Haematopoietic Graft Engineering

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Abstract

Although haematopoietic stem cell transplantation (HSCT) is a powerful treatment modality, it is a blunt instrument against cancer and diseases of the haematopoietic and immune system. Various techniques have been developed to engineer the haematopoietic stem cell (HSC) graft to enable it to perform its task more effectively. These techniques include the removal of cells which cause graft-versus-host disease (GVHD), the eradication of cells which might cause relapse, the expansion of donor cells when there is an inadequate cell dose, and the addition of selected cells to improve graft function. In this review, we will discuss each of these means of haematopoietic graft manipulation in turn and then touch on some regulatory requirements in the field of haematopoietic graft engineering. While the science is still prepubescent, it has passed its infancy and further developments in the next decade or so should allow it to be taken to a wider scale to benefit more patients.

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Introduction

Haematopoietic stem cell transplantation (HSCT) has become an established modality for the treatment of cancer and diseases of the bone marrow and immunological system. Such transplants effect their cures through 3 main avenues: namely, eradication of disease through intensive chemo/radiotherapy, replacement of defective blood-forming cells with normal haematopoietic progenitors and resetting of the immunological rheostat. Firstly, through intensive myeloablative chemo/radiotherapy, residual cancer cells in a patient are destroyed. In addition, abnormal immune cells, which mediate autoimmune disease, can also be eradicated. However, this leads to bone marrow failure and an HSCT is thus necessary. Secondly, in bone marrow failure syndromes like severe aplastic anaemia and in genetic disorders like thalassaemia, an HSCT could lead to the establishment of normal haematopoiesis. Thirdly, the patient may have a defective immune system and lack the immune effector cells necessary to eradicate his own cancer. Alternatively, he could possess an abnormal self-directed autoimmunity. An HSCT allows for a revamping of the immune system to enable it to attack cancer cells or to stop attacking itself. This "redemption" of the immune system is the main means by which transplants not utilising marrow ablative doses of chemo/radiotherapy (the so-called non-myeloablative transplants) work.

Although HSCT is a powerful treatment modality, it is a blunt instrument against cancer and diseases of the haematopoietic and immune system. While allogeneic transplants (with the infusion of haematopoietic cells from another individual) may introduce cells that initiate a graft-versus-tumour (GVT) effect,[1] they may also introduce cells that lead to a potentially fatal graft-versus-host disease (GVHD) where donor cells attack the recipient organs. On the other hand, in autologous transplants, the source of the haematopoietic cells is from the same individual. As such, in autologous HSCT, there is not only the possibility that tumour cells may be present in the graft, causing a relapse, there is also the likelihood that the same patient's immune cells will not be able to recognise and eradicate the tumour.

Consequently, various techniques have been developed to engineer the haematopoietic stem cell (HSC) graft to enable it to perform its task more effectively. These techniques include the removal of cells which cause GVHD, the removal of cells which might cause relapse, the expansion of donor cells when there is an inadequate cell dose, and the addition of cells to improve graft function (Table 1). In this review, we will discuss each of these means of

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Table 1. Strategies Employed in Haematopoietic Graft Engineering

