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STEM CELLS AND STEM CELL TRANSPLANTATION

Carmelo Carlo-Stella, Antonio Tabilio*

*Department of Hematology, Bone Marrow Transplantation Unit, University of Parma, and * Department of Clinical Medicine, Pathology and Pharmacology, Hematology and Clinical Immunology Section, University of Perugia, Perugia Italy*

ABSTRACT

Stem cell transplantation (SCT) is an increasingly used therapeutic approach for the treatment of hematological and non-hematological disease of neoplastic and non-neoplastic origin. How the phenomena controlling blood cell production take place during SCT is still largely unclear. Increasing knowledge of stem cell biology, the availability of large amounts of stem cells due to mobilization techniques, as well as new developments in hematopoietic cell manipulation offer exciting experimental and therapeutical options in the field of SCT and will be reviewed here.

Key words: stem cells, stem cell transplantation

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Stem cell transplantation (SCT) is increasingly used in the treatment of hematologic and non-hematologic disease of neoplastic and non-neoplastic origin.[1,2] Since successful transplant depends on engraftment of pluripotent hematopoietic stem cells in the marrow microenvironment, and the transplant procedure involves infusion of stem cells into the circulation, it is assumed that stem cells are capable of homing to the marrow and docking at specific sites.[3] Therefore SCT exploits a physiological mechanism that plays a role in the regulation of normal hematopoiesis. Although it is known that hematopoietic stem cells circulate physiologically, it has not yet been formally proven whether they can re-enter the marrow.[4] Several lines of clinical and experimental evidence support the idea that stem cells have high migratory potential that allows them to leave and re-enter the marrow cavity, thus providing not only immediate but also sustained output of all types of blood cells in lethally irradiated recipients.[5] The reasons for stem cell circulation are still a matter of hypothesis, including the possibility that stem cells destined for differentiation migrate from a so-called *primary microenvironment* to *secondary microenvironment* areas that allow specific differentiation patterns, or that stem cell circulation is involved in the regulation of the balance of self-renewal versus differentiation.[6] The spatial organization of stem cells in the marrow, mediated by stromal cells and the extracellular matrix, is crucial for their controlled regulation.[7] Although growth factors play a key role in stem/progenitor cell proliferation and differentiation, it seems improbable that hematopoiesis is regulated by a random mix of growth factors and responsive cells. Indeed it is likely that regulatory molecules and localization phenomena within marrow stroma are required to sustain and regulate hematopoietic function.[7] Cell adhesion molecules expressed by stem cells interact with specific structures in the extracellular matrix produced

Correspondence: Dr. Carmelo Carlo-Stella, Cattedra di Ematologia Università di Parma, via Gramsci 14, 43100 Parma, Italy. Tel. international +39.521.290787. Fax: international +39.521.292765 E-mail: ccs@ipruniv.cce.unipr.it.
or Dr. Antonio Tabilio, Dipartimento di Medicina Clinica, Patologia e Farmacologia, Sezione di Ematologia e Immunologia Clinica, Università di Perugia, via Brunamonti 1, Perugia, Italy. Tel. international +39.75.5783990. Fax. international +39.75.5783691.
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by marrow stromal cells. Growth factors, produced either locally by stromal cells or peripherally, may bind to the extracellular matrix and be presented to immobilized target cells which recognize each growth factor through specific receptors.[8] How these tightly regulated and ordered phenomena controlling blood cell production function during transplant is unclear. In particular, it is not known how SCT may take advantage of mechanisms for stem cell localization in the marrow cavity. In addition, it is absolutely unclear whether marrow stroma plays a role in hematopoietic regeneration following SCT and whether it might be involved in immunological reconstitution or minimal residual disease control, thus influencing long-term transplant outcome.

Hematopoietic stem cell

Following the discovery that bone marrow transplantation could be used to rescue irradiated mice, the identification and characterization of the hematopoietic stem cell has become essential in order to achieve new developments in SCT.[9] The potential for using stem cells as vehicles for gene therapy has further increased the efforts of a number of research groups involved in stem cell identification and cloning. The cells of the hematopoietic system are heterogeneous and belong to different lineages in different stages of maturity.[10] Stem cells comprise a relatively small cell population, located mainly in the bone marrow, which can (i) undergo self-renewal to produce more stem cells, or (ii) differentiate to produce progeny which are progressively unable to self-renew, being irreversibly committed to one or another of the various hematopoietic lineages, and able to generate clones of up to 10^5 lineage-restricted cells that mature into specialized cells.[11,12] The decision of a stem cell either to self-renew or differentiate, and the selection of a specific differentiation lineage by a multipotent progenitor during commitment are stochastic events. Self-renewal and differentiation, as well as lineage commitment, proliferation, maturation and survival reflect highly integrated processes under the control of several mechanisms, which can be distinguished as extracellular (regulatory molecules, microenvironment) and intracellular (protooncogenes, tumor suppressor genes, transcription factors, cell cycle regulatory molecules). Regulatory molecules include positive (hematopoietic growth factors) and negative (interferons, TGF-β, MIP-1α) factors which interact in complex ways (synergism, recruitment, antagonism).[13] The microenvironment plays a role on at least three levels, including direct cell-to-cell interactions, interactions of cells with extracellular matrix molecules, and interaction of cells with soluble growth regulatory molecules. Intracellular mechanisms of hematopoietic control result in the repression or de-repression of lineage-specific genes regulating growth factor responsiveness and/or the proliferative capacity of hematopoietic cells.[14]

Overall, stem and progenitor cell behavior is the result of highly integrated activity based on extracellular signals triggering intracellular transduction phenomena. The properties of self-renewal and differentiation give stem cells their remarkable ability to repopulate the hematopoietic tissue of lethally irradiated or genetically defective recipients. Although several progenitor cells at various stages of differentiation have been characterized by using *in vitro* or *in vivo* techniques, the main objective of the study of hematopoietic cell development is the isolation and identification of the pluripotent stem cell.[15,16]

Stem cell purification strategies

Many attempts to purify stem cell populations have used a combination of approaches based on the physical and biological properties as well as the immunophenotype of the target cells.[17] Among the physical techniques, density gradient separation is still commonly used as a pre-enrichment step in stem cell purification. Based on evidence that primitive hematopoietic cells are in the G_0 phase of the cell cycle, pharmacologic cell cycle-specific agents can be used as a pre-enrichment step in stem cell purification strategies. Treatment with 5-fluorouracil or 4-hydroperoxycyclophosphamide results in the elimination of proliferating cells but spares the non-cycling stem cells.[18] However, pharmaco-

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logic treatment may be associated with potentially irreversible damage to primitive progenitors, which would lead to delayed engraftment. Primitive progenitors can also be distinguished from their progeny by their different abilities to bind to cultured stromal layers and to tissue culture plastic.[19,20] The major advance in stem cell purification has been the identification of the CD34 antigen as the marker of a morphologically and immunologically heterogeneous cell population that is functionally characterized by its *in vitro* capability of generating clonal aggregates derived from early and late progenitors and by its *in vivo* capacity to reconstitute the myelo-lymphopoietic system in a supralethally irradiated host.[21-24] The CD34$^+$ cell population contains virtually all the myeloid and lymphoid progenitors as well as a small subset of cells that can initiate and maintain stromal cell-supported long-term cultures. Expression of the CD34 marker has dominated attempts to isolate, purify and characterize human hematopoietic stem cells by a variety of immunological means. Recently, several companies have developed equipment for cell purification using antibody-coated magnetic beads, avidin-coated beads or antibody bound to tissue culture plastic.

Several monoclonal antibodies assigned to the CD34 cluster identify a transmembrane glycoproteinic antigen of 105-120 kd expressed on 1-3% of normal bone marrow cells, 0.01-0.1% of peripheral blood cells and on 0.1-0.4% of cord blood cells.[25] The function of the CD34 antigen is not yet known, although it seems that CD34 is involved in adhesion and signal transduction phenomena. CD34 antigen expression is associated with the concomitant expression of several markers, including the lineage non-specific markers Thy1, CD38, HLA-DR, CD45RA, CD71, as well as T-lymphoid, B-lymphoid, myeloid and megakaryocytic differentiation markers.[25] Analysis of the expression of CD38, Thy-1, CD71, the isoforms of CD45, and uptake of rhodamine-123 have resulted in a consensus stem cell phenotype: CD34^{bright}, Thy-1$^+$, CD38$^-$, CD45RA$^-$, rh-123^{dull}, Lin$^-$. CD34$^+$ cells also express receptors for a number of growth factors classified as *tyrosine kinase receptors*, such as the stem cell factor receptor (SCF-R, CD117), and *hematopoietic receptors* which do not contain a tyrosine kinase domain.[25-29] Stem cell tyrosine kinase receptors (STK) such as STK-1, the human homologue of the murine Flk-2/Flt-3, are of particular relevance since their ligands might represent new factors able to control selectively stem cell self-renewal, proliferation and differentiation.[30-35]

