National Institute of Allergy and Infectious Diseases

BIODEFENSE WORKSHOP SUMMARY
HEMATOPOIETIC STEM CELL EXPANSION AND IMMUNE RECONSTITUTION

May 17, 2004

Bethesda Marriott Hotel
Bethesda, Maryland

Abstract

NIAID convened a workshop on May 17, 2004, on hematopoietic stem cell (HSC) expansion and immune reconstitution to evaluate our present knowledge of bone marrow transplantation as well as the future therapeutic value of *ex vivo* expanded cells. The event was one of several workshops organized by NIAID to determine gaps in research and development, and to identify possibilities to develop medical countermeasures against radiation resulting from terrorist attack or accidental exposure to radiation or nuclear materials.

Introduction

Stem cells are cells that maintain self-renewal activity and can also differentiate into multiple cell types. Embryonic stem cells (ES) derived from the inner cell mass of the developing blastocyst are able to expand indefinitely *in vitro* and differentiate through appropriate stimuli into cells of all three germ layers, endoderm, ectoderm, and mesoderm. Other stem cells, such as HSCs, are lineage specific and may be isolated from various tissues in fetal and adult animals. Bone marrow HSCs self-renew and differentiate to produce all mature blood cell types. Today, the isolation and enrichment of human and mouse HSC are possible because of the availability of reagents, such as specific monoclonal antibodies against cell surface markers.

Growing terrorist threats by radiological means have emphasized the need to develop programs supporting basic and applied research that will result in protective and therapeutic modalities for immediate treatment of at risk populations. It is well established that bone marrow (BM) failure is one of the major causes of death after high-

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Dr. Leif Carlsson, Umea University; Dr. Michael Clarke, University of Michigan; Dr. Willem Fibbe, Universiteit Leiden; Dr. R. Keith Humphries, British Columbia Cancer Research Center; Dr. Peter Lansdorp, British Columbia Cancer Research Center; Dr. Tucker LeBien, University of Minnesota Cancer Center; Dr. Bruno Peault, University of Pittsburgh School of Medicine; Dr. Shahin Rafii, Cornell University Medical College; Dr. Irving Weissman, Stanford University School of Medicine; and Dr. Juan Carlos Zuniga-Pflucker, University of Toronto.

dose total body irradiation, and it has been known for more than fifty years that bone marrow cells can rescue animals irradiated with a lethal dose. Although clonogenic mixed colony formation in the spleen after BM transplantation provided evidence for the involvement of stem cells in rescue from radiation damage, the cell population responsible for the rescue was not known until recent years.

This NIAID workshop, entitled "Hematopoietic Stem Cell Expansion and Immune Reconstitution", focused on the biology of HSC with specific emphasis on a) the ontogeny of HSC, b) factors mediating the mobilization of HSC to the circulation, c) factors involved in HSC expansion, d) HSC differentiation to generate T and B cells, and e) factors maintaining the pluripotential status of the stem cells. The goal of the workshop was to identify gaps in research and opportunities in both basic science and clinical research to create medical countermeasures against radiation threats.

HSC expansion and factors involved in self-renewal

HSCs have the capacity to self-renew and to differentiate into different blood cell lineages to preserve the hemato-lymphoid system. One of the major efforts in stem cell biology is to elucidate the molecular mechanisms that govern the self-renewal function of stem cells. The long-term goal is to develop culture systems to expand HSC *in vitro* for clinical purposes. Several groups have identified pathways and proteins that are involved in this process. However, the relationships among these molecules, their interplay, and the identification of a possible "master coordinator" gene for expansion have not yet been identified. It was recently reported that adult and fetal mouse and adult human HSCs express the proto-oncogene bmi-1, which is essential for the generation of self-renewing adult HSCs. Although the number of HSCs in the liver of bmi-1 -/- fetal mice is normal, they are markedly reduced in the postnatal stage and there is no detectable self-renewal of adult HSCs in these mice (Dr. Michael Clarke). Transcriptional profiling has shown that the expression of stem cell associated genes, such as cell survival genes, transcription factors, and genes modulating proliferation, including $p16^{lnk4a}$ and $p19^{Arf}$, are altered in the bmi-1 -/- mice bone marrow cells. Expression of $p16^{lnk4a}$ and $p19^{Arf}$ in normal adult and fetal mouse and adult human HSCs results in proliferative arrest and p53-dependent cell death, respectively. Mounting evidence indicates that bmi-1 plays a key role in regulating the proliferative activity of normal and leukemic stem cells and primitive progenitor cells, and that common genetic determinants may regulate these cell types.