A) Removal of Cells that Cause GVHD
1. Negative selection of donor T cells
* Soybean lectin agglutinin and E-rosette depletion
* Monoclonal antibodies (mAb) - against CD3+ lymphocytes
- other cell surface markers e.g. CD52
2. Counterflow centrifugation elutriation
3. Introduction of HSV-TK suicide gene for selective killing in the event of GVHD
4. Selective depletion of various lymphocyte subsets
5. Blockade of differential cytolytic pathways
B) Removal of Cells that Cause Relapse
1. Pharmacological purging
* metabolites of cyclophosphamide like 4-hydroxycyclophosphamide (4-HC) and mafosfamide
* pre-treatment with amifostine
2. Immunological methods for tumour cell mAb
* usually conjugated with other agents to increase their effect on the tumour cells, e.g. cytotoxic chemicals, like ricin-A or saporin-6, and serum complement with or without the use of immunomagnetic beads
3. Positive selection for CD34 or CD133 positive haematopoietic precursors
4. Combination of enrichment of CD34+ cells and tumour-directed negative selection
5. Others
* Suicide adenoviral vectors expressing the cytosine deaminase gene (ACD)
* Hyperthermia
* 5-aminolevulinic acid (ALA)-based photodynamic treatment (ALA-PDT)
C) Expansion of Donor Cells
1. Stroma-free liquid suspension cultures employing various cocktails of cytokines
2. Stromal cell-dependent cultures
3. Clinical scale culture utilising
* Gas-permeable Teflon-coated bags
* Artificial 3-dimensional matrix culture systems
* Clinical-scale bioreactor devices - continuously perfused flat culture-plates
- stirred suspension devices
D) Addition of Cells to Improve Graft Function
1. T cell clones are rendered specific for tumour antigens
2. Expanded CD8+ NK-T cells with anti-tumour activity
3. Immunoregulatory cells
* Facilitating cell (FC) populations
* CD4+CD25-regulatory T cells
4. Gene transfer technologies
* Treatment of genetic diseases
* Cancer (gene marking)
* Infectious diseases [e.g. human immunodeficiency virus (HIV)]

haematopoietic graft manipulation in turn and then touch on some regulatory requirements in the field of haematopoietic graft engineering.

Removal of Cells that Cause GVHD

Selective removal of alloreactive T cells has been able to reduce GVHD significantly in animal models and man (Fig. 1).[2,3] This may be done before the infusion of the HSC graft[4] by negative selection of donor T cells or positive selection of CD34-positive HSCs. Negative selection of donor T cells was initially performed by methods like soybean lectin agglutinin and E-rosette depletion,[5,6] but when monoclonal antibodies (mAb) against T-lymphocytes became available, these were used to selectively weed out lymphocytes positive for CD3[7,8] or other cell surface markers.[9] Alternatively, a technique called counterflow centrifugation elutriation (a term used in ore mining whereby one might "purify, separate, or remove...by washing, decanting and settling")[10] has been developed successfully for selective donor lymphocyte depletion.[11,12] However, GVHD often goes hand in hand with a GVT effect,[13] which is also mediated by donor lymphocytes.[14] In fact, donor lymphocyte infusions have led to disease remissions in relapsed or persistent disease post-transplant.[15] Thus, removing donor lymphocytes to reduce GVHD reactions may also remove the lymphocytes necessary to mediate GVT.[16] As such, various strategies have been devised to separate the GVT phenomenon from GVHD. In one such strategy, donor T cells are transfected with herpes simplex virus-thymidine kinase (HSV-TK) gene; these cells are then included in haematopoietic transplantation and the donor T cells allowed to exert their GVT effect. However, if severe GVHD occurs, the T cells may be ablated by the simple administration of acyclovir, which is toxic to the donor lymphocytes carrying the HSV-TK suicide gene.[17] Other strategies have been attempted, which involve the selective depletion of various lymphocyte subsets, including CD4-positive[18] or CD8-positive[19] lymphocytes. While these strategies have met with limited success, refined lymphodepletion strategies with infusion of defined doses of the various lymphocyte subsets may be more successful.[20,21]

Of great interest is the observation that different cytolytic pathways may, at least in part, mediate GVHD and GVT.[22,23] The Fas-Fas ligand (FasL) (CD95-CD178) pathway appears to mainly mediate GVHD[24] while the perforin-granzyme pathway appears to be the major route of GVT.[25,26] While these observations are not absolute and there are many exceptions to the rule, the selective blockade or enhancement of cytolytic pathways could provide an opportunity to separate, to some extent, the desired GVT from the unwanted, and potentially lethal, GVHD.[27]

In short, while the removal of GVHD-causing cells from the HSC graft is not difficult, the greatest challenge being dealt with by many investigators has been to do so without depriving the graft of its potential for a GVT effect.

