Gene transduction of tristetraprolin or its active domain reduces TNF-α production by Jurkat T cells

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Abstract. Tristetraprolin (TTP) is a physiological regulator of tumor necrosis factor (TNF)-α production. It destabilizes TNF-α mRNA by binding to the AU-rich element located in the 3' region of TNF-α mRNA. We wished to determine how transducing the TTP gene or its fragment gene encoding its biological active site, the tandem zinc finger (TZF) domain, affects TNF-α production, cell viability and growth of Jurkat T cells. Jurkat T cells were transduced with either the TTP or the TZF gene using retrovirus vectors. Cell growth and apoptosis was analyzed. Expression of genes before or after appropriate stimuli was measured by real-time PCR. In addition, production of the TNF-α protein was measured by enzyme immunoassay. The transduction of either gene reduced TNF-α mRNA levels under unstimulated conditions, and reduced the response to phytohemagglutinin stimulation. Production of TNF-α protein upon stimulation was also decreased in TTP/TZF-transduced cells. Transduction of either gene also affected the expression of granulocyte-macrophage colony-stimulating factor mRNA in a similar fashion, but not that of c-myc. The growth rate of TTP-transduced Jurkat T cells tended to be slower than that of TZF- or mock-transduced cells. TTP-transduced cells were more susceptible to campthothecin-induced apoptosis than others. Our results indicate that either TTP or TZF gene transduction using retrovirus vectors can reduce the production of TNF-α in Jurkat T cells although some differences were noted between TTP and TZF in cell growth and occurrence of apoptosis. These results suggest that TTP may be a potential target for new therapies against RA.

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Abbreviations: RA, rheumatoid arthritis; TNF, tumor necrosis factor; TTP, tristetraprolin; ARE, AU-rich element; 3' UTR, 3' untranslated region; GM-CSF, granulocyte-macrophage colony-stimulating factor; TZF, tandem zinc finger; DMEM, Dulbecco's modified Eagle's medium; CO_2, carbon dioxide; VSV-G, vesicular stomatitis virus G protein; EGFP, enhanced green fluorescent protein; TTP-EGFP, pGCDNsamIRESEGFP-TTP-His-FLAG; TZF-EGFP, pGCDNsamIRESEGFP-TZF-His-FLAG; PCR, polymerase chain reaction; PHA, phytohemagglutinin; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IRES, internal ribosomal entry site; ELISA, enzyme-linked immunosorbent assay; IL-3, interleukin-3

Key words: AU-rich element, post-transcriptional regulation, retrovirus vector, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is an inflammatory disorder affecting mainly the joint synovium. Continuous inflammation causes joint destruction and greatly impairs the quality of life of the patients. To develop novel therapies for RA, investigations on RA are being performed to elucidate the pathogenesis of RA and the mechanisms that lead to joint erosion and destruction. Although the precise mechanism is still unclear, it is generally accepted that tumor necrosis factor (TNF)-α plays an important role in the pathogenesis of RA, through a number of studies on TNF-α knockout mice, TNF-α receptor knockout mice and TNF-α transgenic mice (1-3).

It is now well-established that the administration of anti-TNF-α antibodies to RA patients or mice with experimentally-induced arthritis results in a marked decrease in the severity of the arthritis (1,4-6). Inhibition of the functions of TNF-α by antibodies or decoy receptors is one of the most important methods currently available to control the activity of RA. Further elucidation of the mechanism that regulates TNF-α production may provide a clue for the development of new therapies of RA.

A number of steps regulate the production of proteins, including TNF-α. Generally, transcriptional regulation is recognized as the main step that controls protein synthesis, and many studies have demonstrated the importance of

transcriptional regulators including nuclear factor-κB, nuclear factor of activated T cells, and activator protein-1 (7-10). However, recently, it has become apparent that post-transcriptional regulation is also important in the regulation of protein production, especially in that of cytokines (11,12).

A number of molecules are involved in post-transcriptional regulation of TNF-α production (13,14). Among those molecules, tristetraprolin (TTP) is one of the most investigated to date (15). TTP is a widely distributed phosphoprotein encoded by an immediate-early gene, *Zfp-36* (16). It has been demonstrated that TTP knockout mice, although appearing normal at birth, eventually manifest marked medullary and extramedullary myeloid hyperplasia associated with cachexia, erosive arthritis, dermatitis, conjunctivitis, glomerular mesangial thickening, and high titers of anti-DNA and anti-nuclear antibodies (17). These symptoms could be prevented by administration of anti-TNF-α antibodies. Thus, the phenotypes observed in these mice are caused mainly by TNF-α overproduction. It was demonstrated that TTP induces destabilization of TNF-α mRNA by binding to the AU-rich element (ARE) in the 3' untranslated region (3' UTR) of TNF-α mRNA, which accelerates mRNA degradation by enhancing the removal of the polyadenylated tail from the mRNA (18), thereby reducing the production of TNF-α protein (19). Granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA also has a similar structure in its 3' UTR (20).

