**Identification and Isolation of Stem Cells in Different Phases of the Cell Cycle.**

**Introduction**

Chronic myeloid leukaemia is a stem cell disease, which is characterized by excessive numbers of circulating intermediate progenitors and mature granulocytes. (Eaves and Eaves 1987). Because stem cells are intrinsically resistant to damage by either cytotoxic drugs or radiation, diseases that originate in a stem cell are difficult to treat. CML has three distinct phases of progression, chronic phase where large numbers of relatively mature granulocytes exist with a typical time scale of 4-6 years see fig?. This is followed by the onset of accelerated phase, which lasts up to 1year, and culminates in blast crisis where the numbers of immature white cells increase proportionately.(Sawyers 1999) At this stage the disease becomes much more resistant to therapy and almost certainly involves further acquired mutations. The patient mortality is 100% within 3-6 months of onset of blast crisis. (Spiers 1977).

If an appropriate therapy could be found for the treatment of CML in chronic phase, progression to fatal blast crisis would be arrested. Since chronic phase offers the best opportunity for successful therapy, the work described here focuses exclusively on this stage of the disease. In an effort to identify possible targets, which could be exploited for therapeutic purposes, the properties of normal and CML stem cells have been investigated.

Normal stem cells are known to be largely quiescent, residing in the G0 phase of the cell cycle, where they can be recruited into division in response to appropriate stimuli.(Bradford, Williams et al. 1997),(Ponchio, Conneally et al. 1995) This has been demonstrated by introducing quiescent cells into NOD/SCID mice, where the cells can exit G0 and repopulate the immune system of the mouse. It was an unexpected finding that alongside the actively cycling malignant population in the chronic phase of CML, there also coexisted a quiescent stem cell fraction. These cells are primitive, expressing the stem cell marker CD34 and are part of the leukaemic clone as verified by FISH (fluorescence in-situ hybridisation) and RT-PCR (Holyoake, Jiang et al. 1999). It has been established that cells expressing CD34 but lacking lineage markers are primitive and able to generate progeny of different phenotypes. However, there is also some evidence that an earlier stem cell exits which doesn’t express CD34 or lineage markers and is able to repopulate the immune system of a myeloablated host. (Bhatia, Bonnet et al. 1997) In order to compare the properties of normal and CML stem cells it was necessary to separate them from the rest of the circulating blood cells prior to subsequent experimentation. Using CD34 as a stem cell marker the cells were isolated by either positive or negative stem cell selection. The following describes the selection process in detail with representative numbers and FACS plots for each system and for normal and CML cells. All of the CML cells used in this study were collected from patients in chronic phase at diagnosis and before treatment.

**Stem cell selection**

Stem cells are a very rare fraction of the circulating progenitors, around 0.1%, and in order to study these cells in culture, it was necessary to significantly enrich the stem cells before FACS sorting specific cell sub-populations. This was achieved by either positive or negative stem cell selection. The starting cell population in the CML patients was collected during leukapheresis, which is primarily done to reduce the numbers of circulating white cells, usually excessive at the time of diagnosis and can lead to further clinical complications. This is done by means of a dedicated leukapheresis machine, which allows red blood cells and plasma to be returned to the patient after removal of a large number of the white cells. Leukapheresis products that are surplus to requirement can be used for research with patient consent. Within the CML patient group there is a large variation in the starting CD34+ percentage from 0.3-50% the majority being greater than 3%, characteristic of a stem cell disease. (Richmond LJ, Alcorn MJ et al. 2002)

For normal collections donors, have taken G-CSF (granulocyte stimulating factor) for 6 days before collection to boost the number of circulating progenitors. There can be large differences in the effectiveness of mobilisation leading to differences in the percentage of CD34+ cells in the starting fraction from 0.3-1.7%. The dose required by the patient for a stem cell transplant is calculated as 6x106 CD34+ cells/kg and, with consent, the remainder can be used for research purposes. The machine settings are operator controlled and the quality and purity of stem cells will be influenced by the settings. For this reason, and because of differences in effectiveness of mobilisation, there is significant variation in the starting CD34+ number and subsequent recovery following selection.

The stem cell populations from both selections are intrinsically different. There is some evidence that a more primitive cell than a CD34 expressing cell exists within the stem cell compartment that can in turn generate CD34+ cells. (Goodell, Rosenzweig et al. 1997) A negative selection would include these cells since they would lack lineage markers, which could explain the lower CD34+ cell percentages using this method. A positive selection would exclude these cells since it only targets cells expressing the CD34 marker. The method of choice is dependant on the final experiments to be performed although they can be used interchangeably if the FACS strategy includes gating on the CD34+ population.