In mice, the number of HSCs increases in the liver during embryonic development, suggesting that genes regulating the self-renewal process are expressed in the fetal liver. Dr. Leif Carlsson found that the LIM-homeobox gene, *Lhx2,* is expressed in fetal liver during active hematopoiesis. Mice with a homozygous mutation in *Lhx2* die *in utero* due to a severe anemia. The mechanism of HSC expansion mediated by *Lhx2* is not known, however it has been demonstrated that embryonic stem (ES) cells or stem cells from adult bone marrow ectopically expressing *Lhx2* gene can generate HSC-like cell lines. These HSC-like cell lines share several characteristics with normal HSCs, including response to specific cytokines/growth factors, expression of transcription factors, patterns of cell surface marker expression and interactions with stromal cells. The HSC-like cell lines

established from adult bone marrow have some radioprotective capacity and can generate erythroid, myeloid, and lymphoid cells following transplantation into lethally irradiated recipient mice. The *Lhx2*-expressing HSC-like cells self-renew by a cell non-autonomous mechanism. This suggests that *Lhx2* regulates genes that are responsible for the secretion of mediators that have the capacity to expand HSC.

Hox genes have important lineage-specific functions in a variety of somatic tissues, including the hematopoietic system. HOX A and B clusters, including HOXB3 and HOXB4, are preferentially expressed in HSC and play a regulatory role in proliferation and differentiation. Dr. Humphries reported that retroviral overexpression of HOXB4 enhances the rate and ultimate level of HSC regeneration both *in vivo* and *in vitro*. Soluble versions of HOXB4 (TAT-HOXB4 fusion proteins) have recently been used instead of gene transfer to stimulate HSC expansion *in vitro*, suggesting a feasible approach for clinical application. The finding that a variety of NUP98-HOX fusion genes are over-expressed in hematologic malignancies suggested an experiment to test whether these might be even more potent for stimulating HSC expansion. Both engineered NUP98-HOXA10 and NUP98-HOXB4 have strong growth promoting effects for HSC with expansion of over 1,000-fold demonstrated in just 7 days in *ex-vivo* culture. Heightened HSC expansion *in vivo* by the HOXB4 gene was not accompanied by identifiable anomalies in the peripheral blood of the mice tested, but the safety of expanded HSC for human use has not yet been studied. Identification of this group of expansion factors that can induce *in vivo* and *ex vivo* production of large numbers of pluripotent and differentiation-competent HSC creates possibilities for new therapeutic interventions.

In addition to Hox genes, other factors, such as fibroblast growth factor-1, thrombopoietin, and wnt-3a, are reported to play a role in maintaining and expanding HSC. The *wnt* signaling pathway involves cell surface *frizzled* receptors and the *lpr* 5/6 co-receptor. Interaction of these receptors with the wnt protein activates the *disheveled* gene product, which in turn inhibits the association of glycogen synthase kinase 3β and axin with β-catenin. Association of β-catenin with the LEF/TCF family of transcription factors results in transcription of downstream target genes leading to HSC expansion. Dr. Irving Weissman reported that ectopic expression of β-catenin in mouse cells expressing Bcl-2 can result in HSC expansion from 100 to 1000-fold *in vitro*. The majority of these cells (70%) retain the stem cell phenotype. Inoculation of 125 of these cells was shown to rescue lethally irradiated mice and to give rise to long-term multi-lineage cells.