Past studies reported that the tandem zinc finger (TZF) region, consisting of two zinc finger motifs, is the biological active site of TTP, and can bind ARE and destabilize the mRNA of TNF-α (21,22). On the other hand, it has been reported that the introduction of the TTP gene induces cell apoptosis (23,24).

In a recent report (25), we investigated the quantity of TNF-α and TTP mRNAs in synovial tissues of RA patients. Interestingly, serum C-reactive protein was significantly increased in patients whose synovium had a lower TTP/TNF-α gene expression ratio. These results implied that individual differences in TTP production influence the activity of RA, and that inappropriate TTP production may be one of the factors that cause higher RA disease activity. We considered that TTP is a suitable potential therapeutic target of RA, and hence it is important to investigate the detailed function of TTP and the consequences of TTP-overexpression.

In this study, we transduced the TTP gene or the TZF gene, to a human T cell leukemia cell line, Jurkat T cells, using retrovirus-derived vectors. We examined the effect of these transductions on the expression of the TNF-α gene and production of TNF-α protein, as well as the effects on cell growth and induction of apoptosis. The results showed that overexpression of TTP or its active domain reduced TNF-α production by Jurkat T cells. The retrovirus-derived vectors showed high efficiencies for the transductions performed in this study, providing a valuable tool for further studies.

Materials and methods

Cell culture. 293gp is a packaging cell line derived from U293 cells. PG13 represents also a packaging cell line derived from NIH3T3 cells. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1,000 mg/l glucose, L-glutamine and sodium bicarbonate (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (BioWest, Loire Valley, France), penicillin G sodium (100 U/ml) and streptomycin sulfate (100 μg/ml) (Invitrogen, Carlsbad, CA). These cells were maintained at 37˚C in a humidified incubator under 10% carbon dioxide (CO_2) in air. Jurkat T cells and their transduced cells were cultured in RPMI-1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich Japan) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (BioWest), penicillin G sodium (100 U/ml) and streptomycin sulfate (100 μg/ml) (Invitrogen). These cells were maintained at 37˚C in humidified incubator under 5% CO_2 in air.

Recombinant retrovirus vector. The structure of GCDNsap and the method to produce recombinant retroviruses packaged in the vesicular stomatitis virus-G (VSV-G) protein have been described previously (26). To construct the GCDN/OVA vector with full-length OVA cDNA and the enhanced green fluorescent protein (EGFP) gene, a *Bam*HI-*Xho*I fragment containing full-length OVA cDNA, and a *Xho*I-*Cla*I IRES/EGFP fragment obtained from GCsamI/E (27) were inserted into the GCDNsap vector. The vectors were converted to the corresponding recombinant retroviruses packaged in the VSV-G by transduction into 293gp (22). The virus titer of GCDN/OVA was 9.0×10^6 infectious units/ml on Jurkat T cells.

Orientation of the cDNA inserts and the integrity of the DNA sequences were confirmed by sequencing using the ABI PRISM Big Dye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) followed by comparison with the published sequences (TTP accession no. MN_003407) on the National Center for Biotechnology Information database. The pGCDNsamIRESEGFP-TTP-His-FLAG (TTP-EGFP) expression construct contains the hTTP coding region (nucleotides 60-1,040) cloned in-frame with the 3' His and FLAG tags ligated in between *Not*I and *Sal*I sites. The pGCDNsamIRESEGFP-TZF-His-FLAG (TZF-EGFP) expression construct contains the fragment, which codes the TZF (nucleotides 315-611), the biological active domain of TTP (24). The TZF fragment was ligated into the vector in-frame with the 3' His and FLAG tags between *Not*I and *Sal*I restriction enzyme sites. TTP and TZF fragments were generated by polymerase chain reaction (PCR) using primers corresponding to the 15 nucleotides at each end of the fragment and flanked by a *Not*I site at the 5' end site and a *Sal*I site at the 3' end site. The sequence is equal to the published TTP gene sequence except for a silent mutation, C→T, at position 368 (Fig. 1). The pGCDNsamIRESEGFP vector without any modification was used as the control mock vector.