There is growing interest in the possibility that different types of progenitors can be measured directly by multiparameter phenotyping of CD34$^+$ cells or subpopulations. This approach has the significant advantage that the results may be quickly available and can be used to guide clinical decisions. However, although empirically useful for some applications, correlations between progenitor cell phenotype and functional activity are not yet refined enough to be clinically applicable. In addition, although CD34 antigen is expressed by virtually all progenitor cells, the percentage of CD34$^+$ cells with clonogenic activity assayable *in vitro* ranges from 10 to 50%. The problem of non-clonogenic CD34$^+$ cells is still open and is not adequately explained by the presence of lymphoid progenitors that are not assayable with currently used *in vitro* systems. Non-proliferating CD34$^+$ cells might represent a subpopulation which is not responsive to conventional myeloid hematopoietic growth factors. The non-proliferating CD34$^+$ subset might require the presence of cofactors, such as the ligand of STK-1 or the hepatocyte growth factor, able to activate stem cell-specific genes whose expression is a prerequisite for responsiveness to conventional growth factors.[34,36,37]

Stem cell assays

With the exception of transplantation of human cells into immune deficient mice, the identification of putative human stem cells has relied on *in vitro* assays. A number of clonogenic assays for short-term marrow or blood culture are now available for quantitative analysis of the various hematopoietic progenitor cell classes.[38] These semisolid assay systems require appropriate nutrients and growth factors and are particularly suitable for measuring quantita-

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tive changes in the different progenitor cell types and for evaluating growth factor responsiveness or investigating differential effects of regulatory molecules on progenitors at different stages of differentiation or on different hematopoietic pathways. However, the short-term assays are not suitable for analyzing self-renewal or interactions of hematopoietic progenitors with stromal cells. By using the long-term culture (LTC) technique, sustained production of myeloid cells can be readily achieved, provided that a stromal layer is present when marrow (or blood) is placed in liquid culture at relatively high cell concentration with appropriate supplements, temperature and feeding conditions.[38] The LTC system, based on *in vitro* re-establishment of the essential cell types and mechanism responsible for localized and sustained *in vivo* production of hematopoietic cells in the marrow, offers an approach able to investigate not only proliferative and differentiative events but also the self-renewal of all clonogenic cell types. Long-term culture systems are generally set up in two stages. The first involves the culture of a confluent layer of stromal cells of human or murine origin. For the second stage, the confluent stromal culture is used as a feeder layer for the hematopoietic sample to be tested. Primitive cells in the test sample migrate into the stromal layer where they proliferate and release CFU-GM in the supernatant medium. Half of the supernatant (medium + cells) is harvested at weekly intervals when the cultures are fed, and its cell content is incorporated into short-term clonogenic assays to provide a measure of the activity of the primitive cell population which was inoculated over the confluent stroma. Obviously, at the beginning of the culture period many of the CFU-GM in the supernatant cannot be attributed to the activity of the cells in the stromal layer, but after 4-5 weeks the pre-existing CFU-GM will have disappeared and all of the CFU-GM will be produced by the primitive cells in the stromal layer. In the case of human bone marrow, a 5- to 8-wk period between initiating cultures and assessing clonogenic progenitor numbers allows quantification of a very primitive cell in the starting population, the so-called *long-term culture-initiating cell* (LTC-IC).[15] Recently, it has been shown that LTC-IC are capable of self-renewal.[16] Provided that LTC-IC are cultured by limiting dilution in the wells of microtitre plates so that their frequency in a sample can be determined using Poisson statistics, quantitative information on the frequency of LTC-IC and their proliferative potential (number of CFU-GM generated by each LTC-IC) can be obtained. Another assay system, the cobblestone area-forming cell (CAFC), uses pre-formed stroma as a support for hematopoiesis.[39] In this system the primitive cells are measured directly by their ability to form characteristic colonies of cells resembling cobblestones. The CAFC assay must be performed at limiting dilution because each primitive cell can form several cobblestone sub-colonies, and without Poisson statistics it is not possible to be certain how many cells contributed to the formation of a group of sub-colonies.

With the possibility of studying not only the differentiation but also the self-renewal of primitive progenitors, the LTC system will play an increasingly important role in the design and assessment of new strategies involving the genetic engineering of hematopoietic cells and marrow stromal cells.

Long-term culture assays and stem cell transplantation procedures

The LTC-IC assay, and a variety of other assays for primitive hematopoietic cells, have been used to measure primitive cell populations in bone marrow, peripheral blood and cord blood. Practically speaking, hematopoietic cells that can generate active hematopoiesis for weeks *in vitro* or months *in vivo* after transplantation are considered stem cells. This seems to be a clinically useful criterion because it characterizes those cells which are important for sustained hematopoietic recovery following SCT. However, it reflects an oversimplification of the rather complex process of hematopoietic function, which is governed by stochastic effects influencing stem cells. In fact, the ability of a cell to provide long-term hematopoietic activity can either be due to a long period of quiescence after

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the initiation of the culture or be a function of the probability of stem cell self-renewal that influences the long-term survival of stem cell clones.[40] Thus the number of primitive cells measured in LTC assays will be the product of the number of stem cells present at the onset of the culture and the probability of stem cell self-renewal. Although LTC assays will likely predict the in vivo repopulating activity of the graft, the clinical definition of a stem cell does not consider those stem cells that differentiate and die soon after transplantation or initiation of a culture.

While primitive progenitors can be purified to apparent phenotypic homogeneity, several experimental observations demonstrate that they do not behave identically when cultured in the assay systems used to detect them. The functional heterogeneity of an otherwise phenotypically homogeneous cell population can be interpreted as a reflection of stochastic effects resulting in heterogeneous proliferative activity by a uniform population of stem cells. Some stem cells will self-renew when they first divide and some will differentiate immediately, resulting in extinction of the stem cell clone. According to stochastic models a certain, and so far unpredictable, degree of clonal extinction is inevitable unless the probability of self-renewal is 1, in which case the size of a given stem cell population would double after the first division. Experimental evidence demonstrating that 100 purified stem cells (Thy-$1^{lo}$$Lin^{-}$$Sca$-$1^{+}$) are required to protect 95-100% of lethally irradiated recipient mice, and that the purified stem cells can provide both early and late hematopoietic cell reconstitution suggests that proliferation by a uniform population of cells reveals biologic heterogeneity, because the same cells are capable of producing mature cells rapidly and reconstituting the stem cell pool.[41] This and other evidence strongly supports the concept that the separation of stem cell subpopulations and the existence of stochastic effects should be considered together when studying stem cell biology and planning SCT strategies.[42] The ability to purify stem cells has already changed clinical transplantation strategies and will modify the use of SCT in the near future with the employment of *ex vivo* engineered and *ex vivo* expanded stem cells. According to stochastic effects, it can be predicted that individual transplanted stem cells will have a small chance of survival in the long-term and that many stem cells must be transplanted in order to ensure long-term polyclonal hematopoietic reconstitution.

Quantitative and qualitative issues in stem cell transplantation

Advances in clinical SCT practice are currently based on the use of a variety of sources of stem cells, including bone marrow, mobilized peripheral blood and cord blood. In addition, purified $CD34^{+}$ cells obtained by a single positive selection step or by combined negative and positive selection steps are increasingly utilized for SCT. The employment of $CD34^{+}$ cell subsets, *ex vivo* expanded cells, and genetically engineered cells is being considered for the near future. Hematopoietic reconstitution following SCT is largely influenced by qualitative and quantitative matters, such as the source of stem cells, their quantity and quality, the quantity and quality of accessory cells (T-lymphocytes, natural killer cells, stromal cells, etc.), and stem cell manipulation procedures.

The traditional source of hemopoietic progenitor cells for both autologous and allogeneic transplants was, until recently, the bone marrow. Autologous bone marrow transplantation, which is not true transplantation but marrow harvested prior to myeloablative radio-chemotherapy and transfused as bone marrow rescue, has been widely used in hematologic and solid malignancies.[43] In allogeneic transplant, the bone marrow is generally employed in syngeneic, allogeneic HLA-matched siblings, and transplant from a matched unrelated donor. Both unmodified and T-lymphocyte-depleted bone marrow have also been employed in mismatched transplants with poor success, because of the high incidence (80%) of severe GvHD and the high risk of graft failure (20%). Since the end of the 1980's peripheral blood stem cells, harvested after chemotherapy alone or after the administration of hemopoietic growth factors during steady-state hematopoiesis or

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after chemotherapy, have largely replaced bone marrow as the preferred autologous stem cell source for rescue following myeloablative therapy.[44] After early attempts to use unstimulated PBSC in matched BMT,[45] studies with allogeneic PBSC were used in cases where the donor was not suited to general anesthesia and bone marrow harvesting,[46] and in the treatment of graft failure after bone marrow transplantation.[47] A number of centers have reported on the use of PBSC in small series of patients, mainly with advanced hematologic malignancies undergoing primary allogeneic transplantation.[48] Allogeneic PBSC transplants seem to have the same advantage over bone marrow that they do in autologous rescue in favoring more rapid hemopoietic reconstitution.

