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PURIFICATION OF RECOMBINANT HUMAN TUMOR NECROSIS FACTOR PRECURSOR FROM *ESCHERICHIA COLI*

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SUMMARY: To study its biological functions, tumor necrosis factor precursor (proTNF) with a molecular size of 26-KDa was obtained as a recombinant protein from *Escherichia coli*. The recombinant proTNF was successfully accumulated in the insoluble form, corresponding to about 10-15% of total *E. coli* proteins. Solubilization, gel filtration and anion exchange chromatography were performed under denatured conditions followed by dialysis in phosphate-buffered saline. These processes removed most of the contaminating bacterial proteins, yielding proTNF with a purity of about 70-80%. This recombinant proTNF is expected to be useful for functional studies on activated macrophages with membrane integrated proTNF.

Tumor necrosis factor (TNF) is a monokine produced by activated macrophages (1). It is believed to be present in two forms: the mature 17-KDa diffusible form (2-4) and the membrane integrated 26-KDa precursor form (5).

Two steps called the 'primed stage' and the 'triggered stage' are necessary for efficient production of mature TNF by macrophages (1). We previously reported that the primed stage of TNF production by macrophages was reversible (6), and that macrophages were primed for this production during the normal development of mice (7) or regeneration of the liver after a partial hepatectomy (8). We also showed that the TNF precursor (proTNF) was present in the primed stage of macrophages (9).

From these results, we hypothesize that primed macrophages carrying the membrane integrated form of proTNF may play a significant role in regulating the homeostasis in normal embryogenesis and thereafter even in adults, and that the membrane integrated form of TNF precursor may be its effector molecule.

The mature form of TNF has been reported to have a wide variety of biological activities *in vitro* and *in vivo* (10,11). However, it is actually detected only in diseases with extreme inflammatory responses *in vivo* such as severe bacterial infection and septic shock (12), suggesting that a large amount of mature TNF is released in a physiologically emergent state and that TNF may act in a membrane integrated form rather than a mature form *in vivo*.

Recently, Kriegler et al. demonstrated that the membrane integrated form of proTNF showed cytocidal activity to certain cells *in vitro* through cell to cell contact (5,26). This strongly suggests the possibility that a wide variety of biological activities may be mediated by proTNF. However,

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synthesis of proTNF has not yet been reported; and consequently, no extensive analysis of proTNF in many biological phases has yet been initiated. In view of this situation, we wanted to establish a system by which purified proTNF could be obtained and report here the successful production and partial purification of the TNF precursor as a recombinant protein from *E.coli*.

MATERIALS AND METHODS

Materials: All restriction endonucleases and modified enzymes used were obtained from Takara Syuzo (Kyoto, Japan) or Nippon Gene (Toyama, Japan). Expression vector with bacteriophage T7 promoter denoted as pET-3a and *E. coli* BL21(DE3) were originally established by Studier et al.(13) and kindly provided by Dr. H. Nishina (Tokyo Institute of Technology). Synthetic oligonucleotides were synthesized by a DNA synthesizer (Model 391, Applied Biosystems, CA, U.S.A.). All other chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Wako Junyaku (Osaka, Japan).

Plasmid construction: The cDNA library was constructed by a λgt10 system from polyA RNA obtained from a phorbol ester-induced human monocyte-like cell line, THP-1, according to the method of Gubler and Hoffman (14). A full-length cDNA clone encoding the proTNF was isolated from this cDNA library by a plaque hybridization procedure using the Xho I-Pst I fragment from subcloned human TNF gene as the probe. Isolated recombinant λDNA was digested with Eco RI, and the cDNA fragment from the 5'-artificial Eco RI site derived from the linker to another Eco RI site in the 3'-untranslated region of TNF cDNA was subcloned into pBluescript (Stratagene, La Jolla, CA, U.S.A.). Apa I (cut at the 5'-terminal coding portion of proTNF cDNA) to Bam HI (derived from the multi-cloning site of pBluescript) fragment was isolated and ligated with synthetic Nde I-Apa I linker which corresponds to the remaining part of the 5' region containing the initiation codon. This ligated fragment was then circularized with an expression cassette of Nde I-Bam HI of pET-3a. After isolation of the correct construct, plasmid DNA was introduced into *E. coli* BL21(DE3).

