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Improved therapeutic efficacy of mammalian expressed-recombinant interferon gamma against ovarian cancer cells

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ABSTRACT

Human interferon gamma (hIFNγ) affects tumour cells and modulates immune responses, showing promise as an anti-cancer biotherapeutic. This study investigated the effect of glycosylation and expression system of recombinant hIFNγ in ovarian carcinoma cell lines, PEO1 and SKOV3. The efficacy of *E. coli*- and mammalian-expressed hIFNγ (hIFNγ-CHO and HEK293, glycosylated/de-glycosylated) on cytostasis, cell death (MTT, and Guava-ViaCount® flow-cytometry) and apoptotic signalling (Western blot of Cdk2, histone H3, procaspase-3, FADD, cleaved PARP, and caspase-3) was examined. Hydrophilic Interaction Liquid Chromatography determined the structure of *N*-linked glycans present in HEK293-expressed hIFNγ (hIFNγ-HEK). PEO1 was more sensitive to hIFNγ than SKOV3, but responses were dose-dependent and expression platform/glycosylation status-independent, whereas SKOV3 responded to mammalian-expressed hIFNγ in a dose-independent manner, only. Complex-type oligosaccharides dominated the *N*-glycosylation pattern of hIFNγ-HEK with some terminal sialylation and core fucosylation. Cleaved PARP and cleaved caspase-3 were not detected in either cell line, but FADD was expressed in SKOV3 with levels increased following treatment. In conclusion, hIFNγ did not induce apoptosis in either cell line. Mammalian- expressed hIFNγ increased cell death in the drug-resistant SKOV3. The presence of FADD in SKOV3, which may inhibit apoptosis through activation of NF-κB, could serve as a novel therapeutic target.

1. Introduction

Human interferon-gamma (hIFNγ) is a cytokine with immunomodulatory properties, vital for innate and adaptive immunity against viral/microbial infections and exhibits cytotoxic/cytostatic activity against cancer cells. Native hIFNγ is naturally synthesised by $CD4^+$ T helper cell type 1 (T_{h1}) lymphocytes, $CD8^+$ cytotoxic lymphocytes and natural killer (NK) cells [1,2].

The active form of native hIFNγ is a soluble homodimer with two *N*-glycosylation sites on asparagines (N^{25} & N^{97}) on the surface of each dimer [1–3]. The glycans at N^{25} are fucosylated and are mainly complex-type sialylated oligosaccharides with the sugar composition of *N*-acetylneuraminic acid, galactose, mannose, *N*-acetylglucosamine, and fucose. In contrast, the glycans at N^{97} are non-fucosylated hybrid high-mannose structures with a sugar composition of *N*-acetylneuraminic acid, galactose, mannose, and *N*-acetylglucosamine [2,4].

Commercial hIFNγ is bacterially derived from *Escherichia coli* (human interferon gamma-1b (hIFNγ-1b), tradename: ACTIMMUNE®), approved for clinical treatment of chronic granulomatous disease and malignant osteopetrosis, with growing prospect for cancer immunotherapy [2]. A short half-life stemming from a lack of glycosylation limits the efficacy of hIFNγ-1b [5] and production is costly due to the formation of inclusion bodies and endotoxin contamination [6]. More cost-efficient production is being explored in eukaryotic systems such as yeast (*e.g. Pichia pastoris*) [7], protozoa (*e.g. Leishmania* sp.), and mammalian cells (*e.g.* Chinese hamster

Abbreviations: **2-AB**, 2-aminobenzoic acid; **3D**, three-dimensional structure; **AU**, arbitrary unit; **BSA**, bovine serum albumin; **CHO**, Chinese hamster ovary; **hIFNγ-CHO**, CHO-expressed hIFNγ; **deglyco-hIFNγ-HEK**, deglycosylated HEK293-expressed hIFNγ; **delgyco-hIFNγ-CHO**, deglycosylated CHO-expressed hIFNγ; **FBS**, foetal bovine serum; **hIFNγ-HEK**, HEK293-expressed hIFNγ; **hIFNγ-R**, hIFNγ receptor; **HEK293**, human embryonic kidney 293; **hIFNγ-1b**, human interferon gamma-1b;; **hIFNγ**, human interferon-gamma; **HILIC**, hydrophilic interaction liquid chromatography; **IRF-1**, interferon regulatory factor-1; **IL1β**, interleukin 1 beta; **NK**, natural killer cells; **NF-κB**, nuclear factor kappa-light-chain-enhancer of activated B cells; **PNGase F**, peptide N-glycosidase F; **FADD**, Fas-Associated death domain; **PARP**, poly-ADP-ribose polymerase; **RIPK3**, receptor-interacting kinase 3; **STAT1**, signal transducer & activator of transcription-1; **TNFα**, tumour necrosis factor alpha

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ovary (CHO), human embryonic kidney 293 (HEK293), mice, rat) [2], with best results to date in mammalian expression systems (higher productivities, similarity to native hIFNγ in glycosylation and protein folding) [2]. Differences exist in the *N*-glycan structure of hIFNγ expressed in CHO; *i.e.* non-human-like sialylation potentially induces immunogenicity and affects stability [2,8].

Glycan residues provide protease resistance, and the type of glycan affects half-life (*i.e.* high mannose-type hIFNγ expressed in insect cells had a shorter half-life) and pharmacokinetics of hIFNγ [3]. Detailed knowledge of the effect of glycosylation on the therapeutic efficacy of hIFNγ is limited, although glycosylation has been proven to be essential for the therapeutic efficacy of protein drugs [9]. This study explored the efficacy of glycosylation status of recombinant hIFNγ from three different expression systems (*E. coli,* CHO and HEK293) against the ovarian cancer cell lines, PEO1 and SKOV3, to evaluate whether or not mammalian expressed recombinant hIFNγ can be a superior substitute for the prokaryotic product.

