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“To condition” or not “to condition”, this is the question:

The evolution of non-myeloablative conditions for transplantation

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Abstract

In 1985, Eugene Cronkite published in *Experimental Hematology* data indicating that five consecutive “transfusions” of high numbers of marrow cells significantly increase the number of donor-derived cells detected by day 10 spleen colony forming assay, the most primitive hematopoietic cells detectable at that time, present in the host for as long as two months post transfusion (1). These data provided the first evidence that donor hematopoietic stem cells (HSC) may at least persist in vivo for some time in recipients that were transfused and not transplanted, i.e. not subjected to treatments that deplete their marrow niches of endogenous HSC. The limited technology available at the time prevented Dr. Cronkite to pursue this observation into the development of non-myeloablative transplantation procedures and his experiment as well as the term “bone marrow transfusion” have since long been forgotten. In recent years, the scientific need to clarify HSC functions in non-stressed hosts and the clinical need to develop transplantation procedures with levels of morbidity/mortality acceptable to cure inherited hematological disorders have inspired the search for non-myeloablative transplantation procedures including methods to “outcompete” endogenous host HSC such as those pioneered by Dr. Cronkite's experiments using high transfusion doses. This review will describe the technical progress since Dr. Cronkite's insightful work that has finally provided a path to the clinic.

Keywords

Hematopoietic stem cells; cKIT; hematopoietic stem cell niches; myeloablation; bone marrow transplantation; competitive repopulation assays

Introduction

The founding fathers of *Experimental Hematology* were all highly dedicated, enthusiastic scientists with a strong belief that collegiality, international collaboration and extreme

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dedication were necessary to move the field forward. For me, and many others, Eugene Cronkite was one such pioneer. When I was a post-doctoral fellow, Gene was the Editor-in-Chief of Experimental Hematology a journal that was considered at that time the place in which everybody that was somebody in the stem cell biology field wished to publish her/his research.

In 1999, after many years of training in several international laboratories, I finally sent my first paper on hematopoietic stem cells (HSC) to Experimental Hematology and Dr. Ronald Hoffman, the editor at that time, was kind enough to accept it (2). This fact made me feel like I was finally been recognized as a “STEM CELL” expert and accepted in the inner circle of the “Few” who knew. And then it came an even greater surprise. On March 3rd 2001, a few years after the publication of my article, “Gene” wrote me a letter (Figure 1) congratulating me for being finally able to demonstrate what he had hypothesized in one of his papers in 1985 (1). The kindness with which Gene, who was known for being harsh, expressed his compliments made me feel more than anything else that the years of hard training and working were finally paying off and that I was being recognized as a scientist who had something to add to the field. The fact that the letter was written only few months before his death that sadly occurred on June 23rd of the same year, proves that Gene was one of the lucky ones who remain scientifically active until the very last.

But what was all the “buzz” that made Gene so excited about his article and mine? The reason is that the two articles address a central question of hematopoietic stem cell (HSC) therapy which has surfaced again in the scientific and clinical arena in recent years. The question is whether conditioning is necessary for exogenous HSC to engraft. This review will summarize how the formulation of this question evolved over time, the technical progress that allowed it to be addressed and how this knowledge has been applied to the development of innovative HSC therapies for inherited hematopoietic disorders.

The scientific question underscored by Dr. Cronkite's experiment

The question raised by Dr. Cronkite's experiment is whether HSC therapies should be considered transplantation or transfusions. In other words: Is it necessary first to deplete the bone marrow of endogenous HSC by myeloablative procedures for donor HSC to engraft (here defined as “bone marrow transplantation”)? Or is it possible to achieve donor HSC engraftment in unconditioned hosts (here defined as “bone marrow transfusion”).

Both hypotheses are based on the HSC-niche model of hematopoiesis in which appropriate levels of blood cells are produced by a defined number of HSC lodged in supportive niches which instruct their fate (3). The term “niche”, originally introduced by Raymond Schofield in 1978 (4), includes all the microenvironmental cues (accessory cells, growth factors and elements of the extracellular matrix) that support HSC and progeny function. The complex interactions occurring between HSC and their natural microenvironment were recently visualized by in vivo imaging technologies (5). Bone marrow transplantation is based on a “tenure-track” model of HSC-niche interaction: The bone marrow contains fixed numbers of niches permanently occupied by fixed numbers of HSC (Figure 2A). Therefore, to allow donor HSC to colonize (to be employed) the niches (the jobs) of the host, the host HSC must

be first eradicated (fired). Bone marrow transfusion is instead based on a “musical chair game” model: HSC-niche interaction is a dynamic process during which HSC continuously leave “one niche” to colonize a new one. Therefore at any given time, the bone marrow of the host contains “free niches” that may be colonized by donor HSC (Figure 2B). In this game of musical chairs, donor HSC with a proliferative or numerical advantage over the host ones will inevitably compete them out of their niches becoming the dominant HSC population that generates all the host's blood cells.

