogen, leading to a loss of platelet aggregation. Thrombasthenia is clinically characterized by mucocutaneous hemorrhage with episodes of intracranial and gastrointestinal bleeding. To develop methods for gene therapy of Glanzmann thrombasthenia, a murine leukemia virus (MuLV)-derived vector, −889P1αβ3, was transduced into peripheral blood CD34+ cells from 2 patients with thrombasthenia with defects in the β3 gene. The human αIIb promoter was used in this vector to drive megakaryocyte-targeted expression of the wild-type β3 subunit. Proviral DNA and αIIbβ3 biosynthesis were detected after in vitro differentiation of transduced thrombathenic CD34+ cells with megakaryocyte growth and development factor. Flow cytometric analysis of transduced patient samples indicated that 19% of megakaryocyte progeny expressed αIIbβ3 on the surface at 34% of normal receptor levels. Treatment of transduced megakaryocytes with a combination of agonists including epinephrine and the thrombin receptor-activating peptide induced the αIIbβ3 complex to form an activated conformation capable of binding fibrinogen as measured by PAC-1 antibody binding. Transduced cells retracted a fibrin clot in vitro similar to megakaryocytes derived from a normal nonthrombathenic individual. These results demonstrate ex vivo phenotypic correction of Glanzmann thrombasthenia and support the potential use of hematopoietic CD34+ cells as targets for αIIb promoter-driven MuLV vectors for gene therapy of platelet disorders. (Blood. 2000;95:3645-3652) © 2000 by The American Society of Hematology

Introduction

Glanzmann thrombasthenia is a rare, autosomal recessive bleeding disorder characterized by an absence or dysfunction of the platelet receptor for fibrinogen, integrin αIIbβ3 (glycoproteins [GP] IIb-IIIa). To date, about 50 distinct mutations associated with Glanzmann thrombasthenia have been localized with relatively equal occurrence on either the αIIb or β3 gene. As in many genetic disorders, the molecular abnormalities range from major deletions and inversions to single point mutations. Although the percentage of abnormal αIIbβ3 expressed on the platelet surface may vary with the type of defect, all thrombathetic platelets are functionally indistinguishable as characterized by the failure of defective αIIbβ3 to bind fibrinogen resulting in the absence of platelet aggregation after stimulation by physiologic agonists.

Clinically, Glanzmann thrombasthenia is characterized by irregular bleeding from mucous membranes with easy bruising, epistaxis, gingival bleeding, and menorrhagia, which usually appears at an early age and recurs throughout the individual’s life. These individuals occasionally experience severe intracranial or gastrointestinal bleeding that may result in death. Platelet transfusions are used to treat severe cases of bleeding associated with Glanzmann thrombasthenia, although many patients become refractory to transfusions.

Glanzmann thrombasthenia occurs at a low rate internationally, but certain geographically restricted groups have a high carrier rate for thrombasthenia including Iraqi Jews, distinct Arab populations of the Middle East, French gypsies, and individuals from southern India. Approximately 2.3% of the 270,000 Iraqi Jews living in Israel are carriers for thrombasthenia. Individuals of Arabic decent also have a prevalence for this disorder, with more than 13 patients identified from 5 kindreds. The discovery of the molecular genetic defects in relatively large, “high-risk” populations has influenced the development of rapid DNA-based diagnostic assays, which provides opportunities for genetic counseling and family planning for suspected carriers of the abnormal αIIb or β3 gene, and could also help to identify prospective candidates for gene therapy.

The CD34+ hematopoietic cells are potential targets for gene therapy strategies because these cells can be safely collected from the body, genetically modified, and reinfused into the patient. This cell population has the capacity to generate an engrafting cell mass capable of establishing hematopoiesis with progeny cells of multilineages expressing the transferred gene for the life span of the graft recipient. The use of CD34+ cells in vitro to develop a strategy for gene therapy of platelet disorders was facilitated by the discovery of techniques that induce pluripotent CD34+ progenitor
cells to proliferate and differentiate into megakaryocytes in vitro and in vivo using c-Mpl-ligand or megakaryocyte growth and development factor (MGDF), 8 AIIb/IIIc ligand, interleukin (IL)-3, IL-6, IL-11, and stem cell factor (SCF). Among other effects, MGDF plays a direct role in increasing the transcriptional activity of the integrin αIIb gene in megakaryocytes; therefore, an αIIb promoter-directed expression system may be activated by MGDF within transduced CD34+ cells. Thus, CD34+ cells can be transduced with genes driven by the αIIb promoter and induced to form megakaryocytes that can be examined for targeted prooviral gene expression.