Throughout the studies presented in this thesis, it was necessary to freeze the cells following stem cell enrichment. Both types of selection involved a considerable time input and further cell sorting required at least 12 hours including staining and cell culture post-sort.

**Positive stem cell selection**

In a positive selection the cells of interest are removed from the rest of the population by labelling with antibody, magnetic beads and selecting using a magnetic column. For the positive selections described here the Isolex® 50 system was used. This was a commercial method used for clinical stem cell selections that was scaled down using a smaller magnetic column for research samples, as the number of total cells/column was critical to recovery and purity.

For positive selection the cells were labelled with a mouse anti-human monoclonal antibody against the CD34 antigen as a stem cell marker and further labelled with a sheep anti-mouse antibody bound to a magnetic bead. The cells were then passed through a magnetic column such that the unlabelled cells were eluted first before a stem cell releasing agent was added in order to detach the bound cells. The releasing agent contains a nano-peptide (PR34®) with a similar sequence to the antigen, which competes for binding of the active site, and because the nano-peptide is in excess the antigen-bound cells are released.

**Example of a CML and a normal positive cell selection**

A diagram of the labelling and elution procedure is shown in Fig ? The stem cells are released with the CD34 antigen intact and available for further labelling. The selected cells were assessed by FACS for CD34 purity and plots of the pre-column and post-column samples for both normal and CML are shown in figure ?

Using side scatter (SSC) to discriminate cells on the basis of their granularity (lower granularity indicates less differentiation) and including a marker for CD34 the more primitive cells can be identified. Fig ? shows that the number of CD34 positive cells has increased in the CML sample from 31.8-98%, and in the normal sample from 1.59-83%. The cells were ≥99% viable post selection in both the normal and CML examples and these were representative for most selections. The final percentage of CD34 positive cells obtained using this method varied between 53-98% for both CML and normal, with recoveries ranging from 36-71% in normal samples and 69-98% for CML samples. The quality of the starting population both in terms of total cell number and percentage of CD34+ cells can markedly influence the efficiency of the selection.

The FACS plots in FIG ? show there is little qualitative difference in the post-selection sample between the normal and CML patient. The number of CD34+ cells recovered and the final purity of the sample (as shown in table ?) are dependent on the starting cell population and not on the disease status of the patient.

Table ? shows an example of the cell numbers obtained from positive stem cell selections for normal and CML. The total cell starting number in the normal example was 1.2x1010 and the total number of CD34 positive cells available was 1.8x108 based on the CD34 starting percentage of 1.53%. The selection produced a cell population which was 85% CD34+ and 62% of the available CD34 cells were recovered from the column giving a total of 1.12x108 cells which were then frozen in liquid nitrogen in aliquots of 20x106/nunc. In the CML example the stem cell selection produced an increase in CD34+ percentage of 4.72-94% with a recovery of 53% of the available CD34+ cells which were 3.44x108 giving a final number of stem cells of 1.84x108, which were frozen in liquid nitrogen at 20x106/nunc. As the selection system used was a unique scaled down clinical system there are no published numbers using this method, however the recoveries and enrichment numbers were within the limits published for the full clinical system, [REF] which uses the same antibody cocktail and methodology but has a different magnetic column.

**Negative stem cell selection**

In negative stem cell selections, cells expressing lineage markers were labelled with a panel of mouse anti-human antibodies including CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, IgE and glycophorin A then further labelled with a sheep anti-mouse antibody bound to magnetic colloid. Table ? showS the antibodies contained in the progenitor enrichment cocktail and the relevant target cells to which they bind.

|  |  |
| --- | --- |
| **Antibody** | **Target cell** |
| **CD2** | **T cells, NK cells** |
| **CD3** | **T cells,** |
| **CD14** | **Monocyte/macrophage** |
| **CD15 (CML)** | **Granulocytes/basophils** |
| **CD16** | **NK cells, granulocytes** |
| **CD19** | **B cells** |
| **CD24** | **B cells** |
| **CD56** | **NK cells,** |
| **CD66b** | **Granulocytes** |
| **IgE** | **Basophils** |
| **Glycophorin A** | **Erythroid cells** |

A schematic outline of the selection procedure is shown in FIG ?. The method was modified when CML cells were processed by the addition of biotinylated anti-CD15 to the cells before the addition of the other antibodies described and anti-biotin included with the magnetic colloid. This step facilitated the removal of the excessive number of granulocytes characteristic of this stage of the disease. The cells were then passed through a magnetic column where the unlabelled stem cells were eluted and the labelled lineage+ cells remained attached to the column. The purity of the selected cells was assessed in the same way as for the positively selected cells, by calculating the percentage of CD34 strongly positive cells that were also low in side scatter before and after selection.

