# Suppression of Erythro-Megakaryocytopoiesis and the Induction of Reversible Thrombocytopenia in Mice Transgenic for the Thymidine Kinase Gene Targeted by the Platelet Glycoprotein αIIb Promoter

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## Summary

The mechanisms that regulate the commitment of a totipotent stem cell to the megakaryocytic lineage are largely unknown. Using a molecular approach to the study of megakaryocytopoiesis and platelet production, mice in which thrombocytopoiesis could be controlled were produced by targeting the expression of the herpes simplex virus thymidine kinase toxigene to megakaryocytes using the regulatory region of the gene encoding the α subunit of the platelet integrin αIIbβ3. The programmed eradication of the megakaryocytic lineage was induced by treating transgenic mice bearing the hybrid construct (αIIbtk) with the antiherpetic drug ganciclovir (GCV). After 10 d of treatment, the platelet number was reduced by >94.6%. After discontinuing GCV, the bone marrow was repopulated with megakaryocytes and the platelet count was restored within 7 d. Prolonged GCV treatment induced erythropenia in the transgenic mice. Assays of myeloid progenitor cells in vitro demonstrated that the transgene was expressed in early erythro-megakaryocytic progenitor cells. The reversibility and facility of this system provides a powerful model to determine both the critical events in megakaryocytic and erythroid lineage development and for evaluating the precise role that platelets play in the pathogenesis of a number of vascular occlusive disorders.

The differentiation of myeloid cells requires a highly complex series of cellular events in which a small population of stem cells generates large populations of mature cells and culminates in the production of circulating blood cells. The molecular mechanisms regulating the proliferation and differentiation of the pluripotent hematopoietic stem cell involve interactions between stromal elements and a wide variety of inducing and inhibitory cytokines. Previously, in vitro clonal assays as well as in vivo studies of bone marrow (BM)[1] cells have suggested a unifying hierarchical model that implicates the commitment of a particular stem cell to specific lineages via a progressive loss of its pluripotentiality (reviewed in reference 1). This model is supported by the observation that several types of multilineage colonies of variable phenotypes can develop directly from a unique totipotent stem cell under the influence of a range of specific cytokines. The existence of such colonies has been documented in mice (2–4) and humans (5–7). The genetic mechanisms controlling the commitment and diversification of cellular lineages, however, are poorly understood. This is particularly the case in megakaryocyte development, which entails the commitment and proliferation of progenitor cells, maturation, and endomitosis, culminating in the production of platelets.

Most of the studies on megakaryocyte development have been performed on ex vivo models using semisolid or liquid culture systems (8, 9). These techniques, however, have their limitations. They do not result in platelet production, and gene transfer experiments are not feasible because of the low representation of megakaryocytes in BM. In addition, the absence of the microenvironment does not permit the analysis of the involvement of both known and unknown factors that

[1] *Abbreviations used in this paper:* AChE, acetyl cholinesterase; BM, bone marrow; BFU-E, erythroid burst-forming unit; CFU-GEMM, granulomonocytic-erythroid-megakaryocytic CFU; CFU-MK, megakaryocyte CFU; EM, erythroid megakaryocytic; GM-CFC, granulocytic colony-forming cells; EPO, erythropoietin; GCV, ganciclovir; MGG, May-Grünwald Giemsa; rHu, recombinant human; rMu, recombinant murine; RT-PCR, reverse transcriptase PCR; tk, thymidine kinase.

exert their effect in vivo. Nor does it take account of the contribution of accessory stromal cells. Thus, a molecular genetic approach addressing the mechanisms that regulate megakaryocytopoiesis requires in vivo studies with appropriate animal models.

With this in mind, our approach has been to target the expression of a toxic gene into megakaryocytes of mice to provide the means by which platelet production may be modified on demand. The successful application of a number of toxigenes as reverse genetic tools in transgenic mice has been reported. Bacterial protein toxins, such as diptheria, cholera, and more recently, tetanus, have been used to eradicate acinar pancreatic cells (10), induce pituitary hyperplasia and gigantism (11), and disrupt spermatogenesis (12). Although the number of transgenic animals described in these studies was apparently normal, the small number of transgenic founders reported in other studies suggests that early lethality may occur because of transient expression of the transgene in the embryos after microinjection. An alternative approach would be to use genes that are not themselves toxic, but sensitize the targeted cells to the effects of drugs. This is the case for the thymidine kinase (tk) gene of HSV-1. Unlike mammalian tk, HSV tk is capable of phosphorylating nucleoside analogues, like ganciclovir (GCV), leading to the inhibition of DNA synthesis in dividing cells (13, 14). This approach has the advantages of its ease of administration and reversibility. Hematopoiesis is a particularly good system for the application of this cell suicide technique because it allows the monitoring of a particular cell type during a complex program of cell differentiation.

The αIIb gene has been shown to be an early marker of megakaryocytopoiesis, and it encodes the α subunit of the platelet integrin αIIbβ3 (15). This molecule functions as a receptor for adhesive proteins and is involved in platelet adhesion (16). While the β3 subunit is synthesized in different tissues, the αIIb subunit is expressed only in megakaryocytes. It has been shown that the gene is active at an early stage of megakaryocyte differentiation (17) and an 813-bp DNA fragment located upstream of the initiation start site of the αIIb gene contains *cis*-acting elements necessary for lineage specific expression in vitro (18, 19). Thus, the regulatory elements of the αIIb gene were used to target the expression of the tk gene to early megakaryocyte progenitors of transgenic mice. The injection or withdrawal of GCV was shown to result in a rapid, severe, and reversible thrombocytopenia.

## Materials and Methods

*Recombinant DNA Construct.* The plasmid pBKtk-1 (20) was digested by BamH1 and blunted. The 3,290-bp DNA fragment containing the HSV-1 tk gene was further digested by HincII to delete the regulatory sequences of the virus, and it was inserted into the EcoR V site of pBluescript (Stratagene, La Jolla, CA), giving the plasmid ptkH. The human αIIb promoter region, extending from +33 to −787 relative to the initiation start site, was excised from the plasmid IIbpBLCAT3 (18) and inserted in the HincII site of ptkH. The resulting plasmid was digested by SacI to excise the 3,599-bp fragment containing the entire αIIbtk gene. For in vitro studies, this fragment was blunted and inserted into a blunt-ended SalI-digested pCEP4 plasmid (Invitrogen, San Diego, CA), an episomal vector containing the hygromycin B resistance gene. For the production of transgenic animals, the 3,599-bp DNA fragment was microinjected directly into fertilized eggs.