Removal of Cells that Cause Relapse

In autologous HSCT, small quantities of tumour cells may be present in the harvested HSC product and get

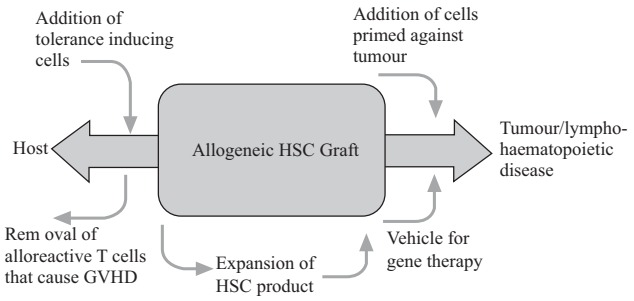

Fig. 1. Engineering the allogeneic HSC graft.

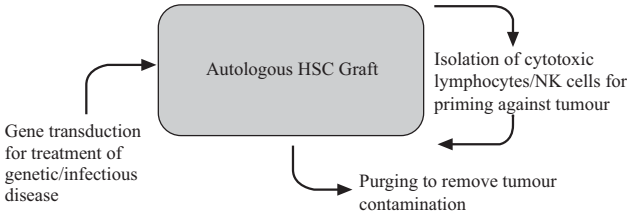

Fig. 2. Engineering the autologous HSC graft.

reinfused into the patient, leading to a relapse of the disease. Consequently, methods to remove these contaminating tumour cells from autologous grafts have been devised (Fig. 2). The most popular methods for this so-called tumour cell purging are through pharmacological and immunological means.

The main agents used in the pharmacological purging of autologous grafts are metabolites of cyclophosphamide like 4-hydroxycyclophosphamide (4-HC) and mafosfamide. Pharmacological methods of purging tumour cells are inexpensive and relatively easy to perform but may be associated with delayed engraftment, due to toxicity to haematopoietic precursors, and development of chemotherapy-resistant tumour cells. Normal cells in the HSC graft may be protected from the toxic effects of pharmacological purging by pre-treatment with amifostine. This organic thiophosphate, also known as WR-2721, was originally designed as a radioprotective agent in the event of nuclear fallout,[28] but later was also found to protect normal tissues and cells from the toxicity of radiation and cytotoxic agents of alkylating and platinum classes.[29] Implanted tumours and leukaemia cells are not similarly protected[30] and studies suggest that the cytoprotection afforded to normal cells is due to the fact that normal cells have higher membrane alkaline phosphatase activity (required for metabolism of the amifostine prodrug to the active thiol WR-1065) than tumour cells.[31] In preliminary studies, amifostine appears to shorten the period of delayed engraftment induced by pharmacological purging.[32] The agent has also been used in vivo in allogeneic bone marrow transplantation to protect the patient against the effects of myeloablative conditioning. We showed in a randomised study that amifostine significantly decreases the duration of mucositis with a consequent improvement in gastrointestinal mucosal integrity and a resultant decrease in infection rate.[33]

Immunological methods for tumour cell purging employ mainly the use of mAb. These mAb may be directed against the tumour cells, and exert negative selection against the contaminating cells. These mAb, which may not be directly toxic to their targets, are usually conjugated with other agents to increase their effect on the tumour cells. These agents include cytotoxic chemicals,[34] like ricin-A or saporin-6,[35] and serum complement with or without the use of immunomagnetic beads.[36] Alternatively, these mAb may perform positive selection for CD34- or CD133-positive haematopoietic precursors and consequently, decrease tumour cell contamination by 2-3 logs.[37] Naturally, tumour cells that may also carry the CD34 surface antigen (e.g., acute myeloid leukaemia) are unsuitable for purging by CD34+ positive selection. More recently, techniques have generally moved from negative selection to a combination of enrichment of CD34+ cells and tumour-directed negative selection, whereby the HSC graft is first subject to positive selection for CD34+ cells and then put through negative selection for tumour cells.[38,39]

