RT-PCR CLONING AND EXPRESSION OF COMPLEMENTARY DNA FOR HUMAN TUMOR NECROSIS FACTOR ALPHA

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ABSTRACT

U-937, a monocytic cell line was induced with Phorbol Myristate Acetate (PMA) for human tumor necrosis factor alpha (hTNF-á) production. An optimized RT-PCR was employed for construction of hTNF-á complementary DNA (cDNA). The resulted fragment was verified by restriction digestion mapping with PvuII.The verified fragment was cloned in pUC18 plasmid and transformants were pelletted onto LB agar medium containing ampicillin, X-gal and IPTG. The resulting white colonies were verified by PCR and cultured in LB medium containing ampicillin and IPTG. The biological activity and the quantity of hTNF-á expression was assessed by an ELISA method using a monoclonal anti hTNF-á antibody together with a bioassay utilizing L-929 line as sensitive cells.

Key words: hTNF-alpha, RT-PCR, cDNA, Gene expression

INTRODUCTION

Tumor Necrosis Factor Alpha (TNF-á) was first described by Carswell, *et.al* in mid 1970's (1) as a substance in the sera of endotoxine treated animals. TNF-á showed a tumor necrosing effect on certain transplanted tumors in mice and cytotoxicity against certain tumor cell lines without any effect on the normal cells (2,3,4). Further studies showed that TNF-á is a non-glycosilated 17 KD protein containing 157 amino acids (2,3,5,6) which is produced and secreted by macrophages, monocytes, T cell, NK cells and neutrophils (2,5) and has a wide range of biological activities in metabolic control processes, proinflamatory reactions, heamatopoisis, immune regulation and cachexia (5,7,8). Due to the importance of TNF-á in pathological processes such as cancer and rheumatoid arthritis, production of a large quantity of recombinant hTNF-alpha is required for studying its biological effect, and as a result number of isolation, cloning and expression of TNF-á gene have subjected of many investigations (2,3,4,6). In this study the result of cloning of TNF-á by RT-PCR and expression of the cloned gene in *Esherschia coli* is described.

MATERIALS AND METHODES

Materials

U-937 (NCBI C130), L-929 (NCBI C161), RPMI 1640, FCS (Fetal Calf Serum)(Gibco), PMA (Phorbol Myristate Acetate, Sigma), GITC (Guanitidine Isothiocyanate), Actinomycine D (Merck), *Eschershia coli* strain DH5á, Pvu II (Cinagene), hTNF-á, AMV reverse transcriptase, Taq DNA polymerase, Klenow fragment, Sma I, Calf Intestine Alkaline Phosphatase, Hind III , T4 DNA Ligase, IPTG (Isopropyl-B-D-thiogalactoside), X-gal (5'-Bromo-4-chloro-3-indolyl-B-D-galactopyranoside), High Pure PCR product purification kit (Cat.No.1 732 668), hTNF-á ELISA(Cat.No.1 425 943) (Roche) were used in this study.

Methods

Induction of TNF-á production: U-937 cell line was cultured in RPMI 1640 plus 10 % FCS until the cell concentration reached to $2x10^6$ cell/ml, the suspension was centrifuged and cells were resuspended at $5x10^6$ cell/ml in the same medium. PMA, at final concentration of 10 ng/ml, was added and incubated for 24 hours (37°C, 5% CO_2). Cells were harvested and lysed with 4M GITC solution and kept at –70 °C until used. The supernatant was used as source of TNF-á.

Assay of Biological Activity: The method of Clemens et al (9) was used for measurement of biological activity as follow: L-929 cell line was cultured at $5x10^4$ Cell/Well in RPMI 1640 plus 10% FCS for 4 hours (37 °C, 5% CO_2). Standard hTNF-á, induced and non-induced U-937 supernatants, and Actinomycine D (1ìg/ml final concentration) were added in triplicate into

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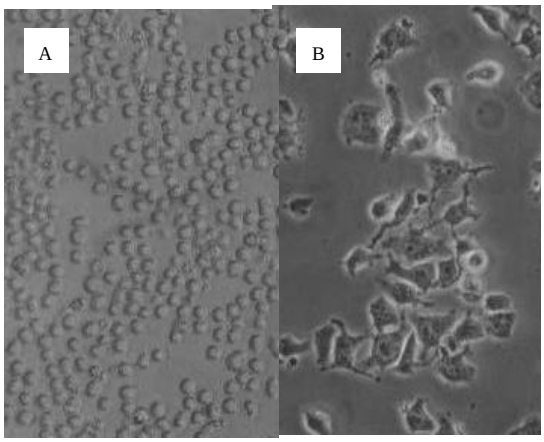

Fig 1: A) U-937 cell line before induction, B) U-937 cell line after induction by PMA

wells of 96-well tissue culture plate and plate incubated at 37 °C and 5% CO_2 for 16 hours. Then cells were fixed with a 33% phenol solution in PBS (Phosphate Saline Buffer, pH 7.4), washed with water and stained with crystal violet (1% W/V). The optical density of the dye exclusion was measured by ELISA reader at 450 nm (9).

