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Ex vivo expansion of haematopoietic stem cells and gene therapy development

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Summary

The results of a research project on ex vivo expansion of human haematopoietic stem cells (HSC) and development of gene therapy, funded by the Swiss National Research Program 46, are summarised and discussed in the context of current progresses and difficulties in these fields. A routine method for ex vivo expansion of human HSC is not yet available. However, stem cell biology has progressed importantly in recent years; ex vivo expansion of human HSC should become possible in the near future. Regarding gene therapy development, we obtained with HIV-1-derived bicistronic lentiviral vectors efficient delivery of genes into immature haematopoietic cells and also primary human B lymphocytes. However, clinical gene therapy still faces a variety of problems. For the (into chromosomes) integrating lentivectors, currently the most promising tools for HSC-based gene therapy, the risks of insertional mutagenesis need to be fully assessed before larger clinical trials can start.

Key words: haematopoietic stem cells; ex vivo expansion; gene therapy; lentivectors; bicistronic vectors; B lymphocytes; insertional mutagenesis

Introduction

A research project, funded by the national research program (NRP) 46, pursued three goals: 1) To develop an *in vitro* assay which can detect how many cells within a mixed cell population are haematopoietic stem cells (HSC). Such an assay would facilitate research on HSC, including the next subproject. 2) To develop a routine method for the *ex vivo* amplification (ie multiplication by cell division *in vitro*) of human HSC. This would enhance the safety of HSC transplantation in many situations and facilitate gene therapy based on these cells. 3) To study delivery of genes into HSC and also B lymphocytes by means of bicistronic lentivectors. Results of this work, and also what we could not do, is summarized below and discussed in the context of present progresses and difficulties in these fields.

Summary of our studies on quantitation and *ex vivo* expansion of human HSC

When we formulated the projects in 2001, reports in the literature suggested that human HSC amplification in cell cultures was possible with cell growth-inducing cytokines known at that time. Human HSC could apparently be quantitated in a surrogate *in vivo* assay using immunodeficient NOD/SCID mice [1]. Because this assay lasts 6 weeks or longer, and is difficult, we attempted to develop a shorter *in vitro* assay. The immature cells capable of forming both lymphoid and non-lymphoid blood cells were believed to be HSC [2]. Such cells were detected in cell cultures which also lasted 6 weeks or more. We isolated with the fluorescence-activated cell sorter (FACS) immature cells from umbilical cord blood ($CD34^+$ $CD38^{low}CD19^-$ cells; approximately 0.4% of total nucleated cells) and dispensed these, one cell per well, into cultures with MS-5 stromal cells and various cytokines [2]. After 10 to 14 days, small cell clones were tested for the presence of myeloid (CD33) as well as B-lymphoid (CD19) markers by

Abbreviations

FACS	fluorescence-activated cell sorter
GFP	green fluorescent protein
HSC	haematopoietic stem cells
IL	interleukin

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reverse transcription-PCR. Approximately 1 in 100 to 1 in 20 of the cultures was found to generate such mixed clones. However, we also found that the numbers of blood cells generated in 6-week-NOD/SCID mouse assays poorly correlated with the numbers of injected immature cells. Thus, we could not validate the putative in vitro HSC assay.

To explore ex vivo amplification of putative HSC, cord blood $CD34^+CD38^{low}$ cells were cultured with stem cell factor, FLT3 ligand, thrombopoietin and interleukin (IL)-3 (all cytokines together or various combinations). Usually after 5 to 7 days, the growing cells were analysed by cell division tracking (with a fluorescent die, CFSE, which becomes diluted two-fold after each cell division) in conjunction with detection of cell maturation markers. We found that cells with a still immature phenotype ($CD34^+CD38^{low}$ and lacking erythroid, myeloid and lymphoid cell lineage markers, ie lin^- cells) had performed up to 9 cell divisions. However, because of the cell death rate during cultures, there was no significant net increase in lin^- cells (except approximately a two-fold increase when all four cytokines were present; unpublished data). We abandoned those studies for the time being, because we had no HSC assay to validate HSC expansion.

