Structure-function analysis of the carbohydrates of human cell expressed EPO: their role 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, due to cancer, or as a result of chemotherapy, infection or inflammation (Smith *et al*, 2001). However, rhEPO is purified from Chinese Hamster Ovary (CHO) cells, and it is becoming apparent that the biological importance of species-specific post-translational modifications, in particular glycosylation, is pivotal to protein function.

Like many other cytokines, EPO is heavily glycosylated, with up to 35% of its molecular weight consisting of N- and O-linked carbohydrate moieties. It has been proposed that glycosylation of EPO is important for secretion, solubility, resistance to proteolysis, immunogenicity, biological recognition, biological activity, *in vivo* stability and clearance (Ergie and Browne, 2001). It has also been suggested that the proportion of sialic acid containing carbohydrates affects the serum half-life and *in vivo* activity of rhEPO (Elliot *et al.*, 2004).

Our aim was to determine whether human cell expressed EPO (EPO^{hcx}) promotes erythroid development from human CD34$^+$ stem cells more effectively than other commercially available sources (expressed in CHO cells). rhEPO was expressed and purified in modified human 293 cells (EPO^{hcx}). We have shown that EPO^{hcx} promotes increased erythroid differentiation, and enables a similar degree of cellular proliferation. We have also demonstrated that removal of the sialic acid moieties from EPO^{hcx} reduces the biological activity compared to normally glycosylated EPO^{hcx}, resulting in a similar effect on erythroid development as CHO-expressed EPO.

MATERIALS AND METHODS

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

rhEPO^{hcx} was expressed in modified human 293 cells cultured in DMEM supplemented with 10% donor calf serum and 4 mM L-glutamine. Transfection was performed using calcium phosphate. Following transfection, conditioned medium was collected and subjected to dye ligand chromatography followed by cation exchange and size exclusion chromatography.

Desialylated EPO^{hcx} was obtained by the treatment of purified EPO^{hcx} with neuraminidase (Roche) at room temperature for 16 hours. The resultant desialylated EPO was purified from the enzyme and carbohydrates by size exclusion chromatography.

Bioactivity of EPO^{hcx}

The biological activity EPO^{hcx} and CHO expressed rhEPO (Peprotech) was compared by examining the effect of these cytokines on the proliferation and differentiation of cord blood CD34$^+$ stem cells. CD34$^+$ stem cells (Cambrex Bio Science Walkersville Inc.) were grown in HPGM (Cambrex Bio Science Walkersville Inc.), supplemented with EPO (50 ng/ml) and SCF (20 ng/ml; SCF^{hcx} or Peprotech) for 7 days at 37˚C. Cellular proliferation was assessed using traditional cell-counting methods, with trypan blue to exclude non-viable cells. Erythroid differentiation was assessed by examining the expression of erythroid specific proteins. Glycophorin A (BD Pharmingen) and the transferrin receptor (CD71; BioLegend) were analysed 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. All data is representative of at least three experiments.

EPO Signalling

CD34$^+$ stem cells were grown in HPGM, supplemented with 15% FCS, EPO (50 ng/ml) and SCF (20 ng/ml) for 10 days at 37˚C. The resulting erythroid precursors were starved for 4 hours, before being stimulated with EPO for the indicated times. Whole cell extracts were subject to western blotting and probed with an antibody recognising the phosphorylated form of STAT5 (Y-STAT5 Tyr694; Cell Signaling Technology Inc.), or stained with Coomassie blue to demonstrate equal protein loading.

RESULTS/DISCUSSION

Structural Analysis of EPO^{hcx}

The carbohydrate structures of EPO are important for its *in vivo* activity, and as glycosylation patterns differ between species, we were interested in comparing the carbohydrate structures of EPO^{hcx} and CHO expressed rhEPO (CellScience). Major differences in carbohydrate content were found. CHO expressed rhEPO migrates as a 30.4 kDa protein, while EPO^{hcx} migrates as a broad band between 25 to 40 kDa. Further, EPO^{hcx} consisted of a mixture of N- and O-linked glycoforms, similar to human serum EPO (Skibeli *et al*., 2001), whereas CHO expressed EPO consisted of a more homogenous oligosaccharide population (Table 1 and Table 2). These results highlight the differences between human and CHO expressed rhEPO, and led us to examine whether these structural differences affect the bioactivity of EPO^{hcx}.

EPO^{hcx} Promotes Increased Erythroid Differentiation in Human CD34$^+$ Cells Compared to CHO Derived EPO.

i) Proliferation

Our initial experiments investigated whether human cell expressed EPO affects CD34$^+$ cell proliferation. CD34$^+$ cells were grown in EPO^{hcx} or commercially available rhEPO for 7 days, and the extent of proliferation was examined. EPO from both sources supported cellular proliferation to a similar degree (Figure 1).

ii) Erythroid Differentiation

The effect of human cell expressed EPO on erythroid differentiation was also examined. CD34+ cells cultured with EPO^{hcx} expressed approximately 2-fold higher levels of the erythroid specific cell-surface markers Glycophorin A and CD71 (Figure 2A and 2B). Further, EPO^{hcx} enhanced the expression of haemoglobin α in these cells after 7 days in culture (Figure 2C). These results provide evidence that EPO^{hcx} is more biologically active, inducing greater erythroid differentiation, than current commercially available sources of rhEPO.

