Volume 314, number 1, 85–88 FEBS 11854 December 1992

Natural human tumor necrosis factor beta (lymphotoxin)

Variable *O*-glycosylation at Thr^7, proteolytic processing, and allelic variation

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Received 5 October 1992

Natural human tumor necrosis factor beta (TNF-β) purified from supernatants of a human B-lymphoblastoid cell line was found to be heterogeneous in molecular mass, with seven components resolved by gel electrophoresis. All components are *N*-glycosylated at Asn^{62}; *N*-glycosylation does not contribute to heterogeneity. In addition, part of the molecules are *O*-glycosylated at Thr^7; *O*-glycosylation is heterogeneous due to variable decoration with neuraminic acid. The four lower molecular mass forms are derived from the full-length protein by trypsin-like proteolytic cleavage in the N-proximal region; these clipped molecules lack *O*-linked carbohydrates. Two allelic variants differing in amino acid position 26 (threonine/asparagine) were identified.

Tumor necrosis factor beta; Lymphotoxin; Glycosylation; Cytokine

1. INTRODUCTION

Tumor necrosis factor beta (TNF-β), or lymphotoxin, is a cytokine produced by activated lymphocytes and some B- and T-lymphoid cell lines [1–4]. Human TNF-β is encoded by a gene located on chromosome 6 adjacent to the gene for TNF-α. The proteins show 29% identity in their amino acid sequences; TNF-β is longer by 17 amino acids at the N-terminal end [5]. Both TNFs bind to the same cell surface receptors and show similar biological activities, including cytotoxic or cytostatic effects on tumor cells, antiviral activities and a wide range of immunomodulatory effects (reviewed in [6,7]).

TNF-β is a homotrimeric protein; the subunit consists of 171 amino acids with a calculated molecular mass of 18,664 Da and has a single potential *N*-glycosylation site [8,10]. Earlier attempts to purify natural human TNF-β from supernatants of lymphoid cell lines have resulted in widely varying estimates of molecular masses between 12 and >200 kDa [9]. Analysis by SDS-PAGE of highly purified preparations also gave inconsistent results, with molecular masses varying between 20 and 70 kDa [2,10,11]. In several studies, heterogeneous preparations were obtained, with up to seven bands with apparent masses between 19 and 80 kDa [12]. Various groups, using radiolabelled preparations, provided evidence that TNF-β is *N*-glycosylated and possibly also *O*-glycosylated [13–15]. The heterogeneity of some preparations thus may be due to post-translational modification. To clarify this situation and to identify the *O*-glycosylation site(s), we isolated natural TNF-β produced by the human B-lymphoblastoid cell line, RPMI-1788, and characterized the protein using HPLC methods, SDS-PAGE, amino acid sequence analysis and deglycosylating enzymes.

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2. MATERIALS AND METHODS

2.1. *Production and purification of natural TNF-β*

TNF-β was purified from supernatants of the human B-lymphoblastoid cell line, RPMI 1788, stimulated with mezerein by serial chromatography on controlled pore glass, Mono Q anion-exchange and concanavalin A columns, as described previously [16]. The resulting material was homogeneous as judged by gel permeation HPLC and reverse-phase HPLC. Recombinant, *E.coli*-derived TNF-β was kindly provided by G. Bodo and by Genentech Inc., San Francisco.

2.2. *SDS-PAGE, HPLC techniques, amino acid sequence analysis and peptide mapping*

Protein samples were electrophoresed in the presence of 0.1% SDS on 15% acrylamide gels according to Laemmli [17]. Reverse-phase HPLC was performed on a Bakerbond WP C18 column (4.6 × 250 mm, particle size 5 μm, pore diameter 300 Å) at 30°C, using the following solvents: solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetronitrile. The following program was used: 0–2 min, 20% B; 2–26 min, 20–68% B (linear gradient); 26–36 min, 68% B (flow rate 1 ml/min). Proteins were detected by their absorption at 214 and 280 nm. Gel permeation chromatography was performed on two Waters Protein-Pak I 125 connected in series (7.8 × 300 mm each, particle size 10 μm), using 0.5 M Na_2SO_4, 0.02 M NaH_2PO_4, pH 7.0, 0.04% Tween 20 in 25% propylene glycol (flow rate 0.5 ml/min). Proteins were detected by their absorption at 214 nm.

