Repetitive Busulfan Administration After Hematopoietic Stem Cell Gene Therapy Associated with a Dominant HDAC7 Clone in a Nonhuman Primate

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Abstract

The risk of genotoxicity of retroviral vector-delivered gene therapy targeting hematopoietic stem cells (HSCs) has been highlighted by the development of clonal dominance and malignancies in human and animal gene therapy trials. Large-animal models have proven invaluable to test the safety of retroviral vectors, but the detection of clonal dominance may require years of follow-up. We hypothesized that hematopoietic stress may accelerate the proliferation and therefore the detection of abnormal clones in these models. We administered four monthly busulfan (Bu) infusions to induce hematopoietic stress in a healthy rhesus macaque previously transplanted with CD34⁺ cells transduced with retroviral vectors carrying a simple marker gene. Busulfan administration resulted in significant cytopenias with each cycle, and prolonged pancytopenia after the final cycle with eventual recovery. Before busulfan treatment there was highly polyclonal marking in all lineages. After Bu administration clonal diversity was markedly decreased in all lineages. Unexpectedly, we found no evidence of selection of the MDS1/EVI1 clones present before Bu administration, but a clone with a vector integration in intron 1 of the histone deacetylase-7 (HDAC7) gene became dominant in granulocytes over time after Bu administration. The overall marking level in the animal was increased significantly after Bu treatment and coincident with expansion of the HDAC7 clone, suggesting an *in vivo* advantage for this clone under stress. HDAC7 expression was upregulated in marrow progenitors containing the vector. Almost 5 years after Bu administration, the animal developed progressive cytopenias, and at autopsy the marrow showed complete lack of neutrophil or platelet maturation, with a new population of approximately 20% undifferentiated blasts. These data suggest that chemotherapeutic stress may accelerate vector-related clonal dominance, even in the absence of drug resistance genes expressed by the vector. This model may both accelerate the detection of abnormal clones to facilitate analysis of genotoxicity for human gene therapy, and help assess the safety of administering myelotoxic chemotherapeutic agents in patients previously engrafted with vector-containing cells.

Introduction

THE POTENTIAL GENOTOXICITY of retroviral vector-delivered gene therapy targeting hematopoietic stem cells (HSCs) has been a significant concern since vector-linked leukemias were reported in humans, nonhuman primates, dogs, and mice (Modlich *et al.*, 2005; Ott *et al.*, 2006; Seggewiss *et al.*, 2006; Hacein-Bey-Abina *et al.*, 2008; Zhang *et al.*, 2008). Mechanisms of oncogenesis associated with retroviral gene therapy may include activation of proto-oncogenes by vector

promoter/enhancer elements (Ott *et al.*, 2006; Seggewiss *et al.*, 2006; Hacein-Bey-Abina *et al.*, 2008), disruption of tumor suppressor genes (Baum *et al.*, 2003, 2006), activation of micro-RNAs (Nair, 2008), generation of fusion or read-through transcripts from the inserted promoter into cellular genes (Benz *et al.*, 1980), and interplay between transgenes and genes activated by vector insertion (Bohne and Cathomen, 2008).

The progression to abnormal hematopoiesis or leukemia is clearly a multistep process, and assessing genotoxic risk of specific vector backbones or transgenes in relevant models is

