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The happy destiny of frozen haematopoietic stem cells: from immature stem cells to mature applications

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Abstract

Forty years ago, van Putten described in the *European Journal of Cancer* (see this issue) quantitative studies on the optimal storage techniques of mouse and monkey bone marrow suspensions. Survival of the animals after irradiation following injection with stored bone marrow cell suspensions was the endpoint. He observed some species differences, but based on the data obtained considered a careful trial of the glycerol-polyvinylpyrrolide (PVP) combination for storage of marrow in man was indicated. In spite of this, dimethyl sulphoxide has become the 'standard' cryopreservant for human marrow stem cells. Over the last 40 years, there has been a tremendous increase in knowledge about haematopoietic stem cells and their use in the clinic. Haematopoietic stem cells are now known to travel between the bone marrow and peripheral blood and are the best-characterised adult stem cells. These cells are currently widely used for transplantations in the clinic and are obtained from a wide variety of sources. These include the bone marrow, peripheral blood, cord blood, autologous as well as allogeneic stem cells from related or unrelated donors. Increasingly, data has become available that adult haematopoietic stem cells can generate differentiated cells belonging to other cell types, a process called "developmental plasticity". Thus, they may contribute to non-haematopoietic tissue repair in multiple organ systems. This has created a whole new potential therapeutic armamentarium for the application of haematopoietic stem cells outside of the area of malignancies and haematopoietic disorders.

Keywords: Haematopoietic stem cells storage; Transplantation; Bone marrow; Blood; Plasticity

1. Introduction

Once upon a time, almost 40 years ago, van Putten, a Dutch investigator described in the *European Journal of Cancer* (see this issue) quantitative studies on the optimal storage techniques of mouse and monkey marrow suspensions [1]. Survival of the animals after irradiation following injection with stored cell suspensions was the endpoint. Some species differences were observed, but, in general, the data supported a careful clinical trial of the glycerol-polyvinylpyrrolide (PVP) combination for bone marrow storage. This report appeared in a time that the storage of human bone marrow cells for transplantation had only provided suggestive evidence for clinical application. Thomas was the first to show that bone marrow cells could be infused and resulted in temporary recovery of haematopoiesis in man [2]. However, it took more than 20 years before allogeneic bone marrow transplantation was used on a large scale, and became standard treatment for a large variety of haematological and immune deficiency diseases [3]. Similarly, stored autologous bone marrow cells seemed attractive tools to treat patients with chemotherapy- or radiation-induced myelosuppression. McFarland *et al.* [4] published in 1959 the first results in this area.

Interestingly, the concept of marrow cell transfusion had already been translated to the clinic at a time when the existence of haematopoietic stem cells and their regulatory factors was completely unknown. Over the last 40 years, there has been a tremendous increase in

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knowledge, while only limited changes in the freezing techniques have occurred. In the 1970s, clonogenic assays were developed [5]. These assays showed that there were specific progenitor cells of neutrophil, macrophages, eosinophils, megakaryocytes and erythroid cells. These short-term cultures reflected matured haematopoietic progenitors. Next, an alternative *in vitro* culture assay was developed by Ploemacher *et al.* [6] in which more immature stem cells could be tested. In the 1980s the genes for various haematopoietic growth factors were cloned. This allowed the production of recombinant human haematopoietic growth factors. The effects of these factors on the bone marrow were extensively tested in the laboratory and the clinic. Of these factors, granulocyte-colony stimulating growth factor (G-CSF) has gained an important position in clinical practice. However, these growth factors have not made the use of haematopoietic stem cells superfluous in the clinic.

Currently, we know that haematopoietic stem cells, defined as cells with extensive self-renewal and pluripotent differentiation potential, give rise to blood cells and comprise only 0.5% of the population of adult bone marrow cells. Haematopoietic stem cells are now known to travel between the bone marrow and peripheral blood and are the best-characterised as adult stem cells. They are currently widely used for transplantations in the clinic [7]. These transplants contain stem cells derived from bone marrow, peripheral blood, cord blood, autologous as well as allogeneic stem cells of related or unrelated donors. Haematopoietic stem cell reinfusion is performed to replace defective haematopoiesis (and lymphopoiesis). In cancer treatments, haematopoietic stem cells are intended to restore bone marrow function following intense radiotherapy and/or chemotherapy.