Establishment of retrovirus-producing cell lines. TTP-EGFP or TZF-EGFP (at 20 μg) and VSV-G (at 10 μg) were co-transfected into 293gp cells using MBS mammalian transfection kit (Stratagene, La Jolla, CA) according to the instructions provided by the manufacturer. Each supernatant was collected, and protamine sulfate was added (final concentration: 10 μg/ml). Two ml of each supernatant with protamine sulfate was added to PG13 cells with 2 ml of

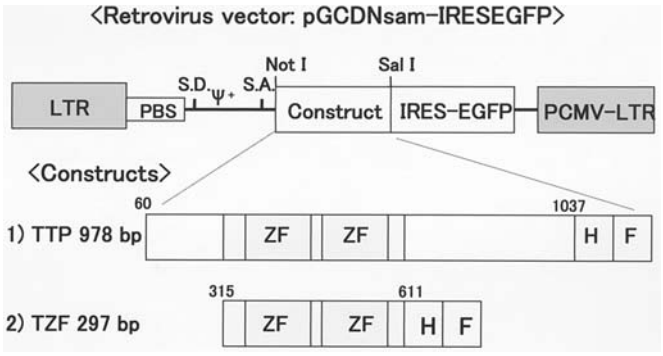

Figure 1. Schematic diagram of the retrovirus vector, pGCDNsamIRESEGFP, and the constructs of tristetraprolin (TTP) and its tandem zinc finger domain (TZF). An internal ribosomal entry site (IRES)-enhanced green fluorescent protein (EGFP) fragment was added downstream of the multicloning site of the retrovirus vector, pGCDNsamIRESEGFP. The TTP construct (total 978 bp: 60-1,037 in MN_0003407) and the TZF construct (total 297 bp: 315-611 in MN_003407) were amplified by PCR, and His6 (H) and FLAG (F) tag sequences were added. Each of these constructs was flanked by a *Not*I site at the 5' end site and *Sal*I site at the 3' end site, and was ligated into the retrovirus vector.

DMEM medium cultured in 6-well plates (Corning, New York, NY). The PG13 cells were centrifuged at 2,000 rpm for 30 min at 32˚C and then incubated at 37˚C in a humidified incubator under 10% CO_2. This process was repeated three times and the cells were incubated for a few days. Limiting dilution was performed for transduced cells, and the supernatant from the wells that contained cell colonies was collected and RNA copies were quantified by Northern dot blot hybridization using ^{32}P-conjugated TZF probe. The infectious efficiencies were analyzed for selected PG13 cells by detecting the inserted EGFP using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ).

Transduction of the TTP or TZF gene to Jurkat T cells. To transduce Jurkat T cells with pGCDNsamIRESEGFP inserted with TTP, TZF or mock, $1.0x10^5$ cells were plated in 4 ml of the culture medium and 1 ml of each supernatant from each pGCDNsamIRESEGFP-transduced cell line, with protamine sulfate, followed by centrifugation at 2,000 rpm for 30 min. Two days after transduction, the cells were harvested and washed twice with phosphate-buffered saline, and subjected to flow cytometric analyses, using a FACScalibur analyzer. After the analysis, limiting dilution was performed using RPMI-1640 medium with hybridoma enhancing supplement (Sigma-Aldrich Japan) to obtain cell clones.

Western blotting. Cloned Jurkat T cells transduced with TTP-EGFP, TZF-EGFP or mock viruses ($1.0x10^7$ cells) were cultured and the medium was changed 1 day before harvesting. Cells were collected, centrifuged at 1,000 rpm for 5 min, resuspended in 100 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.5% NP40, and 2 mM phenylmethylsulfonyl fluoride) and incubated for 5 min on ice. Cell lysates were centrifuged at 15,000 rpm for 5 min. The supernatant was collected and immunoprecipitated using M2 affinity gel (Sigma, St. Louis, MO). The immunoprecipitant was separated on 4-20% gradient SDS polyacrylamide gels (Daiichikagaku-yakuhin, Tokyo), transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and the recombinant TTP or TZF protein expressed in the cells was detected by anti-FLAG M2 peroxidase-conjugated antibody (Sigma).

Cell proliferation assay. The cell growth of the transduced cell clones and control cells was estimated by directly counting the number of viable cells. For each clone, $1.0x10^5$ viable cells were plated in 3 ml of culture medium, and were counted every day for 5 days. Cell culture was performed in triplicate, and the experiment was repeated three times to confirm the data obtained.

Induction and detection of apoptosis. To induce apoptosis of Jurkat T cells, camptothecin (Biovision, Mountain View, CA) was added to the culture medium at a final concentration of 6 μg/ml, and the cells were incubated for 4 h. Annexin V-phycoerythrin-conjugated antibody (Becton Dickinson, San Jose, CA) was used to estimate the percentage of cells undergoing apoptosis by flow cytometry. The assays were performed according to the methods described in the product manuals.

