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Determination of binding site of anti-tumour necrosis factor-α monoclonal antibody using hybrid and mutant proteins

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Abstract In order to map the immunogenic epitope for the monoclonal antibody E7H2 on the human tumour necrosis factor (hTNF-α) molecule, a number of chimeric proteins were developed by in-frame joining segments of the human genes encoding TNF-α and lymphotoxin (TNF-β) as well as by coupling appropriate coding regions for human and mouse TNF-α. High level expression of these chimeric genes was achieved in *Escherichia coli* by placing the coding sequences under control of either *E. coli trp*-promoter or a tandem of bacteriophage T7 constitutive promoters A2 and A3. As revealed by Western blot analysis with monoclonal antibody E7H2 directed against human TNF-α, the region involved in the binding of this antibody includes sequence ValGluLeuArg in the N-terminal part of the TNF-α molecule.

Key words: Tumour necrosis factor (TNF-α); Lymphotoxin (TNF-β); Hybrid proteins; Expression in *E. coli*; Monoclonal antibody; Immunoblotting

1. Introduction

The molecular basis of the exquisite specificity which is characteristic for molecular recognition between certain types of biomolecules still remains an important issue despite many studies in this field. The advent of gene cloning and engineering of vectors for efficient expression made many recombinant proteins of biological interest available, followed by a breakthrough in the structural and functional analysis of bioactive proteins and cell receptors, including facilitated X-ray crystallographic studies of three-dimensional structure of these proteins [1,2]. Monoclonal antibodies are essential tools for these analyses. The functional regions of the protein of interest can be identified by mapping the epitopes recognized by antibodies which are capable of modulating biological activities and, in particular, interact with the receptor protein and inhibit binding to the specific ligand [3].

Previously, mapping of antigenic determinants relied upon the analysis of panels of evolutionarily related proteins. Alternatively, epitopes were mapped using chemical modification or protection of particular amino acid residues [4] or fragments purified by high-performance liquid chromatography after chemical or proteolytic cleavage of the entire antigen molecule [5,6].

Tumour necrosis factor (TNF-α), a pleiotropic cytokine produced mainly by activated monocytes-macrophages, was initially characterized as a protein inducing the haemorrhagic necrosis of certain transplanted tumours in mice [7]. To date, TNF-α has been proved to be a key mediator of inflammation during infectious diseases and exerts strong system effects including the septic shock [8]. In an attempt to gain more insight into the functional topography of the TNF-α molecule, we initiated an epitope analysis using monoclonal antibodies (Mabs) E7H2 [9] which are specific for human recombinant TNF-α [10]. Here, we report the construction of chimeric and mutant TNF molecules and their use for defining the epitope recognized by Mabs E7H2.

2. Materials and methods

2.1. Plasmids and strains

The construction of a semisynthetic gene for human TNF-α (plasmid pTNF331) adapted for bacterial expression has been reported [11]; plasmid pLT21 coding for shortened human lymphotoxin (TNF-β) [12], plasmid pTNF336 [13] with a mutated gene for TNF-α were previously described. Plasmid pmTNF harboring a gene for murine TNF-α under the control of a *trp*-promoter was kindly provided by Drs. V. Kravchenko and S. Nedospasov. *E. coli* strain SG20050 [14] was used for expression of the hybrid and mutant genes.

2.2. Construction of plasmids

General genetic engineering techniques were essentially performed as described in [15]. Enzymes were purchased from Fermentas (Lithuania).

Plasmid pTNF331 was cleaved simultaneously with *Msp*I and *Hin*dIII, and a 504 bp fragment of the human TNF-α gene lacking 32 N-terminal codons was purified by electrophoresis in 8% PAG and then ligated with a *Hin*dIII-*Xho*I fragment (3.2 kbp) of pTNF331 in the presence of excess of 5′-dephosphorylated synthetic 18 bp *Xho*I-*Msp*I duplex. Screening of recombinant clones was carried out by in situ hybridization with 5′-^{32}P-labeled oligonucleotide insert. As a result, plasmid pTNF90 containing the gene for human TNF-α with a deletion of 22 amino acid residues (7–28) in its N-terminal part was developed.

