Human cell-expressed EPO: Analysis of the role of glycans in mediating proliferation and differentiation of CD34+ stem cells.

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INTRODUCTION

Erythropoietin (EPO) is the primary regulator of the survival, proliferation and differentiation of erythroid progenitors. Recombinant human EPO (rhEPO) is used to treat anaemia, resulting from cancer or chemotherapy, kidney disease infection or inflammation[1]. At present, the only commercially available rhEPO is purified from Chinese Hamster Ovary (CHO) cells. However, it is becoming apparent that species-specific post-translational modifications, particularly glycosylation, are pivotal to protein function.

Like many other cytokines, native rhEPO is heavily glycosylated, with up to 35% of its molecular weight consisting of N- and O-linked oligosaccharides. It has been proposed that glycosylation of rhEPO is important for its secretion, solubility, resistance to proteolysis, immunogenicity, biological recognition, biological activity, *in vivo* stability and clearance[2]. It has also been reported that the proportion of sialic acid containing carbohydrates affects the serum half-life and *in vivo* activity of rhEPO[3].

Our aim was to determine whether human cell expressed rhEPO (EPO^{hex}) promotes erythroid development of human CD34$^+$ stem cells more effectively than commercially available non-human cell expressed rhEPO (expressed in CHO cells), and to examine the role of sialic acid residues in mediating the biological effects of EPO.

MATERIALS AND METHODS

Characterisation of Human Cell Expressed EPO (EPO^{hex})

RhEPO (EPO^{hex}) was expressed in modified human HEK293 cells cultured in serum free DMEM supplemented with 4 mM L-glutamine. Following transfection, conditioned medium was collected and subjected to dye ligand chromatography followed by cation exchange and size exclusion chromatography. Glycan structures were analysed by LC-MS and probable structures predicted using GlycosidIQ (www.glycosuite.com). Desialylated EPO^{hex} was obtained by neuraminidase (Roche) treatment of purified EPO^{hex} at room temperature for 16 hours. The resultant desialylated EPO^{hex} was purified from the enzyme and carbohydrates by size exclusion chromatography.

Bioactivity of EPO^{hex}

The biological activities of EPO^{hex} and CHO-expressed rhEPO (Peprotech) were compared by examining the effect of these cytokines on the proliferation and differentiation of cord blood CD34$^+$ stem cells. CD34$^+$ stem cells (Lonza Group Ltd., Switzerland) were grown in HPGM (Lonza), supplemented with rhEPO (50 ng/ml) and SCF (20 ng/ml; either SCF^{hex} or Peprotech SCF) for 7 days at 37°C. Cellular proliferation was assessed by cell-counting in a haemocytometer, with trypan blue to exclude non-viable cells. Erythroid differentiation was assessed by examining the expression of the erythroid specific protein glycophorin A (GPA; BD Biosciences) and the transferrin receptor (CD71; BioLegend), by flow cytometry (FACSCalibur). Haemoglobin α expression was determined by SDS-PAGE followed by western blotting (Santa Cruz), and by observing the cell pellets following centrifugation at the termination of all experiments. For cellular morphology, slides were prepared using a Shandon CytoSpin III Cytocentrifuge, and cells were stained with Wright-Giemsa. All data is representative of three experiments. Statistical significance was determined by a two-tailed t-test, *=p<0.05, ***=p<0.001.

EPO Signalling

CD34$^+$ stem cells were expanded for 7 days in HPGM, supplemented with 15% FCS, rhEPO (50 ng/ml) and SCF (50 ng/ml), at 37°C. The resulting erythroid precursors were serum and cytokine starved for 4 to 6 hours, before being stimulated with EPO^{hex} or CHO-expressed rhEPO for the indicated times. Whole cell extracts were subjected to western blotting and probed with antibodies recognising either the phosphorylated forms of STAT5 (pSTAT5 Tyr694), Akt (pAkt Ser473), MAPK (p44/44 Thr202/Tyr204) or antibodies recognising total STAT5, Akt or MAPK proteins (Cell Signaling Technology Inc.). Densitometric analysis was carried out using Multi-Gauge v3.0 (FujiFilm Corporation, 2007). Jak/STAT (Jak2 inhibitor II), MAPK (PD98059) and PI3K/Akt (LY294002) inhibitors were from Merck Pty. Ltd.