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a significant challenge for the field. The patients in the X chromosome-linked severe combined immunodeficiency (X-SCID) and chronic granulomatous disease (CGD) clinical trials did not progress to leukemia or clonal hematopoiesis for two or more years after transplantation of transduced cells (Hacein-Bey-Abina *et al.*, 2002, 2008; Ott *et al.*, 2006). Fifteen years of prior research in preclinical models did not reveal the risks associated with the use of these vectors to transduce HSC targets. Thus there is a need for new approaches to assess the likelihood of abnormal clonal dominance associated with transduction of HSCs. A number of assays have been proposed, including *in vitro* immortalization of murine bone marrow cells (Modlich *et al.*, 2006), induction of leukemias in tumor-prone mouse models such as Arf or Cdkn2a knockouts (Montini *et al.*, 2006; Shou *et al.*, 2006), cotransplantation of human HSCs and mesenchymal stem cells (MSCs) transduced with viral vectors into immune-deficient mice (Bauer *et al.*, 2008), serial transplantation of murine marrow cells (Kustikova *et al.*, 2005), and achievement of growth factor independence using cell lines (Okuda *et al.*, 1994). However, the relevance of these *in vitro* and rodent assays to human hematopoiesis may be limited, based on the poor predictive value of rodent and *in vitro* assays for HSC gene transfer efficiency, and major differences in the kinetics and clonal patterns of hematopoiesis between small and large animals (Abkowitz *et al.*, 1995; Shepherd *et al.*, 2007). We have used the rhesus macaque model and found it to be highly predictive of outcomes in human clinical trials (Donahue and Dunbar, 2001). In long-term follow-up of a cohort of more than 20 rhesus macaques transplanted with murine leukemia virus (MLV) vector-transduced cells, one animal developed leukemia 5 years after transplantation (Seggewiss *et al.*, 2006).

We hypothesized that under hematopoietic stress, clones might more rapidly dominate. The commonly used alkylating agent busulfan (Bu) is known to have a significant impact on stem cell behavior (Dunn, 1974; Abkowitz *et al.*, 1993; Hsieh *et al.*, 2007). We have previously shown that single doses rapidly but temporarily impacted on vector-containing clonal patterns in rhesus macaques (Kuramoto *et al.*, 2004), and thus we hypothesized that it could function as an effective hematopoietic stressor, uncovering clones with abnormal characteristics linked to vector insertions. In the current study, we repeatedly dosed a rhesus macaque transplanted previously with CD34⁺ cells transduced with a retroviral vector carrying only a marker gene with busulfan. Before busulfan treatment, this animal was found to harbor two independent retroviral vector integrants in the MDS1/EVI1 gene locus in a background of polyclonality (Hematti *et al.*, 2004). Given growing concerns regarding this locus as an integration site (Li *et al.*, 2002; Calmels *et al.*, 2005; Ott *et al.*, 2006), we asked whether application of hematopoietic stress could select for cells with vector activation of this locus and accelerate or initiate leukemogenesis. Although we found no evidence for selection of MDS1/EVI1 clones, a single clone with provirus integration within the histone deacetylase-7 (HDAC7) gene became highly dominant after busulfan treatment, not only accounting for the vast bulk of vector-containing myeloid cells, but also expanding relative to nontransduced hematopoietic cells. The animal then progressed to fatal myelodysplasia/leukemia.

Materials and Methods

Rhesus macaque treatment protocol

Rhesus macaque RQ2297 was transplanted on July 14, 2000 with autologous CD34⁺ cells transduced with retroviral vectors LNL6 and G1Na after total body irradiation (TBI) as described (Takatoku *et al.*, 2001). Busulfan (4 mg/kg, intravenous) was administered 2 years and 3 months after transplantation (Kuramoto *et al.*, 2004), followed by four additional monthly busulfan doses of 4, 4, 6, and 6 mg/kg beginning 4 years after transplantation. All procedures were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute (Bethesda, MD).

Isolation of granulocytes, T cells, and B cells

Isolation of peripheral blood granulocytes and mononuclear cells (MNCs) was performed with lymphocyte separation medium (LSM) according to the manufacturer's instructions (MP Biomedicals, Illkirch, France). Sorting of T and B lymphocytes was performed after staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and phycoerythrin (PE)-conjugated anti-CD20 antibodies (BD Biosciences, San Jose, CA). Post-sort purity was more than 98%.