Synthesis of cDNA for real-time PCR. Jurkat T cells transduced with TTP, TZF or mock viruses were pre-incubated in 6-well culture plates (Corning) ($1.0x10^6$ cells/well suspended in 3 ml RPMI medium). Phytohemagglutinin (PHA) (Wako, Osaka, Japan) was added at a final concentration of 5 μg/ml to the wells and the cells were harvested at the time course indicated in Figs. 4b and 5. Cells were spun down to pellets and total RNA was extracted from the cell pellets using Isogen (Nippon Gene, Tokyo), and cDNA was synthesized using RevertAid first strand cDNA synthesis kit (Fermentas, Hanover, MD), following the instructions provided by the manufacturer.

Real-time PCR. Synthesized cDNA samples were amplified with specific primers and fluorescence-labeled probes for the genes of interest, and accumulation of amplified products was monitored by an ABI 7700 sequence detector (Applied Biosystems Japan, Tokyo). qPCR Mastermix was purchased from Eurogentec (Seraing, Belgium), the magnesium concentration was 5 nM final, the primer concentration was 200 nM final, and the probe concentration was 100 nM final. Thermal cycler conditions were: 50˚C for 2 min and 95˚C for 10 min, then 45 cycles of 95˚C for 15 sec and 60˚C for 1 min. Serial dilutions of standard samples were included and standard curves for the gene of interest and the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene were generated in every assay. All measurements were performed in triplicate. The level of gene expression was calculated from the standard curve, and expressed relative to that of GAPDH gene. The sequences of specific primers and probe are as follows: TNF-α forward, 5'-TGGAGAAGGGTGACCGACTC-3'; TNF-α probe, 5'-CGCTGAGATCAATCGGCCCGACTAT-3'; and TNF-α reverse, 5'-TCCTCACAGGGCAATGATCC-3'. The primers and probe for GM-CSF, C-MYC, and GAPDH were purchased from Applied Biosystems.

Enzyme immunoassays for measurement of TNF-α. Jurkat T cell clones transduced with TTP, TZF or mock viruses were cultured with 5 μg/ml of PHA for 12 h at a cell concentration