Plasmids pTNFab and pTNFba, coding for hybrids between hTNF-α and hTNF-β, were developed by recombining plasmid pTNF336, a derivative of pTNF331 containing a *Pst*I restriction site in the TNF-α gene, with pLT21. For this purpose, a vector prepared by digestion of pTNF331 with *Kpn*I and *Hin*dIII (3.2 kbp) was ligated with a 402 bp *Kpn*I-*Pst*I fragment of pTNF336 and a 342 bp *Pst*I-*Hin*dIII fragment of pLT21. The resulting plasmid was named pTNFab. Similarly, plasmid pTNFba was constructed by ligation of a 3.4 kbp *Hin*dIII-*Kpn*I vector with a 548 bp *Kpn*I-*Pst*I fragment of pLT21 and a 223 bp *Pst*I-*Hin*dIII fragment of pTNF336.

To construct in-frame fusions of the murine and human TNF-α proteins, we took advantage of the presence of restriction cleavage sites common in both genes. For constructing plasmid pmhTNF36, a 1072 bp *Pst*I-*Bgl*I fragment of plasmid pmTNF was inserted into *Bgl*I-*Pst*I sites of plasmid pTNF331. In detail, the *Pst*I-*Bgl*I fragment of pmTNF together with a 492 bp *Bgl*I-*Hin*dIII fragment of pTNF331 were ligated with *Hin*dIII-*Pst*I vector (2.84 kbp) derived from pTNF331. Similarly, plasmid pmhTNF49 was constructed by ligation of a 1111 bp *Pst*I-*Eco*64I fragment of pmTNF with 736 bp *Eco*64I-

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*Nco*I and 2.2 kbp *Nco*I-*Pst*I fragments of pTNF331. Alternatively, plasmid phmTNF36 was obtained by inserting a *Pst*I-*Bgl*I fragment into the corresponding pmTNF vector using the multicomponent ligation as described above.

2.3. Purification procedures

Human and murine TNF and the chimeric proteins were isolated as follows. After growth for 24 h at 37°C, bacteria were harvested by centrifugation, resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% mercaptoethanol and 10 μg/ml PMSF, and disrupted by sonication. After centrifugation (15000 rpm, 15 min), the supernatant was dialyzed against buffer A (50 mM sodium phosphate, pH 7.5, 0.1% mercaptoethanol, 10 μg/ml PMSF) and applied to a hydroxyapatite column (Bio-Rad) equilibrated in the same buffer. Proteins were eluted with a linear sodium phosphate gradient (50–300 mM) in buffer A. Fractions were collected and analyzed for the presence of TNF-α mutant and hybrid proteins by SDS-PAGE. Positive fractions were pooled, dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 0.1% mercaptoethanol, 10 μg/ml PMSF) and applied onto a DEAE-Toyopearl 650 column (Toyo Soda) equilibrated in the same buffer. The target proteins were eluted with a linear NaCl gradient (0–0.15 M) in buffer B. The fractions containing mutant TNF-α protein were pooled and sterilized by filtration through membrane filters (Millipore) with pore size 0.2 μm. The protein concentration was determined by Protein assay kit II (Bio-Rad).

The monoclonal antibodies E7H2 were purified from hybridoma supernatant or ascite fluid by affinity chromatography on antigen-Sepharose CL-4B. For this purpose, recombinant human TNF-α was immobilized on CNBr-activated Sepharose CL-4B (Pharmacia) according to standard procedure [16]. Ascite fluid was diluted 1:10 with 50 mM potassium phosphate, pH 7.4, 0.5 M NaCl and loaded on the immuunosorbent column equilibrated in the same buffer. The column was carefully washed out, and Mabs E7H2 were eluted with glycine buffer, pH 2.8. The fractions containing Mabs E7H2 were immediately neutralized with 1 M Tris, dialyzed against phosphate-buffered saline, aliquoted and stored at −20°C.

2.4. Western blot analysis

SDS-electrophoresis in 13% PAG was performed in accordance with the procedure of Laemmli [17]. Immunoblotting with monoclonal antibodies E7H2 was done essentially as described in [18]. Briefly, the transfer of proteins onto nitrocellulose membrane (Schleicher & Schuell) was carried out in an apparatus for semi-dry blotting (BioTecMed) for 1 h at 0.8 mA/cm^2. Nonspecific protein binding was blocked by the incubation of membranes in buffer TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 1% BSA for 1 h at 20°C. After overnight gentle shaking in a solution of E7H2, the membrane filter was washed with 0.1% Tween-20 in TBS and then incubated with rabbit anti-mouse peroxidase-labeled immunoglobulin for 3 h at 20°C. The staining was performed by treatment with 4-chloronaphthol in TBS containing 20% methanol and 0.03% hydrogen peroxide.