*DNA Transfections.* HEL, K562, U937, and HeLa cells were grown in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% FCS and antibiotics (streptomycin/penicillin) (Boehringer Mannheim GmbH, Mannheim, Germany) and transfected by electroporation, using a gene pulser (Bio Rad Laboratories, Hercules, CA). Each assay was done with 10 μg of the αIIbtk DNA. Cells were grown in RPMI medium supplemented with 10% FCS and antibiotics. After 24 h, hygromycin (50 μg/ml) was added to the medium. The expression of the transfected DNA was analyzed by the reverse transcriptase PCR (RT-PCR) method either 48 h after electroporation or after 3 wk of hygromycin B selection. The cytotoxicity of the nucleoside analogue GCV was determined in vitro on αIIbtk stable transformed cells obtained after 3 wk of hygromycin B selection. Various concentrations of GCV (ranging from 0 to 200 μM) were added to the culture to assess the toxicity of the antiherpetic drug on αIIbtk-transfected HEL cells since it has been found that there are significant differences in the sensitivity to GCV between various cell types (21).

*Production and Screening of Transgenic Mice.* The αIIbtk construct, without any vectorial sequences, was microinjected, using established procedures (22), into fertilized eggs resulting from mating between C57/SJL mice. Transgenic offspring were identified by Southern blot analysis (23) using genomic DNA (10 μg) extracted from tail samples. DNA was digested with PvuII, separated on a 0.8% agarose gel, transfered to Hybond $N^+$ (Amersham International plc, Bucks, UK) and hybridized with a random-primed (24) tk probe.

*RNA Isolation.* Total RNA was prepared from pelleted BM cells using a rapid total RNA isolation kit (5 Prime–3 Prime, Inc., Boulder, CO). RNA isolation from tissues was performed as previously described (22).

*RT-PCR.* 5 μg of total RNA were first treated with 1 U of RQ1 DNase (Promega Corp., Madison, WI) and then denatured (70°C for 10 min) before it was used as a template in a 40-μl cDNA synthesis reaction using random hexanucleotides. 5 μl of the reverse transcription mixture were amplified by PCR (25) in a thermocycler (PHC-3; Techne Corp., Cambridge, MA). PCR reactions were performed under standard Perkin-Elmer Cetus conditions, for 40 cycles (94°C 1 min, 55°C 1 min, 72°C 1 min), followed by 3 min at 72°C. PCR products were run through a 1.5% agarose gel and transfered to Hybond $N^+$. Filters were hybridized with end-labeled internal specific oligonucleotides. Routine controls performed in each experiment included a cDNA reaction mixture without addition of reverse transcriptase as a check against genomic DNA contamination and a PCR control with no template added to control for PCR artifacts caused by contamination.

*In Vivo GCV Administration.* GCV (CYMEVAN; Syntex, Palo Alto, CA) was administrated twice a day by intraperitoneal (i.p.) injection over different time intervals as described later. The dose of GCV was 0.05 mg/d per g body wt.

*Oligonucleotide Synthesis and PCR Primers.* Oligonucleotides used as primers for PCR were synthesized on a DNA synthesizer (381A; Applied Biosystems, Inc., Foster City, CA). The 5′ tk primer was CCCCTGCCATCAACACGCGT and the 3′ tk primer was CGGCGTCGGTCACGGCATAA nucleotides 529–543 and 929–910 from the tk sequence, respectively (26). The 5′ αIIb primer was GGAAGATGGCCAGAGC nucleotides 22–43 and the 3′ αIIb

sequence was GAAGAATTCCAGTGCTGCCAGGGGGC nucleotides 4,013–4,032 (27).

*Growth Factors.* Pure recombinant murine (rMu) stem cell factor and rMuGM-CSF were purchased from Genzyme Corp. (Cambridge, MA). Pure recombinant human (rHu) GM-CSF and pure rHu erythropoietin (EPO) were purchased from Boehringer Mannheim. Pure rMuIL-3 and rHuIL-6 were purchased from PeproTech, Inc. (Rocky Hill, NJ).

*Cell Preparation.* BM was flushed from the femoral cavity with IMDM supplemented with PG-E1 using a syringe with a 25-gauge needle. BM cells were separated from the core matrix by manual pipeting and were washed twice in PBS.

*Megakaryocyte Progenitors (CFU-MK).* BM cells were cultured in triplicate in 35-mm petri dishes (Falcon Labware, Oxnard, CA) in serum-free conditions ($1.5 \times 10^5$ cells per well) in 1 ml IMDM supplemented with various ingredients used in serum-free cultures as previously reported (9), with the exception that 0.3% agar (Difco Laboratories, Detroit, MI) was substituted for methylcellulose. The growth of CFU-MK was stimulated with 50 ng murine IL-3 and 5 ng IL-6/ml. After 7 d of incubation in a $CO_2$ incubator (5% $CO_2$ in air, 37°C), the agar discs were harvested, dessicated on large glass slides, and stained for the megakaryocytic-specific acetyl cholinesterase (AChE) activity (28).

*Erythroid Burst-forming Units (BFU-E).* BM cells ($5 \times 10^4$ cells per well) were cultured in triplicate plastic dishes (Nunclon, Roskilde, Denmark) in 0.25 ml of IMDM supplemented with 20% FCS (Seromed, Berlin, Germany), 1% deionized BSA (Sigma Immunochemicals, St. Louis, MO), 300 μg/ml transferrin fully saturated with $FeCl_3$ (Boehringer Mannheim), 10 μg/ml insulin (Sigma), 0.2 mM crystalline bovine hemin (Sigma), $10^{-4}$ M mono-

