Expression of a Functional Human Tumor Necrosis Factor-α (hTNF-α) in Yeast *Saccharomyces cerevisiae*

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Abstract The recombinant soluble human tumor necrosis factor-alpha (hTNF-α) was expressed in a yeast *Saccharomyces cerevisiae* and its cytotoxicity was evaluated. A cDNA encoding hTNF-α was placed under the control of two different promoters: a glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter and a yeast hybrid *ADH2-GPD* promoter, consisting of alcohol dehydrogenase II (*ADH2*) and the *GPD* promoter. A Northern blot analysis revealed that, although variation in the expression level of hTNF-α existed among transformants, the higher expression was obtained with the *GPD* promoter. Expressed hTNF-α protein (rhTNF-α) was successfully secreted into the culture medium, producing 2.5 mg per liter of culture filtrate, with no changes in cell growth. The bioassay for observing the cytotoxicity to the murine L929 fibroblast cell line, with serial dilution of rhTNF-α, indicated that the secreted rhTNF-α was bioactive and its dose-response was improved eight to ten times over that of the *E. coli*-derived rhTNF-α.

Keywords: human tumor necrosis factor-alpha, hTNF-α, *Saccharomyces cerevisiae*

INTRODUCTION

Tumor necrosis factor-α (TNF-α), homotrimers of 17.4 kDa proteins produced by macrophages and other cells, is a potent lymphoid factor, which exerts cytotoxic effects on a wide range of tumor cells and certain other target cells [1]. As a widely researched cytokine, the anti-tumor effects of TNF-α are known to be not only due to the direct cytotoxicity towards tumor cells, but also to the effective activation of the novel anti-tumor immune reaction *in vivo* [2]. Natural human TNF-α is mainly produced and purified from a monocyte-like cell line [3].

The heterologous expression of a functional recombinant TNF-α in *Escherichia coli* was successfully achieved and stimulated the efforts to produce the protein, as a therapeutic drug on a large scale. However, the high dose of TNF-α when it has to be administered in cancer therapy leads to severe toxicity and side effects in patients [4, 5]. Therefore, it is necessary to enhance the bioactivity or bioavailability of TNF-α *in vivo*, so that the undesirable side effects can be minimized by administration of a low dose.

The expression of foreign proteins in *Saccharomyces cerevisiae* is known to have several advantages over other expression systems. The expressed products can be secreted and yeast is a GRAS (Generally Recognized as Safe) organism, with a long history of applications [6]. Secretion of foreign proteins from eukaryotes is preferable to synthesis in *E. coli*, where foreign proteins may accumulate in inclusion bodies [7]. Moreover, several studies have shown that the specific activity of recombinant cytokine was superior to that of *E. coli* [8,9]. In this study, the mature TNF-α gene was cloned and expressed in *S. cerevisiae*. The protein product was secreted, and the bioactivity of the rhTNF-α was measured and compared with that of the *E. coli*-derived rhTNF-α.

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MATERIALS AND METHODS

Strains and Culture Conditions

Plasmids were maintained and propagated in *E. coli* HB101 or DH5α as described by Sambrook *et al.* [10]. The LPS (20 μg/mL)-stimulated peripheral blood mononuclear cell (PBMC) was used for amplification of the cDNA encoding hTNF-α, and *S. cerevisiae* 2805 (MATα pep4::HIS3 prb1-δ Can1 GAL2 his3 ura3-52) for the heterologous production of hTNF-α [11].

The *S. cerevisiae* was maintained in YEPD medium (yeast extract, 10 g/L; peptone, 20 g/L; dextrose, 20 g/L), with a uracil deficient selective medium (yeast nitrogen base without amino acids, 6.7 g/L; adenine and tryptophan, 0.03 g/L; casamino acid, 5 g/L; dextrose, 20 g/L; agar, 20 g/L) used to screen the transformants at 30°C. Primary inoculum was prepared from 5 mL of

uracil selective medium, cultured for 24 h, with a total of 1×10^7 cells, transferred to a 300-mL Erlenmeyer flask containing 40 mL of YEPD broth. Expression cultures were grown at 30°C, with continuous agitation (200 rpm), after which the culture filtrates were assayed for TNF-α expression, using an enzyme linked immunosorbent assay (ELISA).

