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    The principle investigator, (as well as all coworkers) left Hippie Cancer Research Institute as early as September 1996, and were therefore present and active only for the first three months by which this grant was active. Furthermore, the PI was denied a request to continue the grant in his new laboratory at Lund University Hospital in Sweden. Thus, very little progress was made in the very limited time that this grant was active at Hippie Cancer Research Center.

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13. ABSTRACT (Maximum 200 words)
    The objective of this Breast Cancer Research program awarded from the US Army Medical Research was to investigate whether it might be feasible to expand hematopoietic stem cells to be used for stem cell transplantation. If so, it could reduce the mortality and morbidity of breast cancer patients undergoing high-dose chemotherapy, and represent an approach for purging stem cell grafts for contaminating tumor cells. Based on recent observations made by our group, we specifically proposed to use flt3 ligand, in an effort to expand repopulating stem cells. However, the principle investigator, Dr. Sten Erik W. Jacobsen (as well as all the workers listed in the original grant proposal) left Hippie Cancer Research Institute as early as September 1996, meaning that the PI and his laboratory were present and active only for the first three months of the current grant, and most of the efforts in this brief period was devoted towards successfully establishing the repopulation assay which was based on transplantation of expanded stem cell populations into lethally irradiated syngenic mice. Later (through the support of other grants), this assay helped us to demonstrate that flt3 ligand together with the recently cloned thrombopoietin is indeed effective in expanding long-term reconstituting murine, as well human stem cells, supporting that reconstituting stem cells might be expanded in culture through the use of stem cell active cytokines.

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(5) Introduction:

In the last years promising results have emerged with regard to the ability of high-dose chemotherapy to improve the prognosis of patients with advanced cancer of many types. Accordingly, a high number of clinical high-dose chemotherapy trials are currently running on different cancers. Since the bone marrow toxicity resulting from such treatment is lethal, it is absolutely required to do a hematopoietic transplantation in these patients following the chemotherapy. Bone marrow transplants has increasingly been replaced by stem cell transplants, which are usually collected from the patient (autologous transplantation) prior to the chemotherapy.

In this grant we proposed to optimize such stem cell grafts to improve their ability to reconstitute the bone marrow, and to reduce the tumor cell contamination of grafts, which could otherwise represent a serious source of relapse. Although the ultimate goal of this study was to utilize this technique clinically, we initially wanted to establish the feasibility and safety of this method in an animal (mouse) model for myelosuppression and subsequent stem cell transplantation.

Although promising findings await confirmation from ongoing large randomized multicenter trials, it seems that high-dose chemotherapy might become an important treatment of metastatic as well as advanced localized disease in multiple types of cancer. Currently, the most common way to correct myelosuppression resulting from high-dose chemotherapy is the use of peripheral-blood progenitor cells (PBPC) mobilized with cytokine treatment such as G-CSF. However, there appears to be a period of obligotory neutropenia and thrombocytopenia lasting 9-11 days, which can not be overcome through the use of cytokines or PBPC. Since this is thought to be due to an insufficient number of more mature progenitor cells, increased focus has been directed towards the use of ex vivo expanded hematopoietic progenitor cells (from bone marrow, peripheral blood or cord blood).

Other potential advantages of using ex vivo expanded progenitor cells include:
1. Reduction of the risk of tumor cell contamination of the graft, since bone marrow involvement might contribute to relapses.
2. Generation of a stem cell graft from a low number cells would reduce costs and eliminate need for leukapheresis.
3. Allow repeated high-dose chemotherapy, which hopefully will result in more patients going into complete remissions.