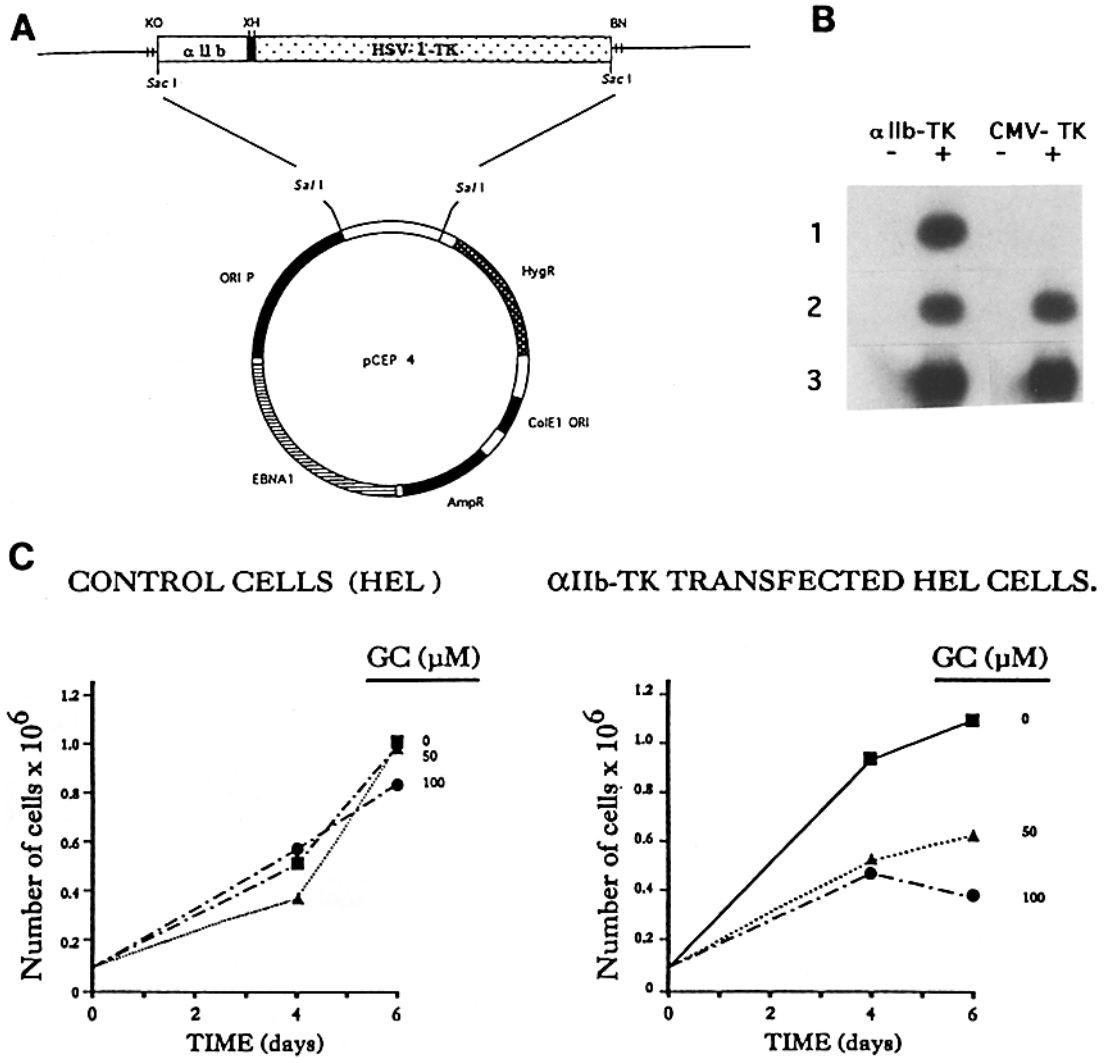


**Figure 1.** In vitro analysis of αIIbtk gene function. (*A*) Diagram of the recombinant plasmid used for transfection. The SalI fragment of the pCEP4 containing the CMV promoter was replaced by the αIIbtk gene excised from pBluescript by SacI digestion. The KpnI (*K*), XhoI (*0*), XbaI (*X*), BamHI (*B*), and NotI (*N*) restriction sites of pBluescript are represented. (*B*) PCR analysis performed on RNA prepared from HEL cells transfected with CMVtk or αIIbtk in the presence (+) or absence (−) of reverse transcriptase: (lane *1*) amplification with the 5′ αIIb primer and the 3′ tk primer, (lane *2*) amplification with 5′ and 3′ tk primers, and (lane *3*) amplification performed with 5′ and 3′ αIIb primers. (*C*) Inhibition of cellular growth by GCV in αIIbtk transfected cells.

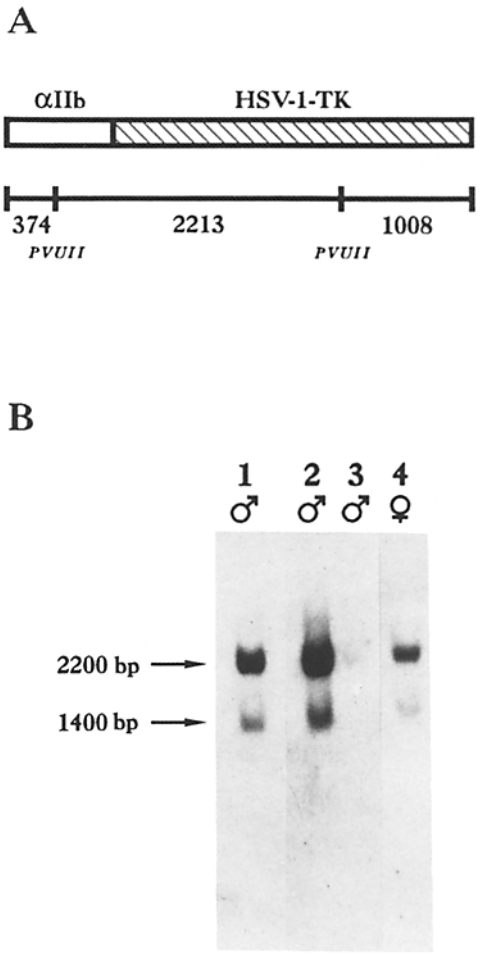


**Figure 2.** Development of mice transgenic for αIIbtk. (*A*) Schematic illustration of the αIIbtk construct. The restriction map shows the PvuII sites and the size (bp) of the restriction fragments. (*B*) Southern blot analysis of DNA prepared from the four founders. As expected, a 2,213-bp fragment was observed after PvuII digestion and hybridization with the tk probe. A second expected 1,382 fragment was obtained when several transgenes are positioned in a head to tail conformation. Mice numbers 1, 2, 3, and 4 had integrated 6, 10, 1, and 3 copies of the transgene, respectively.

thioglycerol, and 1.2% methylcellulose (Fluka AG, Buchs, Switzerland). The growth of BFU-E was stimulated with 1 ng of rMuIL-3 and 1 U of EPO. The cultures were examined after 8 d and aggregates of >100 red cells were considered as a BFU-E.

