Ex Vivo Expansion Of Haematopoietic Cells In The Treatment Of Accidental Irradiation-Induced Aplasia: Feasibility Studies.

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ABSTRACT

The lessons learnt from the treatment of previous radiation accidents using either bone marrow transplantation or growth factor therapy suggest that it is of importance to investigate new therapeutic regimens. Ex vivo expansion of haematopoietic stem cells, precursors and differentiated cells is a new approach of growth factor therapy which may be of interest for the treatment of patients with irradiation-induced bone marrow aplasia. Ex vivo expanded maturing cells could be used to limit the early risks bound to aplasia (infections related to granulocytopaenia, bleedings associated with thrombocytopaenia), whereas expanded immature cells could hasten haematopoietic recovery. Indeed, it is possible to culture from the blood or bone marrow the cells able to proliferate and differentiate. A sufficient quantity of cells to cover the transfusion needs of a radiation victim through an aplasia episode can be produced, in presence of a specific growth factor combination. Qualitative studies shows that the expanded cells exhibit a close to normal functionality. Long-term culture techniques demonstrate the expansion of immature cells. We have set up a high dose total body irradiation non-human primate model in order to study the therapeutic potential of ex vivo expansion of autologous progenitors and differentiating cells. All the steps of the process (sampling, positive selection of the immature cells, ex vivo expansion, irradiation of the animals, reinjection of the cultured cells and study of the outcome) are established. In order to allow the long term follow up of the ex vivo expanded haematopoietic cells (homing to the bone marrow or localisation to specific organs for example), a retroviral gene transfer technique for transduction of green fluorescence protein (GFP) gene toward the selected immature blood or bone marrow cells is under development in this model. Taken together these elements will allow establishing the feasibility of ex vivo expansion of haematopoietic cells for the treatment of accidental irradiation-induced aplasia.
Introduction

Bone marrow aplasia is one of the main syndromes appearing following radiation exposure. As a consequence bone marrow aplasia is the first syndrome threatening the victim’s life because of bleeding related to thrombocytopenia, infection related to leucocytopenia or anaemia. Two therapeutic approaches of the haematopoietic syndrome have been used (in conjunction with antibiotherapy, transfusion and aseptic isolation).
- Allogeneic bone marrow transplantation, to replace the injured organ
- Growth factor therapy to stimulate the residual haematopoiesis

In the seventies and eighties, bone marrow transplantation had become enshrined in the literature as a proposed final successful therapy for the acute radiation syndrome [1,2,3]. There were at least two main reasons sustaining this medical attitude: bone marrow appears as being the most sensitive organ to whole body exposure within survival doses and clinicians had experience with several thousand such procedures, including preconditioning therapy for transplantation. After the Chernobyl accident, which was the first occasion where a large number of heavily irradiated victims had to be treated and where several BMT’s were performed, it has been concluded that indications of BMT were limited [4,5]. In the opinion of the physicians in charge of the victims, this therapy resulted in worsening of the conditions of their patients and they advice against its use. However, it is impossible to say for certain whether the transplantations were warranted, as many cases among the most heavily exposed victims developed complications mainly due to the associated burns. Anyhow, it is recognised that a BMT must be regarded as an exceptional and potentially hazardous measure [6].

Although BMT may not be completely contraindicated, the particular conditions prevailing in accidental overexposure explain the need for exploring other therapeutic approaches such as the growth factor therapy.

Ex vivo expansion of haematopoietic stem cells, precursors and differentiated cells is a new approach of growth factor therapy to stimulate the residual haematopoiesis. In vitro experiments suggest that the effect of numerous growth factors on cell differentiation and proliferation is dependent on the dose, way and schedule of delivery [9]. As a consequence of the clearance properties of the growth factors, high doses of growth factors are delivered systemically to the treated patients. Exposure of numerous cell types to high doses of growth factor may also result in possibly deleterious effects. In the specific situation of the radiation accident growth factor therapy resulted in mitigated success

- because the growth factors might have been administered too late after the accident allowing infection to develop
- because of specific side effects (exacerbation of radiation burns for instance) the used growth factors (GM-CSF, IL-3, G-CSF) might not have a sufficiently broad action on haematopoiesis to trigger the restoration of normal haematopoiesis,
- finally no treatment of specific problems such as thrombocytopenia (and therefore bleeding) is available.

Taken together these points suggest that although a powerful tool, growth factor therapy has not reached its goals and must be improved.

We here report our studies on the feasibility of a new approach of accidental radiation-induced aplasia: the ex vivo expansion of haematopoietic stem cells.

