Rapid and Sensitive Assay of Tumor Necrosis Factor-α Gene Transcription

Xiaoying Zhang,[1] Jianping Ye,[2] Liying Wang,[2] Jiradej Manosroi,[3] Xianglin Shi,[2] and Yon Rojanasakul[1,3]

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INTRODUCTION

Tumor necrosis factor-alpha (TNF-α) is a cytokine that appears rapidly in response to infection and trauma (1,2). It plays a beneficial role as an immunostimulant and important mediator of host defense against infectious agents and malignant tumors (2,3). However, when abnormally regulated, i.e., because of an autoimmune response or tissue injury, TNF-α can cause severe systemic toxicity and even death. High levels of TNF-α have been associated with various pathological states, such as cachexia and sepsis, and transgenic animals overexpressing TNF-α develop pathological inflammatory conditions, including polyarthritis and central nervous system demyelination (4,5). Moreover, neutralization of TNF-α activity leads to improvement in models of inflammatory diseases and in patients with arthritis and sepsis (4,6). Because of the physiologic and pathologic importance of TNF-α, several TNF-α-modulating drugs have been developed for therapeutic purposes. Major therapeutic strategies developed thus far have been focused on TNF-α antibodies and antagonists. Antibodies have several advantages, such as target specificity over other therapeutic agents; however, their use has been largely limited resulting from their potential immunotoxicity. Although great efforts have been made to humanize the antibodies to avoid their immunogenicity, several alternative strategies, including those using small non-immunogenic molecules, increasingly have been investigated as antibody replacements.

A major problem concerning the development of TNF-α-modulating drugs has been the lack of rapid and effective assays for TNF-α. Traditional bioassays and enzyme-linked immunosorbent assay (ELISA), although sensitive, are time-consuming and cost-prohibitive, especially when a large number of drug candidates need to be tested. In addition, these assays do not permit evaluation of drug's action at the gene transcription level in which most developing TNF-α drugs act. Therefore, there is a need for a transcription-specific, more-rapid assay for TNF-α. In this study, we report a new, highly sensitive and specific assay for TNF-α gene transcription. This assay is based on the use of TNF reporter cell line, which was created by stable transfection of a macrophage cell line (RAW264.7) with a TNF-luciferase plasmid vector. The macrophage cell line was chosen because macrophages are the primary source of TNF-α production in the body (7). This reporter cell line provides several potential advantages over existing methods, which include: 1) it can be propagated or maintained indefinitely and is therefore always available when needed; 2) it allows easy automation and high throughput applications, i.e., through the use of 96-well plates; and 3) it permits simultaneous detection of TNF-α gene transcription and protein expression. Our results showed that this reporter cell line was sensitive to TNF-α activation and provided test results that correlate well with the ELISA results.

MATERIALS AND METHODS

Cell Culture and Plasmid Preparation

The macrophage cell line RAW 264.7 was obtained from the American Type Cell Culture Collection (Rockville, MD). The cells were grown in DMEM (GIBCO BRL Life Technologies) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. They were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The reporter plasmid contains a TNF-α promoter fragment (–1260/+60) linked to a luciferase reporter gene (a kind gift from Dr. Peter Johnson, National Cancer Institute, Frederick, MD). The reporter plasmid was introduced into DH5α bacteria using a transformation procedure, and the positive colony was selected in ampicillin containing medium. The colony was expanded in 300 ml of LB medium at 37°C overnight. The cells were collected, and the DNA was extracted and purified using the Qiagen Endofree Maxiprep kit (Qiagen, Chatsworth, CA). The $pCDNA_3$ plasmid containing the gentamicin (G418) resistant gene was prepared similarly. This plasmid was used in co-transfection studies for the preparation of a TNF-α reporter stable cell line.

