Molecular Therapy
Commentary

Gene Transfer to HSCs: Finding the Leukemia in Murine Leukemia Viruses

Kenneth Cornetta[1]

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The modification of hematopoietic stem cells (HSCs) with gammaretroviral vectors (here referred to as retroviral vectors) has been associated with the development of hematologic malignancies in a number of clinical trials. Based on these serious adverse events, the field has moved from using retroviral to lentiviral vectors for the modification of HSCs. To date, malignant transformation has not been reported in clinical trials using lentiviral vectors. In this issue of *Molecular Therapy*, Espinoza et al.[1] document the development of a lethal myelodysplastic/myeloproliferative neoplasm in a rhesus monkey following the transplantation of lentivirally transduced cells. While the backbone of the vector was derived from HIV-1, the long-terminal repeats (LTRs) were derived from the murine stem cell virus, a gammaretrovirus. The malignant clone had 9 vector insertions and the altered expression of *PLAG1* appears to be a major driver of the malignant phenotype. Insertions near other candidate players were found, including *KITLG*.

By using a hybrid vector, do the important findings noted in this paper change the risk of lentiviral gene transfer? The majority of retroviral vectors are based on a subset of gammaretroviruses associated with hematologic malignancies in mice (commonly referred to as murine leukemia viruses). As opposed to acutely transforming retroviruses that contain oncogenes within their genome and which rapidly cause cancer in mice, these slowly transforming retroviruses mediate insertional oncogenesis by altering the expression of endogenous proto-oncogenes with cancer arising months after exposure. Adult mice are usually protected from exposure but newborn mice are susceptible, presumably due to their immuno-compromised state. The promoters embedded in the viral LTRs can alter gene splicing and the enhancer can alter the level of gene expression.[2] There also appears to be a predilection for altering specific proto-oncogenes. In the case of the Moloney murine leukemia virus (MLV), mice that develop lymphoma frequently have insertions near *pim-1*. Additional genetic alterations are required for malignancy; *pim-1* transgenic mice do not have an increased risk of malignancy, but when exposed to MLV they rapidly develop lymphoma with insertions near *c-myc* and *n-myc*.[3] Most tumors have multiple viral integrations consistent with the requirement for alterations of multiple oncogenes and tumor suppressor genes. The enhancer appears to be a major determinant in the type of malignancy that develops. Replacing the Moloney MLV (MMLV) 78 base-pair enhancer with that of the Friend erythroleukemia virus switches the predominant malignancy from lymphoma to erythroleukemia.[4] Not all strains of mice will develop lymphoma, and environmental factors can alter susceptibility. For example, adult mice exposed to MMLV and pristine, a saturated terpenoid alkane known to induce leukocytosis in rodents, develop promonocytic leukemia with virus integrations within the *c-myb* oncogene.[5]

Early studies suggested that replication competent retroviruses (RCRs) that arose from recombination in early vector packaging systems posed a potential significant threat to humans. Vector-associated RCRs caused lymphoma in mice similar to that caused by the parent virus.[6] Most concerning is that lymphoma developed in an immuno-compromised monkey that was inadvertently exposed to an RCR in a bone marrow transplant experiment.[7] The above findings generated specific guidance by the US Food and Drug Administration (FDA) regarding screening of all gene therapy products for RCRs.[8]

The hope that using RCR-free retroviral vectors would prevent insertional mutagenesis events has not been borne out. In 2002, the first report of leukemia was noted in a trial of X-linked severe combined immunodeficiency (SCID), with additional malignancies reported in trials for chronic granulomatous disease (CGD) and Wiskott-Aldrich Syndrome (WAS).[9] Similar to what is seen in mice, there is a propensity for targeting a specific oncogene in a specific cell lineage (in the majority of X-linked SCID it was *LMO*-2 in T cells), but a number of factors appear to be important in disease pathogenesis. For example, X-linked SCID patients lack a functional common gamma chain required for interleukin signaling, resulting in a maturation arrest. This leads to a large pool of relatively undifferentiated lymphoid cells within the marrow that may be particularly sensitive targets to insertional mutagenesis. Interestingly, insertional mutagenesis has not been seen in the treatment of another SCID, adenosine deaminase deficiency (ADA). These children have remained free of malignancy and a retroviral vector expressing ADA has been approved for clinical use in Europe. The role of ADA as a housekeeping gene versus other clinical trials in which the transgene was involved in growth regulation may be a partial explanation for the difference in leukemia occurrence. The various trials also used different backbones with different promoter-enhancers which may also impact cancer risk. Understanding and monitoring for insertional mutagenesis continues to be an area of active investigation.[10] Discussing all issues are beyond the

[1]Medical and Molecular Genetics, Indiana University School of Medicine, Walther Hall 980 W. Walnut Street, R3 C649, Indianapolis, IN 46202-5121, USA

Correspondence: Kenneth Cornetta, Medical and Molecular Genetics, Indiana University School of Medicine, Walther Hall 980 W. Walnut Street, R3 C649, Indianapolis, IN 46202-5121, USA.
E-mail: kcornett@iu.edu

scope of a commentary but based on the above issues, newer HSC trials have turned to lentiviral vectors.

Lentiviral vectors have a clear advantage in gene transfer efficiency in HSCs and other quiescent cells. Moreover, HIV-1 is not directly associated with malignant transformation. Safety comparisons of insertional mutagenesis *in vitro* and *in vivo* have consistently favored lentiviral vectors. There have been no malignancy associated with human lentiviral trials, including trials for SCID, CGD and WAS. This is not to say that lentiviral vectors do not have the potential to alter cell growth. The first clinical example occurred in the treatment of thalassemia where vector insertion generated a truncated RNA in HMGA2 that was insensitive to let-7 microRNA degradation.[11] As a result, there was clonal expansion of red cell precursors and the patient required phlebotomy. More recently, clonal expansion in a T cell modified with a chimeric antigen receptor (CAR-T) protocol resulted from insertional disruption of one methylcystosine dioxygenase TET2 allele in an individual with a hypomorphic mutation in the second TET2 allele.[12] To date, these individuals have not developed malignancy.

So does the finding in this monkey alter our risk assessment of lentiviral vectors? The finding of 9 insertions in the malignant clone does provide support for limiting the number of vector copies per cell to the minimum required to attain a therapeutic benefit. This likely applies to all integrating vectors. As to the major mediator of hematologic malignancy in this animal, the retroviral LTR is the most likely causative factor. Supporting this contention are the known biology of gammaretroviruses, similarities to the human clinical experience with oncogenesis and retroviral vectors, and *in vitro* studies showing that retroviral promoter-enhancers in lentiviral vectors increase the risk of insertional mutagenesis.[13] This has led the authors to conclude that, when possible, strong promoters should be avoided in lentiviral vectors. While this may be the case, the retroviral enhancer could be the major element that should be avoided. The observation in this one animal does not allow us to draw conclusions regarding the risk of lentiviral vectors without MLV regulatory sequences. Furthermore, there will likely be a continued debate on the advisability of using retroviral vectors in the setting of differentiated cell types, particularly for CAR-T and T cell receptor trials where malignancies have not been reported. Overall, this study supports avoiding retroviral promoter-enhancers for any vector used to alter stem or progenitor cells, thereby heeding the warning of “leukemia” in “murine leukemia virus.”

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