This study examined the use of peripheral blood CD34+ cells from patients with thrombasthenia as a model system for gene therapy of disorders affecting platelets. This investigation used the murine leukemia virus (MuLV)-derived vector, −889Plα[2]β3, in which the transcription of β3 is controlled by an 889-nucleotide fragment of the human αIIb promoter. We previously used this vector to direct megakaryocyte-specific expression of the platelet alloantigen 2 (PI(2)) form of β3 in human cell lines and expression of the proovial αIIbβ3 complex in megakaryocyte progeny of transduced CD34+ cells derived from a normal (nonthrombasthenic) individual. 10 Alloantigens were used in that study to allow us to distinguish the biosynthesis of proovial (PI(2)) β3 from endogenous (PI(3)) β3 in the normal human cells. The present study extends the use of the −889Plα[2]β3 vector to examine expression of the integrin αIIbβ3 complex on the surface of megakaryocytes following transduction and MGDF-induced differentiation of thrombasthenic CD34+ cells. Functional studies demonstrated that transduced thrombasthenic cells were capable of agonist-induced αIIbβ3 activation and retraction of a fibrin clot indicating ex vivo phenotypic correction of Glanzmann thrombasthenia. These results indicate that ex vivo gene transfer of a megakaryocyte-targeted gene expression system into peripheral blood CD34+ cells, followed by reinfusion of transduced cells, may be appropriate for gene therapy for disorders of platelets.

**Patients, materials, and methods**

**Patients**

Two patients with type I Glanzmann thrombasthenia, RSΔ9β3,11 and EAY115Cβ3, who is a member of a previously reported kindred,12 volunteered for the study. Both have lifelong bleeding episodes characterized by prominent mucous membrane bleeding and bleeding secondary to surgery or trauma. In each case, the diagnosis of thrombasthenia was established by a prolonged bleeding time, the failure of platelets to aggregate with physiologic agonists (adenosine diphosphate [ADP], epinephrine, collagen, and thrombin), and the failure of platelets to retract a fibrin clot. Platelets from each subject contained less than 5% detectable aggregate with physiologic agonists (adenosine diphosphate [ADP], epinephrine) in vivo using c-Mpl-ligand or megakaryocyte growth and development factor (MGDF), 8 AIIb/IIIc ligand, interleukin (IL)-3, IL-6, IL-11, and stem cell factor (SCF). Among other effects, MGDF plays a direct role in increasing the transcriptional activity of the integrin αIIb gene in megakaryocytes; therefore, an αIIb promoter-directed expression system may be activated by MGDF within transduced CD34+ cells. Thus, CD34+ cells can be transduced with genes driven by the αIIb promoter and induced to form megakaryocytes that can be examined for targeted prooviral gene expression.

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**Antibodies and reagents**

Polyclonal antibodies specific for αIIb and β3 and a monoclonal antibody that recognizes an epitope on β3, AP2,13 were gifts from Dr. Peter J. Newman (Blood Research Institute, Milwaukee, WI). The fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody, PAC-1, which recognizes an epitope on the activated αIIbβ3 complex was purchased from Becton Dickinson (San Jose, CA). The monoclonal antibody, AP2,14 which recognizes an epitope on the αIIbβ3 complex, was provided by Dr. Robert R. Montgomery (Blood Research Institute, Milwaukee, WI). The monoclonal antibody, 6D1, 15 which recognizes an epitope on glycoprotein (GP)Ⅲb was provided by Dr Barry Coller (Mt Sinai School of Medicine, New York, NY). Phycoerythrin (PE)-conjugated anti-GPⅠbα antibody (mouse anti-human CD42b) and isotype standards (PE-IgG, FITC-IgM) were purchased from PharMingen (San Diego, CA). ADP was purchased from Fisher (Pittsburgh, PA), and epinephrine was from Bio/Data Corp (Horsham, PA). Thrombin receptor-activating peptide (TRAP) and an Arg-Gly-Asp-containing peptide (GRGDW) were synthesized at the Blood Research Institute Core Facility (Milwaukee, WI).

**Retroviral construct p-889Plα[2]β3**

As previously described, 10 a fragment of the αIIb promoter beginning at nucleotide −889 was used to drive transcription of complementary DNA (cDNA) encoding the platelet alloantigen 2 (PI(2)) form of β3 (provided by Dr. Peter J. Newman, Blood Research Institute, Milwaukee, WI). 16 The −889Plα[2]β3 DNA cassette was positioned within the MuLV-derived retroviral vector, pHT-SIN, which encodes a 3' terminal repeat (LTR) sequence (provided by Dr Estuardo Aguilar-Cordova, Baylor College of Medicine, Houston, TX) lacking the viral enhancer/promoter so that the αIIb promoter could be used to promote megakaryocyte-targeted gene transcription.