Induction of the expression of recombinant proTNF in *E. coli*: *E.coli* cells that carried the expression vector of proTNF were grown at 37° C in good aeration with NZY medium containing 200 μg/ml of ampicillin. When the optical density of 600nm reached about 1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 1mM, and the cells were further cultured for 90 minutes. After harvesting and resuspension with the lysis buffer containing 1 mg/ml lysozyme, 50mM Tris-HCl (pH 8.0), 1mM EDTA, 100mM NaCl, and 1mM phenylmethylsulfonylfluoride (PMSF), induced *E.coli* cells were sonically disrupted by a sonic homogenizer (Sonifier 250, Branson Ultrasonics Corp. Danbury, CT. U.S.A.). Soluble and insoluble fractions of the lysate were separated by ultracentrifugation at 100,000xg for 60 minutes.

SDS-polyacrylamide gel electrophoresis and Western blot analysis: To analyze the recombinant protein produced by *E.coli* cells, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (15). *E.coli* (whole cells) and soluble or insoluble fractions of cell lysate were solubilized in 63mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, and 0.01% bromo phenol blue (BPB). After heat denaturation, solubilized samples were loaded onto a 15% SDS-polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie brilliant blue R-250, and the band purity was estimated by a scanning densitometer (GS 300, Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). For Western blot analysis, SDS-PAGE was carried out as described above, proteins were then blotted electrophoretically onto a nitrocellulose filter (BioTrace, Gelman Science, U.S.A.), and then probed with anti-human r-TNFα monoclonal neutralizing antibody (developed in our laboratory, unpublished). Immunoreactive bands were visualized non-radioisotopically by a biotin-streptoavidin system (Amersham, U.K.).

Purification of recombinant proTNF from *E. coli*: *E.coli* cells obtained from 2 liters of culture were lysed, and insoluble pellets were obtained as described above. After sequential washings with 3.5M urea in 10mM Tris-HCl, pH 8.0 and 8M urea in 10mM Tris-HCl, pH 8.0, the remaining insoluble pellet was solubilized with 6M guanidium hydrochloride (GdmHCl), 10mM dithiothreitol (DTT), 50mM Tris-HCl, pH 8.0, 1mM EDTA, and 100mM NaCl. Gel filtration was carried out using Sephacryl S100HR in a FPLC system (Pharmacia, Uppsala, Sweden) equilibrated with 6M urea, 10mM Tris-HCl, pH 8.0, 1mM DTT, and 0.2M NaCl. Fractions containing the recombinant proTNF were pooled, diluted, and applied to Q-Sepharose Fast Flow (Pharmacia) anion exchange

chromatography in the FPLC system. The fractions eluted with a NaCl linear gradient in 6M urea, 10mM Tris-HCl, pH 8.0, 1mM DTT were pooled and dialyzed against phosphate-buffered saline containing 0.5mM 2-mercaptoethanol. Protein content was measured according to the method of Bradford (16).

TNF assay: Cytotoxicity to L929 cells was measured as described elsewhere (17).

RESULTS

Expression of Recombinant proTNF in *Escherichia coli.*

In our initial attempt to express the recombinant proTNF (r-proTNF), the *tac* promoter vector system (18) was used because we had previously obtained good results with it in the expression of several recombinant proteins including r-TNF-S series (17). Although we succeeded in constructing a suitable plasmid which was expected to express r-proTNF, we failed to obtain proTNF using this plasmid (data not shown).