In 1985, Gene provided evidence that repeated transfusions of great numbers (200 million each time) of marrow cells into isogenic non-irradiated mice increase by 132% the numbers of HSC (determined at that time as cells capable to give rise to hematopoietic colonies in the spleen of the recipient 10 days after transplantation, day 10 CFU-S) of the host for up to 2 months (1). In this experiment, the advantage was provided by the massive dosage of HSC injected. In fact, the mice received for 5 doses of marrow cells each one >2000-fold greater than the minimal dose necessary to observe engraftment in these recipients under ablative conditioning (approximately $1-2 \times 10^5$ bone marrow cells). In spite of its originality, this study has obvious limitations: day 10 CFU-S correspond to short-term HSC and not to true “HSC” (6); donor and host cells were not marked; there was no proof that donor HSC had contributed to hematopoiesis of the recipients; and, last but not least, the size of the graft used was far greater than that which will ever be available for clinical use.

In the 1980's, the clinical need that drove the development of HSC therapies was to cure leukemia in spite of limited understanding on the immunology of self-recognition. In this context, conditioning (radiation +/- chemotherapy) served several functions including depletion of host HSC and, most importantly, leukemic cells, as well as depletion of immune cells and thereby eradicating the underlying disease and limiting the incidence of the graft versus host disease (GvHD) which is responsible for most of the post-treatment morbidity. For these clinical reasons, in spite of the important theoretical implications of Gene's experiment, transfusion was not further pursued as a means to investigate HSC functions for long time.

The development of “competitive” repopulation assays to identify murine HSC

In the early 1990's, the flow cytometry tools necessary to identify “true” HSC became available inspiring studies aimed at their prospective isolation (7-9) and more precise detection by quantitative and functional transplantation assay. In 1993, Harrison et al developed a competitive repopulation assay suited to measure the functions of limited numbers of HSC (10). In this assay, limited numbers of “HSC” are transplanted with large numbers of short-term HSC (and of hematopoietic progenitor cells, HPC) into irradiated recipients. The long-term and short-term HSC/HPC are labeled with distinctive markers, represented by either an antigen, a fluorescent-tagged protein or a genetic code, that allows the identification of the transplanted population giving rise to the host hematopoietic cells. Short-term HSC/HPC exert radio-protective functions by contributing to the host hematopoiesis in the short run. “True” HSC transplanted at limited dilution, or even as single

cells, may therefore first expand themselves and then generate HPC in numbers sufficient for ready detection in a sustained long term manner. This assay has guided the first prospective isolations of murine HSC (11-13), and is still in use (14-16).

Development of “competitive repopulation assays” in which limited numbers of donor HSC compete the endogenous HSC of non-myeloablated hosts

By the late 1980's, bone marrow transplantation had become firmly established in clinical practice for the treatment of leukemias (17). Based on the clinical success in this oncology setting and on a proof-of-principle obtained in 1978 in Wiskott-Aldrich syndrome (18), Don Thomas hypothesized that transplantation with bone marrow containing normal HSC could cure almost all of the inherited hematologic diseases (19). In fact, in theory bone marrow transplantation is a form of gene therapy in which the patient's own HSC carrying the genetic lesion are replaced with those from a healthy donor. However, the clinical development of this approach was hampered by the debate over whether the levels of morbidity and mortality associated with conditioning regimens developed for treatments targeting leukemia would be acceptable in the context of patients affected by genetic disorders, such as hemoglobinopathies, who are often children and treatable by alternative, but not curative and often cumbersome, therapies. Therefore, cure of inherited hematopoietic disorders by bone marrow transplantation was and is still practiced only in a few centers (20).