Plasmid Construction

The total RNA was extracted from the LPS-stimulated PBMC, according to the procedure described previously [10]. First-strand cDNA was synthesized using the First-Strand cDNA synthesis kit, by following the procedures recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, USA). RT-PCR was performed with pTNF-F (5'-GTCAGATCATCTTCTCGAA CC-3') and pTNF-R (5'-CAGGGCAATGATCCCAAAG-3') primers, which are specific to the 5' and 3' ends of the cDNA sequence for mature TNF-α (GenBank accession number X01394), respectively. The PCR amplimer was inserted into the pGEM-T plasmid, according to the manufacturer's recommendations (Promega, WI, USA). For the construction of yeast expression plasmids, an episomal shuttle vector, YEp352 [12], was used. Since a high level of secretion was observed for the yeast-derived heterologous protein using a signal peptide of amylase 1A (*Ramy1A*) (GenBank accession number X16509) from rice [13,14], the sequence encoding the signal peptide of *Ramy1A* was fused to the cDNA for the mature TNF-α gene, using an overlap extension method [15]. The fused construct was then inserted into *Sma*I-digested pUC18. The resulting plasmid was digested with *Bam*HI, and the 567-bp fragment containing the *rhTNF-α*-derived sequence was placed between either one of the two promoters and the galactose-1-P uridyl transferase (*GAL7*) terminator (GenBank accession number M12348) [12]. The *GPD* (glyceraldehyde-3-phosphate dehydrogenase) promoter (GenBank accession number M13807) and a yeast hybrid (AG) promoter consisting of the upstream activating sequence (UAS) of *ADH2* (alcohol dehydrogenase 2) (GenBank accession number J01314) and the *GPD* TATA element, have been described previously [12]. The resulting plasmids were denoted pYEGTNF and pYEAGTNF, respectively (Fig. 1). *S. cerevisiae* 2805 was then transformed with the expression plasmids, using the lithium acetate procedure [16]. The stability of the plasmids introduced into the yeast was measured as follows: samples grown in non-selective medium YEPD, were serially diluted with sterile H_2O to an expected 50 colony forming unit (CFU) per plate, plated on ura$^-$ selective and non-selective plates and the relative number of CFUs was determined.

Northern Blot Analysis

The transformed yeast cells were lysed using glass beads and the total RNA was extracted, according to the procedure described previously [11]. The amount of RNA was measured by UV-spectrophotometry and the

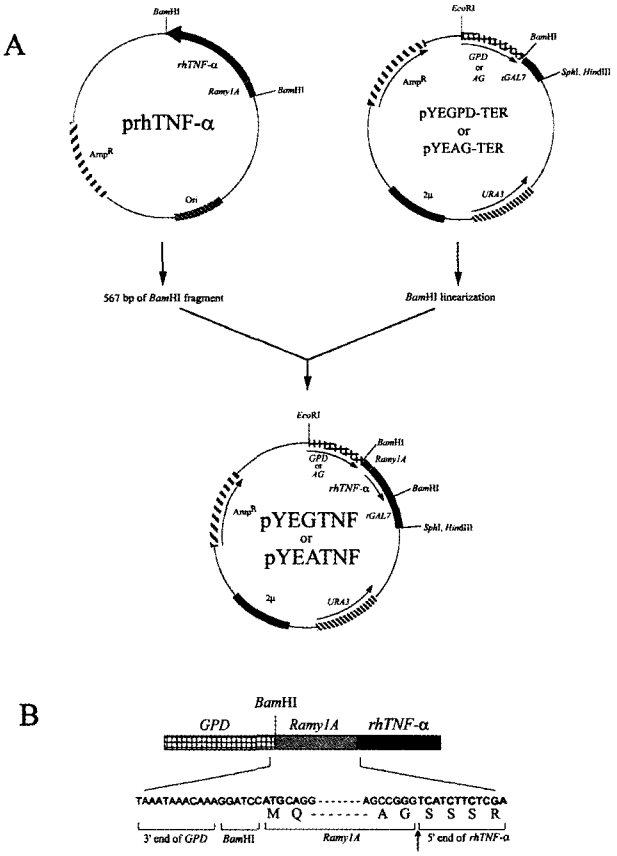

Fig. 1. (A) Schematic diagram of the construction of the transforming plasmids. The boxes represent the genes, or their corresponding functional domains, as described in the text. The panel (B) shows the sequence covering the links of promoter-signal peptide-TNF-α, with the translation start codon represented in bold letters. The arrow indicates the natural cleavage site of the signal peptide of amylase 1A.