Hemopoietic stem cells are also being obtained from other sources such as surgically removed bone marrow (orthotopic transplantation), cadaver bone marrow, umbilical cord blood and fetal liver.[49] Orthotopic transplantation is a different form of donation rather than an alternative source of stem cells. It is performed through a window-cut made in the cortical bone of the ileum.[49] The incidence of GvHD may be decreased using such cells.[50] Experience with hemopoietic cells taken from cadavers is very limited. Eastlund *et al.*[51] estimated the stem cell reserve harvested from cadavers shortly after death, and demonstrated that it could potentially be employed for hematological reconstitution. However, very few reports on allogeneic BMT with cadaver bone marrow are available.[52,53]

Umbilical cord blood is another interesting source of hemopoietic stem cells. The first descriptions of stem cell traffic between the fetus and the placenta during gestation date back more than 30 years, but it was only in the late 80's that attempts were made to employ cord blood stem cells in transplantation, the first being a patient with Fanconi's anemia.[54] The biological bases for the clinical utilization of cord blood are: a) the number of CFU-GM is sufficient for pediatric transplants;[55] b) the proliferative potential is greater than that of adult cells;[56,57] c) it is probably a more complete source of stem cells. Evaluation of progenitor subsets within the CD34$^+$ population indicates that, by extension, cord blood should contain sufficient numbers of these cells for transplantation to adults;[58] d) because of the relative immaturity of the T-cells, cord blood should theoretically reduce the risk of GvHD.[59,60]

All these proposed features seem to be confirmed by experiments in mice,[57,61] and there are a number of reports on the use of cord blood stem cells in allogeneic pediatric transplantation.[62] Nevertheless, a number of aspects of this type of allotransplant still require clarification. For example, the use of a single cord to reconstitute an adult patient, the incidence of GvHD in relationship to the degree of compatibility, and the evaluation of any graft-versus-leukemia effect.

In the embryo, hematopoietic stem cells from the yolk sac and aorta-gonad-mesonephros settle in the fetal liver and hematopoiesis occurs primarily in the liver between the 6th and 22nd weeks of gestation. Thus the fetal liver represents a source of stem cells. In mice the proliferative activity of fetal liver stem cells seems to be greater than that observed in adult bone marrow stem cells. Rebel *et al.*[63] recently reported that individual fetal liver competitive repopulation units (CRU) exhibit greater proliferative activity *in vivo* than similar cells from adult bone marrow. However, notwithstanding these interesting biological properties, the clinical results of human fetal liver stem cell transplants are disappointing. Only 20% of patients with severe combined immunodeficiency syndrome who underwent fetal liver SCT engrafted, and 50% of these developed GvHD.[64] The results obtained in patients with severe aplastic anemia and acute leukemias are much worse; only transient engraftment was observed in these patients.[65,66] Therefore the use of fetal liver hemopoietic stem cells in SCT remains theoretical. However, the high long-term repopulating capacity reported by Rebel *et al.*[63] and the finding that the population of fetal liver cells contains twice as many cells in the S/G_2/M phase as the phenotypically similar population in adult bone marrow[67] make fetal liver cells attractive for stem cell-based therapy strategies.

Traditionally, the quantity of cells necessary

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for a SCT is based on the number of mononuclear cells per kilogram body weight of the recipient. This value provides no information on either the composition of the graft or the stem cell content.

In vitro detection of CFU-GM and flow cytometric evaluation of $CD34^+$ cells have been used for monitoring the stem cell population that is manipulated and eventually infused into the patient. However, neither detection of CFU-GM, which are progenitors committed to the myelomonocytic pathway, nor evaluation of $CD34^+$ cells, which comprise a large majority of non self-renewing cells, is a direct measure of the stem cell content. Due to their methodological complexity, application of stem cell assays to clinical practice seems useless and cannot be proposed. The employment of stem cell assays must be mandatory only for innovative graft manipulation procedures. For standard SCT, including autologous marrow or blood transplantation or allogeneic HLA-identical transplantation, conventional assays, although of limited value, are sufficient for assessing graft viability and function.

Several attempts have been made to correlate the numbers of transplanted CFU-GM and $CD34^+$ cells with clinical outcome.[68] The results of these studies can be summarized by the finding of a threshold number of CFU-GM or $CD34^+$ cells required for optimal engraftment, and the finding that increasing the number of cells above this threshold value does not improve the clinical result.[69] Obviously, the quantity of cells is crucial in determining hematopoietic reconstitution. If it is assumed that 10,000 mononuclear cells (of either marrow or mobilized blood origin) contain 100-3,000 $CD34^+$ cells, the clonogenic potential of such a population is represented by 10-300 CFU-GM. By using stem cell assays, about 5-8 CAFC and 1-3 LTC-IC should be detected within this cell population. Therefore it can be inferred that at least 100 $CD34^+$ cells must be infused to assure the presence of one stem cell.[70] Transplantation of a single stem cell is not sufficient to guarantee engraftment because the probability that any one stem cell will provide long-term hematopoiesis is governed by many factors, including the probability that it will home to the marrow microenvironment, the kinetics of stem cell proliferation during hematopoietic reconstitution and interactions between donor and host cells. Studies in mice suggest that only 1 in 10 stem cells is capable of reaching the hematopoietic microenvironment to home adequately.[71] Thus the minimum requirement for $CD34^+$ cells is increased by 1 log. The kinetics of stem cell proliferation is also influenced by symmetrical vs asymmetrical stem cell divisions, as well as by the probability of differentiation/renewal. Donor-host cell interactions in autologous transplantation are different from those in allogeneic transplant and are also influenced by post-transplant therapy.

A sufficient number of accessory cells, particularly T-lymphocytes, in the graft is also of importance for engraftment. The benefical role that T-lymphocytes have on engraftment has long been known.[72] However, when the number of infused T-lymphocytes is too high, graft-versus-host-disease (GvHD) develops. A clonable T-lymphocyte content of $<1\times10^5$/kg b.w. in the graft is thought to eliminate GvHD in matched allogeneic transplants,[73] and $<3\text{-}5\times10^4$/kg b.w. to bypass the problem in mismatched transplants.[74] But it is not the quantity of T-lymphocytes alone that is responsible for transplant engraftment; their quality is also crucial. The relative immaturity of cord blood T-cells should, in theory, reduce the risk of GvHD,[62] while those in fetal liver seem to lower the incidence of GvHD but raise the incidence of graft failure.[65]

Mechanisms of engraftment

It is becoming increasingly apparent that distinct subsets of stem cells may be responsible for different phases of engraftment after transplantation.[75,76] Murine studies in which isoenzyme analysis and retroviral gene marking of hematopoietic cells have been used to track the fate of stem cells support the existence of *short-term* and *long-term* reconstituting stem cell populations. In mice, a large number of multilineage clones are active immediately after grafting but rapidly decline, with the majority being inactive 12 weeks after transplantation.[77,78] This observa-

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tion indicates the existence of a population of cells with multilineage short-term engraftment potential. Using a large animal transplantation model, it has been demonstrated that multiple clones contribute to short-term engraftment, followed by the sustained contribution of a relatively few stem cells.[79] At least two hypotheses have been proposed to explain the existence of short-lived clones. The first is based on the existence of a large number of clones with equal long-term repopulating potential that could compete for niches within the bone marrow microenvironment, with the eventual dominance of a few clones with an initial self-renewal and proliferative advantage. The second hypothesis is the existence of distinct subsets of totipotent stem cells, one that is short-lived and reconstitutes multiple lineages of transplanted recipients and another that is responsible for life-long hematopoiesis. So far, evidence for the separation of distinct subsets of cells with differing repopulating potentials has not been demonstrated in human subjects. The existence of cells with long-term engrafting potential is evident in allogeneic stem cell transplant recipients who survive for decades with donor-derived hematopoiesis. Functional heterogeneity of the transplantation potential of human stem cells can be illustrated in recipients of mobilized peripheral blood SCT. In fact, in these patients hematopoietic reconstitution is significantly accelerated compared to marrow grafts, suggesting either that mobilized peripheral blood, with respect to marrow, is enriched in short-term repopulating stem cells or receives a substantial contribution of lineage-restricted progenitors (CFU-GM) in the early phase of engraftment. Formal evidence in support of the different hypotheses will only be provided by the infusion of retrovirally-marked hematopoietic cells which can be tracked over time.