*Granulomonocytic Colony-forming Cells (GM-CFC).* BM cells were cultured in triplicate ($5 \times 10^4$ cells per well) in 0.25 ml IMDM supplemented with 20% FCS, 1% deionized BSA (Sigma), $10^{-4}$ M monothioglycerol, and 1.2% methyl-cellulose. The growth of GM-CFC was stimulated by 10 ng/ml rMuGM-CSF. After 7 d of incubation at 37°C in a $CO_2$ incubator flushed with 5% $CO_2$ in humidified air, aggregates of at least 50 cells were considered to be GM-CFC.

*Mixed Progenitors and Granulo-Monocytic-Erythroid-Megakaryocytic CFU (CFU-GEMM).* CFU-GEMM were cultured in quadruplicate in 35-mm petri dishes containing $5 \times 10^4$ BM cells in 1 ml of culture medium that had the same composition as that used for BFU-E. The growth of mixed colonies was stimulated by 50 ng/ml rMu stem cell factor, 50 ng/ml rMuIL-3, 25 ng rHuIL-6, 10 ng rHuG-CSF, and 2 U of EPO. The dishes were incubated for 7–8 d at 37°C in a fully humidified incubator flushed with 5% $CO_2$. The dishes were examined under an inverted microscope. GM-CFC, BFU-E, CFU-MK, and various mixed progenitors including CFU-GEMM were identified and counted. The nature of mixed progenitors was controlled by picking up the colonies from the methylcellulose using an Eppendorf pipette. The colonies were smeared on glass slides, stained with May-Grünwald Giemsa (MGG), and examined microscopically.

*Cell Counting Procedures.* Blood was collected from tail vein bleeds into acid citrate dextrose containing PG-E1 (10 μM), 7:1 blood/anticoagulant (vol/vol). All cell counts were determined manually using a counting chamber and microscopy. Erythrocytes were counted after dilution (1:200) in PBS, whereas leukocyte and platelet counts were performed after dilution (1:20) in Thrombo-Zahl solution (Merck, Darmstadt, Germany). The mean values ($n$ = 10) for the control nontransgenic animals by this method were: platelets = $0.94 \pm 0.24 \times 10^{12}$/liter (SD), leukocytes = $10.19 \pm 3.11 \times 10^9$/liter (SD), erythrocytes = $10.45 \pm 2.13 \times 10^{12}$/liter (SD), and they were within the normal range for nontransgenic mice (29, 30).

## Results

*Analysis of the αIIbtk Hybrid Gene Function.* The αIIbtk plasmid, in which HSV-1 tk expression is directed by 813 bp of regulatory sequences of the megakaryocytic specific promoter αIIb, was introduced into plasmid pCEP4. This is an episomal vector for expression of foreign genes in mammalian cells that contains the hygromycin B gene as the selectable marker (Fig. 1 *A*). To establish whether the expression of the construct was tissue specific, transient transfections of the pCEP4-αIIbtk were performed in megakaryocytic and nonmegakaryocytic cell lines. Tk transcripts were detected only in the transfected HEL megakaryocytic cell line as determined by RT-PCR of mRNA using tk-specific primers (Fig. 1 *B*). The U937 monocytic cell line, the HeLa epithelial cell line, the K562 erythroleukemic cell line, and the nontransfected HEL cells did not demonstrate any detectable tk mRNA. In contrast, when the IIb promoter was replaced by the CMV promoter, tk transcripts were detected in all the cell types examined (data not shown).

To test for the function of the expressed protein and the toxic potential of the hybrid gene in vitro, stable cultures of HEL expressing tk were generated after 3 wk of hygromycin-B selection. At this stage, $10^5$ cells were cultivated in the presence of different concentrations of GCV for an additional 6-d period. As shown in Fig. 1 *C*, only the transfected cells expressing the αIIbtk gene demonstrated reduced growth, while nontransfected HEL cells were unaffected. The results obtained with both transient and stable transfected cells confirmed that the hybrid gene was active in vitro and maintained a tissue-specific activity and thus was suitable for the production of transgenic mice.

*Generation and Characterization of Transgenic Mice.* The αIIbtk transgene (Fig. 2 *A*) was excised from the pCEP4-αIIbtk and microinjected into fertilized eggs. Among the 18 offspring obtained, 4 were found to be transgenic by