Increasing data have become available that adult stem cells generate differentiated cells beyond their own boundaries, a process called "developmental plasticity". This has created a whole new potential therapeutic armamentarium for the application of haematopoietic stem cells outside of the area of malignancies and haematopoietic disorders [8].

In this article, we will describe new knowledge about haematopoietic stem cells and address standard treatment options, as well as new perspectives for their application.

2. Haematopoietic stem cell sources

2.1. Bone marrow

Although van Putten ends his paper with the suggestion to explore the application of a glycerol-PVP mixture for bone marrow storage, dimethyl sulphoxide (DMSO) has become the 'gold standard' cryoprotectant [9]. The stem cells are usually stored in liquid nitrogen, although centres have successfully attempted to store bone marrow cells (or blood stem cells) at 4 °C for periods of up to 9 days [10].

Bone marrow for reinfusion is usually collected under general anaesthesia by multiple marrow aspirations. These cells are now known to remain adequate for safe transplantation after up to 14 years of cryostorage [11]. The freezing of bone marrow is largely replaced by storage of peripheral haematopoietic stem cells. For allogeneic bone marrow transplantation, the donor bone marrow can directly, without freezing, be reinfused in the patient.

2.2. Peripheral haematopoietic stem cells

In the peripheral blood, only very few haematopoietic stem cells are present. These circulating haematopoietic progenitor cells include pluripotent stem cells and, therefore, can also be used for restoring haematopoiesis following myeloablative treatment. Chemotherapy and haematopoietic growth factors can induce a transient shifting of progenitor cells from extravascular sites into the circulation. This enables the collection by apheresis of a sufficient number of progenitor cells to guarantee engraftment. A large volume apheresis further increases peripheral blood progenitor cell procurement efficiency. The frequency of repetitive collections depends on the pre-treatment conditions of the patient.

There are a number of advantages of peripheral haematopoietic stem cells versus bone marrow cells. Randomised trials have demonstrated an enhanced recovery of all cell lineages post-transplantation by using peripheral blood cells compared with bone marrow cells. The collection of the stem cells is considered less burdensome as no general anaesthesia is required.

In the adjuvant breast cancer study of Tallman *et al.* [12] bone marrow was reinfused after high-dose chemotherapy, while in the same setting Rodenhuis *et al.* [12] used peripheral stem cells. In the first study, the occurrence of leukaemia and myelodysplastic syndrome was noticed in the high-dose arm, while no evidence was observed in the other study. It is speculated that the change to develop leukaemia or myelodysplastic syndrome is lower following peripheral stem cell harvest. However, 5–10% of the patients treated with autologous stem cell transplantation for relapsed aggressive lymphoma develop myelodysplasia. Pre-treatment conditions, including radiotherapy and chemotherapy, determines the occurrence of this frequently fatal complication.

For allogeneic transplantation, peripheral blood stem cells are also being used. A disadvantage is the presence of high numbers of T-cells, which increases the risk of graft-versus-host disease (GVHD). Several centres therefore perform T-cell depletion protocols and add a limited number of T cells to the graft.

In spite of these disadvantages, based on the easier procurement of haematopoietic stem cells and advantageous engraftment characteristics, peripheral haematopoietic stem cell reinfusion has mostly replaced the use of bone marrow-derived progenitor cells in autologous and allogeneic transplant protocols.