The competitive repopulation assay developed by Harrison (4), in which low numbers of HSC, thanks to their greater proliferation potential, eventually compete with and replace the by far greater numbers of co-transplanted short-term HSC/HPC suggested to us that an “appropriate” advantage would also allow low numbers of donor HSC to compete with the endogenous HSC/HPC supporting their engraftment into non-myeloablated hosts. The development of this “competitive repopulation assay” in non-myeloablated hosts was inspired by its possible implications for gene therapy of inherited hematopoietic disorders. We were hoping that, by developing this assay, we would gain insights on how to provide to the few in vitro genetically repaired HSC, given the low gene transfer efficiencies at that time (21), a proliferation advantage that would allow them to outcompete those non-repaired when the autologous graft would be transplanted into the patients. Ideally, this proliferation advantage would also allow the repaired HSC to compete against the endogenous non-repaired HSC making it possible to administer gene manipulated grafts to non-myeloablated patients and thus skip the conditioning step responsible for the morbidity experienced by transplanted patients that is otherwise dispensable in autologous settings that do not pose immunological threats.

Because of our long lasting interest in inherited disorders of the erythroid system (22), we turned our attention to mouse models of congenic macrocytic anemia due to defective proliferation of erythroid progenitors. It was long known that mice carrying mutations in the *white* (*W*) and *steel* (*S^l*) locus manifest similar phenotypes which include macrocytic anemia (23). The level of anemia ranges from fetal lethality (23) to mild with normal adult

life-span (24) depending on the severity of the mutation. The recognition that the hematopoietic phenotype of *W* mice is transmissible and curable by bone marrow transplantation while that of *Sl* mice is not had led to the hypothesis that the two mutations affect the relationship between the HSC (*W*) and its niche (*Sl*). Strong support for this hypothesis came finally with the discovery that *Sl* encodes the gene for the HSC-specific growth factor stem cell factor (SCF, also known as Sl-factor, KIT ligand and mast cell growth factor) (25) while *W* encodes cKIT, the receptor for SCF (26) expressed on the HSC surface (27). Further studies identified that SCF does not sustain HSC self-replication (28-30) but is necessary and sufficient for their survival (28) and commitment to multipotent HPC (30,31) and drives in cooperation with other factors (the SDF-1/CXCR4 axis and integrins) homing of HSC in the marrow (32,33).

In 1991, Harrison and Astle had demonstrated that bone marrow from healthy syngeneic mice sustains robust levels of lymphoid and erythroid reconstitution when transfused into non-conditioned syngeneic mice carrying the double *W41J/W41J* or *W41J/W39J* mutation (34), providing proof-of-principle that healthy HSC have a proliferative advantage over endogenous cells with impaired cKIT signaling. We decided to further pursue this hypothesis using as recipients mice carrying the double *W/W^v* mutation which had been developed by Boggs et al as a model to quantify the proliferative potential of HSC and to identify those capable to contribute to long term hematopoiesis (35). The *W* allele is a partial deletion of *cKit* that encodes a protein lacking the SCF binding domain while the *W^v* allele contains a point mutation (C to T) at position 2007 that abolishes the catalytic activity of the first two tyrosine kinase domains of the protein. Although as a consequence the ability of *W/W^v* HSC to respond to SCF is 25% lower than normal, hematopoiesis in these animals is partially normal because of compensation by the high levels of SCF produced in their marrow microenvironment (Joan Egrie, personal communications and Figure 2B). We reasoned that such high SCF levels would provide to normal HSC a proliferative advantage over the *W/W^v* ones allowing them to become the dominant HSC population even if the recipients were not myeloablated. The development of PCR-based genotyping of the *W^v* allele that exploits the unique “restriction site” generated by this mutation (36) and the use of donor and host mice expressing unique Hb markers (2) allowed us to determine the levels of donor-derived hematopoiesis in non-myeloablated *W/W^v* mice transplanted with as few as 250-500 purified HSC. In these experiments, HSC were purified with a flow cytometry method which exploited the antigenic and metabolic profiling of the cells (7) achieving levels of purity comparable to that obtained with other methods available at the time (37). The results indicated that as few as 250 HSC (only 10% of which likely seeded in the marrow) positively competed the total endogenous *W/W^v* HSC population (approximately 2×10^5 cells). In fact three months post-transfusion the recipient *W/W^v* mice expressed 100% of donor-derived red blood cells and 30% of donor derived white blood cells in their circulation. In addition, one year and half after transfusion, the bone marrow of the recipient mice contained as many as 75,000 donor HSC (i.e. one quarter of their HSC were donor derived), providing genetic evidence supporting the original hypothesis by Dr. Cronkite that donor HSC may engraft and self-renew in non-conditioned hosts (1). Last but not least, *W/W^v* mice were cured of their anemia for all their life.