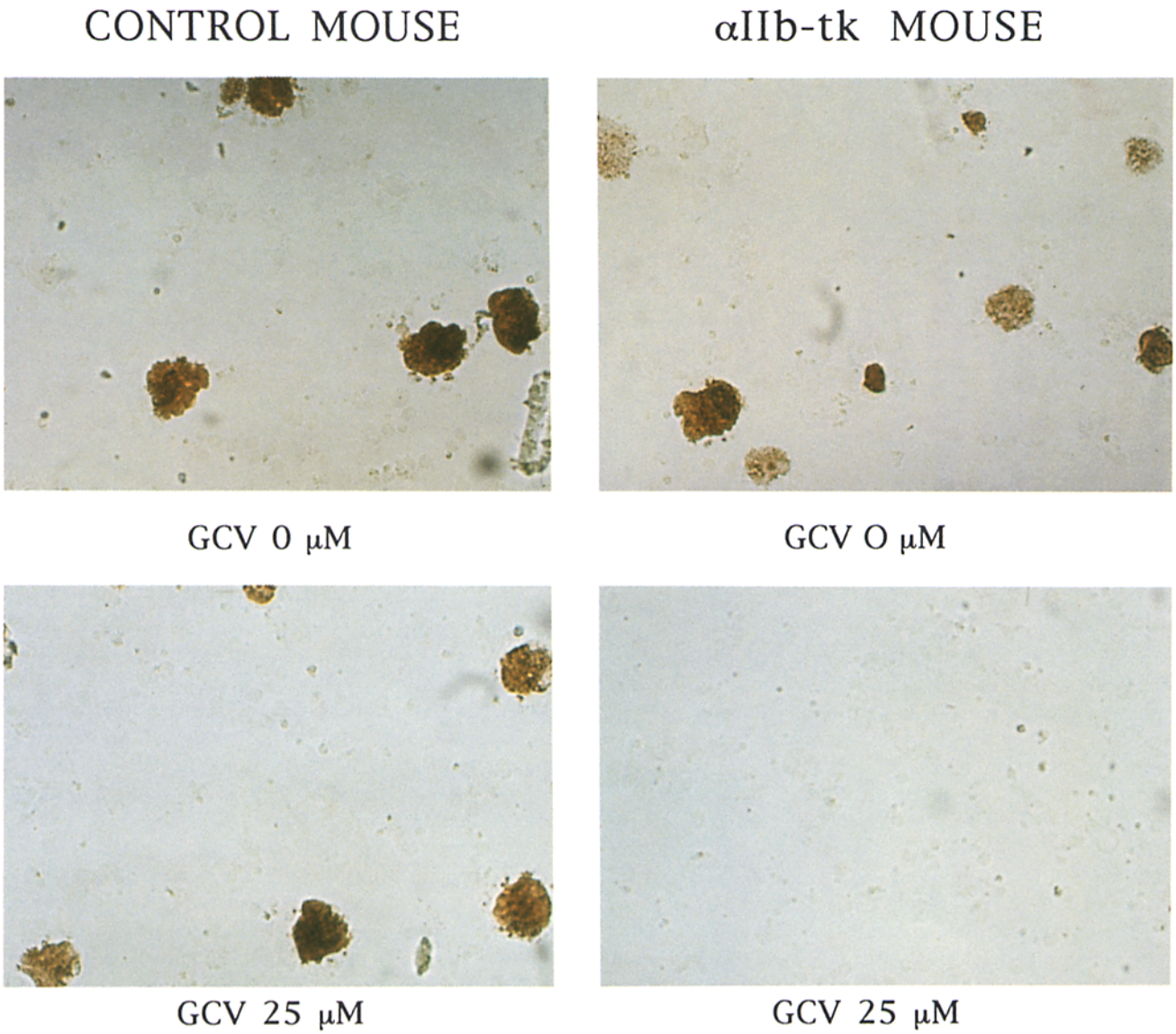


**Figure 3.** Development of megakaryocytes in cultures of BM from transgenic and nontransgenic mice in the presence or absence of GCV. BM isolates were seeded in liquid serum free medium at a concentration of $5 \times 10^5$ cells/ml and were cultured in the presence or absence of 25 μM GCV. After a 5-d culture, megakaryocytes were identified by AChE positivity.

Southern blot analysis performed with the tk probe (Fig. 2 *B*). The number of integrated copies ranged from 1 to 10, as determined after phosphorimager analysis of tk-hybridized blots. All founders transmitted the transgene to their progeny in a Mendelian fashion. In this study, the transgenic line derived from the founder carrying one copy of the transgene was analyzed.

To test for the tissue-specific expression of the chimeric gene, total RNA was prepared from BM, liver, kidney, thymus, spleen, lung, adrenal gland, testis, submaxillary gland, and brain of transgenic and nontransgenic littermates. This was analyzed by RT-PCR using the tk primers. Tk mRNA was found in the BM, adrenal gland, and testis. Previously, the αIIb promoter has not been shown to be active in these last two tissues. The presence of tk transcripts in the adrenal gland cannot be considered as aberrant expression since another megakaryocyte-specific gene, platelet factor 4, has also been reported to be active in this tissue (31). The significance of this observation, however, remains unknown. On the other hand, it was found that all constructs harboring the coding region of the tk gene were expressed at different levels in the testes of all the transgenic males, regardless of the promoter that was used. It was suggested that testicular expression may be controlled by a cryptic TATA box–independent promoter located in the coding region of the tk gene (32).

To test the function of the tk protein produced by the transgene, the toxic effect of GCV on megakaryocytes was examined. Cells isolated from the BM of transgenic and nontransgenic littermates were cultured in serum-free medium containing GCV and supplemented with IL-3 and IL-6 to en-

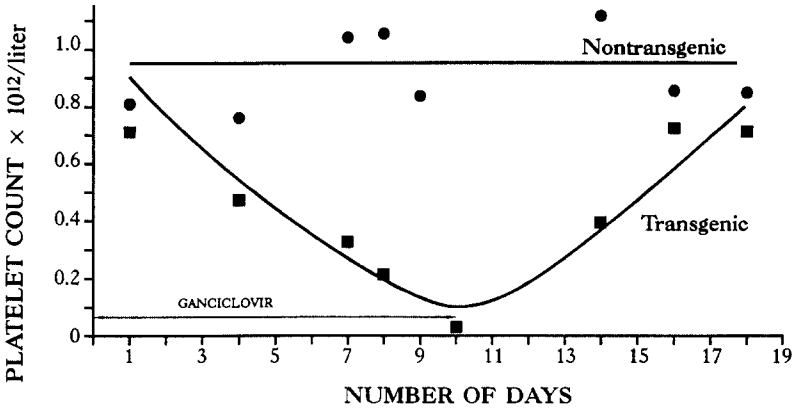


**Figure 4.** Changes in platelet count after GCV treatment of transgenic and nontransgenic mice. Transgenic and nontransgenic littermates were treated with GCV (0.05 mg/g i.p. daily for 9 d). Platelet counts were determined on tail bleeds during and after the suspension of GCV treatment. Each data point shows the mean SD ($n$ = 5).

rich for megakaryocytes (9). The concentration of GCV used (25 $\mu$M) was determined on the basis of pilot studies where varying concentrations (0–200 $\mu$M) of GCV were evaluated. At the concentration chosen, no toxic effects on normal control megakaryocytes were noted (data not shown). After 6 d of liquid culture, no megakaryocytes were detected in the transgenic BM culture, as determined by the megakaryocytic-specific AChE method (Fig. 3). Megakaryocytes of the nontransgenic mice were not affected by the GCV treatment. We concluded that the tk gene was expressed in megakaryocytes under the control of the αIIb promoter and its product, i.e., tk, selectively eradicates tk expressing cells in the presence of GCV.