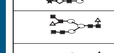
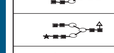
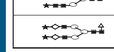
The Molecular Mechanisms Determining Enhanced Erythroid Differentiation Induced by EPO^{hcx}

i) The sialic acid moieties are important for enhanced erythroid development induced by EPO^{hcx}

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^{hcx} and it's desialylated counterpart were compared. Removal of the sialic acid residues from EPO^{hcx} resulted in a lower molecular weight protein, as expected (Figure 3A). Interestingly, CD34$^+$ cells grown in desialylated EPO^{hcx} have an increased proliferative capacity, compared to untreated EPO^{hcx} (Figure 3B). Further, desialylated EPO^{hcx} results in a 2-fold reduction in erythroid marker expression, and decreased haemoglobin expression (Figure 3C and 3D), producing similar results to CHO expressed EPO. These results suggest that the sialic acid residues are important for the enhanced bioactivity of human expressed EPO, particularly its ability to induce erythroid differentiation. This is consistent with findings by other researchers reporting that increased sialic acid content promotes increased *in vivo* activity (Elliot *et al.*, 2004).

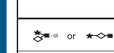
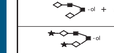
ii) EPO-induced Intraceullar Signalling Pathways

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. The activation of STAT5 was examined to determine whether the differential responses induced by human or CHO expressed EPO were a result of variations in signal transduction. Little difference in STAT5 phosphorylation was detected when cells were stimulated with EPO^{hcx} or CHO expressed rhEPO (Figure 4). As the PI3K/Akt pathway is critical for erythroid differentiation (Sivertsen *et al*, 2006), differences in the activation of this pathway are currently being examined. We are also examining changes in the expression of GATA-1 in response to EPO^{hcx} and CHO expressed EPO, as GATA-1 is a key erythroid transcription factor involved in regulating globin gene expression (Zhao *et al*., 2006).

N-linked Oligosaccharide	Percentage of Total Oligosaccharides	
	EPO^{hcx}	CHO expressed EPO
	22	-
	23	-
	22	-
	33	-
	-	100

■ HexNAc ○ Hex ★ NeuNAc △ Fuc

Table 1. Characterisation of N-linked Oligosaccharides from EPO^{hcx} and CHO expressed EPO.

O-linked Oligosaccharide	Percentage of Total Oligosaccharides	
	EPO^{hcx}	CHO expressed EPO
	-	38
	69	62
	9	-
	22	-

■-ol HexNAc aditol ○ Hex ★ NeuNAc

Table 2. Characterisation of O-linked Oligosaccharides from EPO^{hcx} and CHO expressed EPO.

Figure 1. EPO-induced Cellular Proliferation. CD34$^+$ cells were cultured with EPO^{hcx} or CHO expressed EPO for 7 days. Data indicates the mean +/- standard deviation of two independent experiments.

Figure 2. EPO-induced Erythroid Differentiation. A. Glycophorin A and B. CD71 were analysed by flow cytometry. Data shown is mean fluorescence from four independent experiments and a histogram from a representative experiment. Statistical significance was determined by a two-tailed t-test, *=p<0.05. C. Haemoglobin expression analysed by Western blotting (upper panel) and visualization of the cell pellet (lower panel).

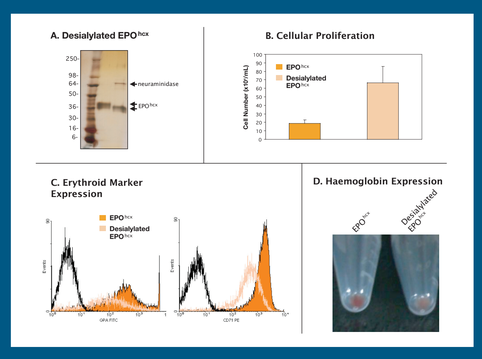


Figure 3. EPO^{hcx} Desialylation Decreases Bioactivity. A. Silver staining of EPO^{hcx} following neuraminidase treatment. CD34$^+$ data represents the mean +/- standard deviation of two independent experiments. Statistical significance was determined by a two-tailed t-test, ***=p<0.001. C. Expression of erythroid markers Glycophorin A (GPA) and CD71. D. Centrifuged cell pellets demonstrating haemoglobin expression.

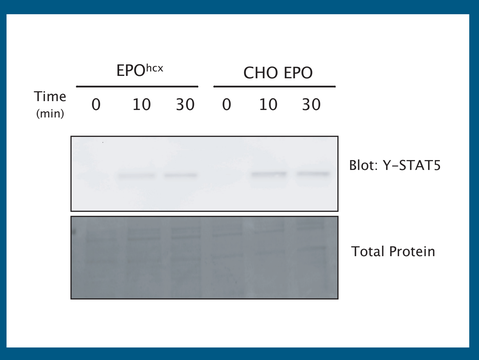


Figure 4. EPO-induced STAT5 Phosphorylation. Western blots were probed using a phospho-tyrosine specific STAT5 antibody (Y694; upper panel). To ensure equal loading, the gel was stained with Coomassie blue (lower panel).

SUMMARY

EPO^{hcx}, with human cell specific glycosylation, promotes greater erythroid differentiation from human CD34$^+$ stem cells, than non-human cell expressed EPO. Further, removal of the sialic acid moieties results in loss of the enhanced bioactivity of EPO^{hcx}. The precise molecular mechanisms responsible for this augmented effect on erythroid development are currently being investigated.

These data may have important clinical implications for the production of a human expressed rhEPO, which may be more effective in treating anaemia. Further, these results may also have implications for enhanced *ex vivo* CD34$^+$ stem cell manipulations for use in transplantation therapy.

References

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