Stable Transfection

Approximately 1×10^6 cells were plated on a 12-well plate and allowed to grow for 24 hours before the transfection. The cells were washed twice with DMEM and incubated for 4 hours in 1 ml of transfection medium containing TNF-luciferase plasmid (1.5 μg/ml), $pCDNA_3$ plasmid (0.5 μg/ml), and LipofectAMINE (GIBCO Life Technologies) (24 μg/ml). After transfection, the cells were washed and cultured in a growth medium containing 10% FBS. Twenty-four hours later, the medium was replaced with a selection medium containing 0.5 μg/ml of G418. The medium was changed every 2 days, and the culture was allowed to continue for 2 weeks.

Cloning of Reporter Cells

After a 2-week culture in the selection medium, survived cells were collected and plated in a 96-well plate at a cell density of 1 cell/well for an additional week. This subcloning process was performed to ensure a homogenous population of the cells. Ten positive colonies subsequently were selected

[1] West Virginia University School of Pharmacy, Department of Pharmaceutical Sciences, P.O. Box 9530, Morgantown, West Virginia 26506.

[2] National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Morgantown, West Virginia 26505.

[3] Chiangmai University, Faculty of Pharmaceutical Sciences, Chiangmai, Thailand 50200.

[4] To whom correspondence should be addressed. (e-mail: yrojanasakul@hsc.wvu.edu)

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and tested for luciferase activity. For TNF-α stimulation studies, cells were treated with lipopolysaccharide (LPS), a known stimulator of TNF-α (8), in the presence or absence of TNF-α inhibitors. At indicated times after the treatment, the cell samples were collected and analyzed for TNF-α gene expression and protein production using luciferase and ELISA assays, respectively.

Measurements of Luciferase Activity and TNF-α Protein Production

Luciferase activity was measured by enzyme-dependent light production using a luciferase assay kit (Promega, Madison, WI). After each experiment, cells were washed and incubated at room temperature for 10 minutes in 250 μl of lysis buffer (Promega). Ten-microliter samples were then taken and loaded into an automated luminometer (BioRad, Hercules, CA). At the time of measurement, 100 μl of luciferase substrate was automatically injected into each sample, and total luminescence was measured over a 20-second time interval. Output is quantitated as relative light units (RLU) per μg protein of the sample. For analysis of TNF-α protein, cell-free supernatants were used. TNF-α levels were determined using a TNF-α ELISA kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Luciferase Activity of Stably Transfected Reporter Cells

The macrophage RAW 264.7 cells were co-transfected with the TNF-luciferase plasmid and the $pCDNA_3$ plasmid that provides a selectable marker. After a 2-week selection period in G418 containing medium, ten colonies of the survived cells were selected by limit dilution. Reporter activity was then determined in the 10 chosen clones using the luciferase assay. The results showed that all 10 clones exhibited a basal luciferase activity, i.e., in the absence of any stimulation (Fig. 1A). In the presence of LPS stimulation, all of the clones exhibited strong and inducible luciferase activities (Fig. 1B). The magnitude of induction by LPS in these clones ranged from 15- to 27-fold over the non-treated controls. These results indicate that all of the chosen clones were successfully transfected with the TNF-luciferase reporter and $pCDNA_3$ plasmid. Clones 5 and 6 exhibited the greatest induction in response to LPS stimulation (27-fold), which suggests that these clones were most efficiently transfected with the TNF-luciferase plasmid. Clone 6 was chosen in all subsequent studies.

Luciferase Activity Parallels TNF-α Protein Production

A key question concerning the use of the reporter cell line as a means to detect TNF-α activation is whether or not