total RNA (30 μg per lane) was separated on a 1% agarose gel (in 2.2 M formaldehyde). Before blotting, the gel was stained with ethidium bromide to confirm that similar amount of RNA had been loaded for each sample. The RNA was transferred onto a Hybond membrane, as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, USA). Hybridization was performed in church buffer [7% (w/v) SDS, 1% BSA, 1 mM EDTA and 250 mM $NaPO_4$, pH 7.2], at 65°C [17]. The probe was labeled with α-[^{32}P]-dCTP, using a random labeling kit (Amersham Pharmacia Biotech, Piscataway, USA) [18].

Quantitative Assay of rhTNF-α by ELISA

The concentration of rhTNF-α in the culture filtrate of *S. cerevisiae*, was measured by sandwich ELISA. In this experiment, a recombinant *E. coli*-derived rhTNF-α, purchased from BD Biosciences Pharmingen (San Diego, CA, USA), was reconstituted to a concentration of 200

μg/mL in pure water, according to the manufacturer's instructions and used as a standard. The culture filtrates of the transformed yeasts were subsequently tested in an ELISA, according to the manufacturer's instruction (BD Bioscience).

Western Blot Analysis

Concentrated culture filtrates [19] were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently blotted onto a nitrocellulose filter. After blocking, the filter was incubated with anti-rhTNF-α antiserum, followed by binding to an anti-mouse IgG conjugated to a horseradish peroxidase as a secondary antibody. 4-Chloro-1-naphthol was applied as a substrate for colorimetric detection by horseradish peroxidase, according to the method of Sambrook *et al.* [10].

Measurement of Biological Activity of hTNF-α

Culture filtrates of the recombinant *S. cerevisiae* were collected, dialyzed twice for 2 h at 4°C against PBS and sterilized by passing through a 0.2-μm syringe filter. The protein concentration in the filter-sterilized culture filtrates was measured by the Bradford assay, using the Bio-Rad Protein Assay Kit [20] and adjusted to 20 μg/mL for further analyses. In order to measure the biological activity, murine L929 cells were resuspended in growth medium at 2×10^5 cells/mL and then 100 μl of the cell suspension was aliquoted into each well of a microtitre plate (Falcon, USA), which was then incubated at 37°C, for 24 hr, in a 5% (v/v) CO_2/air humidified atmosphere. The serial dilutions of the standard: a recombinant *E. coli*-derived rhTNF-α purchased from BD Biosciences Pharmingen (San Diego, CA, USA) or yeast-derived rhTNF-α, were added to the wells containing the cells. Then 100 μL of medium containing 2 μg/mL of actinomycin D was added to the wells, which were incubated for a further 24 hr. After incubation, the cytotoxicity of the TNF-α was then determined by the MTT assay [21], using the CellTiter 96® AQ_{ueous} Assay Kit (Promega, WI, USA).

RESULTS AND DISCUSSION

Growth of Transformed *S. cerevisiae* and Production of rhTNF-α

Several transformants of *S. cerevisiae,* representing each recombinant plasmid, were selected on ura⁻ medium. The plasmid DNAs from these transformations were isolated and reintroduced into *E. coli* to confirm the presence of the recombinant plasmid in the yeast. The *rhTNF-α* expression level was measured at the end of two days of incubation. Northern blot analysis of four cells transformed with either pYEGTNF or pYEATNF revealed all the transformants expressed the sequence for rhTNF-α (Fig. 2). However, there were variations in the transcriptional level of the *rhTNF-α* gene, among the strains transformed with the same expression construct (Fig. 2). A wide variation in the heterologous gene expression level in *S. cerevisiae* is not unusual when episomal 2 μ ori-based plasmids are used, possibly due to variations in the plasmid copy number between the different transformants [22,23]. Thus, transformants TYEGTNF and TYEATNF, harbouring pYEGTNF and pYEATNF, respectively, were selected, as they had the highest expression levels.