RESULTS AND DISCUSSION

Structural Analysis of EPO^{hex}

The glycan structures of EPO are known to be important for *in vivo* activity. As glycosylation patterns differ between species, we compared the major glycan structures of EPO^{hex} and CHO-expressed rhEPO. CHO-expressed rhEPO migrated as a 30.4 kDa protein, while EPO^{hex} migrated as a broader band between 25 to 40 kDa (data not shown), demonstrating a qualitative difference between them. Further, we demonstrated that EPO^{hex} consisted of a mixture of N- and O-linked glycoforms, similar to native human serum EPO[4], whereas CHO-expressed rhEPO consisted of only N-linked oligosaccharides (Figure 1). These results highlighted the differences between human and CHO-expressed rhEPO, and led us to examine whether these structural differences affect the bioactivity of EPO^{hex}.

EPO^{hex} Promotes Increased Erythroid Differentiation

i) Proliferation

Our initial experiments investigated whether human cell expressed rhEPO induced greater CD34$^+$ cell proliferation than CHO-expressed rhEPO. CD34$^+$ cells were grown in EPO^{hex} or CHO-expressed rhEPO for 7 days, and the extent of proliferation was examined. rhEPO from both sources supported cellular proliferation to a similar degree (Figure 2).

ii) Erythroid Differentiation

The ability of EPO^{hex} to promote erythroid differentiation was compared to that of CHO-expressed rhEPO. CD34$^+$ cells grown in EPO^{hex} were morphologically more mature than cells cultured in CHO-expressed rhEPO. Orthochromatic and polychromatic erythroid progenitor cell maturation was induced by EPO^{hex}, compared to a homogeneous population of immature proerythroblasts seen with expansion in CHO-expressed rhEPO (Figure 3A). CD34$^+$ cells cultured with EPO^{hex} expressed significantly higher levels (two-fold, p<0.05) of the erythroid specific cell-surface markers GPA and CD71 (Figure 3B and 3C) than cells cultured with CHO-expressed rhEPO. EPO^{hex} also enhanced the expression of haemoglobin α in these cells after 7 days in culture (Figure 3D). These results provide evidence that EPO^{hex} is more biologically active, inducing greater erythroid differentiation than CHO-expressed rhEPO.

The Molecular Mechanisms Determining Enhanced Erythroid Differentiation Induced By EPO^{hex}

i) The Importance Of Sialic Acid Moieties On EPO^{hex}

Sialic acid moieties are known to affect the serum half-life and *in vivo* activity of rhEPO. To determine whether these moieties influence erythroid differentiation, the biological activity of EPO^{hex} and its desialylated counterpart were compared. Removal of the sialic acid residues from EPO^{hex} resulted in a lower molecular weight protein, as expected (Figure 4A). Interestingly, CD34$^+$ cells grown in desialylated EPO^{hex} had an increased proliferative capacity, compared to untreated EPO^{hex} (Figure 4B). Further, growth in desialylated EPO^{hex} resulted in a two-fold reduction in mature erythroid marker expression, and decreased haemoglobin expression (Figure 4C and 4D), producing similar results to CHO-expressed rhEPO. These results indicate that the sialic acid residues are important for the enhanced bioactivity of EPO^{hex}, particularly its ability to induce erythroid differentiation. These data complement the reciprocal findings by other researchers reporting that increased sialic acid content promotes increased *in vivo* activity[3].

ii) EPO-induced Intracellular Signalling Pathways

Binding of EPO to its receptor initiates several intracellular signalling cascades, including the Jak2/STAT5, MAPK and PI3K/Akt pathways, which promote cell survival, proliferation and differentiation. Our data demonstrate that EPO^{hex} activated STAT5 to a greater extent (over two-fold) than CHO-expressed rhEPO (Figure 5). No differences in the level of MAPK or Akt phosphorylation were detected. As the Jak2/STAT5 pathway is a key mediator of erythroid differentiation[5], these results suggest that increased activation of this pathway may be a mechanism by which the enhanced erythroid differentiation induced by EPO^{hex} is mediated.