Other novel methods for purging tumour cells have also been explored. An interesting strategy has been to use suicide adenoviral vectors expressing the cytosine deaminase gene (ACD) to transduce leukaemia cells followed by the 5-fluorocytosine exposure. In this strategy, the growth of leukaemia cells was inhibited with a minimal effect on normal haematopoietic progenitors.[40] Hyperthermia has also been used as a purging modality in autologous stem cell transplantation on the basis that leukaemia stem cells have an increased hyperthermic sensitivity compared to their normal counterparts.[41] Even 5-aminolevulinic acid (ALA)-based photodynamic treatment (ALA-PDT) appears to be a promising approach.[42] In our laboratories, we have explored the differential sensitivity of tumour cells and normal cells toward ultraviolet (UV) irradiation and also looked into the possibility of using UV-irradiated tumour cells to prime anti-tumour cytolytic T cells.[43]

Thus far, the benefits obtained by HSC purging have been small although sometimes significant. It is important to note, however, that data for the purging of bone marrow (BM) HSC are not necessarily applicable to peripheral blood stem cells (PBSC) products, which typically contain 10- to 100-fold more cells and can be collected over multiple days. As the level of tumour cell contamination appears to be lower with PBSC than BM, the importance of

purging PBSC is less certain. To more definitively evaluate the utility of graft purging, accurate quantitation of tumour involvement by use of high sensitivity immunocytochemical and molecular biology techniques will be required. In this way, the relationship between graft contamination, purging efficacy, and clinical outcome can be studied in a more quantitative and objective fashion.

Expansion of Donor Cells

True HSC appear to have an unlimited capacity for self-renewal. In vitro assays have been used as surrogate assays for stem cells; these include the long-term culture-initiating cell (LTCIC) assay,[44] the cobblestone area-forming cell (CAFC) assay,[45] and the high proliferative potential colony forming cell (HPP-CFC) assay.[46] HSC may be found among the CD34+ cells, which constitute about 1% of the total bone marrow population. Of the CD34+ cells, CD34+CD38- cells[47] constitute a small fraction, of which true pluripotent HSC are believed to be present at a frequency of <1%. These HSC are able to differentiate into all the blood-forming elements while retaining their pluripotency. Some studies have suggested that the CD133 cell surface antigen may represent a more primitive cell population than their CD34+ counterparts.[48,49] In HSCT, higher doses of HSC, measured inferentially by CD34 counts, appear to result in superior transplant outcomes, with even the potential to cross human leukocyte antigen (HLA) histocompatibility barriers.[50,51] Such transplants may even be performed without the need for post-transplant immunosuppression.[52] Since higher doses of HSC may translate to better outcomes, many investigators have attempted to expand HSC in ex vivo culture systems (Fig. 1). This is particularly important with allogeneic cord blood (CB) products as a source of cellular support for patients undergoing high-dose chemotherapy (HDC), where the use has primarily been limited to smaller children (<20 kilograms body weight) due to the small number of cells in CB.[53] Ex vivo expansion of CB cells has been proposed as a method to increase the number of cells for HSCT in adults, especially since nucleated cell dose (>3.7 x 107/kg) in cord blood transplants appears to be one of the most important factors influencing survival.[46]

HSC expansion may be performed in various ex vivo culture systems. These may be stroma-free liquid suspension cultures[54,55] or stromal cell-dependent cultures. The stroma-free cultures employ various cocktails of cytokines like stem cell factor (SCF), interleukin-3, interleukin-6, granulocyte colony stimulating factor (G-CSF), Flt3 ligand, erythropoietin (EPO) and megakaryocyte growth and development factor (MGDF).[56] However, long-term bone marrow cultures appear to benefit from growth with bone marrow stromal cells, which form an adherent layer in tissue culture. These adherent stromal cells[57] appear to contribute by secreting a variety of stroma-produced soluble factors as well as various cell-to-cell interactions with the HSC to promote HSC growth.[58] However, direct contact between haematopoietic and stromal cells may be unnecessary for either differentiation or conservation of primitive haematopoietic progenitors, though essential for the regulated production of diffusible factors (e.g., cytokines) which promote HSC growth.[59] In our own laboratory, we have explored the use of an anti-CD44 mAb for the ex vivo expansion of CD34-selected cord blood progenitors.[60] Whether stromal cells are used or not, there appears to be a requirement for CD34 selection of the starting cells for optimal expansion.[61,62]