Current progresses and difficulties of research on human HSC

HSC transplantation became a treatment for a variety of congenital and acquired blood diseases [3], because of progresses in tissue matching and immunology, before there was much understanding of stem cell biology. In recent years, research on stem cells (of blood and other organs) advanced fast. It has been found that a whole hierarchy of undifferentiated cells with tissue regenerating capacity persists even in adult organisms [4]. Bone marrow too seems to contain some multi-tissue regenerating stem cells, but various controversies persist [3, 5]. *In vivo*, HSC can remain quiescent for many years; they can also multiply without undergoing differentiation, and they can mature (sometimes by asymmetric division, where only one daughter cell matures) into various differentiated cells. This involves sophisticated regulation critically depending on interactions between stem cells and support/regulatory cells in specific niches (microenvironments). A multitude of signalling pathways are now known to be involved, including those depending on WNT, notch, bone morphogenic protein, sonic hedgehog, fibroblast growth factor and other molecules [6–8]. Thus, it became clear that cultures for *ex vivo* HSC expansion – as mentioned above – in the absence of signals blocking cell maturation, lead to various degrees of HSC maturation and, thereby, compromise their tissue regenerating capacity. Such methods must preserve this capacity and this must be validated.

Work with HSC from humans in particular is difficult. First, one can not perform in vivo HSC assays as with animals. Second, it has been reported that various mammalian species all have similar numbers of HSC per organism. In mice, rats and cats approximately 1 out of 2×10^4, 1 of 5×10^5 and 1 of 2×10^6 bone marrow cells, respectively, was found to be an HSC [9–11]. If this is true, ie if indeed increase in body mass during evolution was associated with an increase in numbers of cell divisions during blood cell maturation rather than an increase in numbers of stem cells, then HSC are extremely rare cells in humans. Many earlier data, eg on gene expression signatures of human HSC or on their quantitation in various assays, need to be revised. So far, a routine method for ex vivo amplification of human HSC is not available. In view of new discoveries regarding stem cell regulation, widely conserved in different species, this should be found in the near future [3, 6–8].

Summary of our studies on gene therapy development

In collaboration with Didier Trono's group we studied delivery of genes (gene transduction) into human immature haematopoietic cells and also B lymphocytes, the cells which produce antibody proteins. We utilised HIV-1-derived lentiviral vectors. Briefly, such vectors had been developed by replacing most of the original coding DNA of HIV provirus by a transgene [12]. Viral particles, which contain the RNA copy of the lentivector (analogous to HIV virus) and can infect various cells, are produced in cell cultures. One adds the vector plasmid (a circular DNA construct) as well as other plasmids which encode viral proteins that no longer are encoded by the vector. One of these plasmids encodes an envelope protein (not from HIV but from vesicular stomatitis virus) which can attach to many cells. We utilised a second generation system with the self-inactivating (SIN) modification. This leads to inactivation of the viral gene promoter (its functions in gene expression and virus replication) in the retrotranscribed DNA copies of the vector which insert into chromosomes of infected cells [13]. Therefore, the vector must have an internal gene promoter for the expression of the delivered transgene.

Gene transfer into human immature haematopoietic cells

When the NRP 46 project started, we had already found that HIV vectors expressing as transgene the marker green fluorescent protein (GFP) under the control of the human elongation factor-1α(EF1α) internal promoter, gave at least 10-fold higher percentages of GFP+ cells among transduced human cord blood CD34+ cells than did a murine retroviral vector [14]. Efficient gene delivery occurred during mitogenic as well as non-mitogenic culture conditions. GFP expression persisted after >20-fold expansion of CD34+ cells and their maturation with appropriate cytokines into erythroid, megakaryocytic or various types of myeloid cells [14]. Large quantities of GFP+ dendritic cells could be generated from a few transduced CD34+ cells; no vector toxicity was found [15]. Various groups had also shown GFP expression in NOD/SCID mouse repopulating cells after transduction with lentivectors.