RT-PCR: Total RNA of induced U-937 cells was extracted by the method of Chomczynski (10). cDNA synthesis was carried out by AMV reverse transcriptase at 51°C for 35 minutes, followed by amplification of specific 504 bp hTNF-á fragment using "5'GAATTCCATAT-GGTCAGATCATCTTCT" as forward and "5'CCCCAAGCTTCGTCCTCCTCAC" as reverse primers by Techne thermocycler (94 °C, 20"+64°C, 30"+72 C,60") for 30 cycles. PCR product was subjected to electrophoresis on 1.5% Agarose gel and stained with EtBr (0.5 ng/ml). For further analysis, PCR product was further purified by PCR product purification kit.

Cloning and Expression: The resulting PCR product was blunt ended by Klenow fragment and cut with Hind III. pUC18 was cut by SmaI and HindIII, and dephosphorylated with calf intestine alkaline phosphatase. Ligation was carried out by T4 DNA ligase at 4°C for 16 hours. Competent *E.coli* strain DH5alpha was transformed by recombinant plasmid. The transformed cells were pelleted onto LB-Agar medium containing Ampicillin (100 ìg/ml), IPTG (1mM) and X-gal (40 ìg/ml) and incubated at 37 °C for 24 hours.

The resulting colonies were employed for minipreparation of recombinant plasmids through alkaline lysis of microorganisms by NaOH/SDS , renaturation by potassium acetate (1.35M final concentration) and alcohol percipitation .

Measurement of expressed protein: PCR-Verified white colonies were cultured in LB-medium containing IPTG (1mM) as an inducer for lac Z' promoter, and ampicillin (100 ìm/ml) for 16 hours at 33°C. Bacterial cell lysate was used as source of recombinant TNF-á and evaluated for ELISA test using anti human TNF-á monoclonal antibody of the hTNF-á ELISA kit. Conjugated anti-hTNF-á + Biotin and anti-hTNF-á + Peroxidase were added in to precoated wells with Streptavidine and then 20 ìl of samples were added to each well. After incubation at room temperature with shaking at 200 rpm for 4 hours, 50 il of enzyme substrate (TMB, Tetramethylbenzidine) were added and the samples were incubated for an other 25 minutes at room temperature with shaking at 200 rpm. Following addition of 50 ìl sulforic acid (0.5 M) and shacking for 1 minute for stopping the reaction, the optical density was measured by ELISA reader at 450 nm.

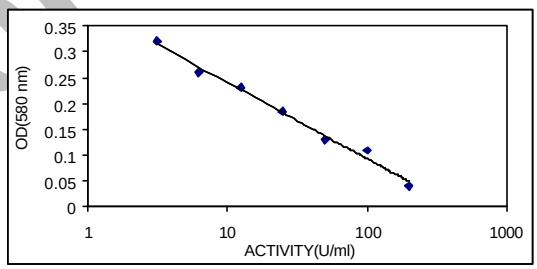

Fig 2: Standard curve of cytotoxic activity of hTNF-alpha on L-929 cell line

Biological activity assay of recombinant hTNF-á: The cell lysates with positive ELISA test, were sterilized and diluted with RPMI 1640 +10% FCS and used for biological activity assay in comparisone with a negative colony as a blank sample by the method which has been described above .

RESULTS AND DISCUSION

Different cells such as peripheral blood cells, U-937, HL-60, cell lines (2,3) have been used for TNF- á production. In some studies, LPS, PMA, and SEB (2,3,5) have been used for induction of U-937, which is a monocytic cell line, for TNF-á overproduction. PMA was used in this study because of its high induction capability. Drastic morphological changes were observed after addition of PMA which is a sign of the cell transformation and production of TNF-á, e.g.

pseudopodia formation (Fig.1-B). Further confirmation of TNF-á production was its cytotoxic effects on sensitive cell lines, i.e.L-929 (2) (Fig.2).

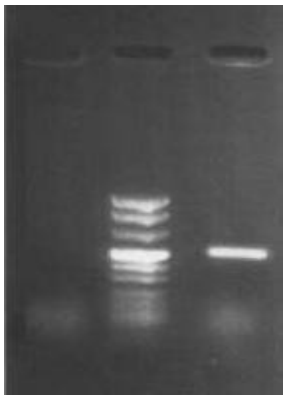

Fig 3: Gel electrophoresis of PCR and RT-PCR products:
Line 1: PCR reaction (without any product). Line 2: Size marker. Line 3: RT-PCR reaction (with a 504-bp fragment)

Actinomycine D was added to enhance the sensitivity of assay (9). Quantification of TNF-á which were produced by induced cells, showed results which were comparable with published data (2). Production of TNF-á in the amounts of 40 units/ml for induced cells and 3-4 units/ml for non-induced ones show the effect of PMA in introducing U-937 cells to cells which produce and secret TNF-á.