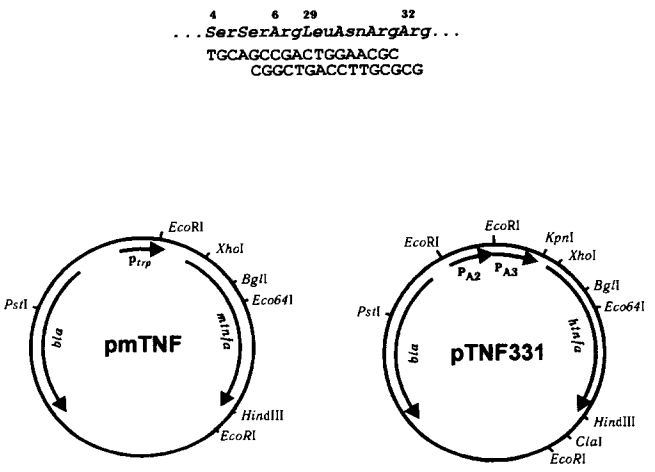

Fig. 1. Structure of synthetic duplex for construction of plasmid pTNF90 and schematic map of plasmids pTNF331 and pmTNF for construction and expression of hybrid TNF-α genes: *htnfa* and *mtnfa*, genes for human and murine TNF-α, respectively; *bla*, *E. coli* β-lactamase; P_{A2} and P_{A3}, transcriptional promoters A2 and A3 from early region of bacteriophage T7, respectively; P_{trp}, *E. coli trp*-promoter.

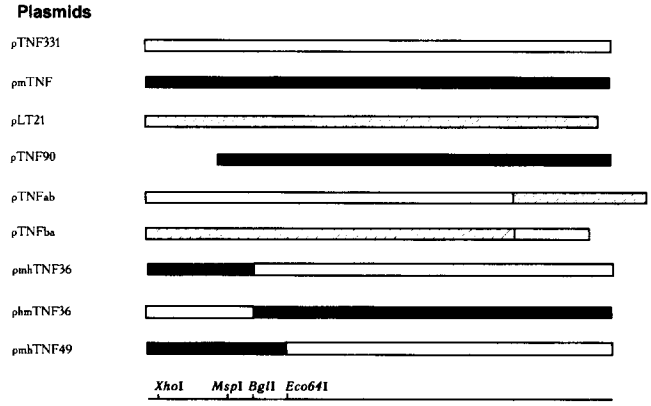

Fig. 2. Schematic presentation of mutant and hybrid proteins. Open and filled bar segments represent murine and human TNF-α coding information, respectively; crosshatched bar segments represent human TNF-β coding sequence. At the bottom, restriction sites used to generate the chimeric proteins are shown.

3. Results and discussion

Using previously prepared recombinant plasmids with artificial genes encoding wild-type human and mouse TNF-α (pTNF331 and pmTNF, respectively) as well as mutant TNF-α (pTNF336) and TNF-β (pLT21), we developed a human TNF-α deletion gene and a number of in-frame chimeric genes encoding hybrids between human and mouse TNF-α and human TNF-α and TNF-β. For constructing a deletion variant of the human TNF-α gene, we took advantage of the presence of a unique *Msp*I restriction site. Therefore, we replaced in plasmid pTNF331 an 81 bp *Xho*I-*Msp*I fragment with a short duplex (Fig. 1). As a result, plasmid pTNF90 encoding human TNF-α protein with deletion of amino acid residues 8–28 was made. The construction of genes encoding the hybrids between human tumour necrosis factor and lymphotoxin was achieved by recombining plasmids pLT21 and pTNF336. Since the plasmids each contain two *Pst*I sites, the construction of pTNFba and pTNFab was done using three-component ligation as indicated in section 2. Plasmid pTNFab encodes a chimeric protein in which the amino acid sequence 3–125 of human TNF-α is fused to the amino acid sequence 125–171 of human lymphotoxin; plasmid pTNFba encodes a polypeptide sequence 22–125 of hTNF-β linked to sequence 127–157 of hTNF-α.

For the construction of in-frame fusion genes encoding chimeric human/mouse TNF-α protein, we took advantage of the presence of restriction sites, *Bgl*I and *Eco*64I common to both genes. Starting plasmids were pTNF331 and pmTNF (Fig. 1), which were previously shown to direct efficient production of human and murine TNF-α, respectively. Depending on the upstream coding sequence, mutant and chimeric genes were expressed either under control of a tandem of two promoters A2 and A3 [19,20] from early region of bacteriophage T7 (in plasmids pTNF331, pLT21, pTNF90, pTNFab, pTNFba, and phmTNF36) or *E. coli trp*-promoters (in plasmids pmTNF, pmhTNF36, and pmhTNF49). Notably, the