Colony-forming unit growth and selection

The MNCs contained in 5 ml of bone marrow obtained via aspiration from the posterior iliac crest were obtained by centrifugation over LSM (MP Biomedicals). MNCs (3×10^5) were seeded in 3 ml of colony assay medium (MethoCult GF⁺ H4435; StemCell Technologies, Vancouver, BC, Canada) without or with added G418 (1.5 mg/ml), and 1 ml was aliquoted per plate. After incubation in 5% CO₂ for 10–14 days, the G418-selected or nonselected colony-forming units (CFUs) were counted, plucked, and washed twice with 1×phosphate-buffered saline (PBS). Total RNA was immediately extracted, using an RNeasy kit (Qiagen, Valencia, CA), and frozen at –80°C.

Retrieval of vector insertion sites

To identify vector insertion sites, linear amplification-mediated polymerase chain reaction (LAM-PCR) was performed as described (Schmidt *et al.*, 2002; Kuramoto *et al.*, 2004) with the following modifications. The nested LAM-PCR amplification products were separated on a 2.5% NuSieve agarose gel, followed by removal of the 193-bp internal band, and cloning of DNA purified from the gel into the TA vector pCR4TOPO (Invitrogen, Carlsbad, CA). The sequences of primers used are listed in Supplementary Table 1 (see www.liebertonline.com/hum). Valid insertions were identified via searching for sequences 5'-AGCAGTTAGG-3' and 5'-TGAAAGACCC-3' specific for the linker cassette and 5'-long terminal repeat (LTR) of the vector, respectively, followed by a rhesus genome BLAT search (<http://genome.ucsc.edu/cgi-bin/hgBlat>), according to criteria previously described (Hu *et al.*, 2008).

Quantitative real-time PCR

To determine the copy number of proviral vectors in peripheral blood cells, genomic DNA was extracted with a DNeasy blood and tissue kit (Qiagen). A set of plasmid

controls used as standards for quantitative real-time PCR (Q-PCR) were constructed with the pCR4-TOPO vector (Invitrogen). Q-PCR was run in triplicate 25- μ l reactions, using 7500 system software (Applied Biosystems, Foster City, CA) under the following conditions: 50°C for 1 min, 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min, run for 50 cycles. The data were analyzed with 7500 sequence detection system software (Applied Biosystems). The copy number of the vector marker gene (bacterial neomycin phosphotransferase) was calculated relative to that of the rhesus albumin (rhALB) gene.

To analyze gene expression at the transcriptional level, 1 μ g of total RNA obtained from CFUs was used for cDNA synthesis, using the SuperScript first-strand synthesis system (Invitrogen). Three microliters of cDNA was used as template for Q-PCR, performed as described previously, comparing expression of HDAC7 mRNA with that of rhesus β -actin (rhACTB) mRNA. The sequences of primer pairs and probes used are listed in Supplementary Table 1 (see www.liebertonline.com/hum).

Southern blot analysis

Southern blot analysis of peripheral blood DNA was performed as described (Seggewiss *et al.*, 2006) with the following modifications. Briefly, 5 μ g of genomic DNA was digested with *Hind*III (10 U/ μ g DNA) at 37°C for 16 hr. Hybridization and detection procedures were performed according to the protocols for the Amersham AlkPhos Di-

rect labeling and detection systems (GE Healthcare Life Sciences, Piscataway, NJ). A neomycin resistance (Neo^R) vector gene fragment was amplified by PCR, using the primers listed in Supplementary Table 1, and used as the Southern blot probe.

Bone marrow histopathological analysis

Bone marrow trephine biopsies were obtained and processed for morphologic evaluation according to standard procedures. Immunohistochemical studies with antibodies were performed using immunoperoxidase staining procedures and an automated immunostainer (Dako, Carpinteria, CA) according to the manufacturer's instructions. Images shown in Fig. 2 were obtained via digital microscopy with an Olympus BH-2 microscope (Olympus America, Melville, NY) equipped with a DPlan $\times$ 40/0.65 numeric aperture objective. Images were captured with an Olympus DP12 digital camera system and recorded on a 3.3V SmartMedia (SSFDC) card. Imaging software was Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

Statistical analysis

All statistical analyses were carried out with Excel software (Microsoft, Redmond, WA). A two-tailed Student *t* test was used to assess the significance of difference between groups of quantitative data, and the chi-square test was used to compare the frequency of HDAC7 insertions before and after busulfan treatment.