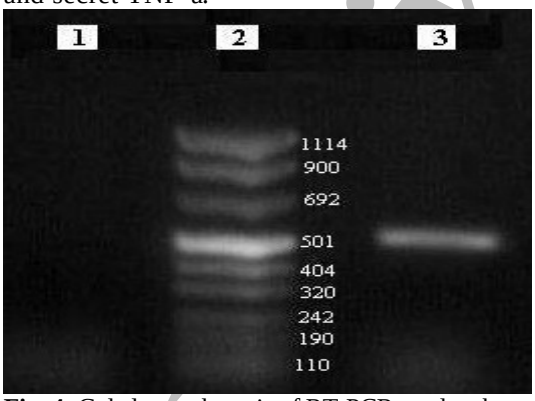

Fig. 4: Gel electrophoresis of RT-PCR product by PvuII restriction enzyme
Line 1: Partial digestion by 10 IU PvuII. Line 2: Size marker. Line 3: Digestion by 20 IU PvuII. Line 4: Blank sample without digestion

There are some literature evidences for cloning and expression of TNF-á. Penica et.al (2) were the first group who cloned and expressed hTNF-á by construction of cDNA library. Other authores by classical cloning methodologies achieved cloning process with a various number

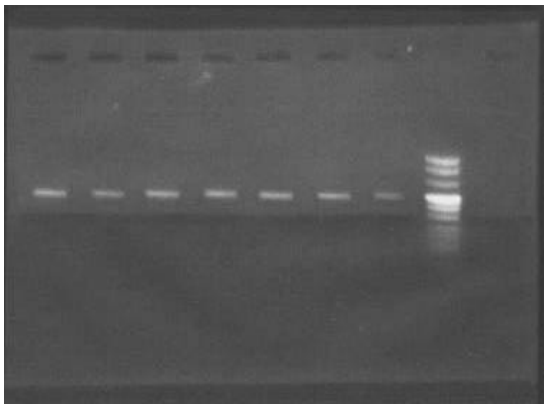

Fig 5: Gel electrophoresis of the PCR products of recombinant microorganisms:
Line 1: PCR of a hTNF-alpha sample. Line 2, 3, 4, 5, 6 and 7: white colonies. Line 8: Size marker. Line 9: A blue colony

of vectors and hosts, such as *E.coli* (2,3,4) as yeast (11). As the construction of cDNA library is a time-consuming and laborious method, it has been substituted with a RT-PCR cloning methodology which is more reproducible, more efficient and faster. PCR cloning of TNF-á of the ovine (12) murine (13) rabbit (14), and human (15) and some others (16,17,18,19) have been reported in literature. In this study, by using specific primers for hTNF-á an optimized one-tube RT-PCR variant was employed. A restriction site for Hind III was placed in reverse primer to obtain a 504 bp fragment corresponding to mature hTNF-á (Fig.3). From the study of RT-PCR cloning of hTNF-á induced HL-60 cell, formation of a 615 bp size is reported. No further studies are available to confirm the correspondence and necessity of this long fragment to mature hTNF-á (15). The sequence of cDNA fragment has been studied by restriction digestion mapping (2,3,6). In this investigate partial and complete digestion of PCR product with Pvu II for 60 minutes were studied. The visualized electrophoretic analysis showed t hree significant fragments (117 bp, 153 bp and 234 bp) on an EtBr stained 1.5% agarose gel (Fig.4). The electrophoretic characteristics of digested fragments collectively were comparable to the published data (2,3).
Transformation of recombinant plasmid r esulted in 6 white colonies, which were verified by PCR (Fig.5). Dephosphorylation of linearized plasmid before ligation step prevented the self-ligation and reduced background. ELISA test on these 6 colonies showed 5 positive colonies with about 125-(pg/g Bacterial wet weight) hTNF-á

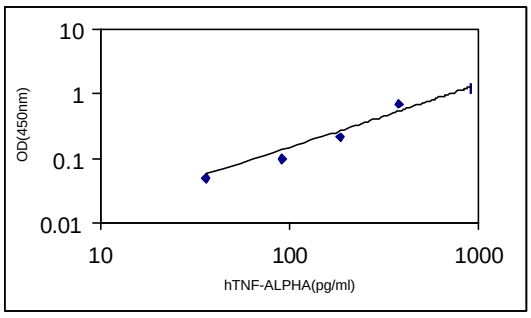

Fig. 6: Standard curve of ELISA test for hTNF-alpha

production (Fig.6). Biological activity assay showed that there were cytotoxic effects in the cell lysates of ELISA positive colonies but no cytotoxic effect was determined for a negative colony, which was not bearing the cDNA for hTNF-á. The activities of the samples were between 420 to 460 (U/g bacteria wet weight). This result shows that only a part of the produced recombinant hTNF-á is biologically active.

In this study, the cDNA for mature human tumor necrosis factor alpha was prepared and successfully cloned in a plasmid vector. The amount of production was small to the weakness of promoter. Subcloning of this cDNA in a potent expression vector will increase the production rate. Since, the native hTNF-á, has lost its therapeutic importance in cancer therapy, there has been many hopes for the preparation of some effective and non toxic analoges of hTNF-á as a potent therapeutic agent against some kind of cancers and tumors. The other advantage is the preparation of analoges which are able to compete with hTNF-á in binding to the hTNF-á receptores.

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