To further examine the role of EPO-induced signal transduction, CD34$^+$ cells were expanded in the presence of chemical inhibitors of the PI3K/Akt (LY294002), MAPK (PD98059) and Jak/STAT (Jak2 inhibitor II) pathways. Cellular proliferation induced by either EPO^{hex} or CHO-expressed rhEPO was reduced by similar extents in the presence of all inhibitors (Figure 6A). However, inhibition of the Jak/STAT and PI3K/Akt pathways reduced GPA expression more than two-fold (Figure 6B). This result further demonstrates the importance of the Jak2/STAT5 pathway in mediating the enhanced bioactivity of EPO^{hex} on erythroid development. Additional investigations are being conducted to determine the precise mechanisms through which this occurs.

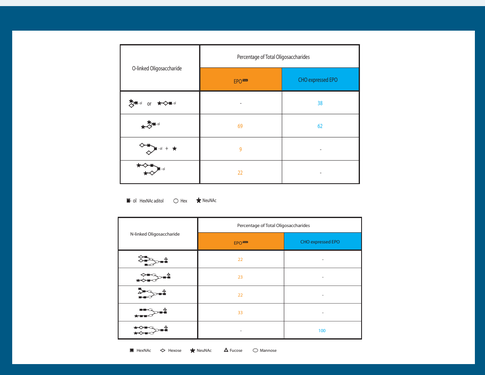

Figure 1. Characterisation of N- and O-linked Oligosaccharides from EPO^{hex} and CHO-expressed rhEPO. Glycan structures were analysed by LC-MS and probable structures predicted using GlycosidIQ (www.glycosuite.com).

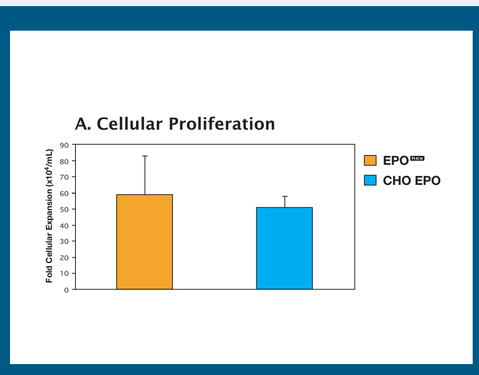

Figure 2. EPO-induced Cellular Prolifration. CD34$^+$ cells were cultured with EPO^{hex} or CHO-expressed rhEPO for 7 days. Data indicates the mean +/- standard deviation of six independent experiments.

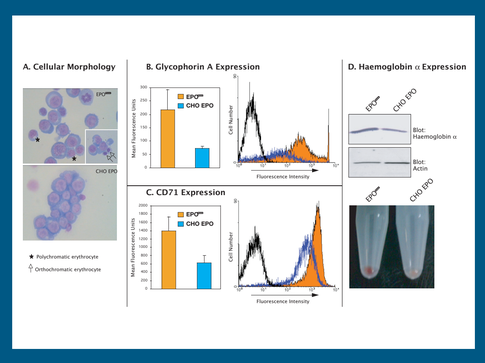

Figure 3. EPO-induced Erythroid Differentiation. CD34$^+$ cells were cultured with EPO^{hex} or CHO-expressed rhEPO for 7 days. **A.** Wright-Giemsa staining of erythroid precursors **B.** Glycophorin A and **C.** CD71 were analysed by flow cytometry. **D.** Haemoglobin expression analysed by western blotting (upper panel) and visualisation of the cell pellet (lower panel).

