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Human Cell-expressed Cytokines Enhance Proliferation and Differentiation of CD34+ Haematopoietic Stem Cells.

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INTRODUCTION

Human haematopoietic stem cells (HSCs) are used clinically for autologous and allogeneic bone marrow transplantation. Generation of sufficient cell numbers of the appropriate cell type for therapeutic use requires stem cells to be expanded *ex vivo*. *Ex vivo* expansion protocols in use at the moment, employ chemically defined, serum-free medium containing an optimal combination of cytokines and growth factors, which may include granulocyte colony stimulating factor (G-CSF) and stem cell factor (SCF) as well as other cytokines.

Currently, recombinant human (rh) G-CSF (rhG-CSF) and rhSCF for therapeutic use, are expressed mostly in *E. coli* and therefore are not glycosylated. In addition, the importance of human-specific glycosylation of cytokines is now being recognised, as species-specific differences in glycosylation can affect the biological properties of a protein. We have expressed rhG-CSF (G-CSF^{hcx}) and rhSCF (SCF^{hcx}) in modified human HEK293 cells, in order to achieve the characteristics of their native counterparts. We have previously demonstrated that G-CSF^{hcx} has the O-linked glycosylation, which is known to enhance its biological stability, compared to non-glycosylated forms[1]. We have also shown SCF^{hcx} to be a highly glycosylated protein, with up to 50% of its molecular weight consisting of carbohydrate.

The aim of this study was to determine whether G-CSF^{hcx} and SCF^{hcx}, with human-cell specific glycosylation, when used in combination, would promote increased CD34+ cell proliferation, differentiation, migration or clonogenic ability compared to non-human cell expressed cytokines. We have found that hcx™ cytokines promote greater proliferation and differentiation of HSCs than recombinant human cytokines expressed in *E. coli*. Furthermore we have demonstrated increased SDF-1α induced migration in HSCs expanded with hcx™ cytokines. These improved responses induced by hcx™ cytokines may have been in part due to effects on signal transduction. In particular, we have observed that G-CSF^{hcx} activates STAT3 and MAPK signalling to a greater extent than *E. coli* expressed cytokines, which are not glycosylated.

MATERIALS AND METHODS

CD34+ cell expansion

CD34+ cells purified from human umbilical cord blood (CB) were obtained from Lonza Group Ltd Switzerland. A total of 1 x 10^4 cells were cultured in HPGM (Lonza) supplemented with 20% FCS, G-CSF (100 ng/ml) and SCF (100 ng/ml) in 24 well plates. *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 measure primitive progenitor cells after *ex vivo* expansion for 7 days. A total of 500 cells per dish were inoculated into dishes of semisolid methylcellulose (1.17%) containing G-CSF and SCF (100 ng/ml each) in IMDM including 20% FCS, 100 U/ml Penicillin, 10 µg/ml Streptomycin and 4 mM glutamax. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and colonies counted 14 days later using an inverted microscope.

Flow cytometry

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, CD33, CD11b, CD15 (BD Biosciences) and CD184 (BioLegend).

Whole cell extracts and western blotting

CD34+ expanded cells were incubated for 2 hours in serum free HPGM medium before stimulation with G-CSF for the indicated times (0-60 minutes). Whole cell extracts were subjected to western blotting and probed with antibodies to the phosphorylated forms of STAT3 (pY705: BD Biosciences), MAPK (pY204: Cell Signaling Technology Inc), total STAT3 (BD Biosciences) and total MAPK (Cell Signaling Technology Inc). Densitometric analysis of western blots and quantification of bands were performed with Multi Gauge software V3.0 (Fuji Film Corporation).

Chemotaxis

Migration of G-CSF and SCF expanded CD34+ cells was assessed using the ChemoTx® System (Neuro Probe, Inc.). Lower wells of the plate contained stromal derived factor-1α (SDF-1α; 0-400 ng/mL). Calcein-AM loaded cells (2.5x10^6/ml) were added to the upper filter frame and incubated at 37°C for 2 hrs. For inhibition studies, cells were pre-incubated with the CXCR4 (CD184) antagonist AMD-3100 (10 µg/ml) for 1 hr at 37°C. Fluorescence was excited with incident light at 480nm and the resulting emitted light measured at 520nm using a BMG FLUOstar plate reader.