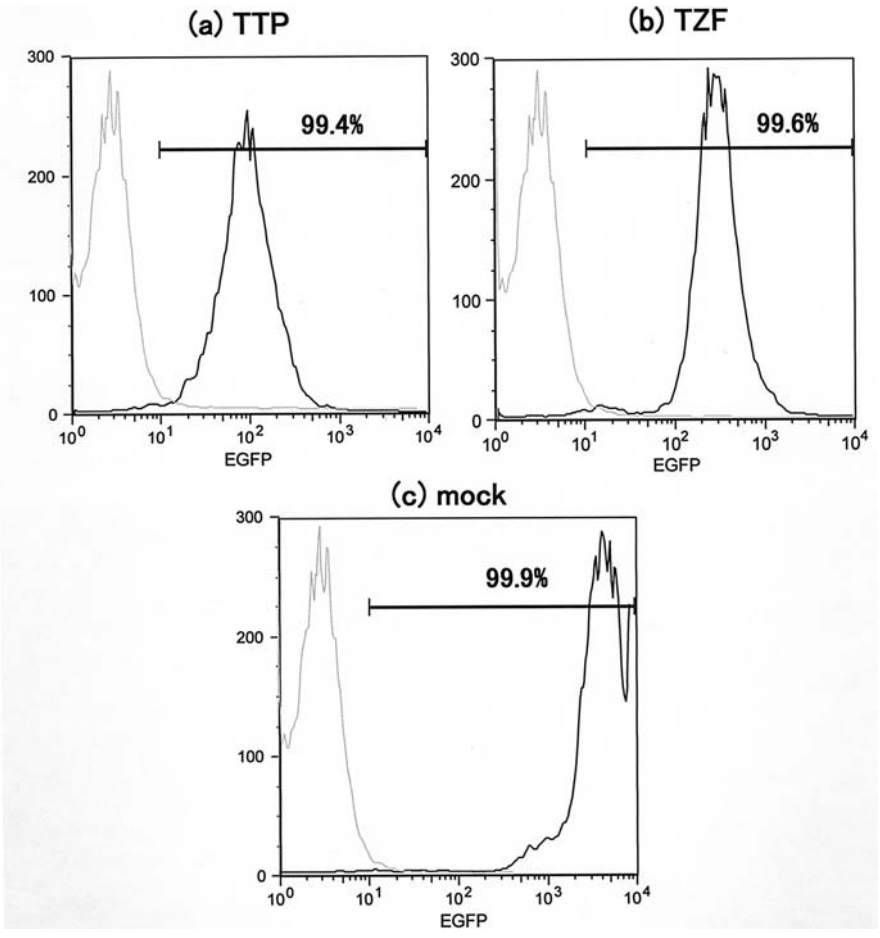

Figure 2. Flow cytometric analysis (a-c) and Western blot analysis (d) of retrovirus vector transduced cells. Jurkat T cells were transduced with retrovirus vector pGCDNsamIRESEGFP containing tristetraprolin (TTP) (a), its tandem zinc finger domain (TZF) gene fragment (b), or no insert (mock) (c). Cell clones were established from transduced cells, and were subjected to flow cytometry. Enhanced green florescent protein (EGFP) was detected in this analysis. Solid line, transduced Jurkat T cells; dotted line, untransduced Jurkat T cells (results representative of three independent clones). Cell lysates were obtained from gene transduced clones and immunoprecipitated (see Materials and methods for details). The immunoprecipitated material was subjected to Western blot analysis using anti-FLAG antibody (d). T, TTP gene transduced Jurkat T cells; Z, TZF gene transduced Jurkat T cells; M, mock-transduced Jurkat T cells; J, untransduced Jurkat T cells; PT, TTP-transfected 293 cells (positive control); PZ, TZF-transfected 293 cells (positive control).

of $1.0x10^6$ cells/ml. The supernatant was collected, concentrated five times by using Centricon (Millipore Corporation, Billerica, MA) and stored at -80˚C. The concentration of TNF-α was measured using Human TNF-α ready-Set-Go Kit (eBiosience, San Diego, CA), according to the protocol provided by the manufacturer. All assays were performed in duplicate.

Statistical analysis. Student's t-test was used to compare the numbers of TTP- or TZF-transduced Jurkat T cells and mock-transduced Jurkat T cells, and was also used to compare the concentration of TNF-α protein between TTP/TZF-transduced Jurkat T cells and mock-transduced Jurkat T cells. All data were expressed as mean ± SD. A P-value <0.05 denoted the presence of a statistically significant difference.

Results

TTP/TZF gene-containing retrovirus vectors are efficiently transduced into Jurkat T cells. We prepared retrovirus vectors inserted with TTP or TZF encoding genes between the *Not*I and *Sal*I sites as shown in Fig. 1. In these retrovirus vectors, the internal ribosomal entry site (IRES) was present between the inserted genes and the EGFP gene. Thus, the inserted and EGFP genes were translated separately and simultaneously. The percentage of EGFP-positive Jurkat T cells after transduction was 80.8% for TTP-EGFP, 92.5% for TZF-EGFP and 95.6% for the pGCDNsamIRESEGFP vector without any modification (mock-EGFP) (data not shown). The transduced cells were submitted to limiting dilution to obtain cells stably expressing TTP or its active fragment. Obtained clones were assayed for

EGFP expression by flow cytometry, to confirm the efficacy of the limiting dilution. Three clones for TTP- and TZF-transduced cells were established, to rule out the possibility that the results shown hereafter are the consequences of the breakdown of a gene by the insertion of the TTP or TZF genes in a particular clone. The percentages of EGFP expressing cells were 99.4%, 99.6%, and 99.8% for the TTP-, TZF-, and mock-transduced cell clones, respectively (Fig. 2a-c, results representative of three independent clones). However, the fluorescence intensity was highest in mock-transduced cells, followed by TZF-transduced cells, while TTP-transduced cell clones tended to have the lowest fluorescence intensity of all clones established. The cell extracts were immunoprecipitated with anti-FLAG antibody-bound beads, and then subjected to Western blot analyses to confirm that the Jurkat T cells transduced with TTP or TZF retroviral vectors indeed expressed TTP or TZF proteins. The protein extract from Jurkat T cells transduced with the TTP-EGFP retrovirus showed a band at approximately 43 kDa when detected by an anti-FLAG antibody, while that from TZF-EGFP retrovirus-transduced Jurkat T cells showed a band at approximately 15 kDa (Fig. 2d). The estimated molecular weights of recombinant products were in accordance with the sizes of the detected bands. These results indicated that the transduction and limiting dilution procedures were efficiently performed and the proteins were properly expressed.