Self-renewal in the strict sense implicates cells capable of unlimited and unaltered proliferative capacity with sustained multi-lineage potential. However, it is currently controversial whether hematopoietic stem cells have such ability to truly self-renew or not. In fact, several in vitro and in vivo observations argue against it. It has been demonstrated that candidate human stem cells isolated from adult bone marrow have shorter telomeric DNA than corresponding cells from fetal liver or cord blood, and that there is a proliferation-associated loss of telomeric DNA which ultimately leads to senescence. In addition, it has been demonstrated that ex vivo expanded murine stem cells at best can maintain the number of stem cells capable of long-term reconstitution. However, it is likely that during potential ex vivo expansion of primitive stem cells a balance exist between proliferation and loss of such cells in culture. Also, studies using cultured retroviral-marked stem cell demonstrated that several mice were repopulated by progeny derived from a single
clone of stem cells, suggesting that at least some stem cells can proliferate in vitro without losing their long term repopulating ability. Accordingly, establishment of cultures capable of better conservation as well as expansion of different subsets of stem cells could eventually result in expansion of long-term repopulating stem cells (LTRC). Yet unidentified soluble or membrane-bound cytokines might prove critical for expansion, and, many studies suggest that stroma might play an essential role in maintaining or expanding primitive stem cells, by providing essential soluble factors and/or direct contact with the stem cells. Direct contact can either be provided by stroma cell to stem cell interaction through integrins such as VLA-4 and VCAM, or through interactions of adhesion molecules on the stem cells with extracellular matrix proteins such as fibronectin. Other studies, suggest that the stroma elements required for expansion/maintenance of primitive stem cells are soluble and that direct contact with stroma might have a negative effect. The observation that hematopoietic stem cells are exhausted following serial transplantations, also argues against hematopoietic stem cells having unlimited proliferative potential. However, it has been argued that the fact that the recipients are irradiated could favor differentiation rather than self-renewal. The potential of mimicking the self-renewal process in vitro could have a great clinical impact, in that the knowledge might be utilized to expand the number of hematopoietic progenitor cells ex vivo to be used in bone marrow or stem cell transplantations as well as in gene therapy. Although it might currently be unrealistic to achieve true stem cell renewal ex vivo, it might be possible to expand or at least maintain the LTRC while expanding the short term repopulating cells (STRC) which also are clinically important.

It is clear that ex vivo expanded progenitor cells contain high numbers of committed and short-term repopulating stem cells which can efficiently restore hematopoiesis in patients receiving one cycle of high-dose chemotherapy. It is however not clear as to what degree such ex vivo-expanded transplants contain long-term repopulating stem cells. It seems that the high dose chemotherapy administered today allows sufficient long-term repopulating stem cells to survive, and that the main purpose of the current ex vivo expansion is to obtain mature short-term repopulating progenitors. However, since multiple cycles of aggressive high dose chemotherapy might be the treatment of choice in the future, it might become critical to maintain the long-term repopulating stem cells in the ex vivo-expanded cultures. This is a big challenge since in general the stem cells appear to also differentiate when proliferating. The magnitude of differentiation depends on the cytokines used for expansion, but no cytokines identified to date have been efficient in maintaining the long-term repopulating stem cells. We had demonstrated that the recently cloned fl3 ligand (FL) potently expands primitive murine bone marrow progenitor cells, and thus it might be unique in its ability to maintain the stem cells in a primitive state (J Exp Med 1995; 181:1357). However, whether FL can promote expansion or maintenance of the "true" stem cell, that is those cells capable of providing long term reconstitution of all hematopoietic cell lineages, remains to be investigated. Accordingly, the proposed studies sought to establish whether FL might be used for ex vivo expansion of short-term repopulating progenitor cells (STRC) without depleting LTRC.
(6) Body:

The principle investigator, Sten Erik W. Jacobsen (as well as all coworkers listed in the original grant proposal) left Hipple Cancer Research Center as early as September 1996 (less than 4 months after the activation of the grant). Furthermore, the PI was denied a request to continue the grant in his new laboratory at Lund University Hospital in Sweden. Also, as far as the PI knows, this project was not continued by other members of the Hipple Cancer Research Center. Thus, very limited progress could be made on this big project during the limited time it was active.

The specific aim was to establish whether repopulating murine stem cells could be expanded, and thus most of the efforts in the brief period of the grant was devoted towards establishing an optimal in vivo reconstitution assay based on transplantation of stem cells into lethally irradiated syngenic mice. Following relocation of the PI's activities to Sweden, we were able (through alternative grant support) to demonstrate that flt3 ligand together with the recently cloned thrombopoietin (TPO) could indeed efficiently expand or maintain long-term reconstituting murine as well as human stem cells. The findings and implications from these studies, although not directly supported through this grant are summarized below.