**Retrovirus production**

Human 293 cells were transiently transfected on 10-cm plates with 15 μg each of pC1-GFP, pC1-VSV-G helper plasmids, and p-889Plα[2]β3 using the Calcium Transfection System (Life Technologies, Gaithersburg, MD). 10 Media containing retrovirus was concentrated 500-fold and resuspended in IMDM. Phycoerythrin (PE)-conjugated anti-GPIbα, PAC-1, which recognizes an epitope on the activated αIIbβ3 complex was purchased from Becton Dickinson (San Jose, CA). ADP was purchased from Fisher (Pittsburgh, PA), and epinephrine was from Bio/Data Corp (Horsham, PA). Thrombin receptor-activating peptide (TRAP) and an Arg-Gly-Asp-containing peptide (GRGDW) were synthesized at the Blood Research Institute Core Facility (Milwaukee, WI).

**Selection of CD34+ cells from peripheral blood**

Peripheral blood collection was performed after obtaining written informed consent from adult Glanzmann thrombasthenic volunteers enrolled in a protocol approved by the University of North Carolina and Johns Hopkins University Committees on the Protection of the Rights of Human Subjects. Subjects were given granulocyte colony-stimulating factor (Amenog, Thousand Oaks, CA) at 10 μg/kg/d subcutaneously for 4 days and peripheral blood cell collection was performed on day 5 using a COBE Spectra Blood Cell Separator. CD34+ cells were immunoselected from the apheresis product on an Isolox 300i Magnetic Cell Separator (Nexell Therapeutics, Irvine, CA), distribution through Baxter Healthcare) as previously described. 19 Cell yields from both patients were approximately 600 million total nucleated cells, with a final recovery of 150 million CD34+ cells (85% CD34+ purity). Selected cells were suspended in X-VIVO 10 (Biowhittaker, Walkersville, MD) containing 1% (v/v) human serum albumin, frozen in 10% (v/v) DMSO at 5 × 10^6 cells/mL, and stored in liquid nitrogen.

**Transduction of CD34+ cells**

Human CD34+ cells were transduced as previously described. 19 Briefly, cells were prestimulated in IMDM containing 20% fetal bovine serum (FBS), 10 U/mL recombinant human (rh) IL-3, 100 U/mL rhIL-6, 30 U/mL recombinant murine SCF (Genetics Institute, Cambridge, MA) and 10 ng/mL flk2/IIIc ligand (R&D Systems, Minneapolis, MN) for 48 hours at 37°C in 5% CO2. Cells were transduced at 5 × 10^6 per well of a sterile, 24-well nontissue culture-treated plate ( Falcon-Becton Dickinson, Franklin Lakes, NJ) coated with 20 µg/cm² RetroNectin (Takara Shuzo, Otsu, Shiga, Japan) with an estimated multiplicity of infection of 10 retrovirus (−889Plα[2]β3) per cell in IMDM plus 20% FCS and rhIL-3, rhIL-6, SCF, and flk2/IIIc ligand. Fresh viral supernatant was added after 2 hours. This procedure was repeated 1 time 24 hours later. Twenty-four hours after the...
final transduction, megakaryocyte formation was induced similar to a previously described method.\textsuperscript{7} Cells were resuspended at $5 \times 10^5$ in IMDM containing 10% platelet poor plasma and rhIL-3, rhIL-6, SCF, flk2/fl3 ligand plus 100 ng/mL rhIL-11 (Genetics Institute) and 100 ng/mL rhMGDF (Amgen) for up to 17 days. Cells were collected, washed twice in phosphate-buffered saline (PBS), and solubilized in 1 mL of lysis buffer and stored at $-80^\circ$C.

Detection of proviral DNA in transduced cells by polymerase chain reaction

Cells (1.2 $\times 10^8$) were split into 3 aliquots and DNA was amplified in 3 separate polymerase chain reaction (PCR) reactions: (1) amplification of cIIB genomic DNA from nucleotides 13 703 to 14 064 (361 base product)\textsuperscript{22} was performed as a positive control to detect genomic DNA from untransduced and transduced samples; (2) PCR of plasmid vector backbone DNA (P-$889P\text{IIb}$) was performed using sense primer 5'-TGACTGTTG-GAGTACTCTAA-3' from nucleotide 1681 to 1880 and antisense primer 5'-TTTACACCCGATACAGGTGCC-3', which consisted of nucleotides 2323 to 2303 (462 base product) outside of the region packaged into retroviral capsids as a negative control that demonstrated retroviral plasmid DNA was not transected into the cells during transduction; and (3) plasmid vector (P-$889P\text{IIb}$) sequence packaged into retroviral capsids was amplified by PCR using sense primer 'OL-psi-1' 5'-T-TGAACTCCTCCT-GTTGAC-3' beginning 111 nucleotides upstream of $-889P\text{IIb}$ DNA cassette and antisense primer 5'-ACTCTCTCCGTCTTGAGCC-3', which consisted of nucleotides $-572$ to $-592$ of the cIIb promoter (428 base product) to detect proviral DNA in transduced samples. The PCR products were separated by electrophoresis on a 2.0% agarose gel and visualized by ethidium bromide staining.