In a following study, we used this approach to identify a Sca1^{pos} HSC population, low numbers of which cured the anemia of non-myeloablated *W/W^v* mice for all their life without generating detectable numbers of transplantable HSC in their bone marrow, providing the first evidence for the existence of two long-term HSC populations, one with unlimited and the other one with limited self-replication potential (38). These results were confirmed in 2014 by Claudia Waskow et al (39) who identified that the two populations are represented, respectively by the resting and cycling HSC populations originally identified by Spangrude and Johnson (40) and that, consistent with the role of SCF in HSC self-replication and commitment discussed above (28-31), the two populations may be discriminated on the basis of the levels of cKIT expression (cKIT^{low} and cKIT^{high}, respectively).

In an attempt to translate these results into a clinically translatable approach we demonstrated that human CD34^{pos} cells expressing reduced levels of cKIT by antisense technology generate long term cultures with a W phenotype. In other words, they have impaired erythroid differentiation but are still capable to generate normal levels of myeloid cells (41). The recognition that *cKIT* was originally discovered as the cellular homolog of the gene transduced by a retrovirus inducing leukemia in cats (42) and emerging evidences that in humans gain-of-function mutations of *cKIT* are associated with tumors, including some forms of leukemia (43-46), discouraged translating these observations into the development of strategies that would provide selective advantages to genetically manipulated human CD34^{pos} cells. However, our original thoughts were recently revitalized by an experiment showing that simian immunodeficiency virus-based vectors including the gene encoding SCF allow long term engraftment of gene marked autologous HSC into macaques subjected to a low conditioning regimen (47). Whether these animals, because of their hyperactive SCF/cKIT axis, will develop leukemia overtime is still unknown.

The combination of a proliferation advantage of donor HSC with ablation of the host immune system allows engraftment without conditioning across histo-compatibility barriers

In 1993, using sensitive molecular techniques allowing discrimination of donor from recipient cells on the basis of the presence of the Y chromosome, Dr. Quesenberry confirmed the original observation by Dr. Cronkite (1) by showing that transfusion of great numbers of male donor cells (5 consecutive daily administration of 10×10^6 bone marrow cells) sustain long term hematopoiesis in non-myelablated syngenic female recipients (48). This result was obtained with host/recipient pairs of multiple strains (Balb/c, BDF1 or CBA-J). Although the clinical translation of this experiment is limited by the fact that cell number alone is not a viable means to provide “advantage” to donor HSC since the size of the grafts available for HSC therapy is often limited and its syngeneic setting limits extrapolation to autologous HSC therapies, this paper was received with great interest (49) and paved the way to additional experiments that explored competitive repopulation assays without the toxicity of myeloablation.

At the beginning of this century, greater understanding of the immune system mediating GvHD provided clues to ways to manipulate the host that would allow “competitive repopulation assays” in non-myeloablated mismatched hosts with reasonable numbers of donor HSC. The strategies investigated at the time were represented either by induction of tolerance of the immune system by pretreating the host with very low numbers of highly purified donor HSC (50) or by devising treatments that would selectively deplete the host of its immune cells. Dr. Quesenberry provided numerous contributions to the development of this second strategy by demonstrating first the possibility to conduct competitive repopulation assays with minimally myeloablated recipients (51) and then by performing experiments indicating that infusion with an antibody blocking the immune-specific CD40 signaling, either in combination with low dose irradiation and induction of tolerance strategies (52) or even by itself (53) allows engraftment of repeated infusions of H2-mismatched bone marrow cells in non-myeloablated hosts. More recently, Waskow et al demonstrated that recipients genetically depleted of their immune cells and harboring proliferation-disadvantaged W/W^v HSC can be used for competitive repopulation assays with numbers of mismatched HSC which may be available in the clinic (54).

Due to the high costs of recombinant human SCF and on the basis of word-of-mouth information that murine SCF is as effective in supporting the growth in vitro of human HPC as human SCF, recently proved by our group (55), numerous investigators stimulated human cultures with media conditioned by a murine fibroblastic cell line molecularly engineered to express murine SCF originally established by Normal Iscove (M von Lindern, personal communication). This knowledge may have inspired in 1998 Geiger et al to demonstrate that human adult HSC injected into murine blastocysts carrying the W/W^v mutation compete with the murine HSC generated in the immune depleted environment of the embryo contributing to its hematopoiesis (56). Based on these premises, Waskow et al very recently tested the hypothesis that human HSC may cross species-barrier and engraft non-conditioned W/W^v mice carrying genetic deletions that impair their immune system (57) providing evidence of the importance of cKIT for engraftment across species and establishing a novel non-stressed animal model to investigate human hematopoiesis.

