Human cell expressed cytokines enhance proliferation of CD34+ haematopoietic stem cells

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INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) are a combination of cytokines used in expansion of haematopoietic stem cells. Expansion of human haematopoietic stem cells is used clinically for autologous and allogeneic bone marrow transplantation.

Currently, recombinant human (rh)G-CSF and rhSCF are produced in *E. coli* and are thus not glycosylated. However, the biological importance of species-specific post-translational modifications, in particular glycosylation, is pivotal to protein function. We have expressed G-CSF (G-CSF^{hcx}) and SCF (SCF^{hcx}) in modified human 293 cells, giving them characteristics of the native (*in vivo*) expressed proteins. SCF^{hcx} is a highly glycosylated protein, with up to 50 % of its molecular weight consisting of carbohydrate. G-CSF^{hcx} has O-linked glycosylation, which is known to enhance its biological stability compared to non-glycosylated forms (Querol *et al*, 1999).

Our aim was to determine whether G-CSF^{hcx} and SCF^{hcx} promote CD34$^+$ cell proliferation, differentiation and clonogenic ability more effectively than other commercially available cytokines. The results show that hcx™ cytokines are more effective in promoting the proliferation of haematopoietic stem cells than recombinant cytokines expressed in *E. coli*. Further, it was determined that the differential response induced by hcx™ cytokines were a result of variations in signal transduction. These variations are under investigation.

MATERIALS AND METHODS

CD34$^+$ Cell Expansion

CD34$^+$ cells purified from human umbilical cord blood (CB) were obtained from Cambrex Bio Science, Walkersville. The cells were cultured in HPGM (Cambrex), supplemented with 20 % FCS in 24 well plates. 10,000 cells were seeded into each well. SCF and G-CSF were added at 100 ng/ml. Commercial *E. coli* expressed G-CSF and SCF were obtained from R&D Systems and Peprotech respectively. After 7 days cells were counted and viability determined by trypan blue exclusion.

Colony forming unit assay

In vitro CFU assays were performed to detect primitive progenitor cells at *ex vivo* expansion day 7. A total of 500 cells were inoculated into semisolid cultures of methylcellulose (1.17 %) using 100 ng/ml each of G-CSF and SCF in IMDM including 20 % FCS, 100 U/ml Penicillin, 10 µg/ml Streptomycin and 4 mM GlutaMAX (GIBCO). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and scored 14 days later with an inverted microscope.

Flow cytometry

Cell differentiation was assessed by examining the expression of specific cell surface proteins by flow cytometry (Becton Dickinson FACSCalibur). Cells were stained with fluorochrome-conjugated monoclonal antibodies to CD34 (BD), CD33 (BD), CD184 (BioLegend), CD11b (BD) and CD15 (BD).

Whole cell extracts and western blotting

To examine phosphorylation of STAT3, expanded CD34$^+$ cells were lysed with passive lysis buffer (Promega) after 7 days culture in SCF and G-CSF. Whole cell extracts were subject to western blotting and probed with antibodies to the phosphorylated form of STAT3 (pY705), and total STAT3 (BD Biosciences). Westerns were analysed with Fuji Film LAS-3000 and quantification of bands was performed with Multi Gauge software.

RESULTS/DISCUSSION

Total cell expansion

To examine differences in biological activity of hcx™ cytokines compared to non-human cell expressed cytokines, after 7 days in culture, CD34$^+$ cell expansion was calculated using trypan blue counting and expressed as fold increase above the initial cell density. The combination of hcx™ G-CSF and SCF induced up to 30% more cell proliferation (Figure 1) compared to the *E. coli* expressed G-CSF and SCF and the difference was significant ($p < 0.05$).

Colony Forming Assay

Colonies grown from the two cell populations generated above were scored on day 14. It was found that the clonogenic ability was also greater when cells were grown in hcx™ expressed cytokines (Figure 2). Furthermore the colonies were larger when G-CSF^{hcx} and SCF^{hcx} were used.

Phenotypic analysis of cells generated on day 7 by flow cytometry

On day 7 of the expansion procedure, a number of stem cell markers differed depending on the source of the G-CSF and SCF. In G-CSF^{hcx} and SCF^{hcx} treated cells, more CD11b$^+$/CD15$^+$ cells were present compared to cells cultured in *E. coli* expressed G-CSF and SCF, indicating more mature granulocytic cells (Figure 3). This correlates with data presented by Querol *et al.* (1999), which demonstrated that glycosylated G-CSF produces more mature granulocytic cells than deglycosylated cells. This suggests that human cell expressed G-CSF^{hcx} and SCF^{hcx} are more biologically active.

CD184 (CXCR4) expression was also found to be higher in cells cultured with G-CSF^{hcx} and SCF^{hcx}, compared to cells cultured with *E. coli* expressed G-CSF and SCF. In a murine model, over-expression of CD184 has been demonstrated to improve stem cell mobility and multi-lineage repopulation, suggesting that higher CD184 expression could be beneficial for proliferation and migration to the bone marrow in transplanted stem cells (Kahn *et al*, 2004).