Immunoprecipitation analysis

Immunoprecipitation analysis was performed as previously described.\textsuperscript{10} Precleared lysates were immunoprecipitated for 1 hour at 25°C with AP2 coupled to Affi-gel HZ (Bio-Rad, Hercules, CA). Immunoprecipitates were electrophoresed on a 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel under nonreducing conditions and proteins were transferred to Immobilon-P (Millipore, Bedford, MA) at 200 mA for 3 hours and blocked for 12 hours at 4°C in 10% FBS in TBS-Tween. Protein was detected using rabbit polyclonal antibodies specific for cIIb (5 µg/mL) and β3 (4 µg/mL) and a peroxidase-conjugated F(ab')\textsubscript{2} fragment donkey antirabbit IgG (H + L) (Jackson ImmunoResearch, West Grove, PA) at 1:20 000 dilution followed by chemiluminescence detection and exposure to autoradiography film from 1 second to 10 minutes.

Indirect immunofluorescence

Transduced and untransduced cells ($5 \times 10^5$) were blocked for 15 minutes in 2% bovine serum albumin (BSA) and 0.1 mmol/L Ca/Mg in PBS and incubated with 5 µg AP2 for 20 minutes at 25°C, then treated with PE-conjugated F(ab')\textsubscript{2} donkey antimouse secondary antibody (Jackson ImmunoResearch) for 20 minutes on ice. Cells were resuspended in 200 µL of 2% formaldehyde, 0.2% glutaraldehyde in PBS and fixed to single wells of a 24-well tissue culture-treated plate for 15 minutes at 25°C while centrifuging at 230g. Positively staining cells were detected and photographed using a Zeiss Axiovert 10 fluorescence microscope at $\times 320$ magnification.

Flow cytometric analysis

Transduced and untransduced cells ($1.5 \times 10^8$) were blocked for 15 minutes in 2% BSA and 0.1 mmol/L Ca/Mg in PBS; incubated with 2.5 µg of AP2, AP3, 6D1, mouse IgG for 20 minutes at 25°C; and treated with PE-conjugated F(ab')\textsubscript{2} donkey antimouse secondary antibody (Jackson ImmunoResearch) for 20 minutes on ice. Cells were resuspended in 200 µL of 1% paraformaldehyde in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson) using CellQuest software. A minimum of $2 \times 10^6$ cells exhibiting light scattering properties of megakaryocytes were used for the analysis. Identification of megakaryocytes in this cell population was further confirmed with antibodies directed against glycoprotein GP Ib. Megakaryocytes expressing cIIb, were determined using samples stained with AP2 and the secondary antibody described above. Cells expressing β3 were determined using samples stained with AP3 and secondary antibody. Negative populations of cells were determined using unreactive isotype-specific antibodies as a control for background staining. Fluorescence contours are shown at 50% log density plots. The efficiency of $-889P\text{IIb}3$ to transduce CD34$^+$ cells was calculated by comparing the percent of the cell population that expressed the cIIb$3$ complex following transduction with untransduced and normal nonthrombasthenic megakaryocytes under identical culture conditions. The mean fluorescence intensity of AP2 and AP3 staining was measured at saturating antibody levels\textsuperscript{11,12} to determine receptor expression level and estimate receptor density on the cell samples.

Agonist-induced cIIb, activation

Culture cells were harvested for physiologic studies of cIIb, function 8 to 13 days after transduction. Cells (1.5 $\times 10^6$/mL) were incubated with PE-GPbox and FITC-PAC1 (5 µg/mL) antibodies in modified Tyrode buffer containing 1 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 50 µmol/L each of TRAP, ADP, and epinephrine for 15 minutes at 25°C. FITC-PAC1 binding was monitored in the FL1 channel of the flow cytometer on the gated subset of cells that expressed GPsIb (FL2). The specificity of FITC-PAC1 binding was determined by co-incubation of samples with a blocking peptide containing Arg-Gly-Asp (GRGDW, 2.5 µmol/L). Samples were diluted 10-fold with buffer and examined using flow cytometry and 2-color analysis performed as described above.

Clot retraction

Clot retraction assays were performed using a slightly modified version of a previously described method.\textsuperscript{22} Ten to 14 days after transduction, cultured cells (1.5 $\times 10^5$/mL) were resuspended in serum-free IMDM containing 60 µg/mL human fibrinogen in a standard aggregometry tube. Clot formation was initiated by the addition of 2.5 U/mL thrombin. Tubes were incubated at 37°C for up to 12 hours and photographed.

