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Research paper

Hydrophobic cavity in C-terminus is essential for hTNF-α trimer conformation

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ABSTRACT

A variety of tumour necrosis factor α (TNF-α) derivatives have been bioengineered to improve antitumour activity and reduce toxicity. The expression of TNF-α in *Escherichia coli* usually yields a mixture of homotrimers and monomers; however, only the trimer shows antitumour activity. TNF-αD10, a bioengineered hTNF-α derivative, demonstrated 10-fold higher cytotoxicity against tumour cells compared to hTNF-α, but the trimer to monomer ratio was 58:42. In the present study, we investigated the structural differences between the trimer and the monomer of TNF-αD10. We found that the chemical shifts of the C-terminal Trp^{114} in the trimer were significantly different from those in the monomer and that the replacement of Trp^{114} with different amino acids remarkably reduced the trimer production. Further analysis of the publicly available X-ray crystallographic data for trimeric and monomeric hTNF-α revealed that the conformation of the U-shaped region formed by the fragment Cys^{101}–Trp^{114} was different between the two forms: a hydrophilic cavity in the monomer and a hydrophobic cavity in the trimer. These findings suggested the potential approaches of molecular and structural modification for future improvement of hTNF-α trimer production.

1. Introduction

Tumour necrosis factor alpha (TNF-α) is a pleiotropic cytokine that plays a critical role in antitumour activity, immune modulation and proinflammatory mediation. This protein exerts a direct cytotoxic effect on a wide range of human and murine tumour cell lines both *in vivo* and *in vitro*. To date, the clinical use of exogenous TNF-α as a therapeutic strategy has been limited due to its side effects, including fever, dose-related hypotension, and hepatotoxicity [1]. To improve the antitumour activity and reduce systemic toxicity, various types of TNF-α derivatives have been designed and prepared by protein engineering techniques [2]. It has been reported that the antitumour active form of TNF-α is a compact, non-covalently linked homotrimer [3,4], and native TNF-α exists as a trimer *in vivo* [5]. However, TNF-α expressed in *Escherichia coli* is usually a mixture of trimers and monomers [6]. The monomer does not have antitumour activity but, instead, displays other properties, such as trypanolytic and sodium uptake activating effects [7,8]. Therefore, increasing the expression of the trimeric TNF-α and addressing the causes of the formation of the monomeric by-product have become areas of great pharmaceutical interest.

TNF-αD10, a hTNF-α derivative in which the N-terminal amino acids Ser^4–Ser^5–Asp^{10} and the C-terminal amino acid Leu^{157} have been replaced by Cys–Thr–Arg and Phe, respectively, has 10-fold higher cytotoxic activity against mouse L929 cells than hTNF-α [9]. Like the other derivatives, TNF-αD10 expressed in *E. coli* is also a mixture of the trimer and the monomer in the ratio of 58:42. Through an *in vitro* denaturation–renaturation cycle, we have found that the monomeric TNF-αD10 cannot be converted into its trimeric form. To address the reason for monomer production, we first speculated that the monomer might result from misfolding features that induce a different conformation than the native trimer form. There are two classes of protein folding catalysts in *E. coli*: (1) disulphide bond reductases and isomerases (Dsb proteins) that catalyse the formation and rearrangement of disulphide bonds and (2) peptidyl–prolyl *cis–trans* isomerases (PPIases) that catalyse the *cis–trans* isomerisation of peptidyl–prolyl bonds [10,11]. It is known that the disulphide bridge between Cys^{69} and Cys^{101} in TNF-α is required for the maintenance of its β lamellar structure but not for its trimeric conformation [12]. However, the C-terminus of TNF-α, which contains six proline residues, has been shown to be important for the maintenance of the trimeric conformation [13]. With these facts in mind, we further speculated that the *cis–trans* isomerisation of peptidyl–prolyl bonds catalysed by PPIases might be involved in the formation of the TNF-α trimer.

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In the present study, we utilised NMR (nuclear magnetic resonance) to investigate the structural differences between the monomer and the trimer of TNF-αD10 due to its ability to provide structural information at atomic resolution under near-physiological conditions [14]. Two sets of proline-containing peptides were obtained from the trimer and the monomer under the same hydrolysis conditions, and their NMR spectra were compared. In addition, using publicly available X-ray crystallography data for wild-type hTNF-α (1TNF) [3], a monomeric hTNF-α mutant (4TSV) [15], and two trimeric hTNF-α mutants (5TSW) [15] and (1A8M) [16], we conducted a structural analysis using the program SPDBV. To further determine the importance of the core conformation in the formation of the trimeric TNF-αD10, we also performed a mutagenesis study.

2. Materials and methods

2.1. Materials

The plasmid pJLA503 from our laboratory was used to express the TNF-αD10 gene. *E. coli* JM103 was purchased from the Institute of Microbiology of the Chinese Academy of Sciences. IBA (2-iodosobenzoic acid), NTCB (2-nitro-5-thiocyanatobenzoic acid), actinomycin D, trypsin and MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma (St. Louis, MO, USA). Chromatography-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Sephadex G-25, DEAE Sepharose CL-6B and Superdex 75 were obtained from Amersham Biosciences (Sweden).

L929 cells (ATCC CCL-929) were obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), non-essential amino acid solution, and penicillin-streptomycin solution were purchased from Gibco Laboratories (Invitrogen Co, Grand Island, NY, USA). Heat-inactivated foetal calf serum (FCS) was purchased from Shanghai Al-Amin Bio-tech Co., Ltd. (Shanghai, China).