For N-terminal amino acid sequencing, proteins were dissolved in 70% formic acid and directly applied to the cartridge of an Applied Biosystems model 477A pulsed liquid-phase sequencer. The sequencer cycles FIL-1, BEGIN-1 and NORMAL-1 were used as suggested by the manufacturer. The phenylthiohydantoin derivatives of the amino

acids from each cycle of the Edman degradation were analyzed on-line by reverse-phase HPLC, using an Applied Biosystems model 120A instrument. The HPLC conditions were chosen according to the recommendations of the manufacturer. Amino acid derivatives were detected by UV absorption at 269 nm. Retention times of the samples were compared to a set of standards.

Peptide maps were generated by treatment of reverse-phase HPLC-purified protein with trypsin (sequencing grade, Boehringer-Mannheim). 100 μg of TNF-β were dissolved in 100 μl 1% NH_4HCO_3, pH 8.5, and 5 μl trypsin (200 μg/ml in 0.01% trifluoroacetic acid) were added. After incubation for 6 h at 37°C additional 5 μl of trypsin were added, and digestion continued for another 18 h at 37°C. Peptides were applied to a Merck LiChroCART 125-4 HPLC Cartridge Superspher 100 RP-18 end-capped column (4 ×119 mm, particle size 4 μm, pore diameter 100 Å). Chromatography was performed at 30°C using the solvents as described above; parameters: 0–70% B (linear gradient), 0–70 min, 1 ml/min. Peptides were detected by their absorption at 214 and 280 nm. The resulting maps were compared with a map of recombinant *E. coli*-derived TNF-β prepared similarly. Peptides derived from natural TNF-β having no counterpart in the map of the recombinant material were sequenced.

2.3. *Deglycosylation*

Deglycosylation experiments were performed with reverse-phase HPLC-purified TNF-β. The protein was dissolved in 50 mM CH_3COONa, pH 5.5, 4 mM $CaCl_2$ and treated with *Vibrio cholerae* neuraminidase (Boehringer-Mannheim; 50 mU/ml, 18 h at 37°C). Additional treatment with *O*-glycosidase from *Streptococcus pneumoniae* (Oxford GlycoSystems; 29 mU/ml, 18 h at 37°C) was performed in 50 mM Na_2HPO_4, 50 mM citric acid, pH 6.0, 0.1% NaN_3). *N*-Linked oligosaccharides were cleaved by incubation with *N*-glycosidase F from *Flavobacterium meningosepticum* (Boehringer-Mannheim; 140 U/ml, 18 h at 37°C) in 100 mM sodium phosphate, pH 8.0, 10 mM EDTA, 0.2% Triton-X-100, 0.1% SDS, 10 mM dithiothreitol followed by reverse-phase HPLC as described above.