Example of a normal and a CML negative selection

In the normal example shown in figure ? the number of CD34 positive cells has increased from 1.23-76.2% and in the CML sample the percentage of CD34 positive cells has increased from 5.9-86%. Since this population also contains some cells that do not express lineage markers or CD34 it would not be expected that the number of CD34 positive cells would be as high as in the positive selection. In practice negative selections produce a lower range of CD34 positive cells typically from 40-88% compared to 53-98% for positive selections. The FACS plots in FIG? show that, as in the case of the positively selected cells, there is little difference between the normal and CML samples in terms of stem cell purity and yield. The addition of anti-CD15 in the CML sample maximises the purity of the stem cells in the selected fraction by removing the large excess of granulocytes typical of CML in chronic phase.

Bhatia, M., D. Bonnet, et al. (1997). Identification of a novel CD34 negative population of primitive human hematopoietic cells capable of repopulating NOD/SCID mice. Blood, W.B.Saunders Co. **1:** 258a.

Bradford, G. B., B. Williams, et al. (1997). "Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment." Exp.Hematol. **25**: 445-453.

Buckle AM, Mottram R, et al. (2000). "The effect of Bcr-Abl protein tyrosine kinase on maturation and proliferation of primitive haematopoietic cells." Mol Med. **10.**: 892-902.

Durand R.E and O. P.L. (1982). "Cytotoxicity, mutageniciy and DNA damage by Hoechst 33342." The Journal of Histochemistry and Cytochemistry. **30**(2): 111-6.

Eaves, C. J. and A. C. Eaves (1987). Cell culture studies in CML. Bailliere's Clinical Haematology. K. Hinton. London, Bailliere Tindall/W.B. Saunders**:** 931-961.

Goodell, M. A., M. Rosenzweig, et al. (1997). "Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species." Nature Med. **3**: 1337-1345.

Holyoake, T., X. Jiang, et al. (1999). "Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia." Blood **94**(6): 2056-2064.

Ladd, A. C., R. Pyatt, et al. (1997). "Orderly process of sequential cytokine stimulation is required for activation and maximal proliferation of primitive human bone marrow CD34+ hematopoietic progenitor cell residing in Go." Blood **90**: 658-668.

Latt, S. A. (1974). "Detection of DNA synthesis in interphase nuclei by fluorescence microscopy." J,Cell Biology **62**: 546.

Loewe, H. and J. Urbanietz (1974). "Basisch substitutierte 2.6-bis-benzimidazol-derivate, eine neue chemotherapeutisch active korperklasse." Arzneimforsch **24**: 478.

Loontiens, F. G., P. Regenfuss, et al. (1990). "Binding characteristics of Hoechst 33258 with calf thymus DNA, poly [d(A-T)], and d(CCGGAATTCCGG): multiple stoichiometries and determination of tight binding with a wide spectrum of site affinities." Biochemistry **29**: 9029-39.

Ponchio, L., E. Conneally, et al. (1995). "Quantitation of the quiescent fraction of longterm culture-initiating cells (LTC-IC) in normal human blood and marrow and the kinetics of their growth factor-stimulated entry into S-phase in vitro." Blood **86**: 3314-3321.

Richmond LJ, Alcorn MJ, et al. (2002). "CML leukapheresis products can be enriched for CD34+ cells and simultaneously depleted of CD15+ cells using a simple Ab cocktail." Cytotherapy. **4**(5): 407-13.

Sawyers, C. L. (1999). "Chronic Myeloid Leukemia." N Eng J Med **340**(17): 1330-1340.

Shapiro, H. M. (1981). "Flow Cytometric Estimation of DNA and RNA content in intact cells stained with Hoechst 33342 and Pyronin Y." Cytometry **2**: 143-150.

Spiers, A. S. D. (1977). "The clinical features of chronic granulocytic leukaemia." Clin.Haematol. **6**: 77-95.