Ex vivo expansion of haematopoietic stem cells, precursors and differentiated cells is a new approach of growth factor therapy which may be of interest for the treatment of patients with irradiation-induced bone marrow aplasia. [9,10]. These studies aim to expand the pool of progenitors and stem cells for transplantation (or to expand differentiated cells for transfusion). Ex vivo expanded maturing cells could be used to limit the early risks bound to aplasia (infections related to granulocytopenia, bleedings associated with thrombocytopenia), whereas expanded immature cells could hasten haematopoietic recovery. This is made possible due to the development of techniques allowing the selection of a population of haematopoietic progenitors and stem cells from the blood (with stimulation by growth factors prior stem cell harvesting) or bone marrow using CD 34+ cell
positive selection. The next step consisting in their culture with combination of growth factors or additional stromal cells is under development. This approach is interesting for the treatment of patients with radiation-induced aplasia, either because the cells necessary for ex vivo expansion have been banked before the radiation accident or if such cells can be found in the blood or in the marrow and harvested in sufficient quantity after the accidental irradiation, and that it is possible to collect them. Such cells could be available in the blood after various types of irradiation as suggested by results on haematopoietic progenitors in the peripheral blood after therapeutic irradiation [11]. They may be also induced to egress from the bone marrow toward the blood through growth factor mobilisation.

The approach has numerous advantages. Ex vivo expansion could be set up as an autologous process (although it can be used in allogeneic settings such as cord blood expansion), thus allowing to avoid the specific problems of allogeneic cell use (GVHD, or graft rejection as well as the side effects of immune suppressive treatment, but also alloimmunisations bound to numerous transfusions).

- Some of the growth factors has restricted use in vivo due to their toxic side effects although their effects on haematopoiesis could be useful. Ex vivo experiments could allow their use without adverse reaction and at high doses, as they are not used in the organism.

The fact that the cells are kept out of the organism protects them from the indirect consequences of the accident such as inflammation or water loss.

By adjusting the conditions of expansions it is possible to modulate the treatment to obtain the production of a specific mature cell type (granulocytes for treatment of infection, platelets to treat haemorrhage) or only immature cells for transplantation.

Ex vivo manipulation of the cells is, in most experiments, a needed step for the transfer of a gene of therapeutic interest

Important research is necessary to adapt the ex vivo expansion of haematopoietic precursors and stem cells for transplantation or differentiated cells for transfusion in the treatment of radiation-induced aplasia, as in vitro cultures are different for both purposes. To be of therapeutic use the cells produced must retain normal function and regulation [12, 13].

We have set up a high dose total body irradiation non-human primate model in order to study the therapeutic potential of ex vivo expansion of autologous progenitors and differentiating cells. All the steps of the process (sampling, positive selection of the immature cells, ex vivo expansion, irradiation of the animals, reinjection of the cultured cells and study of the outcome) are established. In order to allow the long term follow up of the ex vivo expanded haematopoietic cells (homing to the bone marrow or localisation to specific organs for example), a retroviral gene transfer technique for transduction of green fluorescence protein (GFP) gene toward the selected immature blood or bone marrow cells is under development in this model. Taken together these elements will allow establishing the feasibility of ex vivo expansion of haematopoietic cells for the treatment of accidental irradiation-induced aplasia.

EXPERIMENTS

Culture techniques to expand haematopoietic cells

In order to set up a non human primate (NHP)(Macaca fascicularis) pre-clinical model for the therapeutic evaluation of ex vivo expansion of bone marrow mononucleated cells (BMMNC), we assessed the influence of several parameters on the amplification of total cell number, CFU-GM, BFU-E and Cobblestone Areas Forming Cells (CAFC). The parameters that were assayed included the cellular concentration at the initiation of the culture, the use of teflon® culture bags vs. cell culture treated flasks, and 4 different cytokine combinations.

Furthermore, the use of commercially available serum free medium (SFM) was compared to Iscove’s medium supplemented with 10% foetal calf serum (FCS).

Healthy macaques were used in these experiments, according to French regulations for animal experimentation (Ministry of agriculture, act number 87-848, 10/19/1987). Bone marrow (BM) cells were obtained by humeral aspiration. BM mononucleated cells (BMMNC) were isolated by centrifugation onto a Ficoll layer. BMMNC were then washed twice, numerated and viability was assessed by trypan blue exclusion.