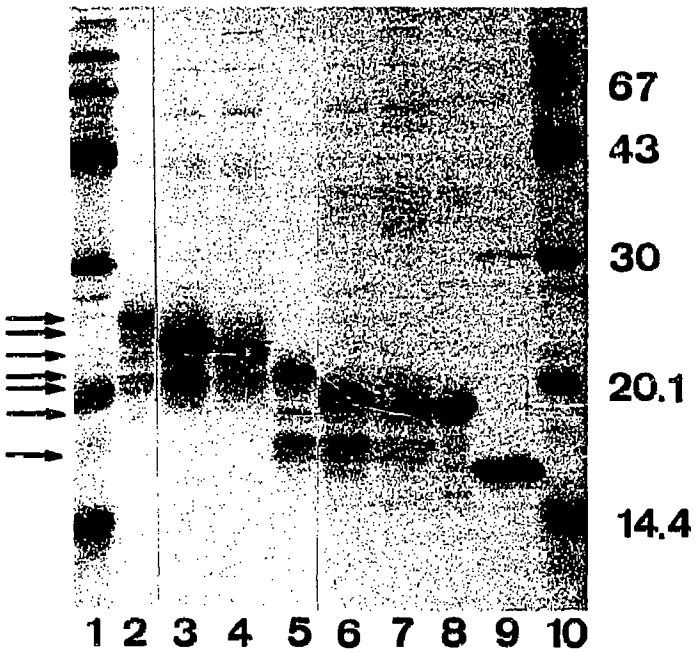

Fig. 1. SDS-PAGE analysis of purified human TNF-β. Samples were separated on a 15% gel. Lanes 1 and 10, marker proteins (mass scale in kDa); lane 2, natural TNF-β (nTNF-β); lane 3, nTNF-β treated with neuraminidase; lane 4, nTNF-β treated with neuraminidase and *O*-glycosidase; lane 5, nTNF-β treated with *N*-glycosidase F; lane 6, nTNF-β treated with *N*-glycosidase F and neuraminidase; lane 7, nTNF-β treated with *N*-glycosidase F, neuraminidase and *O*-glycosidase; lane 8, recombinant (*E. coli*-derived) TNF-β, full length; lane 9, recombinant TNF-β, amino acids 24–171. Arrows point to the seven bands in the original preparation (lane 2).

3. RESULTS AND DISCUSSION

3.1. *Polypeptide structure of natural lymphotoxin*

Natural TNF-β was purified from supernatants of mezerein-stimulated B-lymphoblastoid cells (cell line RPMI 1788) by a series of chromatographic steps as described [16]. The resulting material was homogeneous as judged by gel permeation HPLC (apparent molecular mass, 80 kDa) and by reverse-phase HPLC (data not shown). SDS-PAGE, however, revealed heterogeneity of the preparation, with five major bands with apparent molecular masses of 20.4, 21.1, 22.5, 24.3 and 25.2 kDa, and two additional, faint bands at 18.6 and 19.8 kDa (Fig. 1). The intensity of the four lower molecular mass bands varied in different preparations; moreover, we observed that their intensity increased upon prolonged storage at −20°C, indicating that these bands may represent proteolytic cleavage products.

N-terminal sequence analysis (40 cycles) showed a single major component (about 85–90% of the total protein) corresponding to the expected sequence of the full-length protein. Two further components were also identified, representing proteolytic cleavage products lacking the N-terminal 15 and 19 amino acids, respectively. The cleavage sites correspond to proteolysis by a trypsin-like enzyme (Fig. 2); the shortened proteins most probably correspond to the protein bands with 20.4 and 21.1 kDa. The two minor components with 18.6 and 19.8 kDa were not detectable by this analysis; they probably correspond to fragments lacking 28 and 39 amino acids from the N-terminus (Fig. 2). Note that a preparation of recombinant (*E. coli*-derived, unglycosylated) TNF-β also contains proteolytic cleavage products (Fig. 1, lane 8).

At position 26 of the full-length protein, two amino acids (threonine and asparagine) were identified. Further, sequence analysis of tryptic fragments resolved by reverse-phase HPLC identified two peptides with either threonine or asparagine in position 26, indicating that RPMI 1788 cells express two allelic forms of the TNF-β gene. Indeed, analysis of cDNA clones from human cell lines [18,19] and genomic DNA clones [20] has previously identified sequences coding for both allelic variants, differing in a single base (C/A).

3.2. N- *and* O-*glycosylation*

Analysis of natural TNF-β by SDS-PAGE after cleavage of asparagine-linked carbohydrate residues with *N*-glycosidase F resulted in an increase in electrophoretic mobility of all bands, corresponding to a reduction in molecular mass by about 4 kDa. The overall pattern, however, was unchanged, indicating that the

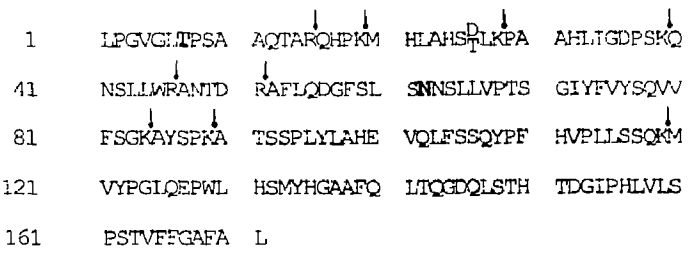

Fig. 2. Amino acid sequence of human TNF-β. Tryptic cleavage sites following amino acids 15, 19, 28, 39, 46, 51, 84, 89 and 119, are indicated by arrows. Sites of *O*-glycosylation (Thr^7) and *N*-glycosylation (Asn^{62}) are bold-faced. In position 26, two amino acids were identified (asparagine, threonine).