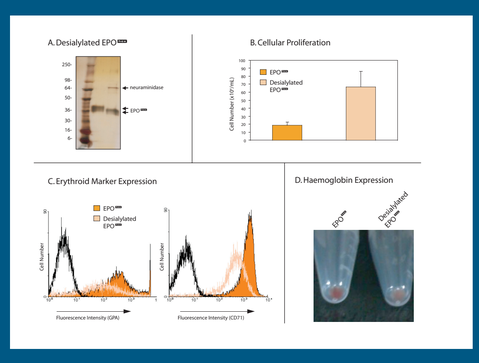

Figure 4. EPO^{hex} Desialylation Decreases Bioactivity. A. Silver stained SDS-PAGE of EPO^{hex} following neuraminidase treatment. CD34$^+$ cells were cultured with EPO^{hex} or desialylated EPO^{hex} for 7 days. **B.** Cellular proliferation was determined by cell counting. Data represent the mean +/- standard deviation of two independent experiments. **C.** Expression of erythroid markers Glycophorin A (GPA) and CD71 were examined by flow cytometry. Data are shown from a representative experiment. **D.** Photograph of centrifuged cell pellets.

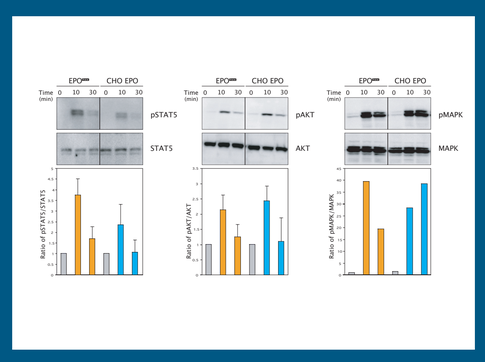

Figure 5. EPO-induced phosphorylation of STAT5, Akt and MAPK. Western blots were probed using phospho-tyrosine specific STAT5, Akt, and MAPK (upper panels). To demonstrate equal protein loading the blots were re-probed with antibodies recognising total STAT5, Akt or MAPK proteins (middle panels). The lower panels demonstrate the relative level of phosphorylated protein as a ratio of total protein from three independent experiments.

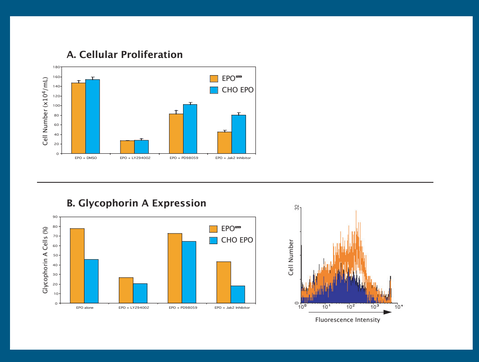

Figure 6. Inhibition of EPO-induced STAT5, Akt and MAPK signalling. A. CD34$^+$ cells were cultured with EPO^{hex} or CHO-expressed rhEPO. Chemical inhibitors were added at Day 3 and 5 of culture, and cells were counted after 7 days. **B.** Glycophorin A (GPA) was analysed by flow cytometry at Day 7. Data shown represent the percentage of GPA positive cells.

SUMMARY

- EPO^{hex}, possessing human cell specific glycosylation, promotes greater erythroid differentiation of human CD34$^+$ stem cells, compared to non-human cell expressed rhEPO.
- Removal of the human-specific sialic acid moieties from EPO^{hex} results in loss of the enhanced bioactivity.
- Amplified activation of Jak2/STAT5 signalling pathway by EPO^{hex} may be, in part, responsible for the augmentation of erythroid development.
- The enhanced activity of EPO^{hex} *in vitro* may translate to more effective *in vivo* activity, thereby improving current therapeutic applications.
- These results may also have implications for enhanced *ex vivo* CD34$^+$ stem cell manipulations for use in transplantation therapy.

References

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