We published three manuscripts in 1997, demonstrating that TPO, alone or in combination with FL, had unique and potent effects on viability (Blood 90: 2282, 1997), growth/expansion (J Immunology 158:5169, 1997), and adhesion (J Immunol 159:1961, 1997) of candidate human bone marrow stem cells, as we had previously demonstrated for candidate murine stem cells (Blood 88:2859, 1996; Blood 88:4481, 1996). In agreement with these data we have previously demonstrated that STRC as well as LTRC can be induced to efficiently cycle in response to combined activation by FL, TO and c-kit ligand (KL) (Jacobsen et al, manuscript in preparation).

The studies on murine stem cells combined with our published studies from 1997 allowed us to conclude that FL, combined with TPO is likely to play a key role in promoting growth of human stem cells. Thus, we have during the last year pursued the ambitious goal of trying to use this cytokine to promote expansion of candidate human stem cells (CD34+CD38−) without eliminating their ability to long-term reconstitute. Such stem cell expansion is an requirement for efficient gene marking of human stem cells with traditional retroviral vectors, and thus for allowing us to address the potential role of tumor purging of autologous transplants.

Although our previously published results had demonstrated that TPO and FL could efficiently recruit early human progenitor/stem cells into proliferation they did not establish how primitive these progenitor/stem cells were, or whether the observed proliferation was associated with stem cell expansion or differentiation. In studies of human stem cells it remains a problem that most of the utilized assays detect progenitor rather stem cells. Thus, during the last year our laboratory has invested a major effort towards establishing and utilizing more sophisticated and predictable human stem cell assays.
One important aspect of these assays, is that they are long-term assays, and accordingly it takes significantly longer time to get sufficient and publishable data than with more traditional "progenitor" assays. The assay we have primarily been working on takes advantage of the unique ability of candidate human stem cells to long-term reconstitute irradiated/ablated human bone marrow stroma cultures. In such cultures it has recently been demonstrated that cells capable of producing committed progenitors for as much as 12 weeks (long-term culture-initiating cells; LTC-IC) are likely to be identical/close to the earliest long-term reconstituting human stem cells. Although we believe this assay will prove to be extremely valuable for evaluation of human stem cells, it is also tedious. First, it takes 4-6 weeks to establish an optimal and functional bone marrow stroma support, and subsequently and additional 12 weeks (long-term culture) + 2 weeks (progenitor assay) to evaluate long-term reconstitution. Although during this 20 week period, the chance of contamination is rather extensive, we have been successful in establishing this assay, which has allowed us to demonstrate that purified human CD34⁺CD38⁻ bone marrow cells expanded in TPO+FL+KL for as much as 7 to 12 days in the absence of stroma and serum results in a 4-6 fold increase in the number of 12 week LTC-IC. In contrast, and of particular interest, CD34⁺CD38⁻ cells cultured for KL+IL-3+IL-6, the cytokine combination utilized in most previous and unsuccessful gene marking protocols are completely depleted in 12 week LTC-IC. Thus, our finding of not only a dramatic increase in 12 week LTC-IC with TPO+FL+KL, but also a complete loss of such candidate stem cells with the more traditionally used cytokines give promise to "our" cytokine combination while (at least in part) explaining why previous gene marking and stem cell expansion protocols have been unsuccessful.
(7) Conclusions

Although the brief grant support from the US Army’s Breast Cancer Research program only allowed us to set up sophisticated methods for evaluating the short and long-term reconstituting ability of FL-expanded murine stem cells, we have been able to pursue this project through alternate grant support in Sweden. Thus, we have now demonstrated that FL and the recently cloned TPO have unique abilities to maintain and probably also expand long-term reconstituting stem cells. However, ultimately the ability of FL and TPO to expand true human LTRC can only be demonstrated in clinical gene marking protocols. Thus, we are currently planning a gene marking protocol to determine whether this approach can be used successfully to provide an efficient and safe graft for cancer patients undergoing high-dose chemotherapy.