2.3. Cord blood

Knudtzon demonstrated in 1974 the presence of relatively mature haematopoietic progenitors cells in human cord blood. However, it took until 1989 for studies to indicate that human umbilical cord blood could be used in the clinical setting. Much effort has been expended on the characterisation of these stem cells. There appear to be clear functional differences between cord and adult bone marrow haematopoietic stem cells [14]. The cord blood stem cells show a higher proliferative capacity and expansion potential. Allogeneic stem cell transplantation can be limited by the lack of suitable bone-marrow donors and the risk of GVHD. Umbilical-cord blood is therefore a third alternative source of haematopoietic stem cells that is currently used. The percentage of stem cells is higher in cord blood than in bone marrow and mobilised blood, but the absolute number is lower than in the two other stem cell sources. Therefore, umbilical cord transplantation is mostly applied in children. An advantage of the umbilical cord blood transplantation is the lower incidence of GVHD. This stem cell graft is therefore also frequently used for matched unrelated donor (MUD) transplantation. Reassuring is the fact that immature human cord blood cells with high proliferative, *ex vivo* expansion and mouse non-obese diabetic-severe combined immunodeficient (NOD-SCID) engrafting ability stored frozen for over 15 years, can be efficiently retrieved, and most likely remain effective for clinical transplantation [15].

Since 1988, over 2000 patients have been treated with cord blood transplants for haematological malignancies, bone marrow failure and inborn disorders. Best results are obtained with cord blood from an human leucocyte antigen (HLA)-identical sibling. However, the broadest applicability of cord blood lies in the use of cord blood for unrelated haematopoietic stem cell transplantations. For this indication, several cord blood banks have been set up. NetCord is an international organisation, which takes care of uniform quality standards for the handling of cord blood for transplants.

3. Purging of haematopoietic stem cell graft of patients with malignancies

Tumour cells frequently contaminate autologous stem cell products of cancer patients [16]. Mobilised peripheral blood stem cells may be less contaminated with tumour cells than bone marrow harvests. Gene-marking studies using retroviral vectors provide evidence that tumour cells contained in autografts contribute to relapse in myeloid leukaemia and neuroblastoma patients. This has stimulated research to clear the tumour cells from the haematopoietic graft. Various techniques have been described, including depletion of tumour cells (purging) and selection of stem cells from the graft. Tumour cell depletion by non-selective chemotherapeutic drugs can, at least partly, eliminate tumour cells, but haematopoietic colony formation is negatively affected. Stem cells selected through enrichment of CD34-positive cells still contains a number of tumour cells. Antibodies and cytokines have also been explored to purge tumour cells. Activation of T cells present in stem cell harvest of breast cancer patients combined with a bispecific antibody has been tested for purging of tumour cells in the harvest. With this approach, specific tumour cell lysis by peripheral blood stem cells was observed, without affecting haematopoietic colony formation [17].

There are until now no randomised trials showing improved (disease-free) survival with purging of autologous haematopoietic stem cells. In the absence of such trials, the contribution of tumour cells in the stem cell autografts to subsequent relapse remains controversial. For this reason, there is currently much less data on purging being published.

4. Indications for autologous and allogeneic haematopoietic stem cell transplantation

The advantage of autologous transplantation is that no GVHD will develop, but the disadvantage is that also no graft versus tumour effect will occur and there is also risk of contamination by tumour cells.

4.1. Autologous stem cell transplantation in haematological malignancy

Autologous stem cell transplantation is increasingly applied as rescue therapy in patients with haematological malignancies. It has become the standard therapy in patients with relapsing aggressive lymphoma and Hodgkin's disease in case the underlying disorder is still chemo-sensitive. In most studies, an overall survival of 50% is obtained in such patients [18]. During recent years, a number of studies have been performed to address the question whether intensive chemotherapy followed by autologous stem cell transplantation is of value in patients with multiple myeloma [19]. Several randomised trials have demonstrated an advantage of the autologous stem cell transplantation versus standard treatment with regard to overall and event-free survival. Based on these data,