Despite these and other advances in understanding the maintenance and expansion of HSCs, many of the molecular mechanisms and pathways by which signaling molecules influence HSCs remain to be elucidated.. Discovery of the interrelations that potentially exist among different signaling pathways is an important step towards achieving a robust protocol for *ex vivo* expansion of HSCs for therapeutic uses.

HSC localization in bone marrow vascular niches and factors mediating mobilization to the circulation

Most hematopoietic stem and progenitor cells reside in defined hematopoietic niches that support their self-renewal, proliferation, differentiation, and mobilization to the peripheral blood. It is known that targeted disruption of vascular niches in the bone marrow results in impaired differentiation and maturation of hematopoietic progenitors. Peripheral blood is the most convenient source of stem cells for treatment of a variety of hematological disorders. However, the number of stem cells in the circulation is very small and their mobilization from organs that are rich in stem cells is critical for success. To induce mobilization, a variety of cytokines and hematopoietic growth factors, such as granulocyte colony-stimulating factor, granulocyte-macrophage colony stimulating factor, interleukin-3, stem cell factor, FLT-3 ligand, and thrombopoietin, have been successfully used. Localization of stem cells in the BM is also influenced by the expression of adhesion molecules, such as the ß1 integrin family of molecules, VLA-4 (CD49d/CD29), VLA-5 (CD49e/CD29), the ß2 integrin molecule, and LFA-1 (CD11a/CD18), on the surface of progenitor cells.

In addition to these factors, Dr. Willem Fibbe found that interleukin-8 (IL-8) plays an important role in rapid mobilization of stem cells. He showed that enhanced mobilization by IL-8 requires the presence of a population of accessory polymorphonuclear cells. Concurrent with IL-8 induced mobilization, a systemic release of the metalloproteinase gelatinase-B (MMPs) of up to a 1,000-fold increase in plasma levels was found within 15 minutes after a single IL-8 injection. MMPs cleave extracellular matrix molecules to which stem and progenitor cells are attached. Neutrophils are responsible for the release of MMPs, making them a major regulator of stem cell mobilization. The role of proteases has been clearly established not only in IL-8 induced stem cell mobilization, but also in G-CSF induced mobilization. For instance, cleavage of VCAM-1 in the bone marrow is observed in mice undergoing G-CSF induced mobilization, and disruption of the VLA-4:V-CAM pathway by antibodies to VLA-4 results in peripheralization of stem cells. Cathepsin-G, elastase, and MMP-9 released from activated neutrophils were found to play crucial roles in these processes. Remarkably, low dose irradiation completely inhibits the mobilization induced by G-CSF or IL-8, possibly due to the induction of protease inhibitors such as alpha-1 proteinase inhibitor.

Dr. Shahin Rafii discussed the mechanism by which stem cells and progenitor cells localize to vascular niches in the bone marrow where they are maintained in an undifferentiated and quiescent state. He showed that chemokines, including stromal derived factor-1 and FGF-4, support the cytokine-independent localization of progenitors to these vascular niches. He also showed that the localization of stem cells to the vascular niche is independent of a chemokine gradient and is mediated through graded "architectonic" activation of adhesion molecules and tethered cytokines that provide directional cues for the localization of cells. Based on these results, Dr. Rafii recently established murine models to identify molecular pathways that are essential for organ-specific stem cell mobilization. Identification of these pathways will lay the foundation for designing strategies to recruit and isolate large numbers of stem cells.

HSC differentiation and the development of T and B cell lineages

One goal of stem cell research is to harness the regenerative potential of these cells to provide an efficient and effective source of lymphocytes. Dr. Carlos Zuniga-Pflucker showed that a simple co-culture system of ES cells and OP9 stromal cells that ectopically express the Notch ligand Delta-like 1 (OP9-DL1 cells) are able to differentiate into hematopoietic cells committed to the T cell lineage with capacity for full differentiation into T cells. These cells undergo stage-specific proliferation and mature into $CD4^+$ and $CD8^+$ T cells *in vitro* and effectively reconstitute the T cell compartment of immune-deficient (RAG-/-) mice. The ES-derived T cells are also able to mount an effective antigen-specific immune response *in vivo*. This approach opens new avenues for the development of immune reconstitution methods using defined sources of human stem cells for therapy.