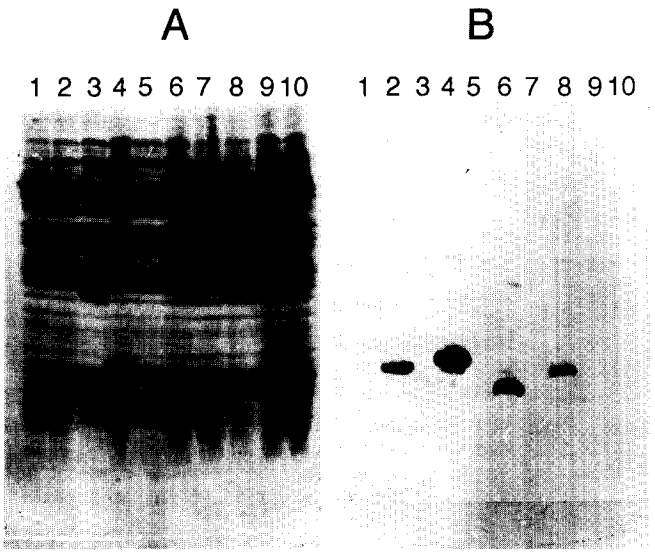

Fig. 3. Electrophoresis in 13% SDS-PAG (A) and Western blot with monoclonal antibodies E7H2 (B) of total cellular protein of *E. coli* SG20050 producing mutant and hybrid proteins: lane 1 — *E. coli* SG20050 without plasmid (control); lane 2 — *E. coli* (pTNF331); lane 3 — *E. coli* (pLT21); lane 4 — *E. coli* (pTNFab); lane 5 — *E. coli* (pTNFba); lane 6 — *E. coli* (pTNF90); lane 7 — *E. coli* (pmTNF); lane 8 — *E. coli* (pmhTNF39); lane 9 — *E. coli* (phmTNF39); lane 10 — *E. coli* (pmhTNF49).

trp-promoter provides constitutive expression of genes in *E. coli* strain SG20050 used in this study. The structures of the chimeric proteins are schematically presented in Fig. 2.

The intact and chimeric TNF proteins were purified following the procedure described in section 2. Remarkably, wild-type murine and human TNF as well as chimeras hmTNF36 and mhTNF36 were well soluble in cytoplasm of *E. coli*, while the hybrid protein mhTNF49 was soluble to only a very small extent. In contrast, the mutant TNF90 and chimeras TNFab and TNFba were completely insoluble. Surprisingly, the chimeric protein hmTNF36 was found to be essentially more active in cytotoxic assay against murine fibroblast cell line L929 than murine wild-type protein (data not shown).

Western blotting with Mabs E7H2 was carried out using either isolated protein (data not shown) or total cellular protein of *E. coli* SG20050 harboring recombinant plasmids (Fig. 3). The antibodies E7H2 did not interact with human TNF-β or with murine TNF-α (Fig. 3b, lanes 3 and 7). At the same time this antibody did bind to chimeric TNFab, but not to TNFba. Together with the finding that E7H2 still bind very well to deletion mutant TNF90, this observation leads to the conclusion that the binding site is located between amino acid residues 30 and 128 of human TNF-α. Replacement of amino acid residues 1–36 of human protein with the murine sequence did not abolish interaction with Mabs E7H2, while the following replacement of amino acids 1–49 resulted in absence of binding. This observation unambiguously indicates that the binding site of anti-human TNF-α Mabs E7H2 is located between amino acid residues 37 and 49. Remarkably, there are only three distinctions between sequence 37–49 of human and murine proteins, namely, Val_{41}, Glu_{42} and Arg_{44}. Finally, the contribution of each of these residues to formation of the antibody binding site may be assessed after further mutation analysis.

It should be noted that the binding of Mabs E7H2 to hybrid protein mhTNF36 was essentially weaker in comparison with wild-type protein or deletion variant TNF90. Probably there are amino acids located between residues 29 and 37 which are not obligatory for formation of the binding site; however, the presence of these residues significantly facilitates the interaction of these Mabs with the antigen. Besides, the location of the Mabs E7H2 binding site explains the failure of this antibody to neutralize a biological activity of hTNF-α. In fact, Mabs E7H2 recognize the amino acid sequence located on the external surface of the TNF-α trimer molecule within a short β-pleated region which is not involved in interaction at least with the second type of TNF receptor (TNFR55) as was shown by analysis of the crystal structure of the soluble human 55 kDa TNF receptor–hTNF-β complex [1].

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