autologous stem cell transplantation belongs to the standard patient care in multiple myeloma cases under the age of 65 years. In view of the encouraging results in multiple myeloma, a number of phase II studies have been performed in patients with primary systemic (AC)-amyloidosis [20]. Although the transplantation related mortality is significantly higher in this group of patients, autologous stem cell transplantation is increasingly applied in a subgroup of the patients, which improves the treatment results when compared with a historical control group. Finally, the value of autologous stem cell transplantation has been tested in the treatment of patients with acute myeloid leukaemia and myelodysplastic syndrome. A prerequisite for performing this type of intervention is to obtain an adequate stem cell graft. Phase II studies have indicated that a significant higher dose of G-CSF is required for obtaining an adequate stem cell graft following intensive chemotherapy in these patients [21]. Whether autologous stem cell transplantation improves the results in acute myeloid leukaemia (AML) patients compared with a standard consolidation course has not been resolved.

4.2. Autologous transplantation in solid tumours

The role of high-dose chemotherapy followed by haematopoietic stem cell reinfusion for solid tumours is still a matter of debate. A potential major indication is breast cancer. At the moment, 14 randomised trials in 5574 patients have studied the role of high-dose chemotherapy as adjuvant treatment for breast cancer. Of these studies, six were "symmetric", answering the question of whether one cycle of high-dose chemotherapy followed by haematopoietic stem cell reinfusion does add to standard (anthracycline)-containing chemotherapy. In all studies, less relapses are observed after high-dose chemotherapy. In the two studies, this did not result in an improved disease free survival due to the relatively high mortality rate in the high-dose arm [12,22]. In the Dutch study, high-dose alkylating therapy improved relapse-free survival among patients with stage II or III breast cancer and 10 or more positive axillary lymph nodes [13]. Based on a retrospective analysis for Her-2 neu, this benefit may well be confined to patients with HER-2/*neu*-negative tumours. Most studies still need maturation to reveal the effect on overall survival. In 2004, a meta-analysis of the adjuvant high-dose breast cancer studies will be performed. In addition, patients with a relapse of a germ cell tumour may benefit from high-dose chemotherapy, although data with improving results of standard dose chemotherapy were also obtained recently. For several solid tumour types, (randomised) studies are still evaluating the precise role of high-dose chemotherapy.

5. Adult haematopoietic stem cells for tissue repair

Recently, it was suggested that adult stem cells originating from bone marrow or peripheral blood contribute to the repair and genesis of cells specific for liver, cardiac and skeletal muscle, gut and brain tissue [8]. It is likely that different types of stem cells will have their selective contribution. Presently, haematopoietic stem cells, endothelial stem cells and mesenchymal stem cells are recognised. The mechanism involved has been named transdifferentiation, although other explanations including cell fusion have been postulated. Below we will address a number of examples.

Korbling *et al.* [23] showed that circulating stem cells could differentiate into mature hepatocytes and epithelial cells of the skin and gastrointestinal tract. Six recipients of gender-mismatched haematopoietic stem cell transplants showed evidence of complete haematopoietic donor chimerism. XY-positive epithelial cells and hepatocytes accounted for 0–7% of the cells in histological sections of the biopsy specimens. These cells were detected in liver tissue as early as day 13 and in skin tissue as late as day 354 after the transplantation of peripheral-blood stem cells.

Under appropriate cell culture conditions, stem cells are also capable of differentiation into cardiac myocytes and endothelial cells [24]. If these results can be reproduced *in vivo*, it may be possible to use stem cells to impede progressive deterioration to heart failure or even to restore cardiac function in patients with damaged myocardium. A number of trials with local injection of mesenchymal stem cells or freshly aspirated bone marrow cells are now underway. Eventually, the final proof of its relevance will have to come from randomised clinical trials.

6. Newly detected characteristics of haematopoietic stem cells

Recently, many new characteristics of haematopoeitic stem cells have been revealed. Using microarray analyses, murine and human haematopoietic stem cells were found to share a number of expressed gene products, which define key conserved regulatory pathways in this developmental system. Moreover, in the mouse, a portion of the genetic programme of haematopoietic stem cells was found to be shared with embryonic and neural stem cells [25]. This overlapping set of gene products represents a molecular signature of stem cells suggesting that the cell environment is of utmost importance in determining which genetic programme is initiated.