Theoretically, subsets of cells with differing proliferative potential may also differ in physical characteristics and therefore may be isolated and functionally defined. As few as 100 Thy-1^{lo}Lin^{-}Sca-1^{+} cells can protect 95-100% of lethally irradiated recipient mice with long-term donor-derived reconstitution.[41] This indicates that this population contains cells with both short-term and long-term engraftment potential and that proliferation by a phenotypically uniform population of cells reveals biological heterogeneity, in that the same cells are capable of producing mature cells and reconstituting the stem cell pool.[41] Through the use of a homogeneous population of primitive human hematopoietic cells, *in vitro* evidence has suggested the existence of a mechanism able to provide both short-term and long-term hematopoietic repopulation.[42] The functional heterogeneity of otherwise uniform populations is in keeping with the existence of stochastic effects. In SCT, the important implication of these observations is that functional activation of primitive hematopoietic cells can produce clones of cells that achieve maturation rapidly or after much longer intervals. However, delineation of the heterogeneity of phenotypically defined human stem cell populations with regard to repopulating potential will require rigorous long-term *in vivo* assays and gene marking studies.

Mechanisms of cure in stem cell transplantation

Long-term remission after SCT is dependent on the direct effect of chemoradiotherapy that not only eradicates neoplastic stem cells but also induces membrane alterations in marrow endothelium facilitating the homing of transplanted cells.[80] The efficacy of presently used regimens cannot be substantially improved by altering drug and radiation doses and schedules.[81] Several lines of evidence from allogeneic transplantation support a potent antileukemic effect mediated by the graft itself, the so-called graft-versus-leukemia (GVL) effect.[82] This evidence includes a correlation between severity of graft-versus-host-disease (GVHD) and leukemia-free survival,[83] a relapse rate in transplantation of twins at least double that in allografts,[84] and an increased relapse rate in some patients receiving T-depleted allografts who do not develop graft-versus-host-disease.[85] The utilization of PBSC may have important implications in triggering a potent GvL. T-lymphocyte content is 1.5-2 logs greater than that in harvested bone marrow, and the natural killer cell content 20 times higher. The question of

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whether this will translate into more potent GvL activity in patients allografted with PBSC as compared with unmanipulated bone marrow cannot be answered at this time, but needs further study. Moreover, the potential use of PBSC manipulated *ex vivo* should lead to more precisely designed allogeneic transplants, with the infusion of antineoplastic lymphocyte clones or non-alloreactive lymphocytes post-transplant in the foreseeable future. At present, there is no clear evidence in autologous SCT of the possibility of inducing an autologous GVL effect.[86] However, two types of manipulation aimed at enhancing the immune system and exerting a GVL effect following autograft with interferon-α, interleukin-2 or other cytokines must be considered an important issue.[87] Another, as yet unappreciated, mechanism that may play an important role in restoring normal hematopoiesis is stem cell dynamics, as shown by long-term sequential analysis of peripheral blood lineages in animals engrafted with genetically marked stem cells.[77] These studies demonstrate that developmental behavior is primarily a function of time. In fact, when retrovirally-marked syngeneic murine marrow is transplanted into recipient animal hematopoiesis, the first 4-6 months is characterized by frequent fluctuations in stem cell proliferation and differentiation and proceeds by the sequential contributions of one of a small number of clones, but this state is in disequilibrium.[78] Following a few months of clonal instability, stable monoclonal or oligoclonal hematopoiesis regularly occurs.[78] This evidence implies that mechanisms regulating stem cell dynamics provide for stability and quiescence in the stem cell pool rather than frequent fluctuation. A number of factors regulating stem cell dynamics through stromal or humoral mechanisms that promote stem cell quiescence are able to modulate the functional activity of stem cells.[6] These mechanisms may explain why autologous SCT can induce long periods of normal hematopoiesis or normal/leukemic mosaicism. As long as the percentage of leukemic stem cells remains small or can be depleted by *in vitro* purging, normal clones can repopulate for various periods of time. Normal hematopoiesis may occur through the sequential functional activity of different stem cell clones, rather than through a static contribution of the entire stem cell pool. Remission after autografting simply implies that normal clones are cycling and thereby maintaining hematopoiesis at the expense of the leukemic clone. Relapse would occur when the leukemic clone is again recruited into cycle. *In vitro* purging prior to autologous transplantation may effectively reset the balance between normal and neoplastic clones and may influence stem cell dynamics by transiently depleting leukemic stem cells, thus favoring the repopulating activity of normal stem cells.

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Factors affecting the outcome of stem cell transplantation

Factors governing the outcome of SCT include the immunosuppressive effects of the conditioning regimens, the degree of compatibility between donor and recipient, the composition of the cells in the graft and the quality of the marrow microenvironment. An inadequacy of any of these factors may lead to graft failure. Both the quality and quantity of hematopoietic stem cells obtained from bone marrow or blood affect the short- and long-term outcome of transplant, in that they affect both hematopoietic engraftment and relapse rate. The short-term results of autologous transplant, usually showing 5 to 25% early mortality, depend on the time required for hematopoietic regeneration, which involves three to four weeks of pancytopenia during which the recipient is at substantial risk of death from infections or bleeding.[88] Indeed autografting-related mortality and morbidity are not only due to hematopoietic but also to non-hematopoietic factors, such as organ toxicity, as well as transplantation procedures. Engraftment and hematopoietic regeneration following autologous transplant may also fail because of defects in the recipient's microenvironment,[19] whose function may also be influenced by the underlying disease.[89] The functional integrity of harvested stem cells may be damaged by prior exposure to radio- or chemotherapy (induction and consolidation therapy, pretransplant conditioning regimens).[90] The extent

of this damage is largely dependent on several factors (chemotherapy schedule, drug combinations, timing of marrow harvest, etc.). The use of systemic alkylating agents, including nitrogen mustards, cytoxan, chlorambucil, is widely recognized to be associated with decreased stem cell function.[91] Finally, marrow collection, Ficoll separation, elutriation, freezing and thawing are causes of damage at various levels. The long-term outcome of autologous transplants is mainly affected by the risk of relapse of the underlying disease, which is the major cause of failure of autologous transplant. Theoretically, two factors may be responsible for relapse: (a) the lack of a graft-versus-leukemia effect; (b) minimal residual disease (MRD), i.e. leukemic cells which survived *in vivo* the intensive conditioning treatment, and any leukemic cells which survived the remission-induction treatment were then taken out and, in spite of marrow purging, were reinfused again with the graft.[92,93]

Conclusions

Advancement of our understanding of stem cell biology associated with the availability of growth factors for clinical use, large quantities of stem/progenitor cells and technologies that allow stem cell purification and transduction of genetic material within hematopoietic cells have dramatically changed our perception of SCT. Several therapeutical approaches are now possible for each patient, often rendering the process of therapeutical decision making very difficult. Adequately planned clinical trials are required to carefully test the different therapeutical options now available for each single disease.

References

1. Appelbaum FR. Marrow transplantation for hematologic malignanices: a brief review of current status and future prospects. Semin Hematol 1988; 25 (suppl 3): 16-22.
2. Borgna-Pignatti C. Bone marrow transplantation for the haemoglobinopathies. In: Treleaven J, Barrett J, eds. Bone Marrow Transplantation in practice. Edinburgh:Churchill Livingstone, 1992:151-9.
3. Gordon MY, Greaves MF. Physiological mechanisms of stem cell regulation in bone marrow transplantation and haemopoiesis. Bone Marrow Transplant 1989; 4:335-8.
4. Micklem HS, Anderson N, Ross E. Limited potential of circulating haemopoietic stem cells. Nature 1975; 256:41-3.
5. Eaves CJ. Peripheral blood stem cells reach new heights. Blood 1993; 82:1957-9.
6. Gordon MY. Physiological mechanisms in BMT and haemopoiesis - revisited. Bone Marrow Transplant 1993; 11: 193-7.
7. Dexter TM. Regulation of hemopoietic cell growth and development: experimental and clinical studies. Leukemia 1989; 3: 469-74.
8. Gordon MY, Riley GP, Watt SM, Greaves MF. Compartmentalization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 1987; 326: 403-5.
9. Scott MA, Gordon MY. In search of the haemopoietic stem cell. Br J Haematol 1995; 90:738-43.
10. Carlo-Stella C, Rizzoli V. Stem cells and stem cell factor(s). Haematologica 1995; 80:1-4.
11. Metcalf D. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. Nature 1989; 339:27-30.
12. Orlic D, Bodine DM. What defines a pluripotent hematopoietic stem cell (PHSC): will the real PHSC stand up! Blood 1994; 84:3919-4.
13. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood 1993; 81:2844-53.
14. Tsai FY, Keller G, Kuo FC, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature 1994; 371:221-6.
15. Sutherland HJ, Eaves CJ, Eaves AJ, Dragowskas W, Lansdorp PM. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood 1989; 74:1563-70.
16. Petzer AL, Hogge DE, Lansdorp PM, Reid DS, Eaves CJ. Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating cells) in vitro and their expansion in defined medium. Proc Natl Acad Sci USA 1996; 93:1470-4.
17. Moore MAS. Clinical implications of positive and negative hematopoietic stem cell regulators. Blood 1991; 78:1-19.
18. Smith C, Gasparetto C, Collins NM, et al. Purification and partial characterization of a human hematopoietic precursor population. Blood 1991; 77:1218-27.
19. Gordon MY. Adhesive properties of haemopoietic stem cells. Br J Haematol 1988; 68:149-52.
20. Carlo-Stella C, Mangoni L, Piovani G, Garau D, Almici C, Rizzoli V. Identification of Philadelphia-negative granulocyte-macrophage colony-forming units generated by stroma-adherent cells from chronic myelogenous leukemia patients. Blood 1994; 83:1373-80.
21. Civin CI, Strauss LC, Brovall C, et al. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 1984; 133:157-65.
22. Andrews RG, Singer JW, Bernstein ID. Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. J Exp Med 1989; 169:1721-31.
23. Sutherland RD, Keating A. The CD34 antigen: structure, biology and potential clinical applications. J Hematother 1992; 1:115-29.
24. Carlo-Stella C, Cazzola M, De Fabritiis P, et al. CD34-positive cells: biology and clinical relevance. Haematologica 1995; 80:367-87.
25. Civin CI, Gore SD. Antigenic analysis of hematopoiesis: a review. J Hematother 1993; 2: 137-44.
26. Lansdorp PM, Sutherland HJ, Eaves CJ. Selective expression of CD45 isoforms on functional subpopulations of CD34+ hematopoietic cells from human bone marrow. J Exp Med 1990; 172:363-6.
27. Baum CM, Weissman IL, Tsukamoto AS, Buckle AM.