Ovarian cancer is the 5th deadliest cancer in women and treatment of ovarian cancer with hIFNγ-1b underwent phase I, II & III clinical trials with mixed results. Some of the identified obstacles were tumour insensitivity to hIFNγ and inability to deliver hIFNγ locally [10–12]. In contrast, preclinical *in vitro* & *in vivo* studies on ovarian cancer cell lines all showed a degree of sensitivity to hIFNγ-1b except for SKOV3 (Table 1).

Anti-cancer efficacy of hIFNγ in ovarian cancer cells was shown to be due to cytostasis, through activation of p53 and p21 leading to cell cycle arrest (Fig. 1) and/or cytotoxicity, causing cell death. The latter was subdivided into pyroptosis through activation of interferon regulatory factor-1 (IRF-1) and caspase-1, and intrinsic apoptotic signalling *via* activation of IRF-1, caspase-8, cytochrome-c release from mitochondria, the caspase cascade and inhibition of poly-ADP-ribose polymerase (PARP) (Fig. 1; Table 1). Treatment of OVCAR3 with hIFNγ-1b led to both, apoptosis and cytostasis. Prior work on PEO1 also showed the possible engagement of both, cytostasis and apoptosis [13,14], which had been suggested based on signal transducer & activator of transcription-1 (STAT1) activation and p53 expression [15]. Therefore, this research aimed to investigate other signalling molecules (cleaved PARP and caspase-3) to provide more conclusive evidence of hIFNγ-induced apoptosis. In contrast, high levels of phosphorylated Fas-Associated Death Domain (FADD), a regulator of cell cycle progression, proliferation, tumorigenesis and necroptosis [16], increased activation of the anti-apoptotic transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a biomarker for aggressive phenotypes in lymphomas, lung and colorectal carcinomas (Fig. 1) [17,18]. hIFNγ, through induction of the Fas pathway and export of FasL [19], can activate FADD (Fig. 1), which may explain h-IFNγ resistance in cancer cell lines like SKOV3. Therefore FADD levels following treatment with hIFNγ were also investigated here in PEO1 and SKOV3.

2. Material & methods

2.1. Ovarian carcinoma cell lines & cultivation

Two ovarian carcinoma cell lines, PEO1 (passage № ≥ 40) (Catalogue № 10032308) and SKOV3 (passage № ≥ 20) (Catalogue № 91091004), were purchased from the European Collection of Authenticated Cell Cultures, UK. Cell lines were maintained at 37 °C in a humidified growth chamber in 5% CO_2 in RPMI-1640 medium supplemented with L-glutamine and sodium bicarbonate (Sigma, R8758, Castle Hill, NSW 1765, Australia) and addition of 10% foetal bovine serum (FBS) (Sigma, F4135; USA origin); penicillins (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}). Cells were subcultured when confluent. For the passage, cells were detached through addition of Gibco® Trypsin-EDTA (0.25%) solution (ThermoFisher Scientific, Newstead, QLD 4006, Australia). Subsequently, trypsin was deactivated by addition of FBS-containing complete medium. Subcultures were seeded with 50 $\times 10^3$ cells per vented T-25 flask (25 cm^2, Orange Scientific, Sydney, NSW, Australia). Culture medium was changed every three days.

2.2. Recombinant hIFNγ

Products from three different protein expression platforms were purchased; hIFNγ-1b from Sigma (SRP3058), CHO-expressed (hIFNγ-CHO) from SinoBiologicals (11725-HNAS, Beijing, China) (Purity: > 92%) and HEK293-expressed (hIFNγ-HEK) from Acrobiosystems (IFG-H4211, Newark, DE 19711, USA). All recombinant hIFNγ products were provided as a lyophilized powder from PBS (pH 7.4) with a purity > 92%, and were tested for endotoxin content (< 0.1 EU μg^{-1}).

Table 1
Summary of preclinical treatments of ovarian cancer cell lines with hIFNγ-1b.

Cell Line	Mechanism of Action	Signalling Molecules Detected	Ref.
2774	Apoptosis	Nitric oxide[a]	[40]
	Pyroptosis	Induction of IRF-1 & activation of caspase-1	[41]
HOC7	Apoptosis	Nitric oxide[a]	[40]
OAW42	Apoptosis	PARP inhibition	[14]
OVCAR3	Apoptosis	Nitric oxide[a]	[40]
	Less sensitive	Induction of IRF-1	[41]
	Apoptosis	PARP inhibition	[14]
	Cytostasis/Apoptosis	Cell cycle arrest; G_1, G_2, and S phases; Induction of p53, Bax, and caspase-3[b]	[42]
	Cytostasis	Increasing p21 mRNA	[15]
OVCAR4, OVCAR5	Cytostasis/Apoptosis	PARP inhibition	[14]
PA1	Pyroptosis	Induction of IRF-1 & activation of caspase-1	[41]
PEO1	Apoptosis/Cytostasis	Depolarization of mitochondrial membrane, release of cytochrome C & activation of caspase-9	[13]
		Induction of caspase-8 & 9	[14]
	Cytostasis	Increasing STAT1 & p21 mRNA	[15]
PEO14, PEO16	Cytostasis/Apoptosis	PARP inhibition	[15]
SKOV3	Insensitive	Initial expression of p21, IRF-1 mRNA detected. However, later the pattern of expression was reduced to the level of untreated cells.	[14,15,41]
SW626	Cytostasis/Apoptosis	PARP inhibition	[14]

[a] Polytherapy of hIFNγ, interleukin 1 beta (IL1β), and tumour necrosis factor alpha (TNFα).
[b] Polytherapy of hIFNγ plus TNFα.