Ex vivo expansion experiments were made using the following growth factors: primate rIL3 5 ng/ml (A gift from G. Wagemaker), IL6 5 ng/ml, G-CSF 20 ng/ml, SCF, TPO and FLT3l 100ng/ml (all from RD system, UK). BMMNC were cultured at a initial concentration of 1x10^6 to 5x10^4/ml, in IMDM supplemented with antibiotics, glutamin and 10% FCS or synthetic mediums : IMDM with 10% BIT (Stem Cell Technologies), Xvivo 15 and Xvivo 20 (Biowhittaker), Stempro 34 SFM (Life technologies), or a serum-free long term culture medium prepared in our lab. BMMNC were cultured in 25cm^2 flasks or in gas-permeable Teflon bags (Vue-life Inc) for
14 days at 37°C and 5%CO₂. On day 7, cultures were either harvested or fed with fresh medium including cytokines in order to augment the culture volume up to 5-fold. On day 0 and on the day of harvest, cells were washed twice, numerated, and viability was assessed by trypan blue exclusion. Cells were then analysed for CFC content. Some cultures were also tested in CAFC assay adapted for monkey BMMNC, in order to define the immature haematopoietic cell content by the end of the culture.

A total of eighteen healthy donors were used in these experiments. In all experiments, the different cultures conditions tested in each experiment were compared to « basic conditions », which are the following :
- culture for 14 days in IMDM with 10% FCS, in flasks,
- an initial cell concentration of 1x10^6 cells/ml,
- a cytokine combination composed of SCF (100ng/ml), IL3 (5ng/ml), IL6 (5 ng/ml), FLT3l (100ng/ml) and TPO (100ng/ml)(S36FT).

We first tested the use of gas-permeable Teflon bags in comparison with flasks for the culture of BMMNC, in « basic » conditions.

The main difference appearing between these two culture systems is that a stromal cell layer developed in flasks, but did not in bags. As a consequence, stromal cell layers were detached by trypsin, and were mixed with non-adherent cells for the evaluation of harvested cells in order to take into account haematopoietic cells that adhere to stromal layers in all following experiments.

Results showed that no significant differences appeared in the amplification factor of total BMMNC, nor in the amplification factor of CFU-GM and BFU-E, indicating that, in our hands, the use of Teflon bags did not modify the ability of BMMNC to expand. This comparison was also made with other culture conditions, and also did not shown any significant differences between Teflon bags and flasks.

However, in the case of large scale ex vivo expansions, Teflon bags are easier to manipulate, and avoid the use of trypsin in order to detach the adherent layer.

SCF, FLT3l, TPO and IL-6 are 4 cytokines known to act on immature haematopoietic cells, hence frequently used for the ex vivo expansion of BM cells. The role of IL-3 and G-CSF remains less clear in ex vivo expansion experiments. We then tested 4 cytokine combinations, including or excluding IL-3 and/or G-CSF, on the expansion of BMMNC in flasks and in Teflon bags.

Results showed that the two combinations without IL-3 were slightly less effective than the two combinations with IL3, for total CMN expansion, as well as for CFU-GM expansion, in flasks as well as in Teflon bags. By contrast, the S36FTG appeared as the most effective combination, with respect to total CMN and CFU-GM expansion, in flasks as well as in Teflon bags.

However, these cytokine combinations were not effective in expanding the BFU-E. This was probably due to the absence of EPO in the cytokine combination tested.

The use of ex vivo expansion in human therapeutic needs the use of serum free mediums (SFM). We then tested 4 commercially available SFM, and a SFM that was previously published, as compared to the classical medium IMDM 10% FCS.

Results showed that none of the mediums tested were more efficient than IMDM 10%FCS, in increasing the total CMN expansion, and that one of the medium was far less effective than the others.

Results also showed that none of the mediums gave significant differences in CFU-GM and BFU-E expansion, as compared to IMDM 10%FCS. Moreover, a very high individual variability in the results was observed, rendering the comparison quite difficult.

The cell concentration at the initiation of the culture appeared as an important parameter. We thus tested different initial cell concentrations in basic conditions. Results of Figure 1 showed that the amplification factor of BMMNC increased with the decrease in initial cell concentration, with a plateau appearing for 2x10⁵ cells/ml and lower concentrations. However, a different effect appeared on CFU-GM, with a peak of amplification factor for 5x10⁵ cells/ml, and a plateau for lower concentrations. For BFU-E expansion, no significant difference appeared for any of the initial cell concentration tested.

Thus, for the total BMMNC expansion, the optimal initial concentration is at 2x10⁵ cells/ml, while for CFU-GM, the optimal cell concentration is rather 5x10⁵ cells/ml.
The duration of the culture is also a parameter that may influence the expansion of BMMNC. We then tested two culture durations, namely 7 days and 14 days. Results showed that a 14-day culture is more efficient for total CMN and CFU-GM expansion, but not on BFU-E expansion. We also tested in these experiments the CAFC content of expanded cells. Results showed that the 7-day expansion induced an increase in CAFC frequency (leading to a mean 8-fold amplification of CAFC), while a 14-day expansion induced a decrease of CAFC frequency. This indicated that the increase in CFU-GM observed in 14-day expansion was mainly due to the differentiation of CAFC in our system.