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Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci USA 1992; 89:2804-8.
28. Huang S, Terstappen LWMM. Lymphoid and myeloid differentiation of single human CD34+, HLA-DR-, CD38-hematopoietic stem cells. Blood 1994; 83:1515-26.
29. Gunji Y, Nakamura M, Osawa H, et al. Human primitive hematopoietic progenitor cells are more enriched in KITlow cells than in KIThigh cells. Blood 1993; 82:3283-9.
30. Rosnet O, Marchetto S, deLapeyriere O, Birnbaum D. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. Oncogene 1991; 6:1641-50.
31. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell 1991; 65: 1143-52.
32. Small D, Levenstein M, Kim E, et al. STK-1, the human homologue of Flk-2/Flt-3, is selectively expressed in CD34+ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. Proc Natl Acad Sci USA 1994; 91:459-63.
33. Lyman SD, James L, Van den Bos T, et al. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. Cell 1993; 75: 1157-67.
34. Lyman SD, James L, Johnson L, et al. Cloning of the human homologue of the murine flt3 ligand: a growth factor for early hematopoietic progenitor cells. Blood 1994; 83:2795-801.
35. Hannum C, Culpepper J, Campbell D, et al. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. Nature 1994; 368:643-8.
36. Kmiecik TE, Keller JE, Rosen E, Vande Woude GF. Hepatocyte growth factor is a synergistic factor for the growth of hematopoietic progenitor cells. Blood 1992; 80: 2454-7.
37. Metcalf D. Hematopoietic regulators: redundancy or subtlety? Blood 1993; 82:3515-23.
38. Eaves CJ, Cashman JD, Eaves AC. Methodology of long-term culture of human hematopoietic cells. J Tiss Cult Meth 1991; 13:55-62.
39. Breems DA, Blokland EAW, Neben S, Ploemacher RE. Frequency analysis of human primitive haematopoietic stem cell subsets using a cobblestone area forming cell assay. Leukemia 1994; 8:1095-104.
40. Gordon MY, Amos TAS. Stochastic effects in hemopoiesis. Stem Cells 1994; 12:175-9.
41. Uchida N, Aguila HL, Fleming WH, Jerabek L, Weissman IL. Rapid and sustained hematopoietic recovery in lethally irradiated mice transplanted with purified Thy-1^{lo}Lin^{-}Sca-1^{+} hematopoietic stem cells. Blood 1994; 83:3758-79.
42. Gordon MY, Blackett NM, Lewis JL, Goldman JM. Evidence for a mechanism that can provide both short-term and long-term haemopoietic repopulation by a seemingly uniform population of primitive human haemopoietic precursor cells. Leukemia 1995; 9:1252-6.
43. Armitage JO. Bone marrow transplantation. N Engl J Med 1994; 330:827-38.
44. Haas R, Murea S. The role of granulocyte colony-stimulating factor in mobilization and transplantation of peripheral blood progenitor and stem cells. Cytokines Mol Ther 1995; 1: 249-70.
45. Kessinger A, Smith DM, Strandjord SE, Landmark JD. Allogeneic transplantation of blood-derived, T cell depleted hemopoietic stem cells after myeloablative treatment in a patient with acute lymphoblastic leukemia. Bone Marrow Transplant 1989; 4:643-6.
46. Russell NH, Hunter AE, Rogers S, et al. Peripheral blood stem cells as an alternative to marrow for allogeneic transplantation. Lancet 1993; 34:1482.
47. Dreger P, Suttorp M, Haferlach T, et al. Allogeneic granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells for treatment of engraftment failure after bone marrow transplantation. Blood 1993; 81: 1404-7.
48. Majolino I, Aversa F, Bacigalupo A, Bandini G, Arcese W, Reali G.Allogeneic transpants of rhG-CSF-mobilized peripheral blood stem cells (PBSC) from normal donors. Haematologica 1995; 80:40-3.
49. Hillyer CD, Wells SJ. Alternative sources of hematopoietic stem cells for bone marrow transplantation and rescue. J Hematother 1993; 2:491-9.
50. Saunders EF, Kapelushnik J, Solh H, Freedman MH. Graft vs host disease (GvHD) is reduced in allogeneic bone marrow transplantation (BMT) using marrow obtained surgically (Abstract). Blood 1990; 76(Suppl 1):563a.
51. Eastlund T, Cahill D, Fautsch S, Bennett S. Hematopoietic progenitors from human cadaveric vertebral body marrow. Preliminary results (Abstract). Blood 1990; 76(Suppl 1):536a.
52. Blazar BR, Lasky LC, Perentesis JP, et al. Successful donor engraftment in a recipient of bone marrow from a cadaveric donor. Blood 1986; 72:1655-60.
53. Lucas PJ, Quinones RR, Moses RD, et al. Alternative donor sources in HLA-mismatched marrow transplantation: T cell depletion of surgically resected cadaveric marrow. Bone Marrow Transplant 1988; 3:211-20.
54. Gluckman E, Broxmeyer HE, Auerbach AD, et al. Hematopoietic reconstitution in patients with Fanconi anemia by means of umbilical cord blood from an HLA identical sibling. N Engl J Med 1989; 321:1174-8.
55. Broxmeyer HE, Douglas GW, Hangor G, et al. Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc Natl Acad Sci USA 1989; 86:3828-32.
56. Harrison DE, Astle CM, Stone M. Numbers and functions of transplantable primitive immunohematopoietic stem cells. J Immunol 1987; 142:3833-9.
57. Broxmeyer HE, Hangoc G, Cooper S, et al. Growth characteristics and expansion of human umbilical cord blood and estimation of its potential transplantation in adults. Proc Natl Acad Sci USA 1991; 89:4109-13.
58. Payne A, Traycoff CM, Laver J, et al. Phenotypic analysis of early hematopoietic progenitors in cord blood and determination of their correlation with clonogenic progenitors: relevance to cord blood stem cell transplantation. Bone Marrow Transplant 1995; 15:187-92.
59. Gerli R, Bertotto A, Crupi S, et al. Activation of cord blood T lymphocytes. I. Evidence for a defective T cell mitogenesis induced through the CD2 molecule. J Immunol 1989; 142: 2583-9.
60. Harris DT, Schumacher MJ, Locascio J, et al. Phenotypic and functional immaturity of human umbilical cord blood T lymphocytes. Proc Natl Acad Sci USA 1992; 89:1006-10.
61. de La Selle V, Gluckman E, Bruly-Rosset M. Newborn blood can engraft adult mice without inducing graft-versus-host-disease across non H-2 antigens. Blood 1996; 87:3977-83.
62. Almici C, Carlo Stella C, Wagner JE, Rizzoli V. Umbilical cord blood as a source of hematopoietic stem cells: from research to clinical appliocation. Haematologica 1995; 80: 473-9.
63. Rebel VI, Miller CI, Eaves CJ, Lansdorp PM. The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their adult bone marrow counterparts. Blood 1996; 87:3500-7.
64. O'Reilly RJ, Pollack MS, Kapoor N, et al. Fetal liver transplantation in man and animals. In: Gale RP, Recent Advances in Bone Marrow Transplantation. New York:Alan R Liss,