In vitro techniques have been developed that make use of the newly detected stem cell properties of these cells to extrude toxic compounds. The genes responsible for these effects include the ABC transporters.

Haematopoietic stem cells can, based on these properties, be functionally characterised by a low accumulation of the fluorescent dyes Hoechst 33342 or rhodamine 123 (Rh123) and are therefore called 'side population' cells. Both compounds are a substrate for the ABC transporter P-glycoprotein encoded by the multidrug resistance (MDR1)-gene.

Another multidrug resistance protein, belonging to the ABC family of transporters, the breast cancer resistance protein (BCRP) is expressed especially in the dye effluxing side population of normal haematopoietic stem cells [26]. BCRP expression appeared to be uniquely restricted to immature haematopoietic progenitor cells that can contain $CD34^-$ and $CD34^+$ cells. Transporter proteins turn out to have important functional effects in the stem cell compartment by affecting the differentiation programme of the primitive stem cell population. It has been suggested that high expression of the ABC transporters is critical for maintaining the quiescent stem cell state by extruding regulatory molecules, such as steroids, required for differentiation, which is consistent with the lipophilic substrate specificity of for e.g. P-glycoprotein and BCRP.

7. Homing of the haematopoietic stem cells

Just as in the days of van Putten's study, factors affecting the homing of stem cells are currently attracting a lot of attention. Plett *et al.* [27] tracked phenotypically-defined populations of bone marrow cells in lethally-irradiated or non-irradiated mice during the first few hours after transplantation. At all time points, recovery of crude, non-purified as well as highly purified transplanted cells was higher in the bone marrow of non-irradiated mice. When 50–100 of these bone marrow-homed cells were examined in serial transplantation studies, bone marrow-homed cells from initially non-irradiated mice were enriched 5–30-fold for cells capable of long-term haematopoiesis in secondary recipients. These data suggest that homing or survival of transplanted cells in irradiated recipients due to radiotherapy effects on the microenvironment is less efficient than that in non-irradiated recipients.

Additional new light was shed on the issue of homing and haematopoietic stem cell pool composition by the group of Mazurier *et al.* [28]. They studied the effect of intrafemoral stem cell injection. Thus far, haematopoietic stem cell repopulation assays have been based on intravenous (i.v.) injection of stem cells. This process requires circulation through blood, recognition and extravasation through bone marrow vasculature, and migration to a supportive microenvironment. Thus, some classes of haematopoietic stem cells may remain undetected. By direct intrafemoral injection, rapidly repopulating cells within the Lin-CD34+CD38loCD36- subpopulation were identified. Those cells efficiently generated high levels of human myeloid and erythroid cells within the injected femur and migrated to the blood and colonised individual bones of non-obese diabetic (NOD)-SCID mice within 2 weeks after transplantation. Lentivector-mediated clonal analysis of individual rapid repopulating cells revealed heterogeneity in their proliferative and migratory properties. The identification of a new class of haematopoietic stem cells and an effective intrafemoral assay provide the tools required to develop more effective stem cell-based therapies that rely on rapid reconstitution.

8. Conclusions

Van Putten's bone marrow study was performed in mice and monkeys. Studies in monkeys are nowadays considered to yield hardly any additional information. Even further, the question addressed by van Putten, namely what is the optimal freezing technique for bone marrow cells would nowadays merely be answered by tissue culture assays instead of experiments in mice. However, studies in mice, as shown in this article, are still an ongoing source for new knowledge on haematopoietic stem cells. Since the publication of van Putten, haematopoietic stem cells have been shown to play an important role in the clinic. Because of the exciting data currently becoming available on the role of haematopoietic stem cells in tissue regeneration it may well be that haematopoietic stem cells can live 'happily ever after' in various tissues in the body where they can contribute to repair processes physiologically or as an intervention.

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