HSC transfusion in the clinic at last: Mini-transplants to cure thalassemia and other inherited hemoglobinopathies

These murine experiments had demonstrated that transfusion is suitable to replace host hematopoiesis with cells from an allogenic donor provided that 1) the donor HSC have an advantage (that may be represented by either greater numbers, a greater response to a microenvironmental cue or even a greater survival of their progeny) over the host ones and 2) the recipients are depleted of their immune cells (either by induction of tolerance, deletion of genes that regulated their maturation or treatment with cytotoxic specific antibodies). Although in the context of HSC therapy of inherited disorders, healthy HSC have by definition an advantage over the diseased ones, viable methods to deplete immune cells in humans were still lacking. A clinical breakthrough was represented by the development of the anti-CD52 antibody alemtuzumab. This antibody specifically depletes human lymphocytes and has been successfully used to treat GvHD and chronic lymphocytic

leukemia (58). In a recent trial, Dr. Rondelli at the University of Illinois at Chicago coordinated over the course of 3 years the transplantation of 13 adult patients with sickle cell disease with stem cells mobilized in the peripheral blood with granulocyte-colony stimulating factor from matched-related donors who in two cases were ABO incompatible (59). The patients were non-conditioned but received alemtuzumab plus low dose total body irradiation to reduce their immune cells. They also received sirolimus as immunosuppression post-transfusion. One year post-transfusion, all 12 of the patients who remained sirolimus compliant expressed stable mixed-donor chimerism and were cured with normal hemoglobin levels, improved cardiopulmonary and quality of life parameters (59).

These results were published at a time when there was great excitement surrounding the success of retroviral strategies suited to repair clinically significant numbers of autologous HSC for gene therapy of hemoglobinopathies (60) and of gene editing methodologies holding greater promise of success than classical gene therapy strategies (61). However, gene therapy, as well as traditional bone marrow transplantation, are expensive procedures requiring sophisticated equipment and may be performed mainly in developed countries. By contrast, hemoglobinopathies are mostly distributed in third world countries (62) where the allocation of the resources necessary to develop these therapies is challenging. This problem is only partially solved by the international programs that allow the transfer of patients from underdeveloped to developed countries to be cured such as the Mediterranean Transplantation Program that allows hemoglobinopathy patients from the Middle East to be transplanted in Italy (63). For obvious reasons, the number of patients that may benefit from these programs is limited. By contrast, mini-transplants, such as those finally established by Dr. Rondelli (59) are technically simple (they involve a transfusion) and both the harvesting of the graft, by mobilization of a suited relative, and its administration may be performed in day hospital in non-specialized centers. Therefore, this strategy finally opens the way for treatment of large numbers of hemoglobinopathy patients in the country where they live.

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Highlights

- In 1985, Dr. Cronkite published that HSC may engraft transfused mice
- Since then, transfusion has represented the “holy grail” of HSC therapies
- Finally this year, 12 Sickle Cell Anemia patients were cured by HSC transfusions

Figure 1. Left: Copy of Dr. Cronkite's letter addressed to Dr. Migliaccio commenting her manuscript published in *Experimental Hematology* in 1999 (2). Right: Front page of Dr. Cronkite's paper in *Experimental Hematology* discussed in this review (1).

Figure 2.

Scheme of the dynamics of donor HSC interactions with host niches in myeloablated (A, transplantation) and non-myeloablated (B, transfusion) recipients. In a transplantation setting, donor HSC (indicated by $+/+$) interact with niches that had been previously emptied by the conditioning regimens (radiation plus chemotherapy). In a transfusion setting, donor HSC compete with the host HSC (indicated by W/W^v) which have left their niche to colonize new ones. If donor HSC have an advantage over the host ones (that may be indicated by either greater numbers, or a more ready response to microenvironmental cues or by longer survival of their healthy progeny) they will compete the host HSC becoming the dominant population that generates all the host's blood cells. The symbols indicate the relative distribution of SCF bound to the membrane of niche cells and of cKIT on the membrane of HSC of healthy (transplantation) and W/W^v (transfusion) microenvironments and of healthy (donor HSC for both transplantation and transfusion) and W/W^v (transfusion) HSC.