RESULTS AND DISCUSSION

Total cell expansion

After 7 days in culture, CD34+ cell expansion with G-CSF^{hcx} and SCF^{hcx} was compared to that with *E. coli* expressed rhG-CSF and rhSCF. Results are expressed as the fold increase above the initial cell density. The combination of G-CSF^{hcx} and SCF^{hcx} induced up to 30% more cell proliferation (Figure 1) compared to the *E. coli* expressed human G-CSF and SCF. These data were significantly different ($p < 0.05$) as measured using a two-tailed t-test.

Colony forming assay

Colonies were scored on day 14. The average clonogenic ability when cells were cultured with hcx™ cytokines was 33% higher than cells cultured with *E. coli* expressed human cytokines (Figure 2). Furthermore, the percent of large colonies was consistently higher (63%) when G-CSF^{hcx} and SCF^{hcx} were used compared to *E.coli* expressed cytokines (54%). The data presented in Figure 2 is one representative experiment, of three experiments.

Granulocytic differentiation

The ability of G-CSF^{hcx} in combination with SCF^{hcx} to induce granulocytic differentiation was also compared to rhG-CSF and rhSCF expressed in *E. coli*. CD34+ cells cultured with G-CSF^{hcx} expressed 2-fold more CD11b and on average the expression of the mature granulocytic marker CD15 was also higher (Figure 3). This resulted in a higher CD11b/CD15 ratio for the hcx™ treated cells indicating a more mature granulocytic phenotype[1]. CD184 (also known as CXCR4) expression was also found to be higher in cells cultured with hcx™ cytokines (Figure 3). CXCR4 is the receptor for SDF-1α (CXCL12), which is believed to be responsible for homing and migration of cells to bone marrow.

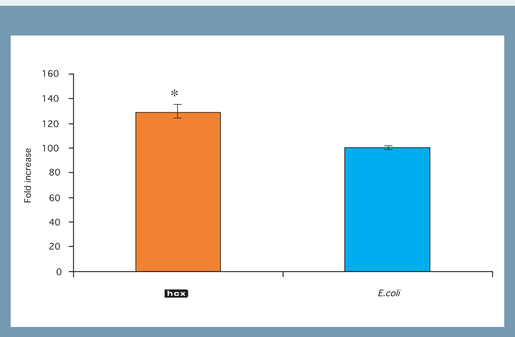


Figure 1: Cell Proliferation of CD34+ cells

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

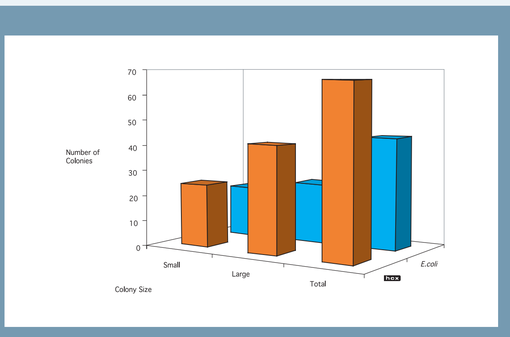


Figure 2: Clonogenic activity of CD34+ expanded cells by G-CSF and SCF

CD34+ expanded cells were inoculated into semisolid cultures of methylcellulose containing G-CSF^{hcx} and SCF^{hcx} or *E. coli* expressed human G-CSF and SCF. Cultures were incubated at 37°C and scored 14 days later using an inverted microscope. Data are from one representative experiment of three experiments.

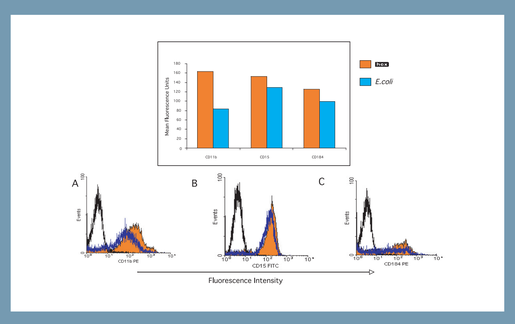
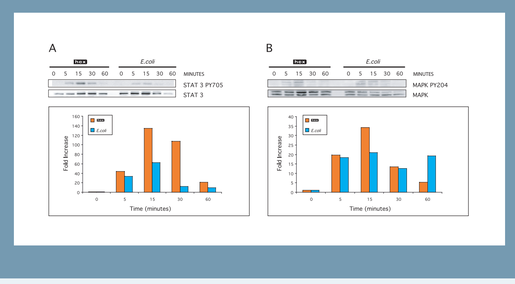


Figure 3: Phenotypic Analysis

CD34+ cells were cultured with G-CSF^{hcx} and SCF^{hcx} or *E. coli* expressed human G-CSF and SCF for 7 days. Expression of cell surface markers was analysed by flow cytometry using fluourescent antibodies to **A.** CD11b, **B.** CD15, **C.** CD184. Data shown are from a representative experiment.