N-linked carbohydrate moiety does not contribute to the heterogeneity (Fig. 1, lanes 2 and 5). Sequence analysis of tryptic peptides identified Asn^{62} as the *N*-glycosylation site, as expected from the cDNA sequence (data not shown).

In contrast, treatment of the preparation with neuraminidase affected the mobility of the two upper bands only, reducing their mass to result in a single new band with 23 kDa (Fig. 1, lanes 2 and 3); additional treatment with *O*-glycosidase resulted in further reduction to about 22.5 kDa (lane 4), equivalent to the 22.5 kDa band in the original preparation (lane 2) which was unaffected by *O*-glycosidase treatment. This band thus most likely corresponds to an *N*-glycosylated, but not *O*-glycosylated protein. Equivalent changes were seen in a preparation that had previously been treated with *N*-glycosidase F (Fig. 1, lanes 5–7). The four low molecular mass bands of the original preparation corresponding to N-terminally cleaved proteins were unaffected by neuraminidase/*O*-glycosidase treatment, indicating that the *O*-glycosylation site(s) are located within the 15 N-terminal amino acids of the molecule. Indeed, careful re-examination of the sequence analysis data revealed that the cycle yield of Thr^7 was much lower than expected, indicating that this amino acid is modified in part of the molecules (data not shown). Further, sequence analysis of tryptic fragments identified both a peptide with modified (and therefore undetectable) and with unmodified Thr^7. Evidence for further *O*-glycosylation sites was not obtained.

Taken together, our results explain the heterogeneity of our TNF-β preparation as follows.

(i) Natural human TNF-β produced by B-lymphoid cells is *N*-glycosylated at Asn^{62}. *N*-Glycosylation does not contribute to the observed heterogeneity in molecular mass.

(ii) The majority of full-length molecules is *O*-glycosylated at Thr^7 (SDS-PAGE bands with masses of 24.3 and 25.2 kDa); a minor proportion of protein lacking *O*-glycosylation can be detected by SDS-PAGE (22.5 kDa) and sequence analysis. *O*-Glycosylation is heterogeneous due to variable decoration of the core carbohydrate by neuraminic acid (probably one and two residues, respectively).

(iii) Additional heterogeneity of the protein is caused by trypsin-like proteolytic cleavage that removes peptides from the N-proximal region of the molecules (SDS-PAGE bands with masses between 18.6 and 21.1 kDa). Although proteolysis may be an artefact of the purification procedure, these cleavage sites are obviously accessible for the protease and may also be recognized in vivo. TNF-β lacking the 23 N-terminal amino acids was previously isolated from RPMI 1788 cells [10]; this type of cleavage, however, cannot be due to a trypsin-like protease.

Recent work has elucidated the closely related three-dimensional structures of both TNF-α and TNF-β [8]. In both molecules, the polypeptide chains fold to give a compact, bell-shaped trimer composed of β-pleated sheets. The N-terminal ends of the proteins protrude from the compact core (10 amino acids in TNF-α, 27 in TNF-β); they apparently do not form well-defined structures. It is thus not surprising that the long N-terminal end of TNF-β is easily accessible to proteolytic enzymes, whereas the compact core is highly resistant.

(iv) Our TNF-β preparation does not contain detectable components with a molecular mass higher than 25 kDa. However, bands that most likely correspond to dimers are detectable in samples that were incubated for several hours at 37°C during the deglycosylation reactions (Fig. 1, lanes 3,4,6,7). TNF-β components with high molecular weight observed by others working with iodinated protein [12] thus most likely also represent artefacts of the preparation.

To explain the heterogeneity of TNF-β isolated from human lymphoid cells, Rubin et al. [12] have postulated that these proteins are encoded by more than one gene. However, molecular cloning has so far identified only a single TNF-β gene in the genomes of humans and other mammalian species. The results presented here indicate that post-translational modifications of a single protein, including variable glycosylation, proteolysis, and aggregation, can fully account for the heterogeneity of natural TNF-β preparations.

Acknowledgements: We thank D. Jobstmann and J. Hoffmann for excellent technical assistance.

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