Results

Formation of the cIIB3 complex following transduction of thrombasthenic CD34$^+$ cells

Patients R.S.\textsuperscript{11} and E.A.\textsuperscript{12} have type I Glanzmann thrombasthenia due to defects in β3 associated with undetectable surface expression of the cIIb, complex, failure of platelets to bind fibrinogen, absence of platelet-platelet aggregates, and inability to retract a fibrin clot. Patient R.S. has a previously reported single nucleotide substitution that affects the splice-donor site of exon 9 of β3 resulting in the deletion of 45 amino acids (RSΔ9β3), and patient E.A. has a novel nucleotide substitution of β3 resulting in a single amino acid substitution of tyrosine to cysteine at residue 115 (EAY115Cβ3). To assess the feasibility of human gene therapy of Glanzmann thrombasthenia, granulocyte colony-stimulating factor mobilized, peripheral blood CD34$^+$ cells were collected from each patient and transduced with MuLV-derived vector $-889P\text{IIb}3$. The transduced CD34$^+$ cells were subjected to in vitro expansion and differentiation with IL-3, IL-6, IL-11, flk2/fl3 ligand, SCF, and MGDF, and examined for the presence of proviral DNA by PCR analysis. After 10 days of cytokine treatment, successful transduction of CD34$^+$ cells from RSΔ9β3 and EAY115Cβ3 was indicated by the detection of proviral DNA by PCR in $-889P\text{IIb}3$-transduced cells but not in untransduced cells (not shown, see “Materials and methods”).

Immunoblot analysis was performed to determine if the cIIb, complex was synthesized in megakaryocyte progeny of
−889PΔ3β3-transduced CD34+ cells from RSΔ9β3 and EAY115Cβ3. On days 10 and 15 after differentiation, respectively, the αIIbβ3 receptor was immunoprecipitated from cellular lysates with an αIIbβ3 complex-specific monoclonal antibody (AP2) and detected using a mixture of well-characterized polyclonal antibodies specific for the αIIb and β3 integrin subunits. Both αIIb (Mr = 145 000) and β3 (Mr = 95 000) were detected in the β3-transduced cells (Figure 1) indicating that there was proviral-derived, αIIb promoter-directed synthesis of PIΔ2β3 resulting in formation of the αIIbβ3 complex. The αIIbβ3 complex was not detected in untransduced thrombocytopenic CD34+ cells (Figure 1) nor in cells transduced with a similar MuLV-derived vector (−889nLacz), which encoded the β-galactosidase gene in place of PIΔ2β3 (not shown).

Surface expression of αIIbβ3 on transduced thrombocytopenic megakaryocytes

Indirect immunofluorescence was performed to examine expression of αIIbβ3 on the surface of megakaryocytes following −889PΔ3β3 transduction of CD34+ cells from RSΔ9β3 and EAY115Cβ3. Five to 10 days after ex vivo expansion and differentiation of CD34+ cells, megakaryocytes were identified in cell cultures by detection of the megakaryocyte-specific glycoprotein (GP)Ib using the monoclonal antibody 6D1. GP Ib was detected on the surface of megakaryocytes in untransduced thrombocytopenic, transduced thrombocytopenic, and normal (nonthrombocytopenic) samples (Figure 2, top row), and cells transduced with −889nLacz (not shown). Only megakaryocytes derived from −889PΔ3β3-transduced CD34+ cells expressed αIIbβ3 receptors on the cell surface. This expression was qualitatively similar to megakaryocyte progeny of CD34+ cells from a normal nonthrombocytopenic individual as demonstrated by detectable AP2 staining (Figure 2, bottom row).

To quantitate the efficiency of transduction and estimate αIIbβ3 receptor density on megakaryocytes, flow cytometric analysis was performed following transduction of patient CD34+ cells with −889PΔ3β3. After day 9 of ex vivo cellular expansion and differentiation, typically 38% of the large cells from each culture sample differentiated to megakaryocytes expressing GP Ib (not shown). In Figure 3, megakaryocytes that expressed αIIbβ3 on the surface were identified on contour plots as the cells that emitted a

**Figure 1.** Immunoprecipitation analysis of −889PΔ3β3 transduced CD34+ cells from patient RSΔ9β3 and EAY115Cβ3. Cells (5 × 10⁶) were collected 10 and 15 days, respectively, after transduction and detergent lysates were immunoprecipitated with 10 µg of an αIIbβ3 complex-specific antibody (AP2). The complexed proteins were separated on a 7% SDS-PAGE gel under nonreducing conditions. Immunoprecipitation using polyclonal antibodies to αIIb and β3 followed by chemiluminescence detection showed that transduction with the −889PΔ3β3 vector resulted in the synthesis of detectable levels of β3 and αIIb (arrows on right), whereas untransduced samples did not have detectable protein. Molecular mass markers are in kilodaltons (left). Additional bands appearing equally in each lane are nonspecific background resulting from chemiluminescence detection using a murine monoclonal antibody for immunoprecipitation and rabbit polyclonal antibodies and a horseradish peroxidase-conjugated donkey antirabbit antibody for analysis.

