Enhanced biological properties resulting from human cell expression of recombinant human Interleukin-4

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INTRODUCTION

Recombinant human proteins expressed in human cells differ from those expressed in non-human systems as they undergo human cell-specific post-translational modifications (PTMs), including glycosylation.

Many cytokines and growth factors are heavily glycosylated, with up to 75% of their mass consisting of carbohydrate moieties. Traditionally, human cytokines have been expressed in non-human cells, including bacteria, yeast and murine expression systems. However, the biological importance of species-specific post-translational modifications, in particular glycosylation, is increasingly being recognised as pivotal to protein function.

Distinctly different biological properties between human proteins expressed in human or non-human cells have been identified. Those differences include mis-folding, aggregation, non-glycosylation, addition of different sugar structures, and other abnormal post-translational modifications of proteins expressed in non-human cells.

Furthermore, it has been proposed that glycosylation is important for secretion, solubility, resistance to proteolysis, immunogenicity, biological recognition, biological activity, *in vivo* stability and clearance of glycoproteins including cytokines and growth factors (Okamoto *et al*, 1991).

We have purified recombinant human interleukin-4 expressed in modified human HEK 293 cells (IL-4hcx). *In vitro* comparisons of the biological activity of IL-4hcx with IL-4 expressed in other species including *E. coli*, CHO and *Pichia pastoris* revealed that glycosylated forms of IL-4 are more stable.

We have also demonstrated that non-glycosylated IL-4 produced either by treating cells with tunicamycin or removing carbohydrate moieties with PNGase F has reduced activity and stability compared to glycosylated IL-4hcx.

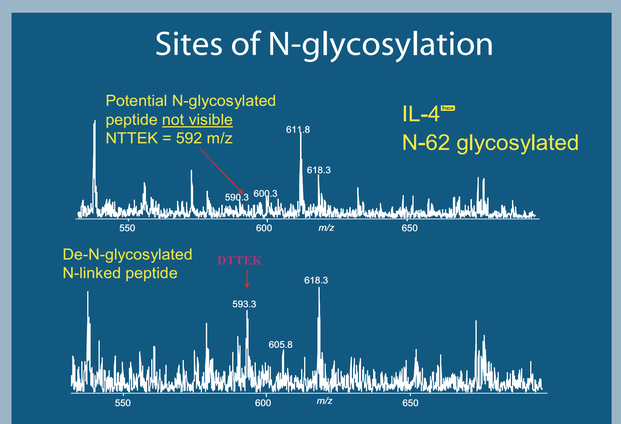

Fig 1. Mass spectrometry peak demonstrating N-glycosylation of IL-4hcx.

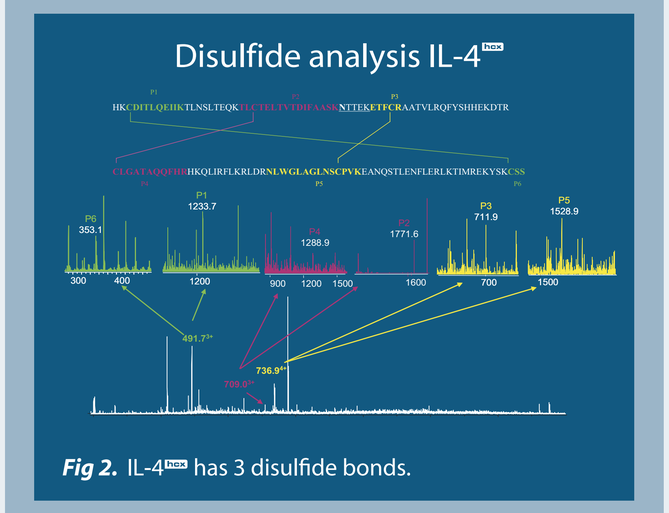

Fig 2. IL-4hcx has 3 disulfide bonds.