*Effect of GCV Treatment on Blood and BM Cellularity.* To study the in vivo effect of GCV treatment on platelet production, 5-wk-old transgenic and non transgenic mice were injected i.p. with GCV (0.05 mg/day per g body wt) for 10 d. Blood samples were drawn from the tail vein and cell counts were determined by microscopy. The mean platelet count ($n$ = 5) in the transgenic mice was reduced from 0.82 × $10^{12}$ to 0.045 × $10^{12}$/liter by day 10 of GCV treatment, corresponding to a >94.6% decrease in circulating platelet number. In contrast, the platelet counts of the nontransgenic littermates were unaffected by the GCV treatment. Leukocyte and erythrocyte counts were unaffected in both groups of animals under these conditions. When the administration of GCV was interrupted, the process was reversed and the platelet counts had returned to normal values by 9 d (Fig.4). Qualitative changes in platelet morphology were observed by microscopy during the induction and reversal of thrombocytopenia during GCV treatment and its withdrawal, respectively. There was a gradual overall reduction in platelet size as thrombocytopenia developed, and this was reversed and larger than normal platelets were observed during the recovery phase. Qualitatively, this is in line with the expected changes in platelet size during supression of thrombopoiesis and its recovery. Unfortunately, the methodology used, i.e., manual cell counting, did not lend itself to accurate determination of mean platelet volume. In future studies, this will be addressed using automated cell counting techniques which provide accurate estimates of this parameter (33).

The precise stage of megakaryocytic differentiation at which the expression of the hybrid gene appeared was next examined in BM after 10 d of GCV administration. As shown in Fig. 5, GCV treatment resulted in a complete eradication of AChE-positive megakaryocytic cells from the BM of αIIbtk mice. Surprisingly, however, microscopic examination of BM cytospins stained with MGG revealed that erythroid cells were also reduced to 1.5% of total BM cells compared to 24% in GCV-treated nontransgenic mice, suggesting an increased sensitivity of the erythroid lineage to the drug in the transgenic animals. In contrast, the myelomonocytic and lymphoid lines were unaffected. Although this may disagree with the observation that the circulating erythrocyte counts were unchanged after 10 d of GCV treatment, this may be explained by the longer half-life of the erythrocyte, which is ~25 d (29). Hence, >10 d treatment with GCV may be required to induce a significant decrease in the number of circulating erythrocytes. To test this hypothesis, animals were treated for 23 d and the cell counts followed. Under these conditions, the mean erythrocyte count decreased from 10.9 × $10^{12}$ to 2.2 × $10^{12}$/liter, but it remained unchanged in the treated nontransgenic mice. Similarly, the mean platelet count decreased from 1.2 × $10^{12}$ to 0.016 × $10^{12}$/liter. Leukocyte counts were unaffected (Table 1).

These data demonstrated that the expression of the tk gene in the megakaryocyte lineage induced a severe but reversible thrombocytopenia caused by a failure of thrombopoiesis. This raised the possibility that these animals might be used to assess the effect of cytokine administration on platelet production. To evaluate this, the platelet kinetics of thrombocytopenic animals were monitored after treatment with IL-6 and IL-11 (0.08 $\mu$g/g per d). As shown in Fig. 6, the rate of recovery was increased with the administration of IL-11, resulting in complete normalization within 2 d, whereas with IL-6, no improvement over untreated thrombocytopenic controls was noted.

*Cytotoxic Effect of GCV on Myeloid Progenitor Cells.* BM cells of control and αIIbtk mice treated for 10 d with GCV were cultured using individual semi-solid progenitor cell assays. The number of megakaryocytic colonies was evaluated in situ

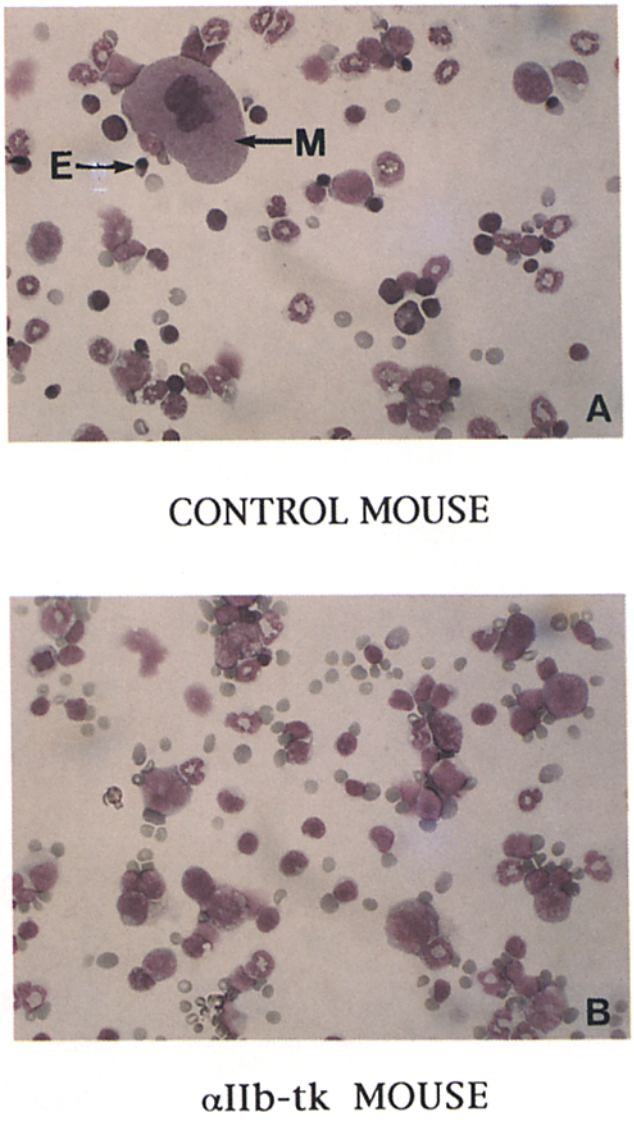


**Figure 5.** Effect of GCV treatment on BM cellularity of transgenic and nontransgenic mice. Transgenic and nontransgenic littermates were treated with GCV (0.05 mg/d per g i.p.) for 10 d. Cytospin preparations were stained with MGG. ×100. (*A*) BM from a treated nontransgenic animal. (*B*) BM from a treated αIIbtk mouse. Mature megakaryocyte (*M*) and erythroid cells (*E*) are seen in the BM of the control nontransgenic mouse but are absent in the transgenic animal.

on dried agar gel disks stained by the AChE method. The development of CFU-MK and BFU-E colonies in cultures of BM cells from GCV-treated αIIbtk mice was drastically affected and colonies were barely detectable, whereas the number and size of granulomonocytic colonies from the BM of the same mice were in the control range (Table 2). The inhibition of CFU-MK and BFU-E was confirmed in a separate set of experiments where early myeloid multipotent progenitor cells were tested in methyl-cellulose. In this assay, a mixture of several hematopoietic growth factors was used to obtain all the combinations of mixed myeloid colonies. As shown in Table 3, a drastic reduction (Experiment 1) or a total disappearance (Experiment 2) of megakaryocytic, erythroid, and various mixed colonies in the αIIbtk BM cell cultures was observed. Only colonies of granulomonocytes and macrophages, in numbers and size comparable to those observed in nontransgenic controls, were seen. These data were also obtained in liquid cultures of BM cells from the αIIbtk mice treated with GCV in vivo, confirming the eradication of megakaryocytic and erythroid cells but maintenance of granulomonocytic cells (data not shown).