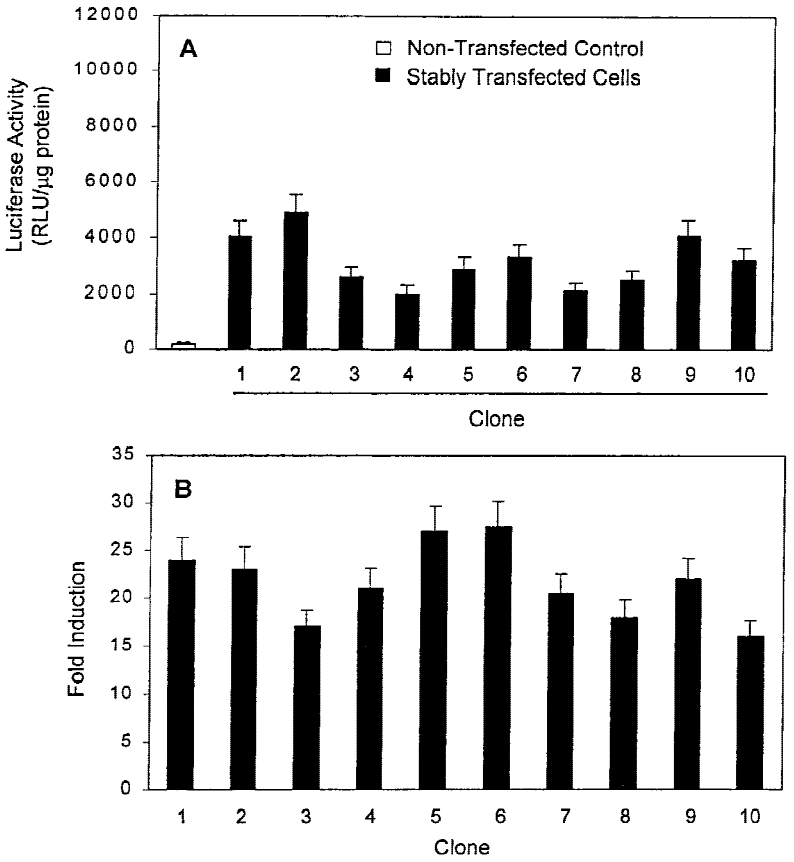

Fig. 1. TNF-α luciferase activity in stably transfected RAW 264.7 cells. The cells (10^5/well) were plated in a 96-well plate for 1 day, after which they were treated with LPS (1 μg/ml) for 6 h at 37°C. (A) Basal luciferase activity. (B) Fold induction of luciferase activity in the presence of LPS. Data are shown as the mean ± SEM, $n = 4$.

this method actually reflects TNF-α expression. To address this question, both TNF-α production and luciferase activity were simultaneously determined after LPS treatment. The reporter cells were treated with LPS for up to 6 hours, after which cell supernatants were harvested and analyzed for TNF-α protein using ELISA. The cellular portions of the samples were also collected, lysed, and the lysates were analyzed for TNF-α transcription activity using the luciferase assay. The results showed that TNF-α protein production and luciferase activity exhibited similar kinetic patterns in response to LPS (Fig. 2, A and B). Because LPS is known to stimulate TNF-α production via transcriptional activation of the TNF-α gene promoter (8), our results therefore indicate that the luciferase activity of the reporter cell line reflects the cellular state of TNF-α gene activation.

Inhibition of Luciferase Activity and TNF-α Protein Production by Nuclear Factor-κB Inhibitor

Gene expression in eukaryotic cells is governed by nuclear transcription factors. These are proteins that interact with specific DNA regulatory elements, i.e., gene promoters, and induce or retard the transcriptional rate. The nuclear factor-κB (NF-κB) is a key transcription factor involved in the activation of TNF-α gene (8). Inhibition of NF-κB would therefore inhibit TNF-α gene expression and thus protein production. In this study, we tested whether an investigational TNF-α-modulating agent, caffeic acid phenethyl ester (CAPE), a compound known to inhibit NF-κB activation (9), could inhibit LPS-induced TNF-α gene and protein expression in the reporter cell line. Cells were treated with LPS in the presence or absence of CAPE, and TNF-α protein production and luciferase activity were determined. The results showed that CAPE was able to inhibit both the TNF-α production and luciferase activity (Fig. 3, A and B). Parallel cytotoxicity studies using lactate dehydrogenase assay indicated that this compound was not toxic to the cells under the experimental conditions (results not shown). Thus, our results demonstrated that the reporter cell line could be used to assess the inhibitory effects of TNF-α-modulating agents.