**Figure 2.** Indirect immunofluorescence analysis of thrombocytopenic CD34+ cells transduced with −889PΔ3β3. The RSΔ9β3 and EAY115Cβ3 CD34+ cells were transduced with −889PΔ3β3, induced to form megakaryocytes ex vivo, and then examined by indirect immunofluorescence analysis for αIIbβ3 surface expression. Cells were blocked in 2% BSA, and incubated with 5 µg monoclonal antibody 6D1 that recognizes megakaryocyte-specific glycoprotein (GP)Ib (top panels) or 5 µg AP2, which recognizes αIIbβ3 (bottom panels), and detected with a PE-conjugated F(ab′)2 donkey antimmunosecondary antibody. Five to 10 days after transduction, megakaryocytes were present in untransduced and β3-transduced cell cultures as demonstrated with the anti-GP Ib antibody; however, only β3-transduced cells demonstrated detectable αIIbβ3 complex on the surface of derived megakaryocytes similar to cultured megakaryocytes from a normal individual (control). There are at least 3 cells in each field of untransduced thrombocytopenic cells stained with AP2 for αIIbβ3.

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**Figure 3.** Flow cytometric analysis following −889PΔ3β3 transduction of thrombocytopenic CD34+ cells. Untransduced and transduced cells were induced to form megakaryocytes for 9 days ex vivo, and then examined by flow cytometric analysis for surface expression of the β3 subunit. Shown in the first set of panels are megakaryocytes expressing αIIbβ3 as detected with complex-specific antibody AP2 and a PE-conjugated F(ab′)2 donkey antimmunosecondary antibody. The second set of panels is cells expressing the β3 subunit as detected with monoclonal antibody, AP3, and secondary antibody. Fluorescence contour plots are presented for normal nonthrombocytopenic cells (upper right), untransduced and transduced cells from EAY115Cβ3 (middle panels), and untransduced and transduced cells from RSΔ9β3 (lower panels). The x-axis depicts cell size as measured with forward scatter on a linear scale, and the y-axis is relative fluorescence intensity of PE-AP2 or PE-AP3. Megakaryocytes that expressed αIIbβ3 on the cell surface were detected in the upper right quadrant as were cells that expressed β3. Normal and patient cells incubated with an isotype nonspecific antibody and secondary antibody were presented as controls for background staining (upper left panels).
high fluorescence intensity with AP2 at saturating antibody levels. An unreactive isotype-specific antibody was used as a control for background staining of normal and patient cells. Normal nonthrombocytopenic CD34+ cells were induced to form megakaryocytes with detectable αIIBβ3 in 41% of the cell population (Figure 3), whereas untransduced CD34+ cells from EAY115Cβ3 and RSΔ9β3 showed no detectable αIIBβ3 expression above background levels (Figure 3). Megakaryocytes expressing αIIBβ3 were detected in 10% and 7% of the transduced cell population from EAY115Cβ3 and RSΔ9β3, respectively (Figure 3). This indicates that approximately 19% of patient megakaryocytes were transduced with −889Plα2β3, when the percent of megakaryocytes expressing αIIBβ3 in the transduced cell populations were compared with untransduced and normal nonthrombocytopenic cultures (Figure 3). Transduced patient megakaryocytes had a mean fluorescence intensity of AP2 staining that was 34% of αIIBβ3 receptor expression level on normal (nonthrombocytopenic)-derived megakaryocytes indicating a reduced receptor density on transduced patient cells (Figure 3). Flow cytometric analysis using a monoclonal antibody (AP3) specific for the β3 subunit demonstrated β3 expression on approximately 5% of transduced patient cells at 28% of normal receptor density (Figure 3). Because the percent of the hematopoietic population expressing β3 (which normally pairs with αV on monocytes and lymphocytes as well as megakaryocytes) is nearly identical with data obtained for αIIBβ3 expression on transduced patient cells, these results suggest that the αIIB promoter directed megakaryocyte-specific expression of the β3 subunit.