FIG. 1. Hematologic parameters over time in rhesus RQ2297. (A) Total white blood cell and granulocyte counts. (B) Hemoglobin concentration and platelet counts. Bu, busulfan; Gr, granulocytes; Hb, hemoglobin; Plt, platelets; WBC, white blood cells.

FIG. 2. Pathology of bone marrow biopsies 3.8 years after busulfan administration (A) and at the time of autopsy (4.8 years postbusulfan) (B). Arrow shows small, mononuclear megakaryocytes. H&E, hematoxylin- and eosin-stained sections; MPO, myeloperoxidase immunostaining. Original magnification, $\times 400$.

Results

Impact of busulfan on peripheral blood counts and bone marrow function

Animal RQ2297 was chosen for long-term follow-up. This animal was originally highly polyclonal, but with two MDS1/EVI insertions detected on several instances up to 2 years posttransplantation, before busulfan treatment (Calmels *et al.*, 2005) and further hematopoietic stress. As reported previously, after a single dose of busulfan (4 mg/kg) 2 years posttransplantation, counts fell transiently, and the number of contributing clones decreased by 60% and then recovered (Kuramoto *et al.*, 2004). To apply further hematopoietic stress, four monthly busulfan treatments were given

beginning 4 years posttransplantation, resulting in prolonged pancytopenia (Fig. 1A and B), which persisted for 15 weeks after the final dose followed by a slow recovery toward normal leukocyte counts and hemoglobin levels but persistent moderate thrombocytopenia. Peripheral blood DNA was obtained for analysis after normalization of counts and for full analysis beginning 2.5 years after the final dose of busulfan. Eight years and 9 months after transplantation and 4 years and 10 months after the final dose of busulfan the platelet count rapidly fell to less than $20,000/\mu\text{l}$, and the animal developed diffuse hemorrhage, necessitating euthanasia.

Bone marrow aspirate and biopsy performed 7 years and 9 months posttransplantation, 3 years and 10 months after the final busulfan treatment (Fig. 2A), at the time the animal had

FIG. 3. Impact of busulfan on long-term clonal diversity. Numbers of independent retroviral integration sites (RIS) were identified in granulocytes, T cells, and B cells by linear amplification-mediated polymerase chain reaction (LAM-PCR) and shotgun cloning, before and after busulfan treatment.

moderate thrombocytopenia but otherwise stable blood counts, showed no evidence of leukemia, but was hypocellular, with erythroid predominance, decreased atypical small megakaryocytes, and decreased left-shifted myeloid maturation, consistent with the development of a myelodysplastic process. Bone marrow aspirate showed no increase in blasts. Bone marrow biopsy at the time of autopsy revealed severe megakaryocytic depletion, and a markedly left-shifted myeloid lineage with approximately 20% myeloid blasts, almost no myeloid maturation, and a decreased myeloid-to-erythroid ratio consistent with ongoing bleeding and stimulation of erythropoiesis. The marrow findings were consistent with

progression to acute leukemia (Fig. 2B). On immunostaining, blasts were myeloperoxidase positive, and CD117 stained both the blasts and the expanded erythroid compartment.

Analysis of clonal diversity

The pattern of retroviral integration sites (RIS) in peripheral blood cell populations was evaluated by LAM-PCR and shotgun cloning. For each time point and cell source, LAM-PCR was performed on eight replicates of 100-ng samples. The number of independent RIS detected decreased markedly in granulocytes after busulfan treatment, compared with the number of RIS detected before all busulfan treatments (Fig. 3). The number of RIS detected in T and B cells decreased to a similar degree after busulfan treatment (Fig. 3). Thus, after repeated cycles of moderate-dose busulfan, clonal diversity decreased significantly, with no recovery of polyclonality by more than 4 years after the final dose of drug.