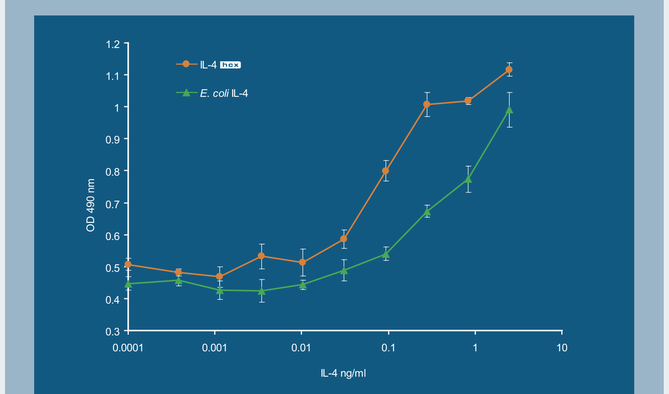

Fig 3. IL-4hcx is more stable in cell culture than *E. coli* expressed IL-4.

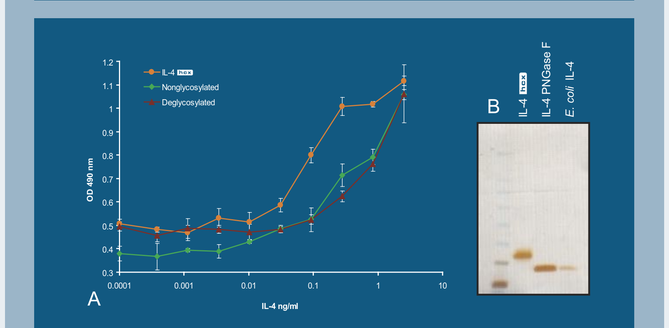

Fig 4. Removal of N-linked glycosylation from IL-4hcx reduces its stability. B. Coomassie blue staining of IL-4 glycoforms. **1**- mw markers, **2**- IL-4hcx, **3** - deglycosylated IL-4, **4** -rhIL-4 from *E. coli*.

RESULTS AND DISCUSSION

CHARACTERISATION OF IL-4hcx

i) Site of N-linked glycosylation

IL-4hcx was analysed by mass spectrophotometry with and without N-glycosidase treatment to determine the site(s) of N-linked glycosylation. Following N-glycosidase treatment, the appearance of a peak at 593 m/z indicated a site of N-linked glycosylation at N62 (Figure 1).

The presence of an N-linked glycosylation site corresponds with the higher molecular weight observed for IL-4hcx when compared to non-glycosylated IL-4 (Figure 4B).

ii) Disulfide analysis

As we were interested in the stability of IL-4hcx, the presence of disulfide bridges between cysteine residues was investigated. Human IL-4 has previously been shown to have 3 disulfide linkages between 6 cysteine residues (Tsarbopoulos *et al*, 1994).

Analysis of the non-reduced, trypsin digested IL-4hcx revealed 3 resistant peptides as indicated in Figure 2. Subsequent analysis following reduction and alkylation to destroy the disulfide bonds revealed 6 constituent peptides (3 pairs), with the mass of each pair equivalent to the corresponding individual masses of the 3 peptides detected from the non-reduced sample.

BIOACTIVITY OF IL-4hcx: COMPARISON OF IL-4 GLYCOFORMS

Our initial experiments were intended to investigate whether human cell expression enhances the biological activity of cytokines and growth factors. Comparison of IL-4hcx with commercially available rhIL-4 (expressed in *E. coli*) following 4 days pre-incubation in cell culture medium (RPMI 1640/10 % FBS) at 37°C was 7-fold more active than *E. coli* expressed rhIL-4, demonstrating it has greater stability in cell culture medium and serum (Figure 3 and Table 1).

As *E. coli* does not glycosylate proteins, this lead to the speculation that glycosylation confers increased stability on human cell expressed proteins. To examine the effect of glycosylation on IL-4 stability, non-glycosylated and de-glycosylated IL-4 (Figure 4B) were generated as described in Materials and Methods.