αIIBβ3 signaling in β3-transduced thrombocytopenic megakaryocytes

Inside-out signaling in β3-transduced cells was measured by performing agonist-dependent binding assays with the fibrinogen mimetic antibody, PAC1, to determine if αIIBβ3 could be induced to form an activated conformation capable of binding fibrinogen. Activation of αIIBβ3 was measured 9 days after ex vivo cellular expansion and differentiation using megakaryocytes selected with a PE-GPIbα antibody following stimulation of cells with TRAP, ADP, and epinephrine. Results of these studies are shown in Figure 4 and demonstrate that in the presence of agonist, β3-transduced megakaryocytes bound FITC-PAC1 at a peak value that was on the average 10-fold above levels from untransduced patient cells. Similar to Figure 3, β3-transduced megakaryocytes bound antibody at a reduced fluorescence intensity that was approximately 9% of the peak value level on normal control megakaryocytes (Figure 4). FITC-PAC1 was inhibited from binding β3-transduced and normal control megakaryocytes in the presence of an Arg-Gly-Asp–containing peptide that is known to block the specific-binding of αIIBβ3 to PAC1 and fibrinogen (Figure 4). In contrast, untransduced thrombocytopenic megakaryocytes did not bind FITC-PAC1 in the presence of the inhibitor peptide (Figure 4). These results indicate that αIIBβ3 expressed on β3-transduced megakaryocytes functions similar to the integrin complex on normal nonthrombocytopenic megakaryocytes because excitatory agonists stimulated PAC1 binding and an inhibitory peptide blocked antibody recognition.

Transduced thrombocytopenic cells retract a fibrin clot

To further identify the function of the expressed αIIBβ3, CD34+ cells from RSΔ9β3 and EAY115Cβ3 were transduced with −889Plα2β3, treated with cytokines for 10 days to form megakaryocytes, and examined for the ability to retract a fibrin clot (Figure 5). The −889Plα2β3 transduced cells mediated clot retraction (middle panels) that was similar to cultured CD34+ cells from a nonthrombocytopenic individual (normal control, right panels), whereas untransduced thrombocytopenic cells were unable to retract a fibrin clot (left panels). The data suggest that the expressed αIIBβ3 receptors are functional in mediating clot retraction, implying an ex vivo correction of the Glanzmann thrombasthenia phenotype.

Discussion

The results of this investigation demonstrate successful transduction of peripheral blood CD34+ cells from 2 patients with...
Glanzmann thrombasthenia using the MuLV-derived vector, −889PlA2β3. Integrin β3 subunit synthesis, αIIbβ3, complex formation, and surface expression were demonstrated on transduced megakaryocytes derived from thrombasthenic patients R.S. and E.A. Although FACS analysis indicated a subnormal αIIbβ3 receptor density on the surface of megakaryocytes, these −889PlA2β3 transduced cells demonstrated function by mediating retraction of a fibrin clot and binding the αIIbβ3 activation-dependent PAC1 antibody on cellular stimulation with agonists, which is consistent with the ex vivo correction of the Glanzmann thrombasthenic phenotype. Proplatelet formation was observed in transduced cell culture, but the quantity of platelets were too few to test aggregation. Our in vitro results show that despite suboptimal transduction efficiency of CD34+ cells and reduced αIIbβ3 receptor density on patient megakaryocytes, there is correction of Glanzmann thrombasthenia. This is consistent with the fact that genetic carriers for Glanzmann thrombasthenia (about 50% normal αIIbβ3 receptor density levels) are disease free with normal platelet aggregation and clot retraction. We have previously observed measurable platelet aggregation and retraction of a fibrin clot using a mixture of 10% normal platelets with 90% thrombocytopenic platelets in vitro (unpublished data). Thus, we might expect to see in vivo correction of thrombasthenia with expression of αIIbβ3 on less than 20% of transduced megakaryocytes as demonstrated in vitro; however, if transduced progenitor cells were delivered to patients in the absence of total marrow ablation, the actual percentage of corrected megakaryocytes could be greatly reduced in the total cell population. Nevertheless, our data suggest that compared to uncorrected platelets, a circulating population of platelets derived from β3-transduced megakaryocytes have an increased potential to aggregate at the site of vascular injury due to the expression of αIIbβ3 that can be induced by agonist to become activated and bind fibrinogen.