When the location of the insertion sites persisting after busulfan treatment was analyzed, the previously detected MDS1/EVI1 RIS were not found in granulocytes, T cells, or B cells by repeated LAM-PCR and shotgun cloning. Q-PCR using allele-specific primers also failed to detect these clones after busulfan treatment (data not shown). However, a clone containing an insertion in the HDAC7 gene locus became extremely dominant (Fig. 4A). The proviral integration was located at position 44873297 in forward orientation, within intron 1 of the HDAC7 gene (also called LOC706667) (Fig. 4B). In granulocytes this clone accounted for only 3% of shotgun-cloned LAM-PCR insertions before busulfan treat-

FIG. 4. Overrepresentation of the dominant clone. (A) Proportion of the HDAC7 insertion compared with total insertions retrieved by LAM-PCR and shotgun cloning over time. * $p < 0.01$, compared with the frequency of HDAC7 clone before Bu treatment. NA, not available. (B) Integration site of retroviral vectors within the HDAC7 gene locus. (C) HindIII sites within the HDAC7 gene containing RIS. Arrow indicates expected band resulting from HDAC7 vector insertion clone. (D) Southern blot analysis. Five micrograms of genomic DNA was digested with HindIII. A fragment of the Neo^R gene was used as a probe. Gr, granulocytes; MNC, mononuclear cells.

FIG. 5. Q-PCR analysis for vector copy number per cell in peripheral blood. Bu, busulfan; NA, not available.

ment, but increased to 71 and 94% of total shotgun-cloned insertions 2.5 and 2.8 years after busulfan treatment, respectively. This progression to marked clonal dominance was limited to granulocytes, with the HDAC7 RIS accounting for only 4.3 and 9% of RIS in T and B cells, respectively, 2.5 years after busulfan treatment (Fig. 4A).

Dominance of the HDAC7 clone was confirmed by Southern blot analysis. A 3.5-kb vector genomic fragment was predicted for the HDAC7 clone after digestion with *HindIII* and probing with Neo^R vector sequences, based on the next closest site in genomic DNA (Fig. 4C). Before busulfan treatment, no dominant clonal band was detected in granulocytes or MNCs (lymphocytes); however, 2.8 years after busulfan treatment, the predicted 3.5-kb band was present in granulocytes but not in primarily lymphoid MNCs (Fig. 4D).

To assess whether the dominance of the HDAC7 clone simply reflected loss of the majority of vector-containing clones, or actual expansion compared with remaining non-transduced marrow hematopoietic stem and progenitor cells, we analyzed the overall level of vector-containing cells contributing to hematopoiesis over time. The copy number was assessed by Q-PCR for Neo^R sequences. The Neo^R copy number in granulocytes increased significantly in granulocytes by 2.5 and 2.8 years after busulfan treatment, in contrast to the relatively stable overall copy number in T and B cells before and after busulfan treatment (Fig. 5). Just before death, the copy number in circulating granulocytes had decreased, but there was little ongoing myeloid maturation and the animal was neutropenic, perhaps suggesting that the abnormal clone was no longer able to

produce mature myeloid cells. At autopsy, the marrow mononuclear cell fraction, which contained 15–20% blasts assessed morphologically on cytospin, had a copy number of 0.11 per cell. Unfortunately, the blasts could not be sorted from the autopsy specimen, so we cannot unequivocally confirm that the leukemia was vector associated; however, there were no mature myeloid elements in the marrow, and the copy number in T and B cells was much lower, so it is probable that the vector signal originated from the blasts.