## Discussion

These studies describe the generation of transgenic mice in which a severe and reversible thrombocytopenia could be produced on demand. This inducible thrombocytopenia was achieved by the expression of the tk gene directed by the regulatory elements of the gene encoding the platelet-specific αIIb integrin subunit. The tk protein was toxic to the early megakaryocytic progenitors, leading to a complete eradication of the lineage within a week. The toxic effect of GCV was reversible and a normal platelet count was restored as soon as 7 d after the termination of the GCV injections. These mice also developed an erythropenia when drug administration was maintained for >20 d. A detailed analysis of the toxic effect of the drug on the BM of GCV-treated mice showed a near total inhibition of the growth of CFU-MK, BFU-E, and CFU-GEMM for the αIIbtk mice, whereas the nontransgenic littermates were normal. Only GM-CFC were observed in the BM cultures of GCV-treated αIIbtk animals. Leakage of tk phosphorylated metabolites from the primary tk expressing cells, i.e., those of the megakaryocytic lineage, could account for the erythroid effect, i.e., a bystander effect on neighboring cells as described by others (34, 35). However, this would seem a most unlikely explanation given the clear demonstration that the effect was completely limited to megakaryocytic and erythroid lineages while GM-CFC were maintained.

Different hypotheses can be proposed to explain the persistence of normal granulomonocytopoiesis despite the absence of multipotent progenitors. The αIIb gene may be expressed at the level of CFU-GEMM, resulting in the eradication of mixed colonies of all kinds. This would have to assume that GM-CFC are derived from a totipotent stem cell via an alternative and direct pathway of differentiation. This is consistent with a model for hematopoiesis that proposes a random commitment of a strictly multipotent to a monopotent stem cell (36) and is supported by data from Fraser et al. (37), demonstrating that rabbit antiserum against human platelets was also significantly toxic to human CFU-GEMM. Its validity is questioned, however, by the finding that the message for αIIb was not found in human $CD34^+$ cells treated with mafosfamide, which spared quiescent early stem cells while killing stem cells in cycle (17).

An alternative hypothesis is that the αIIbtk gene is expressed either at the stage of a restricted erythroid-megakaryocytic (EM) stem cell or at the level of the monopotent

**Table 1.** *Effect of GCV Treatment on Blood Cell Counts*

| Control mouse | | | αIIbtk mouse | | |
|---|---|---|---|---|---|
| D0 | D10 | D23 | D0 | D10 | D23 |
| 10.91 ± 2.3 | 10.93 ± 1.13 | 10.88 ± 0.5 | 10.69 ± 3.053 | 9.23 ± 0.59 | 3.12 ± 0.88 |
| 10.19 ± 1.5 | 12.33 ± 4.0 | 13.00 ± 1.0 | 8.33 ± 4.27 | 8.15 ± 1.65 | 13.59 ± 14.08 |
| 0.80 ± 0.09 | 0.93 ± 0.18 | 0.77 ± 0.14 | 0.73 ± 0.12 | 0.045 ± 0.012 | 0.02 ± 0.007 |

Mean peripheral blood cell counts (± SD) of control and transgenic αIIbtk mice ($n$ = 5) before (*D0*), 10 d (*D10*), or 23 d (*D23*) of GCV (0.05 mg/g per d) treatment. The counts shown are per liter of blood volume.

erythroid BFU-E and CFU-MK. In this case, as soon as an erythroid or a megakaryocytic cell component differentiates into any type of mixed colony, it would be eradicated by the toxic effect of GCV, resulting in the selection of only granulomonocytic cells. This assumption is consistent with a model that supports the differentiation of multipotent progenitors through a stochastic and progressive restriction in cell lineages (5, 38). The existence of a bipotent EM stem cell in the mouse BM has been demonstrated by cell culture experiments using a sexual marker (39) and from studies on the effects of high doses of EPO on murine EM progenitors (40). In addition, most human leukemic megakaryocytic cell lines exhibit concomitant erythroid markers such as globin and glycophorin A (41, 42) or transcriptional factors such as GATA-1, which acts in combination with other factors to control αIIb promoter activity (43–45). Finally, the expression of the αIIb in separate committed progenitors CFU-MK and BFU-E is also supported by other published work. Berridge et al. (46) reported that the preincubation of mouse BM cells with complement and rabbit anti–mouse platelet serum, containing anti-αIIbβ3 antibodies, inhibited the growth of spleen colonies (CFU-S) as well as those of CFU-MK in vitro. Other studies have shown that the αIIb gene is expressed in human erythroleukemic cells (47) and Okumura et al. (48), using immunocytochemistry to detect αIIbβ3, demonstrated its presence in one of the two daughter cell doublets, resulting from the division of a single stem cell, whereas the other developed into BFU-E in semisolid culture. Thus, there is ample evidence that the expression of the αIIbβ3 is maintained during megakaryocyte and platelet production and is turned off in late erythroid differentiation. A repressor element, active in erythroid cells and recently described in the IIb promoter (49), might be responsible for the final tissue-specific expression of this gene in megakaryocytes and platelets. Although the fragment of the αIIb promoter used in our experiments contained this repressor element, we cannot exclude the possibility that other unknown sequences, required for a restricted megakaryocytic expression, might be absent.

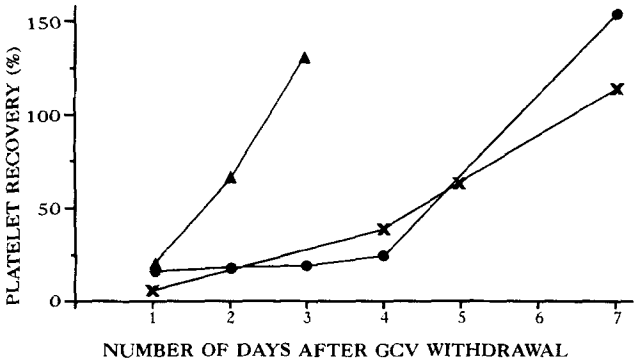


**Figure 6.** Effect of cytokine administration on thrombocytopenia in GCV-treated transgenic mice. Transgenic mice were treated with GCV (0.05 mg/d per g i.p.) to produce stable thrombocytopenia. The effect on platelet number of concurrent treatment with IL-6 (●) and IL-11 (▲) compared with untreated thrombocytopenic control animals (X) is shown. The dose of IL-6/IL-11 was 0.008 g/g per d.