Our new project was to test bicistronic vectors, ie vectors which code for a transgene of interest expressed from the internal promoter and – downstream of an internal ribosomal entry site (IRES) – a transduction marker, such as GFP. We constructed in our laboratory a vector expressing IL-4 and GFP (ie EF1α promoter – IL-4 – IRES – GFP, using the vector plasmid pWPTS-GFP which also carries a cPPT element enhancing nuclear localisation and a WPRE element enhancing transgene expression; see vector map at www.tronolab.epfl.ch). IL-4 was chosen to obtain a secreted protein which could be dosed and which was not present in our cell cultures. After exposure of cord blood CD34+ cells to a vector dose which resulted in approximately 4.5 GFP DNA copies per diploid genome according to quantitative PCR, we found 20% GFP+ cells on average (sometimes up to 40%). Because GFP was encoded downstream of the internal ribosomal entry site (IRES), the mean GFP fluorescence intensity was approximately 2.5-fold lower with this vector than with the monocistronic GFP vector. IL-4 was detectable with a fluorescent monoclonal antibody and correlated with GFP expression in individual cells. The IL-4 secretion rate, measured in cells which had undergone several cell divisions, was 1.2 ng/10^5 cells/24 hours on average. This was measured in cells which had divided several times. This IL-4 production integrase enzyme-dependent, ie vector integration into chromosomes was required. These data (published together with the B cell data [16]) showed that CD34+ immature haematopoietic cells were efficiently transduced by a bicistronic HIV vector carrying a WPRE element (woodchuck hepatitis virus post-transcriptional regulatory element). An at least 10-fold lower cytokine secretion rate has been reported for human leukaemia cells transduced with an HIV vector lacking such an element [17]. Previously, by directly comparing different monocistronic vectors, we had found that the WPRE element enhanced GFP expression in CD34+ cells [14].

Gene transfer into human primary B lymphocytes

With the lentivectors we obtained for the first time – and for any gene delivery method, viral or non-viral – an efficient gene transduction into primary (ie "normal", not immortalised) human B lymphocytes. Previously, only tumour B cell lines or Epstein-Barr virus-immortalised B cells had been significantly transduced with murine retroviral vectors. The first B cell transductions were performed in a culture system using a murine helper T cell line for B cell activation [18]. The cytomegalovirus (CMV) promoter, which had a very weak activity in CD34+ cells [14], was better than the EF1α promoter in B cells. For B cells we could not establish whether cell division was required for lentiviral transduction or not, because these cells (unlike CD34+ or T cells) could not be maintained in a non-dividing, but activated, state. Proliferating B cells are very sensitive to all kinds of cell death inducing stimuli. Compared to CD34+ cells or primary T lymphocytes, an optimal function of the virus producing 293T cell-system was particularly critical for efficient B cell transduction.

For development of gene therapy it was important to avoid the helper T cell line with its potential risks regarding propagation of infectious agents or generation of recombinant viruses. B cells stimulated with CD40 ligand were very poorly transducible. However, we then found that naive and memory B cells were efficiently transduced in a culture system using immunostimulatory oligonucleotides (CpG DNA), anti-immunoglobulin antibody (only required for activation of naive B cells), IL-2 and IL-10 [16]. In the B cells too, GFP expression was approximately 2.5-fold lower with bicistronic than with monocistronic vectors. Therefore, in B cells the fluorescence peaks of GFP+ and GFP– cells became partially overlapping; we obtained 32% GFP+ cells with monocistronic, and 14% with bicistronic, vectors on average. With the IL-4 expressing bicistronic vector, B cells showed the same IL-4 secretion rate as CD34+ cells (using the optimal promoters as mentioned above, respectively, for these cells). We also studied the effect of a known anti-apoptotic protein to obtain proof of principle for the detection of a biological function of a delivered transgene. A bicistronic vector expressing a viral FLIP molecule protected 50% of FACS-sorted GFP+ B cells, but not GFP– B cells, against Fas ligand-induced cell death. A control vector gave no protection. The viral FLIP was detectable in the GFP+ B cells, which had undergone several cell divisions, by flow cytometry or Western blot, confirming that the transgene was effectively expressed [16]. Thus, our data showed that B cells were efficiently transduced with HIV vectors. Such vectors and the above culture system should also be useful for the testing of various gene functions in B cells.