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1983; 799-830.
65. Meng PL, Frei RG. Gu DW, et al. Allogeneic fetal liver transplantation in acute leukemia. In: Gale RP, Tourraine JL, Lucarelli G, eds. Fetal liver transplantation. New York:Alan R. Liss, 1985; 281-91.
66. Prummer O, Fliedner TM. The fetal liver as an alternative stem cell source for hemolymphopoietic reconstitution. Int J Cell Cloning 1986; 4:237-49.
67. Fleming WH, Alpern EJ, Uchida N, et al. Functional heterogenity is associated with the cell cycle status of murine hematopoietic stem cells. J Cell Biol 1993; 122:897-902.
68. Siena S, Bregni M, Brando B, et al. Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide treated patients: enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. Blood 1989; 74:1905-14.
69. Demirer T, Bensinger WI. Optimization of peripheral blood stem cell collection. Curr Opinion Hematol 1995; 2:219-26.
70. Gordon MY, Blackett NM. Some factors determining the minimum number of cells required for successful clinical engraftment. Bone Marrow Transplant 1995; 15:659-62.
71. Coggle JE, Gordon MY. Quantitative measurements on the haemopoietic system in three strains of mice. Exp Hematol 1975; 3:181-6.
72. Kernan NA, Flomenberg N , Dupont B, et al. Graft rejection in recipients of T-cell-depleted HLA-nonidentical marrow transplants for leukemia. Transplantation 1987; 43: 842-7.
73. Kernan NA, Collins NH, Juliano L, et al. Clonable T lymphocytes in T-cell-depleted bone marrow transplants correlate with development of graft-v-host-disease. Blood 1986; 68: 770-3.
74. Aversa F, Tabilio A, Terenzi A, et al. Successful engraftment of T-cell depleted haploidentical three loci incompatible transplants in leukemia patients by addition of rhG-CSF mobilized peripheral blood progenitor cells to bone marrow inoculum. Blood 1994; 84:3948-55.
75. Fraser CC, Hoffman R. Hematopoietic stem cell behavior: potential implications for gene therapy. J Lab Clin Med 1995; 125:692-702.
76. Jones RJ, Wagner JE, Celano P, Zicha MS, Sharkis SJ. Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. Nature 1990; 347:188-9.
77. Lemischka IR, Raulet DH, Mulligan RC. Developmental potential and dynamic behaviour of hematopoietic stem cells. Cell 1986; 45:917-27.
78. Jordan CT, Lemischka IR. Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Develop 1990; 4:220-32.
79. Abkowitz JL, Linenberger ML, Newton MA, Shelton GH, Ott RL, Guttorp P. Evidence for the maintenance of hematopoiesis in a large animal by sequential activation of stem cell clones. Proc Natl Acad Sci USA 1990; 87:9062-6.
80. Tavassoli M, Hardy CL. Molecular basis of homing of intravenously transplanted stem cells to the marrow. Blood 1990; 76:1059-70.
81. Aurer I, Gale RP. Are new conditioning regimens for transplants in acute myelogenous leukemia better? Bone Marrow Transplant 1991; 7:255-62.
82. Slavin S, Ackerstein A, Naparstek E, Or R, Weiss L. The graft-versus-leukemia (GVL) phenomenon: is GVL separable from GVHD? Bone Marrow Transplant 1990; 6:155-61.
83. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N Engl J Med 1979; 300:1068-73.
84. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood 1990; 75:555-62.
85. Mitsuyasu RT, Champlin RE, Gale RP, et al. Treatment of donor bone marrow with monoclonal anti-T-cell antibody and complement for the prevention of graft-versus-host disease. Ann Intern Med 1986; 105:20-6.
86. Barrett AJ, Jiang YZ. Immune response to chronic myelogenous leukemia. Bone Marrow Transplant 1992; 9:305-11.
87. Bianco JA, Appelbaum FR. Biotherapy after marrow transplantation and the use of hematopoietic growth factors. Curr Opinion Oncol 1990; 2:289-96.
88. Champlin RE, Gale RP. The early complications of bone marrow transplantation. Semin Hematol 1984; 21:101-9.
90. Gordon MY, Dowding CR, Riley GP, Goldman JM, Greaves MF. Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia. Nature 1987; 328:342-4.
91. Betticher DC, Huxol H, Mller R, Speck B, Nissen C. Colony growth in cultures from bone marrow and peripheral blood after curative treatment for leukemia and severe aplastic anemia. Exp Hematol 1993; 21:1517-21.
92. Li S, Champlin R, Fitchen JH, Gale RP. Abnormalities of myeloid progenitor cells after "successful" bone marrow transplantation. J Clin Invest 1985; 75: 234-41.
93. Martens ACM, Van Bekkum DW, Hagenbeek A. The BN acute myelocytic leukemia (BNML) (A rat model for studying human acute myelocytic leukemia (AML)). Leukemia 1990; 4:41-57.
84. Schultz FW, Martens ACM, Hagenbeek A. The contribution of residual leukemic cells in the graft to leukemia relapse after autologous bone marrow transplantation: mathematical considerations. Leukemia 1989; 3:530-4.

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Appendix

Abstracts from the *Discutiamone Insieme Meeting on* "Ricostituzione ematologica ed immunologica dopo trapianto di cellule staminali" *held in Florence, November 16, 1995.*

HEMOPOIETIC RECOSNTITUTION AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

A. Tabilio,* F. Aversa,* E. Regazzi,° F. Falzetti,* D. Garau,° V. Rizzoli,° M.F. Martelli,* C. Carlo Stella°
**Dept. of Clinical Medicine, Pathology and Pharmacology, Hematology and Clinical Immunology section, University of Perugia, Perugia; °Bone Marrow Transplantation Unit, University of Parma, Italy*

Matched allogeneic bone marrow transplantation (BMT) is the best therapeutic option for many patients with hematological malignancies. We have recently reported that the combination of peripheral blood stem cells (PBSC) and bone marrow leads to rapid reconstitution of hematopoiesis in two-three loci-incompatible haploidentical transplants (*Aversa et al., Blood 1994; 84:3948*).

Several investigators have shown that hematopoietic reconstitution is delayed up to several years after allogeneic BMT despite a trend toward normalization of peripheral cell counts. The present study evaluated hemopoietic reconstitution in 16 patients who received a hapolidentical BMT (MM-BMT) and 7 given T-cell-depleted matched BMT (M-BMT). Sixty bone marrow donors served for normal reference values. Long-term culture-initiating cells (LTC-IC) were assessed by placing $2x10^6$ marrow mononuclear cells on an irradiated murine fibroblast feeder layer consisting of M2-10B4 cells engi-

neered by retroviral gene transfer to produce IL-3 and GM-CSF, and CFU-GEMM, CFU-GM, BFU-E, measured in IMDM supplemented with IL-3, G-CSF, GM-CSF and erythropoietin in methylcellulose. Patients selected for the study had a similar follow-up: a median of six months after BMT (range 2-24). The results are reported in the table.

	LTC-IC	CFU-GEMM	CFU-GM	BFU-E
MM-BMT patients	42±62	1.4±1.3	92±64	32±25
M-BMT patients	76±63	1.9±1.8	137±44	53±17
Normal donors	299±538	5.7±5.3	221±138	73±47

The results indicate that at least at a median of 6 months hemopoietic reconstitution after T-cell-depleted allogeneic haploidentical BMT is comparable to that seen after T-cell-depleted matched BMT. This demonstrates that the bone marrow reserve is remarkable.

SELECTIVE GRAFT-VS-LEUKEMLA EFFECT, DISTINCT FROM GVHD, OF HLA-INCOMPATIBLE BONE MARROW TRANSPLANTS

Nicola Albi, Loredana Ruggeri, Franco Aversa, Massimo F Martelli, Andrea Velardi
Sez. di Ematologia e Immunologia Clinica, Univ. di Perugia, Italy

We have shown that T-cell-depleted, three loci HLA-incompatible bone marrow transplantation for acute leukemia can be successfully accomplished by the addition to the marrow inoculum of G-CSF-mobilized peripheral blood progenitor cells (*Aversa et al., Blood 1994; 84:3948-55; Reisner, Martelli, Immunol Today 1995; 16:437-40*). The event-free survival of patients at high risk for leukemia relapse prompted the present investigation on the anti-tumor potential of this transplant. Tumor-cell lysis mediated by NK cells is regulated by multiple receptors for HLA class I that, upon recognition of the specific alleles, convey an inhibitory signal that blocks NK-cell lytic activity (*Moretta et al., Adv Immunol 1994; 55:341*). In particular, two 58kD, p58 molecules (GL183 and EB6) have been described that function as receptors for HLA-C locus alleles. After HLA-incompatible BMT, we observed the unexpected emergence of a large population, barely detectable in normal subjects, of donor-type $CD3^+/CD8^+$ TcR-ab T-cells that express p58 receptors. Functional analyses of >900 T-cell clones revealed that there are two distinct sets of $p58^+/CD8^+$ T-cells: one in which expression of the NK receptors for HLA class I is associated with an NK-like function of these cells, and the other in which this function is not exerted. Clones of the former cell population lysed HLA class I-negative targets and were functionally blocked by HLA class I alleles on target cells. In particular, as occurs for NK cells, giving HLA haplotypes conferred protection from lysis in a dominant fashion (and susceptibility to lysis is a recessive character). In addition, masking of HLA class I by mAb reconstituted lysis of autologous HLA-protected normal cells. Class I-mediated negative signaling through NK receptors also predominated over stimulatory signaling through TcR, i.e. it blocked TcR-triggered cytotoxicity and may thus impair antigen-specific responses. However, the NK-like recognition system allows these cells to discriminate between normal cells, which were protected from lysis (i.e. from the GvHD), and leukemic cells, which were lysed and may therefore be targets for a selective GvL effect.