Next, we tried to express r-proTNF using pET-3a carrying the promoter of bacteriophage T7 and *E.coli* BL21(DE3), the lysogenic host of T7 RNA polymerase, as the host strain, both of which were established by Studier et al.(13). A 26-KDa band appeared on stained SDS-polyacrylamide gel when recombinant *E.coli* was induced with IPTG (Figure 1A, lane 2). This 26-KDa band was stained specifically by Western immunoblot analysis using anti-TNFα monoclonal antibody (Figure 1B), and this expression system successfully produced 26-KDa r-proTNF. The amount expressed as judged by densitometric scans of the stained gel was about 10-15% of the total bacterial proteins. Most of the

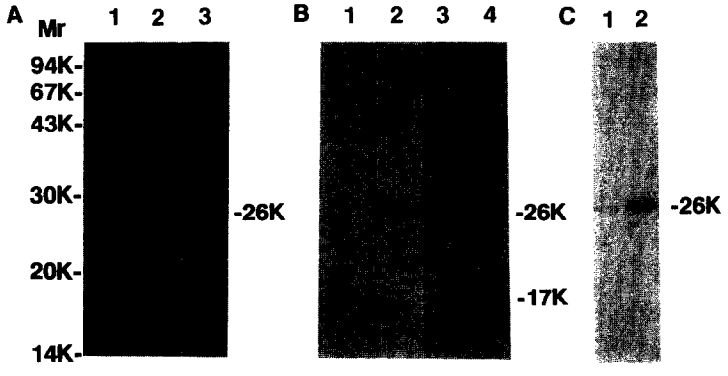

Figure 1. SDS-PAGE and Western blot analysis of r-proTNF produced by *E. coli.* Lysis of bacterial cells and separation of soluble and insoluble fractions are described in Materials and Methods. Samples were subjected to electrophoresis on a 15% SDS-polyacrylamide gel under reduced conditions as described by Laemmli(15). Western blot was carried out with anti-rTNFα monoclonal antibody as described in Materials and Methods. A). SDS-PAGE analysis of r-proTNF production in *E.coli.* Whole *E.coli* cells were solubilized and subjected to SDS-PAGE followed by Coomassie blue staining. Lane 1: *E.coli* carrying pET-3a vector, lane 2: IPTG-induced *E.coli* carrying r-proTNF expression vector, lane 3: uninduced *E.coli* carrying r-proTNF expression vector. B). Western blot analysis of r-proTNF production in *E.coli* probed with anti-human r-TNFα monoclonal antibody. Lane 1: purified r-TNFα, lane 2: uninduced *E.coli* carrying r-proTNF expression vector, lane 3: IPTG-induced *E.coli* carrying r-proTNF expression vector, lane 4: *E.coli* carrying pET-3a vector. C). Western blot analysis of soluble and insoluble fractions of *E.coli* lysate for r-proTNF probed with anti-human r-TNFα monoclonal antibody. Lane 1: soluble fraction of r-proTNF producing *E.coli* lysate, lane 2: insoluble fraction of r-proTNF producing *E.coli* lysate.

Table 1. Summary of the purification of r-proTNF

	Total protein (mg)	r-proTNF content (%)#	r-proTNF amount (mg)	Yield (%)
Total insoluble protein	180	25.4	40	100
Washed pellet	119	30.4	36	78
Sephacryl S100 HR pooled fractions	8.9	50.1	4.5	12.5
Q-Sepharose/ Dialyzed fractions	2.2	75.7	1.7	3.7

r-proTNF content was estimated by densitometric scanning of the dye stained SDS-polyacrylamide gel.

immunoreactive band of 26-KDa was detected in the insoluble fractions when SDS-PAGE and Western blot analysis were done after the separation of soluble and insoluble fractions of the lysate (Figure 1C). This showed that r-proTNF was accumulated in the insoluble form in *E.coli* cells.

Partial Purification of Recombinant proTNF.