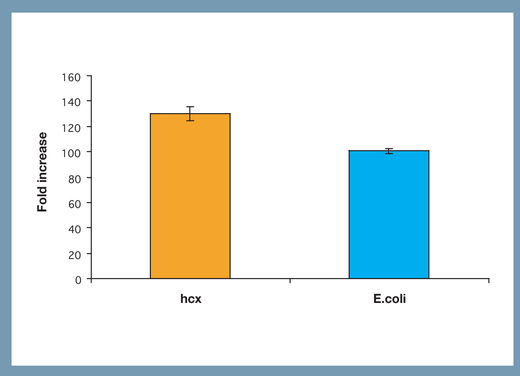

Figure 1: Cell Proliferation of CD34+ cells

CD34+ cells were cultured with G-CSF^{hcx} and SCF^{hcx} or *E. coli* expressed G-CSF and SCF for 7 days. Data indicates the mean ± standard deviation of a representative experiment.

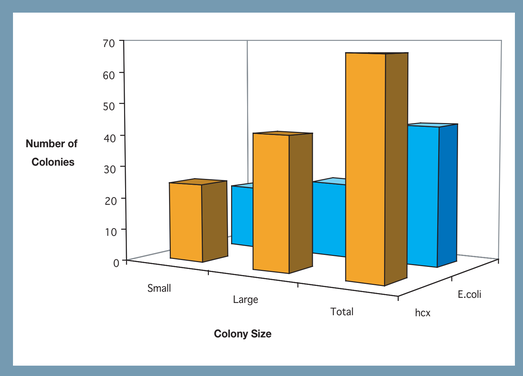

Figure 2: Clonogenic activity of CD34$^+$ expanded cells by G-CSF and SCF.

CD34$^+$ expanded cells were inoculated into semisolid cultures of methylcellulose using 100 ng/ml each of G-CSF and SCF. The cultures were incubated at 37°C and scored 14 days later with an inverted microscope. The graph is a representation of three experiments.

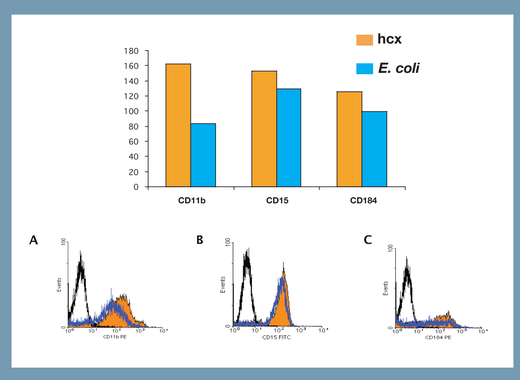

Figure 3: Phenotypic Analysis

CD34$^+$ cells were cultured with G-CSF and SCF for 7 days and expression of cell surface markers was analysed by flow cytometry A. CD11b, B. CD15, C. C. CD184. Data shown is from a representative experiment.

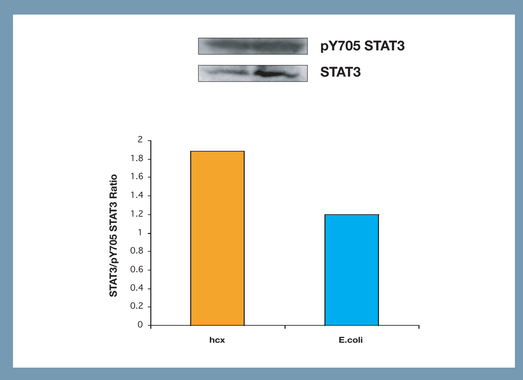

Figure 4: STAT3 Phosphorylation

STAT3 phosphorylation (Y705) was examined by western blotting of cell lysates extracted after 7 days growth in G-CSF and SCF. The result is a ratio of pY705STAT3/ STAT3 as determined by densitometry using Multi Gauge software.

Analysis of STAT3 Phosphorylation

As STAT3 has been shown to be a key mediator of G-CSF signalling in haematopoietic cells (Duarte *et al*, 2000), we investigated STAT3 activation induced by hcx™ cytokines compared to *E. coli* expressed cytokines. Higher STAT3 phosphorylation was detected in cells treated with hcx™ cytokines compared to cells treated with *E. coli* expressed cytokines (Figure 4). Densitometry of STAT3 and pY705STAT3 bands obtained by western blotting was used to obtain their expression ratios. The ratio of STAT3 phosphorylation/total STAT3 for hcx™ cytokine treated cells and the *E. coli* cytokine treated cells were 1.9 and 1.2 respectively. This indicates that G-CSF^{hcx} and SCF^{hcx} are a more biologically active combination than *E. coli* expressed cytokines. Further, the result suggests that the observed increased proliferation and different differentiation is related to STAT3 phosphorylation.

CONCLUSION

This study demonstrated that G-CSF^{hcx} and SCF^{hcx} when used together form a powerful combination for inducing CD34$^+$ stem cell expansion *in vitro*. The results of this study have important clinical applications for the more efficient expansion of CD34$^+$ stem cells for autologous and allogeneic bone marrow transplantation.

Our preliminary data indicates that the hcx™ cytokines are more active at the molecular level. Further studies are ongoing to investigate the intracellular pathways mediating this enhanced proliferation, to elucidate the unique features of our G-CSF^{hcx} and SCF^{hcx}.

References

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