Subsequent studies were performed on a clinical scale using optimal culture conditions in gas-permeable Teflon-coated bags,[63,64] artificial 3-dimensional matrix culture systems,[65] or even clinical-scale bioreactor devices. These HSC bioreactors may be flat plate devices, where cells are grown on flat culture-plates which are continuously perfused with fresh culture medium,[66] or stirred suspension devices, where cells are grown in a suspension system of stirred flask bioreactors.[67]

Expanded HSC products have been used successfully in mice to engraft lethally irradiated mice and were capable of sustaining long-term haematopoiesis in these animals.[68] The expansion of these HSC did not appear to adversely affect the proliferative capacity and lineage potential of the stem cell compartment.[47] In baboons, those receiving expanded CD34+ cells in HSCT had a significantly shorter duration of neutropenia and significantly higher white blood cell count and polymorphonuclear cell nadirs compared with those which did not.[69] In humans, the use of ex vivo expanded HSC has also enhanced neutrophil recovery in HSCT[70] and there are currently many ongoing studies to explore this approach. It is important to note, however, that there appears to be a requirement for the administration of growth factors to the patient after the transplantation of the ex vivo expanded cells, otherwise the expanded cells undergo apoptosis when they are infused into the patient and removed from their cultured cytokine milleu.[69]

In summary, the expansion of HSC progenitors for transplantation can result in faster haematopoietic recovery but, thus far, there have been no randomised studies to show improved survival outcomes. Furthermore, widespread clinical application has been limited by the need for high-level facilities, present in very few centres, to culture these cells for re-infusion into patients.

Addition of Cells to Improve Graft Function

Cells may be added to the transplanted graft to improve clinical outcomes in HSCT. These could either be

immunogenic cells primed against tumour cells, immunoregulatory cells that act to induce graft tolerance or autologous HSC transduced with genes to treat genetic diseases or cancer.

The tumour-reactive lymphocytes may be specifically primed against tumours and infused with the HSC graft or infused later as a donor lymphocyte infusion for eradication of minimal residual disease. Usually T cell clones are rendered specific for tumour antigens (TAs), and these redirected cytotoxic T lymphocytes (CTLs) are infused for the selective augmentation of graft-versus-leukaemia to decrease relapse rates. One such approach is by genetic modification of CTLs to express a chimeric immunoreceptor composed of CD19-specific single-chain immunoglobulin extracellular targeting domain fused to a CD3-zeta intracellular signalling domain.[71] NK cells, which are a small population of peripheral blood lymphocytes with the ability to kill foreign cells in a human leukocyte antigen (HLA)-unrestricted manner, may also be generated for in vivo use to suppress malignant haematopoiesis without affecting normal cells.[72] In a study, large numbers of activated and expanded CD8+ NK-T cells with anti-tumour activity were generated after culture with interferon, cross-linking of antibodies to CD3 and interleukin-2.[73] Dendritic cells, which are professional antigen presenting cells (APC) specialising in stimulating T cell immunity, may also be selectively expanded in or added to HSC grafts by various cytokine combinations including granulocyte macrophage colony stimulating factor (GM-CSF), tumour necrosis factor (TNF), and interleukin-4 (IL-4).[74] Such cultured dendritic cells have been shown to effectively present tumour antigen[75] and were capable of stimulating appropriate anti-tumour T cell immunity.[76] Both allogeneic (Fig. 1) and autologous HSC (Fig. 2) grafts have been primed in this fashion.

Of great interest is the development of various tolerance-inducing immunoregulatory cells, like the facilitating cell (FC) populations for the tolerisation of haematopoietic and organ grafts (Fig. 1). The discovery of graft FC populations has resulted in the restoration of the engraftment potential of purified HSC between genetically disparate individuals. In fact, the addition of FC to T cell-depleted BM grafts resulted in allogeneic engraftment without GVHD or graft failure.[77] However, there have been problems in reproducibly isolating these populations and another approach has been to use CD4+CD25-regulatory T cells. In organ transplantation, the generation of lympho-haematopoietic chimerism by HSCT has led to organ tolerance and an obviation of the need for long-term immunosuppression. These donor-derived haematopoietic stem cells have been used successfully in organ transplantation, but there are many technical aspects and hurdles to be cleared. For cadaveric donors, vertebral bodies provide the best source of these HSC; for live donors, collection of HSC from the peripheral blood after mobilisation with cytokines is preferred.[78]