Introduction of TTP and TZF genes has a small effect on the growth of Jurkat T cells. It is reported that full-length TTP induces apoptosis through the mitochondrial pathway when overexpressed, while TZF does not exhibit such a property (24). To evaluate whether the introduction of the TTP or TZF gene has such an effect on Jurkat T cells, we directly counted the number of cells every day for 5 days to estimate the cell growth (Fig. 3a). At day 5, the mean ± SD number of Jurkat T cells transduced with TTP-EGFP (53.2±20.5 cells/μl) tended to be smaller than that of mock-transduced cells (104.2±23.1 cells/μl) (P=0.0457 by Student's t-test). Cells transduced with TZF-EGFP (65.2±14.0 cells/μl) showed a similar trend, although to a smaller extent (P=0.0665 by Student's t-test). Such tendencies were repeatedly observed in several independent experiments. Next, we detected cells undergoing apoptosis by flow cytometry, using anti-annexin V antibody. The cells were analyzed at both unstimulated and stimulated conditions (final concentration of camptothecin, 6 μg/ml). After 4 h of stimulation, TTP-transduced Jurkat T cells showed the highest percentages of Annexin V-positive cells, suggesting that these cells are more sensitive to camptothecin-induced apoptosis (Fig. 3b).

Introduction of TTP and TZF genes reduces the expression of TNF-α mRNA and the production of TNF-α protein. To evaluate the effect of introducing the TTP or TZF gene into Jurkat T cells, we first measured the amount of TNF-α mRNA in non-stimulated conditions. The expression levels of TNF-α mRNA were lower in TTP-EGFP- and TZF-EGFP-transduced cells, compared with mock-transduced cells (Fig. 4a). However, TNF-α mRNA expression levels were extremely low in all cell groups. Next, we examined the effect of PHA stimulation on those cells. Stimulation of the cells with 5 μg/ml PHA for 1, 6, or 24 h reduced the level of TNF-α mRNA expression in TTP- and TZF-introduced Jurkat T cells at 1 and 6 h after stimulation, compared with mock-transduced cells. In TTP-