Impact of integration of retroviral vector on HDAC7 expression

Considering that the HDAC7 clone accounted for 94% of total insertions isolated by shotgun cloning, and accounted for a dominant band detected on Southern blot, we asked whether vector insertion upregulated HDAC7 expression. We compared HDAC7 mRNA expression via Q-RT-PCR on vector-containing G418-resistant RQ2297 bone marrow CFUs with that of control animal marrow CFUs and CFUs from RQ2297 grown without G418. Relative HDAC7 expression was significantly higher in the G418-selected CFUs from RQ2297 than in nonselected CFUs (Fig. 6).

Discussion

The ability to predict genotoxicity of retroviral vectors is of great importance for gene therapy applications. In clinical trials and large-animal studies, the long incubation period, generally years, required before dominance of abnormal clones is uncovered *in vivo* has limited our ability to anticipate the risks of retroviral vectors (Ott *et al.*, 2006; Seggewiss *et al.*, 2006; Hacein-Bey-Abina *et al.*, 2008). In this study, we used busulfan, an alkylating agent with pronounced activity against HSCs (Dunn, 1974; Abkowitz *et al.*, 1993; Hsieh *et al.*, 2007), to induce hematopoietic stress after gene therapy and determine whether dominant clones emerge.

Our previous study showed that a single dose of busulfan (4 mg/kg) administered 2 years after transplantation reduced hematopoietic clones contributing to granulocytes and lymphocytes by 60–80% in the first 3–4 months followed by complete recovery of polyclonal hematopoiesis (Kuramoto *et al.*, 2004). Two clones containing an RIS at the MDS1/EVI1 locus were detected at that time. The MDS1–EVI1 locus has been demonstrated as a “hot spot” for retroviral vector integration (Calmels *et al.*, 2005; Metais and Dunbar, 2008; Modlich *et al.*, 2008) and was one of three activating insertions in a gene therapy trial for chronic granulomatous disease (Ott *et al.*, 2006; Modlich *et al.*, 2008). Moreover, retroviral insertion in the EVI1 gene may induce leukemogenesis (Modlich *et al.*, 2008). Genetic alterations in the MDS1–EVI1 gene complex may result in abnormal expression of MDS1 and/or EVI1 gene expression and tumor formation (Lugthart *et al.*, 2008; Metais and Dunbar, 2008; Park *et al.*, 2008). In the current study, four additional doses of busulfan were given. Surprisingly, the two MDS1/EVI1 clones and most other clones previously detected disappeared by 2.5 years after busulfan administration. Instead, a new clone with an RIS in the HDAC7 gene was detected. This clone expanded and became highly dominant in myeloid cells. These data are consistent with progression to clo-

FIG. 6. Q-RT-PCR analysis for expression of HDAC7 mRNA in CFUs. * $p < 0.05$, ** $p < 0.01$.

nal hematopoiesis accelerated by initial clonal depletion and increased proliferative stress.

The finding of a marked increase in copy number in myeloid cells after hematopoietic stress imposed by chemotherapy indicates that caution is required in the interpretation of copy number increases after drug selection when vectors carrying drug resistance genes are used. Apparent *in vivo* drug "selection" for primitive progenitor cells containing the drug resistance gene may instead simply reflect dominance of clones with vector insertion sites activating genes that provide a proliferative or survival advantage in the setting of chemotherapy. Analysis of clonal insertion patterns after drug selection will be important for understanding the role of actual drug selection versus clonal selection, and thus far the limited data available suggest that at least with the *O*⁶-methylguanine-DNA methyltransferase (MGMT) drug selection strategy, polyclonality can be preserved after stable and successful selection in dogs, but possibly not in nonhuman primates (Neff *et al.*, 2005; Larochelle *et al.*, 2009).