2.2. Construction of pJLA503-TNF-αD10

TNF-αD10 cDNA was obtained by the site-directed mutagenesis of the parental hTNF-α using the following primers: sense (5′-CGCCATATGGTCAGATCATGTACTCGAACCC-3′; NdeI recognition site underlined) and antisense (5′-CCGGAATTCTCAGAAGGCAATGATCCCAAAGTA-3′; EcoRI recognition site underlined). The N-terminal amino acids Ser^4—Ser^5—Asp^{10} and the C-terminal amino acid Leu^{157} were replaced with Cys^4—Thr^5—Arg^{10} and Phe^{157}. The TNF-αD10 cDNA was then inserted by ligation into the pJLA503 expression vector between the NdeI and EcoRI sites. After the transformation into JM103 cells, the positive clone was selected and confirmed by sequencing.

2.3. Expression and purification of TNF-αD10

A single colony of the JM103 cells containing pJLA503-TNF-αD10 was inoculated into 2xYT medium and then incubated at 30 °C for 2 h and at 42 °C for additional 6 h. After centrifugation at 5000 × g for 20 min at 4 °C, the cells were resuspended in 10 ml of TBS buffer (0.2 M NaCl, 0.1 M Tris, 0.04 M EDTA, pH 7.5) and disrupted by ultrasonication. After centrifugation at 10,000 rpm for 20 min at 4 °C, the supernatant was heat-treated at 60 °C for 30 min prior to a second centrifugation under the same conditions. The supernatant was then submitted to ammonium sulphate fractional precipitation. After being dissolved in Tris–HCl buffer (20 mM, pH 8.0), the crude proteins were desalted on a Sephadex G-25 column and purified on a DEAE Sepharose CL-6B ion-exchange column using elution Tris–HCl buffer (20 mM, pH 8.0) containing sodium chloride (45 mM).

2.4. SDS-PAGE and western blotting analysis

The protein fractions containing TNF-αD10 were denatured in Laemmli buffer and examined using SDS-PAGE (20%), which was stained with Coomassie Brilliant Blue G250 (Amersham Biosciences). The target protein, TNF-αD10, was identified by western blotting using TNF-α-specific antibodies (Sigma).

2.5. Cytotoxicity measurement

The cytotoxic activities of the monomeric and trimeric TNF-αD10 were measured with actinomycin D-treated murine L929 cells according to the literature [17]. Briefly, L929 cells were seeded at 1×10^4 cells/well into a 96-well microtiter plate in DMEM containing 2% foetal calf serum. Eighteen hours later, medium containing 1 μg/ml actinomycin D and various concentrations of TNF-αD10 was added to the cell culture. The cells were grown for an additional 18 h at 37 °C prior to adding 10 μl of MTT (5 mg/ml) to the culture. After 6 h of incubation at 37 °C, the supernatants of the culture were removed and replaced with 100 μl of DMSO. After the MTT formazan was completely dissolved, the absorbance (A) was measured using an automated microplate reader at 490 nm. The cytotoxic activities were calculated as IC_{50}s (50% cell viability concentration).

2.6. Separation of the trimer and the monomer

The monomeric and trimeric TNF-αD10 forms were initially purified by ion-exchange chromatography (DEAE Sepharose CL-6B) and then by a 25 ml Superdex 75 FPLC column (Pharmacia) using the Biologic Duoflow™ chromatography system (BioRad). In sum, 200 μl of the sample was loaded onto a Superdex 75 column, and the proteins were eluted with PBS buffer and lyophilised. The molecular weight of the trimer or the monomer was calculated using standard protein weights. The standard proteins for molecular weight calibration were cytochrome C (MW 12500), BSA (MW 67000), *Bacillus subtilis* enzyme (MW 1450), amino acetic acid–amino acetic acid-tyrosine-arginine (MW 451) and amino acetic acid–amino acetic acid–amino acetic acid (MW 189). A linear relationship existed between the molecular weight and retention time. The regression equation was log MW = 7.34–0.24 T, $r = 0.9938$, $P < 0.01$ (MW: molecular weight, *T*: retention time).

2.7. Hydrolysis of the trimer and monomer

Initially, both the monomeric and trimeric TNF-αD10 were hydrolysed with IBA (2-iodosobenzoic acid) and NTCB (2-nitro-5-thiocyanatobenzoic acid) into five peptides: T1, T2, T3, T4 and T5. The T3 peptide was then hydrolysed with trypsin to give the K1, K2 and K3 peptides, and the T5 peptide was hydrolysed with formic acid to give the K4, K5 and K6 peptides [18–21]. In sum, a protein sample was dissolved in reaction buffer (200 mM Tris, 1 mM EDTA, pH 8.0) containing 1 mM DTT to give a final sample concentration of 5 pmol/μl, and the mixture was equilibrated at room temperature for 2 h prior to centrifugation. NTCB was then added to the supernatant to a final concentration of 20 μg/ml. The reaction was carried out in a closed vessel at 37 °C for 20 min, and the product was isolated by a protein gel tube and then lyophilised. The lyophilised powder was dissolved in water to a concentration of 5 pmol/μl, and IBA was added to give a final concentration of 10 μg/ml. The reaction mixture was kept in a closed vessel at 47 °C for