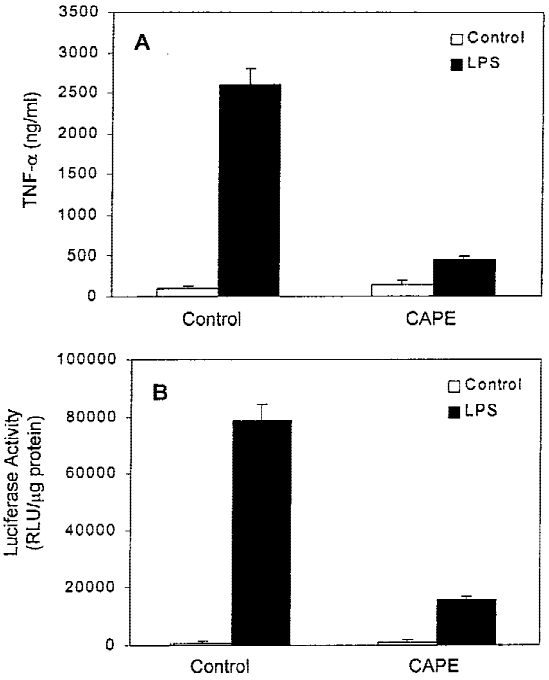

Fig. 3. Inhibition of TNF-α protein production and luciferase activity by NF-κB inhibitor. Stably transfected RAW 264.7 cells (10^5/well, clone 6) were plated in a 96-well plate for 1 dayafter which they were treated with LPS (1 μg/ml) for 6 hours in the presence or absence of the NF-κB inhibitor CAPE (5 μg/ml). (A) TNF-α protein levels determined by ELISA. (B) Transcription activity determined by luciferase assay. Data are shown as the mean ± SEM, $n = 4$.

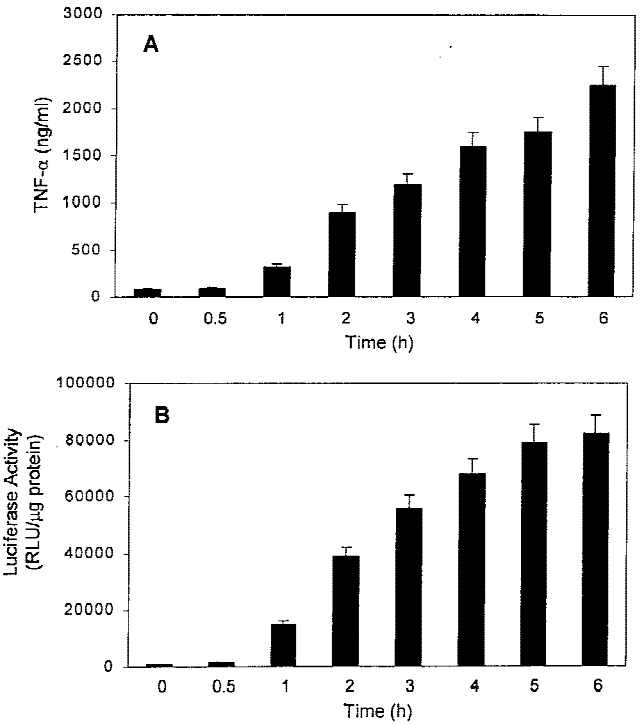

Fig. 2. Time course of TNF-α protein production and transcription activity in LPS-treated cells. Stably transfected RAW 264.7 cells (10^5/well, clone 6) were plated in a 96-well plate for 1 day after which they were treated with LPS (1 μg/ml) at 37°C. A) TNF-α protein levels determined by ELISA. B) Transcription activity determined by luciferase assay. Data are shown as the mean ± SEM, $n = 4$.

In summary, we have developed a highly specific and sensitive assay for TNF-α gene transcription. This assay is based on the use of a macrophage cell line, which was stably transfected with the TNF-luciferase reporter plasmid. This assay was successfully used to assess the stimulatory and inhibitory effects of TNF-α-modulating agents. Because of its potential for high throughput applications and automation, this method should prove useful in testing and screening a large number of investigational agents.

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