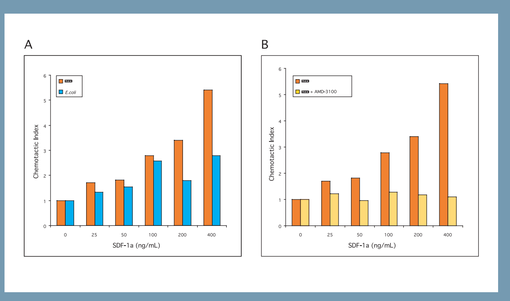


Figure 4: SDF-1α induced chemotaxis

A. The ability of SDF-1α to induce migration of the expanded CD34+ cells was examined. **B.** ADM-3100 (10 µg/ml) inhibition of SDF-1α induced migration.

Figure 5: STAT 3 and MAPK phosphorylation

A. STAT3 phosphorylation and **B.** MAPK phosphorylation was examined by western blotting of cell lysates extracted after stimulation with G-CSF^{hcx} or *E. coli* expressed G-CSF at the indicated times. The results are presented as the relative level of phosphorylated protein as a ratio of total protein as determined by densitometry using Multi Gauge software.

SDF-1α mediated cell migration

As CD184 (CXCR4) expression was found to be consistently higher in cells grown in G-CSF^{hcx} and SCF^{hcx}, than *E. coli* derived human G-CSF and SCF, *we* hypothesised that SDF-1α-mediated migration would be higher in cells grown in hcx™ cytokines than cells grown with *E. coli* expressed human cytokines. Migration of cells expanded with G-CSF^{hcx} and SCF^{hcx} was approximately 2-fold higher at 200 and 400 ng/ml SDF-1α than cells expanded with *E. coli* expressed human cytokines (Figure 4A). To confirm that cell migration was in fact mediated by CD184 expression, cells were pre-incubated with the ADM-3100, a CD184 antagonist. ADM-3100 inhibited chemotaxis in both cell populations (Figure 4B), confirming the role of CD184.

The enhanced ability of cells cultured in hcx™ cytokines to migrate to SDF-1α has significant clinical implications. In both autologous and allogenic bone marrow stem cell transplantation, the ability to migrate to SDF-1α correlates with improved engraftment[2]. Hence the use of hcx™ cytokines to expand stem cells before transplantation could improve engraftment rates.

Analysis of STAT 3 and MAPK phosphorylation

Binding of G-CSF to its receptor induces the phosphorylation of a number of cellular proteins and activates signaling cascades including the signal transducer and activator of transcription (STAT) and mitogen-activated protein kinase (MAPK) pathways[3]. The activation of these pathways was examined to determine whether the differential responses induced by G-CSF^{hcx} or *E. coli* expressed G-CSF were a result of differences in signal transduction. STAT3 activation was up to 9-fold higher (30 minutes activation) in response to G-CSF^{hcx} compared to cells treated with *E.coli* expressed human G-CSF (Figure 5A). Similarly, the MAPKs ERK1/2 were also activated to a greater extent by G-CSF^{hcx} with the greatest difference in phosphorylation observed at 15 minutes (1.6-fold; Figure 5B). These results suggest that the higher STAT3 and MAPK activation elicited by G-CSF^{hcx} may contribute to its increased biological activity. Consistent with our findings, others have demonstrated that STAT3 transduces the proliferation and differentiation signal of G-CSF and that MAPK is responsible for most of the proliferation activity of HCSs[4].

CONCLUSION

- This study demonstrates that G-CSF^{hcx} and SCF^{hcx} together form a more powerful combination for inducing greater human haematopoietic stem cell expansion *in vitro*.
- G-CSF^{hcx} and SCF^{hcx} produced greater numbers of larger colonies, containing more mature granulocytes, compared to *E. coli* expressed human cytokines.
- Our data indicate that the increased biological response observed with G-CSF^{hcx} may be in part mediated by increased STAT3 and MAPK activation.
- Human haematopoietic stem cells expanded in hcx™ cytokines have a greater capacity to migrate to SDF-1α compared to cells grown in *E. coli* expressed human cytokines.

The results of this study have important clinical implications, suggesting hcx™ cytokines may be more efficient for expansion, migration and engraftment of CD34+ stem cells in clinical settings such as bone marrow transplantation.

References

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