B cells differentiate from HSC in a stepwise, programmed fashion through a series of developmental checkpoints. Pro-B cells are the first readily identifiable B-lineage committed precursors that exhibit CD19 and CD34 surface markers. Pre-B cells are defined by cytoplasmic expression of μ heavy chain and have the $CD19^+$ $CD34^-$ phenotype. Dr. LeBien focused on the development of human B-lineage cells from HSC. He showed that development of mature B cells from progenitors is a complex process governed by sequential changes in gene expression. Human B-lineage cells are present in multiple tissue sites in early fetal development. However, from mid-gestation through the eighth decade of life, the BM is the exclusive site of B lymphopoiesis. Pre-B cells are present in 7- to 8-week gestational age fetal liver and 10-week gestational age fetal omentum. Dr. LeBien also investigated common lymphoid progenitors with the capacity to develop into T, B, or natural killer (NK) cells, but little or no capacity to develop into nonlymphoid lineages. These cells are identified as $CD34^+/CD10^+/CD45RA^+$ BM progenitors that do not express T, B, or NK lineage surface markers (i.e., CD2, CD4, CD8, CD16, CD19, CD20, and CD56). Dr. LeBien also discussed the IL-7-mediated enhanced proliferative capacity of $CD19^+$ $CD34^+$ pro-B cells grown on human BM stromal cells. Such cultures result in a specific increase in cell-surface CD19 on human pro-B cells and a decrease in RAG-1, RAG-2, and TdT messenger RNA (mRNA) levels. However, the critical cytokine (or cytokines) that promotes development of CD19+ B-lineage cells from common lymphoid progenitors and promote expansion of pre-BCR+ cells remains uncharacterized.

HSC ontogeny and the development of lymphoid stem cells in the human and mouse

Blood cell formation in humans is similar to other higher vertebrates and begins outside of the embryo in the yolk sac, then in liver, and finally becomes established in bone marrow. In humans, bone marrow hematopoiesis begins during the first trimester of gestation. This is in contrast to mice, which utilize the liver as the source of blood cells for up to two to three weeks after birth. Dr. Peault developed an experimental system to determine whether HSCs generated inside or outside the embryo have the same developmental potential in terms of myelopoiesis and lymphopoiesis. He demonstrated that myeloid cells and NK cells can be generated from both locations, but only the intra-embryonic location produces B cells. Similarly, T cell differentiation was not observed in

yolk sac hematopoietic cells. Dr. Peault also presented evidence of other sites for hematopoiesis in humans including the splanchnopleura on the floor of the dorsal aorta. Hematopoiesis at this site originates from local endothelial cells at day 27 of human gestation. The cells then expand rapidly to form clusters of several thousand cells at day 35, which disappear at day 40. These cells resemble primitive hematopoietic progenitors: $CD34^{++}$, $CD45^{+}$, $CD31^{+}$, $CD43^{+}$, $CD44^{+}$, $CD164^{+}$, $CD38^{-}$, and Lin^{-}. They also express proto-oncogenes and transcription factors that typify the initial stages of hematopoietic development, such as c-myb, SCL/Tal-1, c-kit, Flk-1, GATA-1, and GATA-3. Hence, hematopoietic cells develop from intra-embryonic mesoderm. This implies that hematopoietic cells emerge not only from yolk sac mesoderm in early human ontogeny, but also within the embryo proper. Endothelial cells as an origin for HSC opens new avenues in the quest for sources for HSC for therapeutic purposes.