A plethora of gene transfer technologies is now available and used in various pioneering clinical trials for the treatment of genetic diseases and cancer. After the first attempt to transfer the ß-globin gene into the bone marrow cells of patients with beta thalassaemia, the National Institutes of Health Recombinant DNA Advisory Committee (NIH-RAC) set up the Human Gene Therapy Subcommittee to regulate the science of gene transduction technologies.[79] Thereafter, genetic diseases like severe combined immune deficiency disorder (SCID), resulting from a genetic deficiency of adenosine deaminase (ADA);[80] Gaucher disease,[81] where glucocerebroside accumulates because of a deficiency of glucocerebrosidase; and chronic granulomatous disease[82] have become targets of various clinical trials. In the treatment of malignancy, gene transfer technologies were initially used to evaluate the persistence of tumour infiltrating lymphocytes (TIL) in patients with cancer.[83] The gene therapy approach has also been used to generate genetically modified, human immunodeficiency virus (HIV)-specific, autologous cytotoxic T-lymphocytes. However, the effectiveness of this strategy is limited by T cell-mediated rejection of the gene-modified HIV-specific cytotoxic T lymphocytes.[84]

While these strategies to improve graft function in tumour eradication or disease control are very promising, it has been difficult to move these pre-clinical findings forward to result in any significant clinical benefit at the present time because of numerous regulatory requirements, which will be discussed in the next section.

Regulatory Requirements

The engineering of HSC grafts has enormous potential in improving the outcome and availability of HSCT. Nevertheless, ex vivo processing of BM, PB or CB grafts poses numerous challenges to graft engineers. In autologous graft processing, important choices include graft source, necessity for purging, purging techniques, cryopreservation strategy and post-transplant immunotherapy. Also important is the need to quantify purging efficiency and monitor residual tumour burden. In allogeneic grafts, HSC expansion techniques, choice of cell surface antigen for selection strategies and method of anti-tumour effector cell priming are just a few of the many decisions the graft engineer will have to make. Furthermore, despite careful attention to sterile procedures, low-level contamination of haemato-poietic stem cell components can be introduced before or during manipulation as well as thawing.[85] As such, processing laboratories are now expected to operate according to good manufacturing practices (GMP)[86] and

good tissue practices (GTP) protocols. In addition, the requirements of the Foundation for the Accreditation of Cell Therapy (FACT)[87] and the American Association of Blood Banks (AABB) will have to be met.[88] Deficiencies commonly found during inspections by these organisations include missing validation procedures and/or missing records; failure to perform or document ABO/Rh testing of the HSC component and/or the comparison of the results with prior test results; labels in use not meeting standards; incomplete quality control documentation; unavailable graft infusion documents; incomplete HSC storage policies and procedures; non-documented or untracked adverse reactions to graft infusion; and failure to track engraftment by the laboratory.[89] These measures, though necessary, require rigorous documentation, validation of procedures, competency and proficiency testing, filing of applications with the US Food and Drug Administration (FDA),[90,91] as well as extensive quality assurance and quality control. Naturally, they add considerable cost to the procedure. While the haematopoietic graft engineer needs to meet these regulatory expectations, which are important for patient protection, it is also important to strive to deliver these technologies in as cost-effective, yet qualitative, a manner as possible.

Conclusion

The engineering of the haematopoietic stem cell graft opens up an interesting and exciting avenue for improving the outcome of haematopoietic stem cell transplantation and for the treatment of cancer and diseases of the lympho-haematopoietic system. It could potentially enable us to manipulate the haematopoietic graft to "Do good, not harm" through the selective engineering of its cellular elements. While the science is still prepubescent, it has passed its infancy and further developments in the next decade or so should allow it to be taken to a wider scale to benefit more patients.

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