Activity of IL-4hcx did not change following 4 d pre-incubation, whereas the non-glycosylated forms were 5- and 11- fold less active following incubation at 37°C (Figure 4 and Table 1), indicating that glycosylation confers stability and hence enhances the biological activity of IL-4.

IL-4 from *P. pastoris* was also less stable. IL-4 from CHO, which is also glycosylated, had similar stability to IL-4hcx, again demonstrating the effect of glycosylation on stability.

IL-4 Source	Treatment	ED_{50} (ng/ml)	Fold Difference	p<0.05
IL-4hcx	none	0.13	0.61	No
	37°C	0.08		
Deglycosylated	none	0.18	11.2	Yes
	37°C	2.10		
Non-glycosylated	none	0.77	5.17	Yes
	37°C	4.01		
E. coli	none	0.22	6.99	Yes
	37°C	1.54		
P. pastoris	none	0.30	17.01	Yes
	37°C	5.08		

Table 1. *Comparison of stability of rhIL-4 from different expression sources.*

MATERIALS AND METHODS

CHARACTERISATION OF IL-4hcx

IL-4hcx was expressed in HEK293T 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 size exclusion chromatography using a Superdex 75 column (Amersham Biosciences).

The deglycosylated form of IL-4 was obtained by the treatment of purified IL-4hcx with N-glycosidase F (Roche) at 37°C for 16 hours. The resultant deglycosylated IL-4 was purified away from the enzyme and the carbohydrates by size exclusion chromatography. To obtain non-glycosylated IL-4, the glycosylation pathway was inhibited with 5 mM tunicamycin (Sigma) in the collection media. The non-glycosylated IL-4 was purified in the same manner as IL-4hcx.

N-linked glycosylation of IL-4hcx was examined by mass spectrometry. IL-4hcx, with and without N-glycosidase F treatment, was subjected to LC-MS (C18). For disulfide bond analysis, 2 µg of protein was treated with trypsin. Half of this was then subjected to LC-MS (C18) and peptide mass was analysed. The remaining protein was reduced with DTT and alkylated with iodoacetamide, followed by LC-MS analysis.

BIOACTIVITY OF IL-4hcx

Bioactivity of rhIL-4 was measured in cell proliferation assays using the human TF-1 cell line. IL-4 was pre-incubated with cell culture medium (RPMI/10 % FBS) for 4 days at 37°C before addition to the assay. Cells were subsequently incubated in 96-well tissue culture plates with serial dilutions (0-2.5 ng/ml) of the pre-incubated IL-4 for a further 3 days at 37°C, after which cell viability was measured using the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay: Promega) according to the manufacturer's instructions. Absorbance was read at 490 nm.

ED_{50} and p-values were determined from triplicate data points using the statistical software package "R" with the add-on called "drc" (Ritz and Streibig, 2005). rhIL-4 (*Pichia pastoris*) was purchased from Endogen, rhIL-4 (*E. coli*) was from R&D Systems. CHO rhIL-4 was a WHO standard. All data is presentative of at least 3 experiments.

SUMMARY

Human cell expressed IL-4, with human cell specific glycosylation, has greater biological activity and stability in cell culture than non-human cell expressed proteins.

These data have important implications for *ex vivo* procedures such as derivation of dendritic cells, where IL-4 in the culture medium must be replenished several times during the process of dendritic cell generation. As this requires up to 14 days in cell culture, the increased stability of human cell expressed proteins could facilitate fewer additions of cytokines.

Furthermore, it is likely that PTMs such as glycosylation enhance other biological properties of human cell expressed cytokines.

References **1.** Okamato *et al*. 1991. Arch. Biochem. Biophys. 286, 562-568. **2.** Tsarbopoulos *et al*, 1994. Anal. Biochem. 66, 2062. **3.** Ritz and Streibig 2005. J. Statisitical Software. 12, 1-22.