IMMUNODEFICIENCY FOLLOWING ALLOGENEIC HEMOPOIETIC STEM CELL TRANSPLANTATION (HSCT): $CD34^+$ CLL COUNTS PREDICT TRANSPLANT-RELATED MORTALITY (TRM)

A. Bacigalupo
Div. di Ematologia II, Centro Trapianti Midollo Osseo, Ospedale S. Martino, Genoa, Italy

We prospectively monitored 300 patients weekly after HSCT until day +100 for surface markers (CD3, CD4, CD8, CD56) by flow cytometry, and then at 6-month intervals. Median CD4 counts/cmm, on day +20, +50, +100 and +150 were 64, 115, 103, 218, respectively. Transplant mortality for patients not achieving these levels was signifcantly increased in univariate analysis (p= .001, .004, .0004, .0001, respectively). CD3 counts were also predictive, but not CD8 or CD56 counts. We then ran a multivariate analysis with the following variables: gender, age, diagnosis, phase of the disease, type of transplant (unmanipulated HSCT from HLA-identical siblings vs. others), year of transplant, interval between diagnosis and HSCT, together with transplant variables such as grade of acute graft versus host disease (aGVHD) and number of cytomegalovirus antigen (CMVAg) positive cells (< 5 vs. ≥ 5 positive cells; p = 0.01, $CD4^+$ cell counts on day +20 and transplant type (= 0.05) were also variables predictive of TRM after step-down analysis. By combining low CD4 counts before day 20 and high CMVAg load (>4 cells) one can identify 3 groups of patients: the first (n=24) has both negative factors and a very high risk of dying of transplant-related complications (65%); the second (n=119) has one risk factor and a TRM of 37%; the third (n=128) has neither and a TRM risk of 19%. This study suggests that absolute $CD4^+$ cell counts may be helpful for assessing the degree of immunodeficiency and the risk of mortality after allogeneic HSCT. TRM can be further predicted by combining CD4 counts with MVAg load.

HEMATOPOIETIC AND IMMUNE RECOVERY AFTER TRANSPLANTATION OF CORD BLOOD PROGENITOR CELLS IN CHILDREN

R. Maccario, F. Locatelli, F. Bertolini,*° P. Comoli, G. Sirchia,* F. Severi
*Clinica Pediatrica, Università di Pavia; *Centro Trasfusionale e di Immunologia dei Trapianti, Ospedale Maggiore Policlinico Milano. Current address: Div. Oncologia Fond. S. Maugeri, Pavia, Italy*

In the last few years matched related cord blood transplantation (CBT) has been successfully used to rescue patients undergoing myeloablative therapy. However, few data are available on the kinetics of the hematological and immunological reconstitution of CBT recipients. We investigated the hematological engraftment and immune recovery following related CBT in three patients affected by acute lymphoblastic leukemia, aged 10, 9 and 7 years and with a body weight of 31, 40 and 25 kilograms, respectively. All patients engrafted and none of them experienced acute or chronic graft-versus-host disease. The time needed to achieve more than 0.5×10^9/L granulocytes was 13, 26 and 29 days, respectively, and platelet recovery occurred in 28, 49, and 51 days. The quickest recovery was documented in the child who also received marrow progenitors. All patients presented a marked increase in HbF, with the values observed being much greater than those documented in patients given marrow transplantation and comparable to those observed in normal children during the first year of life. The recovery of T cell immunity as well as that of natural killer subpopulations mimicked that described in bone marrow transplant recipients, with a quicker retum of $CD8^+$ T cells determin-

ing the characteristic inversion of the CD4/CD8 ratio. An impressive increase in the percentage and absolute number of B lymphocytes, apparently not related to viral infections, was demonstrable in all three cases. These data suggest that CBT recipients may experience a slight delay in hematological recovery in comparison to patients given BMT. The reconstitution of erythropoiesis seems to recapitulate the ontogenetic pattern and the kinetics of immune system recovery reproduces what is observed after BMT, with the peculiarity of B cell expansion in peripheral blood.

LONG-TERM HEMATOPOIETIC RECONSTITUTION FOLLOWING MYELOABLATIVE CANCER THERAPY AND AUTOLOGOUS BLOOD CELL TRANSPLANTATION

S. Siena, M. Bregni, M. Di Nicola, F. Ravagnani, A. M. Gianni

Division of Medical Oncology, Istituto Nazionale Tumori, Milan, Italy

Background. It remains uncertain whether hematopoiesis reconstituted by autografting with only peripheral blood hematopoietic progenitors (CPCs) after high-dose myeloablative radiotherapy and/or chemotherapy is durable and capable of coping with the increased demands made by boost radiotherapy, surgery, or infection.

Patients and Methods. The durability of hematopoiesis was evaluated in 34 consecutive cancer patients treated with myeloablative total body irradiation (n=17; median follow-up 3 years, range 3-49 months) and/or alkylating agent chemotherapy (n=17; median follow-up 8 months, range 6-41 months), and then autografted with CPCs because bone marrow autografting was contraindicated. CPCs (≥ 8×10^6 CD34$^+$ cells/kg) had been collected during mobilization into the circulation in response to previous anticancer therapy and hematopoietic growth factor(s).

Results. Following brief temporary pancytopenia, all patients achieved normal and durable hematopoiesis. The newly reconstituted hematopoietic system was capable of reacting favorably to stressful and noxious events such as surgery, radiotherapy, and varicella-zoster infection. No secondary irreversible failure of blood cell production occurred.

Conclusions. The documentation of the durability of normal hematopoiesis following myeloablative cancer therapy and autografting with mobilized CPCs implies that the latter procedure, rather than being solely an alternative to bone marrow autografting, represents an advantageous tool of choice that permits substantial amelioration of the therapeutic index of high-dose cancer therapy.

HEMATOPOIETIC RECONSTITUTION FOLLOWING AUTOLOGOUS TRANSPLANTATION OF ENRICHED PERIPHERAL BLOOD CD34$^+$ STEM CELLS IN MULTIPLE MYELOMA PATIENTS

R.M. Lemoli, A. Fortuna, D. Raspadori, A. Ventura, M. Cavo, S. Tura

Institute of Hematology "L. & A. Seragnoli", University of Bologna, Bologna, Italy

In this study we evaluated PB samples from 27 pretreated MM patients after the administration of high-dose cyclophosphamide (Cy; 7 g/m^2 or 4 g/m^2) and granulocyte colony-stimulating factor (G-CSF) for collection of circulating stem cells (PBSC) to support hematopoietic reconstitution following myeloablative radio-chemotherapy. Twenty-three patients showed adequate mobilization of CD34$^+$ progenitor cells and were submitted to PBSC collection. In 10 patients the circulating hematopoietic CD34$^+$ cells were highly enriched by avidin-biotin immunoabsorption and cryopreserved. The median purity of the enriched CD34$^+$ cell population was 89.5% (range 51-94%) with a 75-fold increase compared to the pretreatment samples. The median overall recovery of CD34$^+$ cells and CFU-GM was 58% (range 33-95%) and 45% (range 7-100%), respectively. Myeloma patients were reinfused with enriched CD34$^+$ cells after myeloablative therapy consisting of total body irradiation (TBI, 1000 cGy) and high-dose melphalan (140 mg/m^2). They received a median of 4×10^6 CD34$^+$ cells/kg and showed rapid reconstitution of hematopoiesis: the median time to 0.5×10^9 neutrophils and 50×10^9 platelets/L of PB was 10, 11 and 12 days, respectively. When we analyzed the immunological reconstitution of this group of patients, we observed a rapid and full recovery of total lymphocyte and NK cell counts, although the absolute CD4$^+$ cell counts were much lower than normal controls. These results, as well as other clinically significant parameters, did not significantly differ from those of patients (=13) receiving unmanipulated PBSC following the same pretransplant conditioning regimen. In summary, our data demonstrate that positive selection of CD34$^+$ progenitor cells provides a cell suspension capable of restoring normal hematopoiesis after a TBI-containing conditioning regimen.

EFFECT OF INDUCTION AND CONSOLIDATION THERAPY FOR ACUTE MYELOGENOUS LEUKEMIA ON THE RECONSTITUTIVE POTENTIAL OF AUTOLOGOUS MARROW.