Our results showed that:

- the use of Teflon bags did not change the expansion of BMMNC. However, Teflon bags are easier to use in large-scale ex vivo expansion.
- The use of IL-3 and G-CSF in combination with SCF, FLT3-l and TPO slightly augment the CFU-GM and total CMN expansion.
- The use of synthetic mediums did not statistically change the expansion of BMMNC. However, great donor variability was observed in the results, suggesting that other synthetic mediums may give better results.
- The use of a $2 \times 10^5$ cell/ml initial concentration augment the total CMN expansion, but do not change the CFU-GM expansion. A $5 \times 10^5$ cell/ml initial concentration was more efficient for CFU-GM expansion, and is obviously cheaper!
- A short time of ex vivo expansion appeared to amplify the immature compartment, while a longer time of expansion induced a high increase in CMN number, but also a maturation of the cells.

Overall, the study of these parameters indicated that the increase in the amplification of total CMN was inversely correlated with a maturation of the cells in the culture [14].

Quality control of the maturing expanded cells

Ex vivo expanded CD34+ progenitor cells from fresh or cryopreserved non-human primate bone marrow, induced to granulocytic differentiation with growth factors, were investigated to determine whether myeloid cells
produced in liquid cultures have normal biological functions needed for the treatment of patients with granulocytopenia following accidental radiation exposure. Simian (macaques) CD34+ cells were cultured with granulocyte-colony stimulating factor (G-CSF), stem cell factor (SCF), interleukin-1, 3 and 6 (IL-1, IL-3 and IL-6), and assessed at 14 days of culture for their capacity to respond to different functional tests. Immunostaining revealed that human ex vivo expanded cells contained Myeloperoxidase (MPO, 82 ± 8%) and Lactoferrin (LF, 30 ± 6%) in their granules. Maturation of cultured cells was associated with stimulated chemotactic responsiveness and respiratory burst activity (superoxide anion or –Figure2- hydrogen peroxide production through DCF fluorescence analysis) in expansions from macaque CD34+ progenitor cells. Mature cells obtained from ex vivo expansion of selected cryopreserved bone marrow CD34+ cells presented reduced but significant functional activities (chemotactic responsiveness and hydrogen peroxide production) when compared to peripheral blood neutrophils. The validation of non-human primate ex vivo expansion system may permit their use as models of irradiation. The feasibility of ex vivo expansion from cryopreserved bone marrow cell samples may offer large perspectives in strategies of banking bone marrow for autologous transfusion.

**Figure 2**: production of hydrogen peroxide following ex vivo expansion of non human primate cells, DCF fluorescence

Haematopoietic cell labelling through retroviral gene transfer for the follow up of ex vivo expanded cells.

We have developed a retroviral gene transfer technique for transduction of green fluorescence protein (GFP) gene toward the umbilical cord blood cells, in order to allow the follow up of the ex vivo expanded haematopoietic cells.

Non-human primate CD34+ cells are positively selected and prestimulated for 48 hours in serum free medium (10^5 cells/ml, SCF, TPO, FLT3-l, 100 ng/ml each). The cells are then transduced twice for 48 hours in the presence of the supernatant of PG13 cell line producing the GFP encoding gene GALV vector (serum free medium, CH296 fibronectin fragment coated plates). Qualitative and quantitative analysis of the expansion product are performed, transduced CD34+ cells are assessed through flow cytometry analysis. Clonogenicity is also assessed through 14 days methylcellulose cultures is assessed and the number of cells and colonies which have integrated the transgene is evaluated.

The use of retroviral vector allows transducing up to 50% of the non-human primate CD34+ cells. Clonogenicity assays show that 15 % of the CFU-GM (Figure 3) and 60% of the BFU-E colonies are producing GFP. The developed transduction protocol allows preserving a high level of CD34+ cells. The proliferation and differentiation of the cells are similar to the one of the expanded non-transduced controls. Such a protocol would allow to establish a long term follow up of injected expansion products [15].

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Figure 3 Haematopoietic progenitor colony labelled through GFP retroviral gene transfer

CONCLUSION

In order to assess the feasibility of \textit{ex vivo} expansion of haematopoietic cells for the treatment of accidental radiation induced aplasia we have studied all the aspects of the proposed approach.

- the condition of expansion of maturing haematopoietic cells suitable for transfusion purposes are defined,
- the functionality of these cells is established,
- the conditions of production of immature cells, which can be used to hasten haematopoiesis recovery, are explored.
- The labelling of the reinfused cells through GFP gene transfer for the follow-up of the whole process is under development.

Taken together these preliminary data suggest that by studying the effect of various dose, dose rate and heterogeneity of irradiation we will then try to define the type of accidents which can benefit from that therapeutic approach.

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