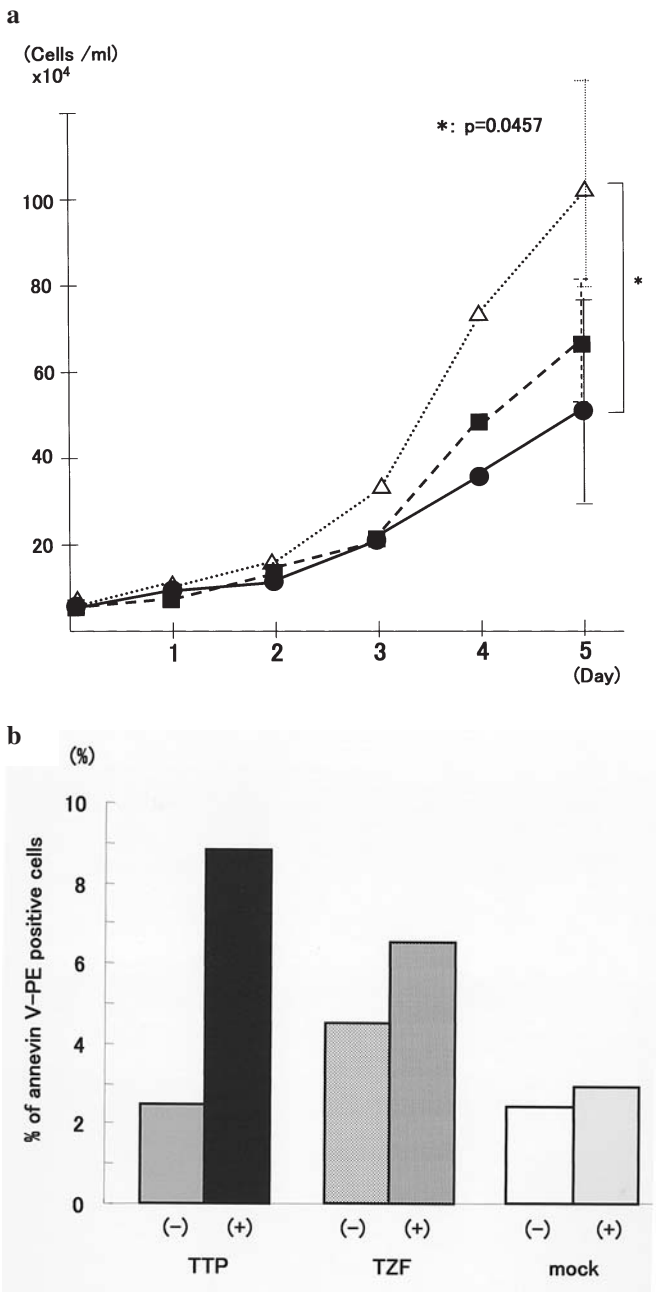

Figure 3. Growth and apoptosis of Jurkat T cells transduced with tristetraprolin (TTP) gene or its tandem zinc finger domain (TZF) gene. (a) Growth of TTP- or TZF-transduced Jurkat T cells. Cells were cultured starting from 1.0x10^5 cells in 3 ml of medium, and the number of live cells was counted daily for 5 days. Results are representative of three independent experiments. Each of the gene-transduced cells were equalized to 3.3x10^4 counts/ml. P-values by Student's t-test. Closed circles, TTP gene-transduced Jurkat T cells; closed squares, TZF gene-transduced Jurkat T cells; open triangles, mock-transduced Jurkat T cells. (b) Induction of apoptosis of TTP- or TZF-transduced Jurkat T cells. Apoptotic cells were detected using phycoerythrin-conjugated anti-annexin V antibody and were analyzed by flow cytometry, either unstimulated, or stimulated with camptothecin (6 μg/ml) for 4 h. Similar results were obtained in three independent experiments. TTP, TTP gene-transduced Jurkat T cells; TZF, TZF gene-transduced Jurkat T cells; mock, mock-transduced Jurkat T cells; (-), unstimulated cells; (+), cells stimulated with camptothecin (6 μg/ml) for 4 h.