C. Carlo-Stella, A. Tabilio, E. Regazzi, D. Garau, R. La Tagliata, S. Trasarti, M. Martelli, V. Rizzoli, G. Meloni

Dept. of Hematology, University of Parma; Dept. of Hematology, "La Sapienza" University, Rome; Dept. of Hematology, University of Perugia, Italy

Although defective colony formation following chemotherapy for acute myelogenous leukemia (AML) is a well-known finding, autologous marrow is successfully used to reconstitute hematopoiesis in AML patients receiving high-dose chemo-radiotherapy. Recently, AML patients included in the AML10 pilot study receiving the ICE/NOVIA regimens as induction-consolidation therapy were reported to have reduced marrow cellularity, often preventing marrow harvest, and markedly delayed engraftment. It was therefore the aim of the present study to investigate marrow clonogenic activity and microenvironment function in AML patients (n= 29) included in the AML10 protocol. To evaluate marrow clonogenic activity, the number of primitive (LTC-IC) and committed (CFU-Mix, BFU-E, CFU-GM) progenitors was determined after consolidation therapy and at marrow harvest. Marrow microenvironment was studied by evaluating the quantity of fibroblast colony-forming cells (CFU-F) and the function of autologous marrow stroma. Sixty bone marrow donors served as controls. Compared to controls, AML patients studied after consolidation therapy (n= 11) and at marrow harvest (n= 18) showed a significant reduction of LTC-IC ($p \leq .03$ and $\leq .002$), CFU-Mix ($p \leq .003$ and $\leq .0001$), BFU-E ($p \leq .004$ and $\leq .0001$), and CFU-F ($p \leq .008$ and $\leq .002$). CFU-GM growth was significantly reduced after consolidation therapy ($p \leq .01$), but not at marrow harvest ($p \leq .09$). When patients studied at harvest were stratified according to inclusion in the pilot or the randomized AML10 trial, the incidence of marrow LTC-IC was significantly higher ($p \leq .04$) in those (n= 9) randomized to receive ICE/IDIA or MICE/NOVIA or DCE/DIA as compared to the ones (n= 9) receiving the ICE/NOVIA therapy. The function of marrow stroma, evaluated as time to reach confluence, adipocyte formation, and the capacity

to support allogeneic LTC-IC, was significantly deranged in all patients, irrespectively of different induction-consolidation regimens and study time. When engraftment data from 18 patients were correlated with progenitor cell growth, no correlation was observed between *in vivo* hematopoietic recovery and the number of reinfused committed progenitors. Interestingly, an inverse correlation was observed between the number of LTC-IC and neutrophil and platelet recovery. In conclusion, this study demonstrates that: (i) induction-consolidation chemotherapy regimens used for AML patients induce a significant, although partially reversible, reduction in LTC-IC, CFU-Mix, BFU-E, CFU-GM and CFU-F growth, associated with severe derangement of stroma function; (ii) both these alterations might reduce the reconstitutive capacity of autologous marrow; (iii) the degree of LTC-IC damage might be dependent on different chemotherapy combinations; (iv) the number of reinfused LTC-IC, but not CFU-GM, seems to correlate with engraftment.

IMMUNE RECONSTITUTION FOLLOWING POSITIVELY SELECTED CD34$^+$ PROGENITOR CELL (PBPC) TRANSPLANTATION: A FLOW CYTOMETRIC STUDY

S. Rutella,* C. Rumi,* S. Sica, B. Etuk,* L. Teofili, E. Ortu La Barbera, P.L. Puggioni,* G. Leone
Center for the flow cytometric study of hematic cells and Chair of Hematology, Catholic University, Rome, Italy

The recovery of lymphocyte count, T cells, CD4$^+$ and CD8$^+$ (cytotoxic and suppressor) T cell subsets, activated T cells, NK and B lymphocytes was evaluated in a group of 7 patients (pts, group A) affected by resistant lymphoproliferative disease and submitted to transplantation with immunoselected CD34$^+$ PBPC (CEPRATE®, Bothell, Washington, USA). Blood samples were obtained weekly from the beginning of the conditioning regimen (BuCy2) to day +70 from transplantation. All determinations were performed using Simultest IMK (Becton Dickinson, CA, USA) and a FACScan flow cytometer (BD). All pts received rhG-CSF (5 mg/kg/day s.c.) in the post-transplant period. Results were compared to data obtained in 10 pts submitted to transplantation with unfractionated PBPC (group B) for hematological malignancies and comparable in terms of conditioning regimen. Normal values for lymphoid subsets were collected from 25 healthy blood donors. Absolute lymphocyte counts recovered more quickly in group B (>1200/μL by week +4) than in group A (>1200/μL by week +7; p<0.01). In group A, CD3$^+$ cells remained below the normal range throughout the study period, CD8$^+$ cells exceeded 500/μL by week +7 and NK cells were over 100/μL by week +6; in group B, CD3$^+$ cells reached values >1000/μL by week +6 (p<0.001 compared to group A), CD8$^+$ cells >500/μL by week +6 and NK cells >100/μL by week +2. In both groups, CD3$^+$ cells displayed an activated phenotype and coexpressed HLA-DR starting from week +3. CD4$^+$ and CD19$^+$ cells remained below normal values throughout the study period. The normal ratio between cytotoxic (CD8$^+$CD11b$^-$) and suppressor (CD8$^+$CD11b$^+$) T cells was preserved in both groups. The most impressive finding was the remarkably low number of CD4$^+$ cells in group A (<200/μL throughout the study period; p<0.001 compared to group C). Contemporarily to this observation, there was an unexpectedly high incidence of infectious complications in group A compared with pts transplanted with unfractionated PBPC, including viral (1 CMV and 1 adenovirus), fungal (*Candida sp*) and bacterial infections (1 Gram$^-$, 3 Gram$^+$). These findings should induce a prudential clinical attitude towards these patients with such immunological fragility. Nevertheless, long-term follow-up is warranted.

IMMUNE RECOVERY AFTER BLOOD PROGENITOR CELL AUTOTRANSPLANTATION.

A. Olivieri, M. Brunori, M. Offidani, L. Ombrosi, P. Scalari*, C. Regnery*, P. Leoni
Clinica di Ematologia e Servizio di Immunologia Clinica e Tipizzazione Tissutale, Università di Ancona, Italy*

Immune recovery (IR) after bone marrow transplantation can play a critical role not only in the incidence of severe infections but also in relapse and in second tumor development. Only a few experiences have been reported regarding the kinetics of IR after blood progenitor cell (BPC) autotransplantation. The aim of this study was to evaluate the kinetics of lymphoid subsets and identify factors affecting their behavior. We studied 40 patients affected by hematologic and non-hematologic malignancies; 30 were autotransplanted with BPC alone and 10 with BPC together with bone marrow; the median follow-up was 6 months (range 1-24). Total lymphocyte and T-lymphocyte count (CD3$^+$) recovered very quickly. In particular, the CD8$^+$ (suppressor/cytotoxic) subset showed rapid reconstitution and its count remained above the normal range until the third month; only within the 6th month did CD8$^+$ cells reach normality. Most of these cells coexpressed CD57 antigen (i.e. cytotoxic cells). By contrast, the CD4$^+$ (helper/inducer) lymphocyte count showed a serious depression in all patients (< 500 cells/μL) during the first 15 months and remained under the normal range after 24 months. The CD3$^-$/CD16$^+$ (NK) cell absolute count was slightly reduced until the 24th month, while the B lymphocyte count increased slowly with complete normalization at the 15th month. The probability of reaching a count of >200 CD4$^+$ cells/μL, a significant threshold for the incidence of opportunistic infections in AIDS patients, was 75% at the first month and 100% at the 12th month after transplantation.

As regards the CD21$^+$ subset, the probability of normalization was 20% at the first month, 50% at the 3rd month and 95% at the 15th month. However, we did not observe any severe opportunistic infections after transplantation in our 40 patients, so a threshold of CD4$^+$ >200/μL is probably sufficient to avoid severe opportunistic infections and antibody production after the first year following BPC transplant.

Univariate and multivariate analysis were performed using as dependent variable the time to reach 200 CD4$^+$ cells/μL and a normal value of CD21$^+$ cells; the independent variables were age, sex, diagnosis, previous chemo-radiotherapy, pretransplant status, kind of priming, conditioning regimen, source of stem cells (BPC alone or BPC plus bone marrow), sepsis incidence and the number of MNC/Kg, CFU-GM/kg and CD34$^+$/kg reinfused. None of the above factors significantly influenced the rate of IR. Our results demonstrate that the kinetics of IR following BPC autotransplantation is independent of the clinical features and of the amount of progenitor cells infused, whereas it is probably related to immune system ontogenesis.

Finally, even though the cytotoxic subset is expanded early after transplantation, the NK compartment does not reflect this behavior; therefore enhancement of this lymphocyte subset with immunotherapy should be explored.

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