One key aspect of this work is the use of a fragment of the αIb promoter to target gene expression to human megakaryocytes. Regulatory elements of the αIb promoter necessary for high level, megakaryocyte-specific gene transcription have been localized to the first 800 nucleotides of the human αIb promoter using cell lines,24-27 transfected rat primary cells,28 and transgenic mice.29,30 Megakaryocyte progenitor cells express GATA and Ets factors that bind within this region to induce a high level of transcription of the αIbβ gene,31 while as yet undefined factors that play a role in restricting transcription to developing megakaryocytes have been localized between nucleotides −80 and −130 of the αIb gene.32,33 An MGDF-responsive element has also been recently identified that increases the transcriptional activity of the integrin αIbβ gene in megakaryocytes.9 Investigations with transgenic mouse models have demonstrated megakaryocyte-targeted transcription in vivo when the transcriptional activation of an 800-nucleotide fragment of the human αIbβ promoter directed expression of the thymidine kinase gene in multipotent hematopoietic cells leading to sustained expression in megakaryocyte progeny and down-regulated expression during erythroid and myeloid lineage differentiation.29,30 We have recently observed supporting results when the MuLV-derived expression vector, −889PlA2β3, selectively targeted expression of β3 to transduced promegakaryocyte cell lines, and a similarly controlled construct, −889nlacZ, preferentially directed β-galactosidase activity to megakaryocytes following transduction and MGDF-induced differentiation of human CD34+ cells.10 The current investigation demonstrates the use of an 889 nucleotide fragment of the human αIbβ promoter for targeted gene expression to correct diseased human megakaryocytes following transduction and MGDF induced differentiation of thrombasthenic hematopoietic CD34+ cells. We detected levels of integrin β3 expression nearly equal to αIbβ3, by flow cytometric analysis suggesting megakaryocyte-specific expression of the transgene in transduced patient cells. The cumulative data indicate that the MuLV-derived αIbβ promoter expression system used in this report has the capacity to direct expression of the β3 gene with selective, MGDF-inducible expression in megakaryocyte progeny suggesting this system may be ideal for gene therapy for platelet disorders such as Glanzmann thrombasthenia.

Two potential challenges arise with our proposal to use the MuLV construct, −889PlA2β3, which expresses the rare PlA2 alloantigen form of β3 for genetic correction of thrombosthenia. First, individuals have become refractory to infused donor platelets due to the production of antibodies against mismatched alloantigenic determinants of β3, resulting in clearance of the transfused platelets34; therefore, epitopes of β3 may have to be identically matched to result in acceptance of corrected platelets as an additional requirement for gene therapy of thrombosthenia. We speculate that further experimentation with different platelet alloantigens of β3 for gene therapy of Glanzmann thrombasthenia may help us overcome some principal problems of transfusion medicine concerning antigen tolerance and correction of platelet refractoriness. This could ultimately lead to a definitive strategy that will pose as a viable alternative to the current treatment of platelet transfusions for bleeding episodes.35 Second, individuals suffering from acute coronary thrombosis have been noted with an increased prevalence for the presence of the PlA2 allelic isoform of β3 on their platelets36,37; alternatively, the PlA1 form of β3 may be used for gene therapy of Glanzmann thrombasthenia. These results indicate that expression of different PlA1 or PlA2 forms of β3 in transduced thrombocytopenic cells may lead to changes in the ability of platelets to activate and aggregate.

The results of this investigation suggest that transduction of altered forms of integrin subunits into CD34+ cells of thrombosthenics may allow elucidation of events important for integrin expression and intracellular signaling in primary human hematopoietic...
cells. Cultured cell lines transfected with recombinant αIIbβ3 subunits have been traditionally utilized to identify key steps that take place during integrin biosynthesis, intracellular signaling, and receptor activation. Although these studies have advanced our understanding concerning fundamental concepts of integrin biology, data suggest that β3, β2, and β1 integrins may behave differently in transfected cells compared to natural host cells due to the influence of cell type-specific cytosolic proteins in governing signal transduction. Information gained from our study may inspire new strategies for investigating integrin-mediated events via megakaryocyte-targeted gene expression during megakaryocytopenesis of primary human hematopoietic cells.

The αIIb promoter controlled expression system, −889P1[β3], was developed to test thrombosthenics with β3 defects; however, this system may be used for gene therapy of other molecular genetic defects of platelets. The system could potentially be used for gene therapy of thrombosthenics with αIIb defects or individuals with defects characterized in (GP)IIb-V-IX complex with Bernard Soulier syndrome, platelet-type von Willebrand disease, and pseudo von Willebrand disease. Other diseases may also benefit from the expression of novel proteins in megakaryocytes, because this study suggests that a platelet could potentially deliver other therapeutic agents to the site of a vascular injury during a primary hemostasis response.

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References
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Erratum

In the article by Urashima et al entitled “An oral CD40 ligand gene therapy against lymphoma using attenuated Salmonella typhimurium,” which appeared in the February 15, 2000, issue of Blood (Volume 95:1258-1263), a correction should be noted. Under “Materials and methods, Salmonella strain and CD40L,” the name of the auxotrophic ST aroA+ strain donated by Dr Bruce A. D. Stocker and grown in 100 mL L broth should have been given as SL7207.