Current progresses and difficulties in gene therapy development

Gene therapy aims at treatment of not only of genetic, but also many acquired diseases, such as cancers, tissue degeneration, HIV infection, etc. Depending on the planned treatment duration, the targeted cells, their location, and whether they undergo cell divisions or no longer divide, one can utilise either non-viral methods, non-integrating viral vectors, or integrating viral vectors (ie vectors which can insert into chromosomes and, thus, also replicate during cell divisions). However, in recent years the clinical applications advanced slower than expected for different reasons.

Non-integrating viral vectors and non-viral methods

Regarding adenoviral vectors, which do not (or almost never) integrate into chromosomes, the problem of boosting strong anti-vector immune responses by repeat administration still needs to be better solved. These vectors also showed dose-dependent toxicity. Adeno-associated virus vectors, which remain mostly non-integrated (ie episomal), have a small DNA transport capacity and, unexpectedly, also elicited immune responses in humans (which had not been found in animal studies) [19]. Non-viral methods for delivering either DNA or RNA (eg small inhibitory RNA inhibiting oncogene expression or viruses), which comprise electric current, physical pressure, liposomes and now also multicomponent nanoparticles, are still less efficient than viral methods [19, 20].

Integrating retroviral and lentiviral vectors

The integrating viral vectors are derived either from the murine simple retroviruses (retroviral vectors) or the more sophisticated lentiviruses (HIV-1 and other lentiviruses, lentivectors) [19, 21]. Studies with lentivectors still are at the preclinical stage (except for one study, see below); most of the data were obtained with HIV-1-derived vectors. Lentivectors are clearly more efficient than retroviral vectors for gene delivery into many human cells, as was also shown by our own data on $CD34^+$ cells and B lymphocytes. In addition, only lentivectors can enter into the cell nucleus and integrate into chromosomes even in non-dividing cells, such as neurons [12]. However, transgene expression in such post-mitotic cells has also recently been obtained with a non-integrating lentivector [22]. In contrast, for HSC and other stem cells which must divide to regenerate blood or other organs (and other dividing cells, such as lymphocytes), an integrating vector is required. Only with integrating lentivectors can one stably integrate a transgene into chromosomes of HSC ex vivo under non-mitogenic conditions. For retroviral vectors one needs mitogenic conditions which (at present, see discussion of ex vivo amplification above) have a risk of exhaustion of stem cell function. Improved integrating vectors regarding regulation of gene expression and biosafety are being developed [21]. However, the most urgent task is to evaluate the risks of insertional mutagenesis of different integrating vectors, ie the risks of overexpression of genes (including oncogenes), or inactivation or disruption of genes (including tumour suppressor genes), by vector inserting (semirandomly, see below) into chromosomes [23].