**Table 2.** *Number of GM-CFC, BFU-E, and CFU-MK in Individual Semisolid Assays from BM Cells of Control and αIIbtk Mice Treated with GCV*

| | GM-CFC | BFU-E | CFU-MK |
|---|---|---|---|
| Experiment 1 | | | |
| Control | 15 ± 2 | 13 ± 2 | 10 ± 2 |
| αIIb-tk transgenic | 10 ± 1 | 0.3 ± 0.1 | 0 |
| Experiment 2 | | | |
| Control | 42 ± 2 | 16 ± 6 | 44 ± 9 |
| αIIb-tk transgenic | 43 ± 4 | 1.5 ± 1 | 6 ± 1 |
| Experiment 3 | | | |
| Control | 38 ± 5 | 13 ± 3 | 30 ± 8 |
| αIIb-tk transgenic | 23 ± 4 | 1 ± 1 | 0 |

Marrow cells (5 × $10^4$ per well) from control and αIIbtk mice treated with GCV (1 mg/d for 10 ds) were cultured in 0.25 ml complete IMDM supplemented with predetermined optimal concentrations of cytokines as described in Materials and Methods. GM-CFC and BFU-E were cultured in methyl-cellulose and counted after 7 d of incubation under the inverted microscope. CFU-MK were cultured in agar for 7 d and identified after desiccation of the gel and specific staining for acetylcholinesterase. Each number (± SD) are the mean of three wells in three different experiments.

**Table 3.** *Number of Monopotent and Mixed Myeloid Colonies from BM Cells of Control and αIIbtk Mice Treated with GCV*

| | GM-CFC | BFU-E | MK | BFU-E-MK | GEM + GEMK + GMMK | GEMMK |
|---|---|---|---|---|---|---|
| 1 | 64 ±11.5 | 12 ± 1.8 | 4 ± 1.7 | 4.5 ± 1.1 | 8.5 ± 3.6 | 2.2 ± 0.8 |
| | 61 ± 3.3 | 0.6 ± 0.5 | 1.4 ± 0.9 | 0.6 ± 0.9 | 1.6 ± 1.5 | 0.4 ± 0.5 |
| 2 | 71 ± 8.5 | 8 ± 3.3 | 9 ± 2.2 | 2.2 ± 0.8 | 4 ± 1.6 | 2.2 ± 1 |
| | 59 ± 7.4 | 0.75 ± 0.5 | 0.25 ± 0.5 | 0 | 0 | 0 |

Marrow cells ($5 \times 10^4$/ml per dish) from control and αIIbtk mice treated with GCV (1 mg/d for 10 d) were obtained by femoral aspiration and plated in 1 ml of culture medium as described in Materials and Methods. The number of colonies was the mean SD of five identical dishes. Mixed colonies consisted of bilineage BFU-E-MK (erythroid and megakaryocytic), trilineage GEM (granulocytic erythroid macrophagic), GEMK (granulocytic erythroid megakaryocytic), GMMK (granulocytic macrophagic megakaryocytic), and multipotent GEMMK (granulocytic erythroid macrophagic and megakaryocytic).

It is also possible that the effect noted on erythroid progenitors may reflect a secondary effect of adrenal suppression given the demonstration that the message for tk was also found in this tissue. Thus it would be possible that, after GCV treatment, tk toxicity of adrenal cells may occur resulting in the loss of hormonal output and a secondary suppression of hematopoietic activity. Although hormone levels were not measured, there was no evidence of adrenal atrophy. Moreover, given the relatively slow turnover of adrenal relative to hematopoietic cells, such an effect would appear most unlikely.

The biological activity of thrombopoietin, the hormone that specifically promotes the proliferation and maturation of megakaryocytes, is mediated by a specific receptor encoded by the c-*mpl* protooncogene (50–53). The effects of c-*mpl* knockout in mice have been reported recently (54). Although exhibiting severe and irreversible thrombocytopenia, the animals were otherwise healthy. The fact that they were not totally thrombocytopenic (15% of normal platelet count), suggests that, in addition to c-*mpl* expression, other factors are required for the complete development and maturation of the megakaryocytic lineage. The present study demonstrates that a severe thrombocytopenia may be produced on demand by the administration of GCV to αIIbtk transgenic mice. Moreover, this effect may be completely reversed by the removal of the inciting treatment. Consequently, this model allows the study of the megakaryocyte lineage by providing the means by which the kinetics of differentiation and maturation may be modified to order and at a given point in time of lineage development. It should also be possible to use this method to target other genes that may modify the activity of the cell in different ways and promote the synthesis of factors that may modify megakaryocyte maturation and/or function subsequently.

The model may also be used to study potential therapeutic interventions in the management of thrombocytopenic disorders since the duration of GCV treatment may be used to dissociate, in large part, the effect on thrombopoiesis over erythropoiesis. For example, in preliminary studies reported here, it was demonstrated that the rate of recovery from thrombocytopenia could be modulated by the administration of cytokines to the thrombocytopenic animals. These studies support the view that this model may have pharmacological relevance in addressing such issues in vivo.

Platelets are directly or indirectly involved in a number of vascular diseases, including thrombosis, coronary artery restenosis, and atherosclerosis. A number of different transgenic mouse models with an atherosclerotic phenotype have recently been developed (55, 56). Thus, using this approach, it is now feasible to create a second generation of atherosclerotic animals with controlled thrombopoiesis. Similarly, the role of platelets in arterial restenosis is still unclear. A model for arterial injury in the mouse has been reported recently (57). Its use in transgenic animals such as those described here would facilitate the study of whether or not adequate platelet numbers influence the outcome of vascular insult.

In conclusion, the αIIbtk mice described in the present study afford a particularly flexible system for inducing thrombocytopenia on demand. This should prove suitable both for deciphering the fundamental mechanisms that underlie thrombocytopoiesis and also for the study of vascular occlusive diseases, where it has been suggested that platelets may play a significant pathogenetic role.

All the animal studies reported were performed in accordance with current French government regulations (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture).

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