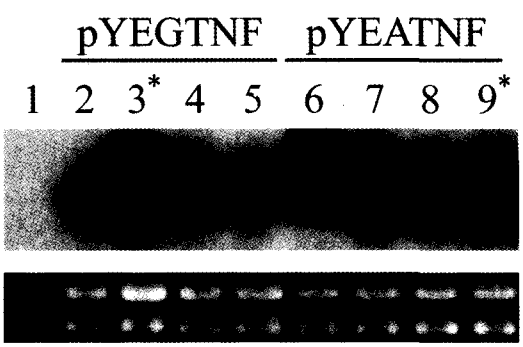

Fig. 2. Northern blot analysis of *rhTNF-α* in transformed yeasts. The yeasts transformed with pYEGTNF and pYEATNF, were probed with labeled *rhTNF-α*. Lane 1 contains RNA of the recipient strain and lanes 2~9 that of eight different transformants. Asterisks indicate the selected transformants of TYEGTNF and TYEATNF. Before blotting, the gel used for the blot in the upper panel was stained with ethidium bromide, to indicate that a similar amount of RNA had been loaded for each sample.

Both the transformants were cultured for three days to obtain growth curves. The observed growth patterns were similar to those determined in previous studies on transformed yeasts [11,12]. After forty hours of incubation, their growth entered a stationary phase with no growth abnormalities of the transformants, due to synthesis of rhTNF-α, being observed (Fig. 3).

The production of rhTNF-α protein was examined using an ELISA and Western Blot analysis. The maximum yield of rhTNF-α was found in the stationary phase for TYEGTNF, which occurred 40 h after cultivation and in the mid log phase for TYEATNF, which occurred 24 h after cultivation; the yields were 2.5 mg/L for the *GPD* promoter and 1 mg/L for the AG promoters (Fig. 3). SDS-PAGE and Western Blot analysis determined that the molecular mass of secreted rhTNF-α is 18 kDa, which is in good agreement with the expected molecular mass of the rhTNF-α gene (Fig. 4). Since the upstream activating sequence (UAS) of a hybrid promoter (pYEGAG) was known to cause repression, as well as, derepression, due to the presence of an excess amount of glucose and ethanol, respectively, and 2% (w/v) glucose and 1.5% (v/v) ethanol supplements gave the best results for the production of foreign product in our previous study [11], the rhTNF-α produced was also analyzed in YEPD medium supplemented with ethanol. The AG promoter directed the below level of 1 mg/L after 24 h of culture, was lower than that of the *GPD* promoter. The protein level decreased rapidly after 24 h could be degradation of the protein by protease or decrease of stability of the pro-

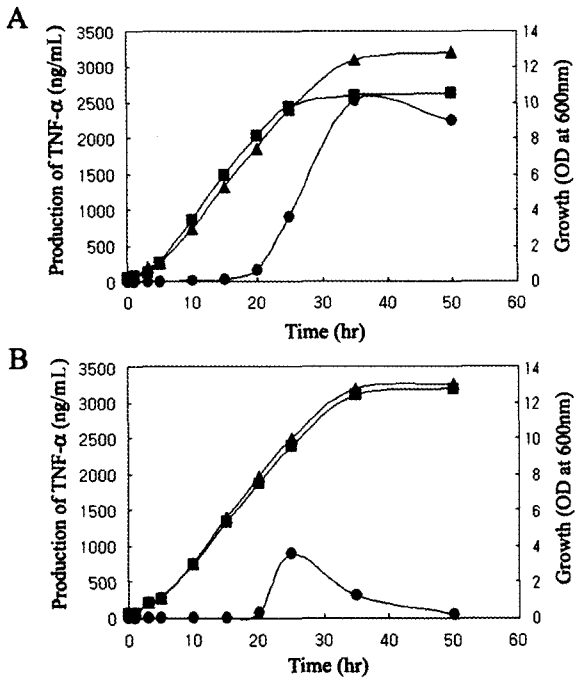

Fig. 3. Time courses of the cell growth (filled triangles, non-transformant; filled squares, transformant) and production of rhTNF-α (filled circles) from TYEGTNF (A) and TYEATNF (B). Data is shown as a representative profile, based on two experiments conducted in triplicate.