To obtain a larger amount of r-proTNF, we next purified r-proTNF from the insoluble fraction of induced *E.coli* cells. Purification was carried out according to two criteria: 1) Increase of the content of the 26-KDa band on SDS-polyacrylamide gel as judged by densitometric scanning; 2) increase of the intensity of the immunostained band on the Western blot using anti-rTNFα monoclonal antibody.

Based on the results of preliminary experiments with various solubilizing agents, a buffer containing 6M GdmHCl, and 10mM DTT was used to solubilize the insoluble materials. Gel filtration and anion exchange chromatography then separated the recombinant protein from most of the bacterial proteins under denatured conditions. The fractions containing 26-KDa r-proTNF were pooled and dialyzed against phosphate-buffered saline with 0.5mM 2-mercaptoethanol. Following these steps, about 2.2mg of partially purified r-proTNF (about 75% purity, as judged by densitometric scanning) was obtained from 180mg of total insoluble proteins. The recovery of r-proTNF was about 3.7% (Table 1). The specific immunoreactivity of this preparation determined by Western immunoblot analysis with anti-TNFα monoclonal antibody was almost the same level as that of purified 17-KDa r-TNFα (Figure 2). On the other hand, the cytotoxic activity of this preparation on L929cells was about 12 units/mg protein, very low compared with that of purified r-TNFα whose specific activity was calculated as about 2×10^6 units/mg protein(17). This result is reasonable because 26-KDa proTNF synthesized in the *in vitro* translation system using rabbit reticulocyte lysate showed no cytotoxicity to L929 cells (5,9).

DISCUSSION

This is the first report of successful expression and purification of recombinant TNF precursor (r-proTNF). We succeeded in the production of r-proTNF and obtained a sufficient amount of partially purified material to study the characteristics and biological functions of proTNF.

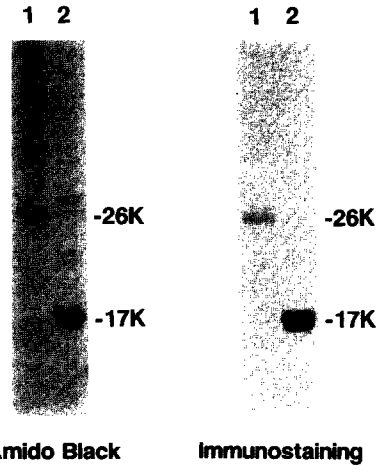

Figure 2. Western immunoblot analysis of partially purified r-proTNF. SDS-PAGE and Western blot were carried out as described in the legend of Figure 1. Duplicate filters were stained with amido black(left) and anti-human r-TNFα monoclonal antibody(right), respectively. Lane 1: partially purified r-proTNF, lane 2: r-TNFα.

Interleukin 1 (IL-1) is another monokine that is known to have a membrane bound form (19). r-proIL-1 has been produced by several groups (20-23) and employed primarily to analyze the processing proteases involved in the post-translational processing mechanisms of IL-1 production (20-25). However, there have been few reports about the biological significance of the membrane bound form of cytokine precursors (5,26,27).

We previously suggested the significance of the 'primed' macrophages in the homeostatic response of the host (7,8) and are now studying the possibility of restoring a distorted state of host homeostasis such as that seen in intractable disease by inducing macrophages to the primed stage of TNF production (to be published elsewhere). We hypothesize that the membrane integrated form of the TNF precursor present in the primed macrophage may play a significant role in this homeostatic response. This hypothesis is partly supported by the report of Peck et al.(28), in which it was suggested that the membrane bound TNF has different biological activities from those of mature TNF.

We expect that the biological functions of the membrane integrated TNF precursor and, furthermore, the significance of primed macrophages in the homeostatic response will be clarified by using this r-proTNF. For further analysis, however, it will be necessary to reconstitute the membrane integrated pro-TNF using artificial membranes such as liposomes.

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