Risks of insertional mutagenesis with different integrating viral vectors

A clinical study in France, performed with a retroviral vector, strikingly demonstrated that the severe combined immunodeficiency (SCID-X1), which results from absence of T cells due to a deficiency of the common γ-chain of the IL-2-family cytokine receptors, can be corrected by ex vivo transfer into immature haematopoietic cells of the missing γ-chain gene. However, 3 of 11 treated children developed T cell lymphoproliferative disease [3, 24]. Although the alternative treatment by HSC transplantation too has its risks, this was a hard blow for gene therapy. In the abnormally proliferating T cells the vector had inserted close to a known T-leukaemia oncogene [24]. It was then established that murine retroviruses insert preferentially into promoter regions of active genes [23]. But in two other, similarly sized SCID gene therapy trials using such vectors no complications of vector insertion were found. Thus, critical variables in different therapy contexts need to be identified. In the SCID-X1 patients, very few gene-transduced T cell progenitors developed the whole T cell system, thereby performing many more cell divisions than do T progenitors in normal individuals. This, in conjunction with transfer of a gene which itself enhanced cell proliferation (the cytokine receptor) and the activation of an oncogene by vector insertion, may have contributed to a multi-step tumorigenic process.

Self-inactivating (SIN) vectors usually have a weaker or more cell-type specific internal promoter than the original viral promoter and thus, should give lower oncogene activation. Also, HIV-vectors were found to insert preferentially into gene ends rather than promoter regions [23]. This should also reduce the risk of oncogene activation. But in theory HIV vectors might inactivate tumour suppressor genes by insertion. Natural HIV infection causes no directly HIV-related tumours. Moreover, in a sensitive, tumour-prone, mutant mouse model, transduction of HSC with retroviral vector, but not with HIV vector, enhanced tumorigenesis [25]. In the single clinical (phase I) lentivector study performed so far, T cells from

five therapy-resistant HIV patients were in 2003 transduced ex vivo with an HIV vector, with enhanced biosafety features, which expresses antisense RNA against the HIV envelope RNA, ie which blocks envelope protein synthesis [26]. No serious side effects, rather a lower HIV titre, were reported. A larger phase II study testing four ex vivo T cell transductions for each patient has started (see press releases at www.virxsys.com). It is important to fully assess the risks of insertional mutagenesis of the lentivectors, the currently most promising tools for HSC-based gene therapy, in large animals and then in humans. This will determine whether, and in what clinical contexts, they can be utilised. In addition, research progresses; artificial virus-inspired vectors and enzymatic nanomachines which repair gene defects in situ are on the agenda of gene therapy research.

Conclusions

We could not reach our goals regarding quantitation and *ex vivo* amplification of human HSC, but research in the field of stem cell biology currently progresses fast; *ex vivo* expansion of HSC should become feasible soon. On the other hand, we could demonstrate efficient delivery of genes into immature human haematopoietic cells and also primary B lymphocytes with HIV-1-derived bicistronic lentivectors, but the risks of insertional mutagenesis of this promising tool must be further assessed, before larger clinical studies can be performed.

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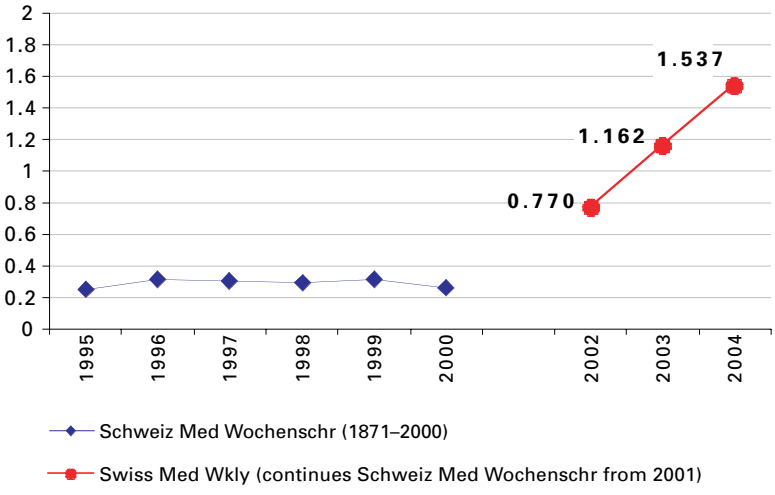

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