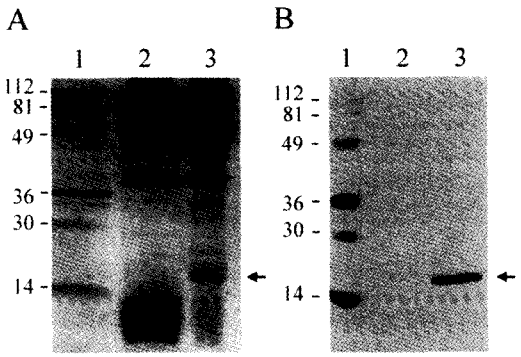

Fig. 4. SDS-PAGE and Western blot analysis of rhTNF-α from a *S. cerevisiae* culture filtrate. A, Commasie blue stained SDS-PAGE of the yeast culture filtrate. B, antigen-antibody reactions of the corresponding yeast gels. Lane 1, molecular weight markers; lane 2, 20 μL of concentrated culture filtrate of the recipient strain; lane 3, 20 μL of concentrated culture filtrate of the transformed strain. Numbers on the left refer to the estimated sizes, in kDa and the arrows indicate the secreted rhTNF-α.

tein itself. The production of foreign protein in this study differed slightly from the previous one [12], in that the hybrid promoter gave the highest expression level of the heterologous gene. However, considering that the previous studies on IL-18 expression showed similar results

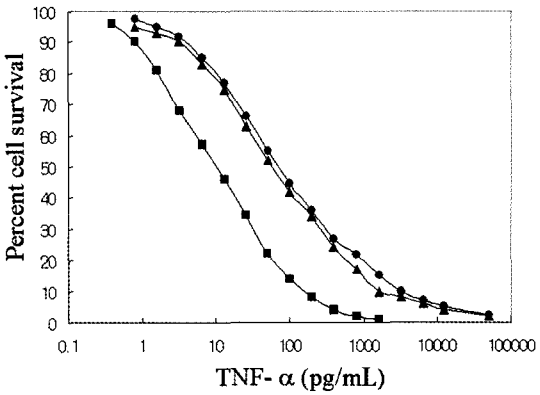

Fig. 5. Dose-response curves for erhTNF-α (filled circles), erhTNF-α diluted with culture filtrate of mock transformant (filled triangles) and yrhTNF-α derived from transformant (filled squares), in murine L929 fibroblast cells, as described in Materials and Methods. Assays were carried out contemporaneously and results are the means of triplicate samples. The X axis represents the concentration of rhTNF-α, diluted with PBS, as a logarithmic scale.

for protein production [11], the difference in the production of foreign protein in same promoter could have been due to the gene of interest, rather than the intrinsic differences in the promoter strength or the plasmid copy number.

Measurement of Biological Activity of rhTNF-α

The biological activity of the rhTNF-α protein from the culture filtrates of the recombinant TYEGTNF, was analyzed by measuring the cytotoxicity to the murine L929 fibroblast cells from serial dilutions of the *E. coli* derived rhTNF-α (erhTNF-α), purchased from BD Biosciences Pharmingen (San Diego, CA, USA), used as a standard, and yeast derived rhTNF-α (yrhTNF-α). Both of the negative controls, which included culture filtrate from a recipient strain and a mock transformant, had no effect on the cytotoxicity (data not shown). In contrast, the standard erhTNF-α, the erhTNF-α, and the culture filtrates of TYEGTNF affected the survival of the L929 fibroblast cells. These results indicated that the rhTNF-α was expressed and secreted in a biologically active form from the TYEGTNF. Moreover, the dose-responsible cytotoxicity of yrhTNF-α, for murine L929 fibroblast cells, was eight to ten times higher than that of erhTNF-α (Fig. 5). The reason for this significant difference in the activities might have been due to the concentration of bioactive form of yrhTNF-α itself, as it is known that the biologically active form of TNF-α, a 17.4 kDa protein, may exist as a dimer, a trimer or even the higher oligomer [3,4]. Although the molecular details of the improved activity of rhTNF-α from yeast remain to be elucidated, it is suggested that the activity of the yeast-expressed rhTNF-α was improved due to a preferred protein folding and/or oligomerization. However, it is still conceivable that other posttranslational modifications, which were not tested for

in this study, might be involved.

The improved specific activity of rhTNF-α produced in yeast is a very promising outcome from the present analysis. Cytokines are known to have many pleotrophic effects on immuno-responses, and clinical applications of many recombinant therapeutic human cytokines have been hampered due to the side effects of overdose [24-26]. Thus, an increase in specific activity is important for the clinical application of many recombinant cytokines. This study has clearly shown that rhTNF-α, from transformed yeast, can be successfully produced and secreted into the culture medium as a biologically active protein and that its specific activity is greater than that for the protein derived from *E. coli*.

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