Insertion of a retroviral vector into intron 1 of the HDAC7 gene resulted in overexpression of this gene, presumably by viral enhancer or promoter activity. HDACs catalyze the deacetylation of lysine residues in the N-terminal tails of core histones as well as of nonhistone proteins, and, together with histone acetylases, determine acetylation status of substrate proteins, particularly core histones (H2A, H2B, H3, and H4), and thereby regulate gene transcription (Grunstein, 1997; Shahbazian and Grunstein, 2007). In addition to the critical action on core histones, HDACs also exert other biological functions as part of a protein complex in which each member has its specialized functions via two mechanisms: direct repression of transcriptional factors or interaction with corepressors in nucleus, and cytoplasm-nucleus shuttling (Verdin *et al.*, 2003; Gallinari *et al.*, 2007). Hyperacetylated histones are generally found in transcriptionally active genes and hypoacetylated histones in transcriptionally silent regions (Grunstein, 1997; Shahbazian and Grunstein, 2007). Eighteen human HDACs have been identified, and grouped into 4 classes (I–IV) according to their homology to *Saccharomyces cerevisiae* HDACs (Gregoret *et al.*, 2004). HDAC7 is found in class II HDACs. Unlike the ubiquitous expression of class I HDACs, HDAC7 (and most class II HDACs) is largely expressed in limited tissue types, including heart, lungs (Kao *et al.*, 2000; Fischle *et al.*, 2001), thymocytes (Kasler and Verdin, 2007), as well as vascular endothelium (Chang *et al.*, 2006), osteoblasts (Jensen *et al.*, 2008), and hematopoietic organs (<http://www.genecards.org>), and thereby is involved in several physiological processes, including vascular integrity (Chang *et al.*, 2006; Mottet *et al.*, 2007; Martin *et al.*, 2008; Wang *et al.*, 2008), thymocyte development (Parra *et al.*, 2007; Martin *et al.*, 2008), osteoblast maturation (Jensen *et al.*, 2008), B lymphocyte function (Matthews *et al.*, 2006), and muscle differentiation (Dressel *et al.*, 2001). HDAC7 overexpression has been documented in pancreatic adenocarcinomas (Ouaissi *et al.*, 2008) but the anticancer activity of HDAC inhibitors (HDACi) has been demonstrated clinically in multiple types of cancers, including hematopoietic malignancies (Bruserud *et al.*, 2006; Kouraklis, 2009). The U.S. Food and Drug Administration-approved HDACi vorinostat, and another HDACi, depsipeptide, were shown to selectively suppress HDAC7 expression with little or no effect

on the expression of other class I or class II HDACs in 14 cell lines, including normal, immortalized, genetically transformed, and human cancer-derived cell lines (Dokmanovic *et al.*, 2007), indicating an association of HDAC7 overexpression with the cancer phenotype.

The preferential survival or proliferation of the HDAC7 clone in myeloid cells (granulocytes) but not lymphoid cells suggests a possible role of HDAC7 in myeloid fate choice. One study has shown that mouse myocyte enhancer factor-2c (Mef2c), a member of the Mef2 family of MADS-box transcription factors with known function in skeletal muscle formation, is required to promote lymphoid and suppress myeloid differentiation of multipotent hematopoietic progenitor cells (Stehling-Sun *et al.*, 2009). Because the N-terminal 121 amino acids of Hdac7, constituting repression domain-1, are known to directly interact with and repress Mef2 (Mef2-A, -C, and -D) (Dressel *et al.*, 2001), Hdac7 may thus promote myeloid differentiation and repress lymphoid differentiation.

In conclusion, our data indicate that induction of hematopoietic stress in a large-animal model after gene therapy is a feasible approach to predict the genotoxic potential of retroviral vectors targeting HSCs, and assess the safety of administering myelotoxic chemotherapy in patients previously engrafted with vector-containing cells. The HDAC7 RIS clone became markedly dominant and was responsible for the expansion of vector-containing cells, and this may be linked to an impact of overexpression of the HDAC7 gene on clone survival, or conversely may simply have resulted from stochastic events due to overall depletion of HSCs.

Author Disclosure Statement

No competing financial interest exists.

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