Stability of HSC and factors regulating telomere length

The DNA of human chromosomes terminates in several kilobases of telomere repeats that are gradually lost with age and with replication *in vitro*. Defective telomere maintenance has been causally linked to cell cycle exit and apoptosis. The telomere length together with telomerase level may predict the proliferative potential of HSC. Peter Lansdorp presented studies on the genetic factors that regulate telomere length. He identified a gene named *Rtel* (Regulator of telomere length) in the mouse that is similar to a DNA helicase-like gene in *C. elegans* that is essential for maintenance of telomere length. This finding could be developed as a potential diagnostic assay to predict HSC status regarding long term potential for self-renewal. Dr. Lansdorp also determined that telomere length in T cells and granulocytes declines significantly with age in both humans and baboons. Furthermore, humans that are haploinsufficient for telomerase typically succumb to the consequences of defective HSC and T cell proliferation before the age of 50. Thus, attempts to expand HSC or other cell types for therapy need to consider the possible loss of regenerative potential due to telomere attrition.

Recommendations

Given the urgent need to develop radiation exposure countermeasures, the NIAID has outlined a comprehensive implementation plan focused on deliverables such as biomarkers of exposure dose, biodosimetry devices, radioprotectants, chelators, and treatments, including the establishment of clinically validated HSC transplant technologies.

The workshop participants acknowledged the need to address the broad range of radiation types and exposure levels that terrorists might use, with the potential for internal or environmental contamination resulting in long-term effects such as cancer. The development of biomarkers and biodosimetry devices for the early identification and decontamination of exposed individuals was considered to be a very high priority.

It was also recommended that methods to purify, expand, and bank HSC on a large scale be explored for feasibility. The participants recognized a need to establish core facilities on a national scale to perform phenotypic analyses on HSC, to verify the identification of long term repopulating cells, and to standardize procedures for collection, expansion, storage, and administration to irradiated recipients. Development of surrogate markers for *in vivo* engraftment and differentiation into blood lineages was also proposed as a high priority.

The development of tolerance inducing regimens would be important for the application of allogeneic stem cell transplantation after radiation exposure. Immunological conditioning could potentially facilitate engraftment without the need for chemotherapy. Such protocols have not been fully successful in the past, and further research is needed.

There was consensus that bone marrow transplantation technologies, including transplant across HLA barriers, are adequately developed. However, the current capacity of the best transplant centers is approximately 250 transplants per year, resulting in an insufficient capacity to treat all relevant patients in a large casualty situation. In addition, the cost of transplantation in large numbers of individuals may be prohibitive. The identification of specific victims who will benefit from HSC transplantation remains a major task. This is a particularly challenging issue because radiation exposure due to a terrorist attack is expected to be non-homogeneous. Physicians who currently employ irradiation for therapy have little experience with such scenarios, and transplantation with too little information may create graft-versus-host reactions. In fact, the Chernobyl experience demonstrated only a limited use for bone marrow transplantation.

The ability to greatly expand purified HSC for long-term storage may alleviate some of these issues. Some, but not all of the molecular regulators for HSC expansion are known. Large-scale robotics approaches were suggested to identify new compounds and small molecules that induce stem cell expansion. Furthermore, pharmaceutical companies with access to large libraries of compounds, small molecules, and natural products should be recruited to identify products with HSC growth promoting effects.

Immune reconstitution follows successful bone marrow transplantation. However, many of the regulators controlling lineage differentiation are not yet known and protocols to enhance the rate of reconstitution are not available. For example, both T and B cell immunodeficiencies persist for one to two years or even longer after bone marrow transplantation. Basic research in this area should be supported to identify growth factors and cytokines responsible for immune cell maturation to promote much more rapid functional reconstitution of immune potential.

Strategies to accelerate recruitment of stem cells from the marrow offer an effective means to force a rapid reconstitution of hematopoiesis. Introduction of stem cell active chemokines, including Stromal Derived Factor-1 (SDF-1) and Fibroblast Growth Factor-4 (FGF-4), facilitates recruitment of the hibernating stem cells from the osteoblastic niche, thereby accelerating hematopoietic recovery. This approach will allow for the

enhanced reconstitution of the myelomonocytic and synergy with other lineage-specific cytokines including G-CSF and Erythropoietin.