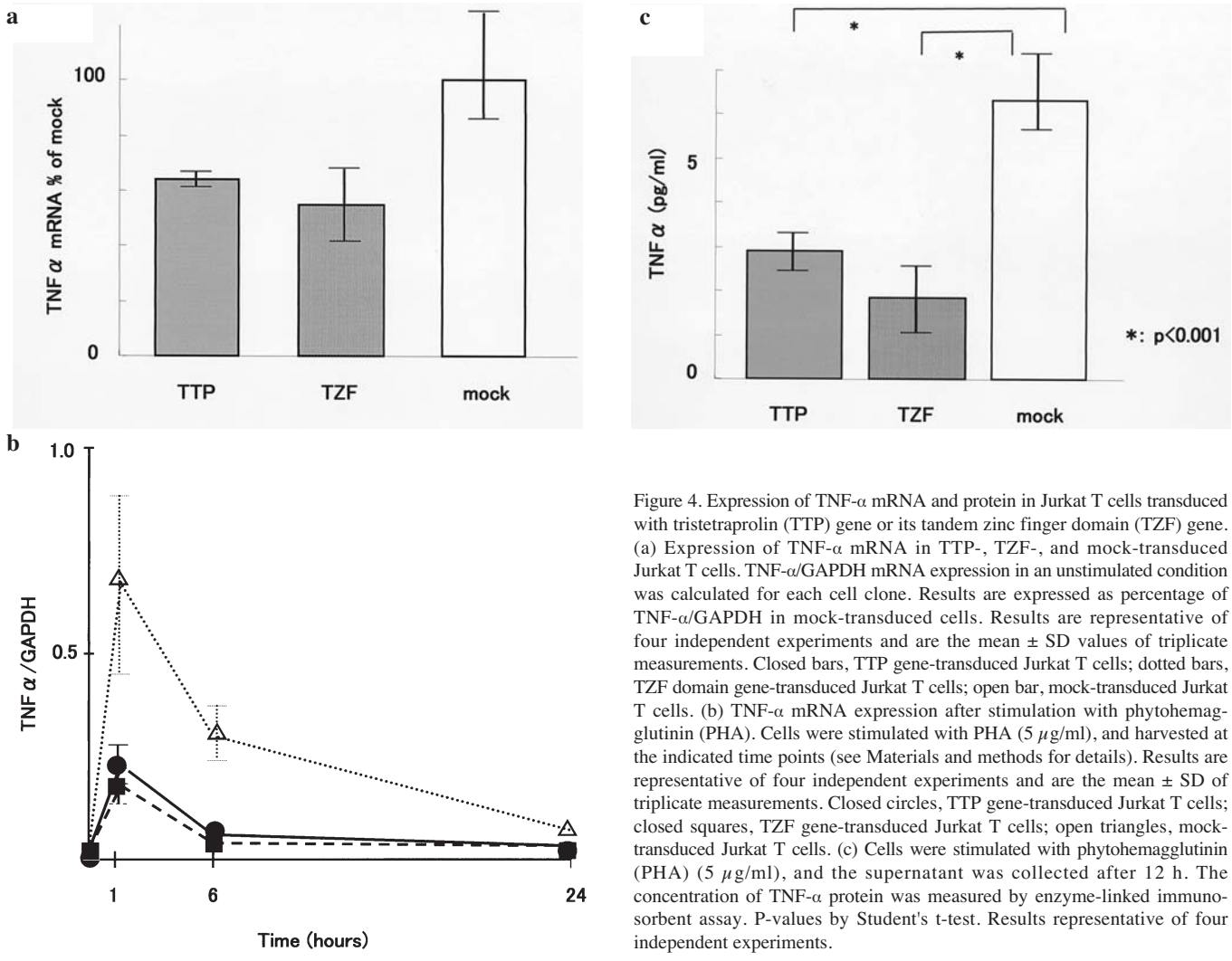

Figure 4. Expression of TNF-α mRNA and protein in Jurkat T cells transduced with tristetraprolin (TTP) gene or its tandem zinc finger domain (TZF) gene. (a) Expression of TNF-α mRNA in TTP-, TZF-, and mock-transduced Jurkat T cells. TNF-α/GAPDH mRNA expression in an unstimulated condition was calculated for each cell clone. Results are expressed as percentage of TNF-α/GAPDH in mock-transduced cells. Results are representative of four independent experiments and are the mean ± SD values of triplicate measurements. Closed bars, TTP gene-transduced Jurkat T cells; dotted bars, TZF domain gene-transduced Jurkat T cells; open bar, mock-transduced Jurkat T cells. (b) TNF-α mRNA expression after stimulation with phytohemagglutinin (PHA). Cells were stimulated with PHA (5 μg/ml), and harvested at the indicated time points (see Materials and methods for details). Results are representative of four independent experiments and are the mean ± SD of triplicate measurements. Closed circles, TTP gene-transduced Jurkat T cells; closed squares, TZF gene-transduced Jurkat T cells; open triangles, mock-transduced Jurkat T cells. (c) Cells were stimulated with phytohemagglutinin (PHA) (5 μg/ml), and the supernatant was collected after 12 h. The concentration of TNF-α protein was measured by enzyme-linked immunosorbent assay. P-values by Student's t-test. Results representative of four independent experiments.

or TZF-transduced cells, TNF-α mRNA expression levels returned to pre-stimulation levels, although the expression level in mock-transduced cells was still high at 6 h after PHA stimulation (Fig. 4b). Similar results were obtained using 2 additional clones for both TTP- and TZF-transduced cells (data not shown). Finally, we measured the amount of TNF-α protein by enzyme-linked immunosorbent assay (ELISA). After 12 h of PHA stimulation, TNF-α protein could be detected, although at low levels. TTP-EGFP- (2.90±0.48) and TZF-EGFP-transduced cells (1.87±0.82) produced significantly less amounts of TNF-α protein, compared with mock-transduced cells (6.33±0.87) (Fig. 4c, P<0.001 by Student's t-test).

TTP/TZF gene transduction affects GM-CSF but not c-myc mRNA expression. It is reported that similar to TNF-α mRNA, the expression of GM-CSF mRNA is regulated by ARE binding proteins (20). Thus, we quantified the GM-CSF mRNA expression levels with the same cDNA samples. The expression levels of GM-CSF mRNA were extremely low in all cell groups before PHA stimulation. The GM-CSF mRNA expression level of TTP-EGFP-transduced cells was slightly higher than TZF-EGFP- or mock-transduced cells at 1 h after PHA stimulation. Similar to TNF-α, the expression levels of GM-CSF mRNA in mock-transduced cells were higher than in TTP-EGFP- and TZF-EGFP-transduced cells at 6 and 24 h after PHA stimulation. GM-CSF mRNA expression levels returned to pre-stimulation levels by 24 h after PHA stimulation in TTP-EGFP- and TZF-EGFP-transduced cells (Fig. 5a). As a control, we measured the expression of c-myc mRNA, which is not influenced by regulation though ARE (28). The expression levels of c-myc mRNA in mock-transduced cells tended to be slightly lower at all time points compared with TZF-EGFP-transduced cells, which showed low expression levels at only 6 and 24 h after PHA stimulation. Unlike TNF-α and GM-CSF, we could not detect clear differences in c-myc mRNA expression among TTP-, TZF- and mock-transduced Jurkat T cells (Fig. 5b). Similar results were obtained using 2 additional clones for both TTP- and TZF-transduced cells (data not shown).

Discussion

The importance of TNF-α in the pathogenesis of RA is well-established, and the results of our recent study indicating that the expression level of the TTP gene in the synovial tissues correlates with the severity of RA (25), prompted us to conduct this study to determine whether TTP has the potential to

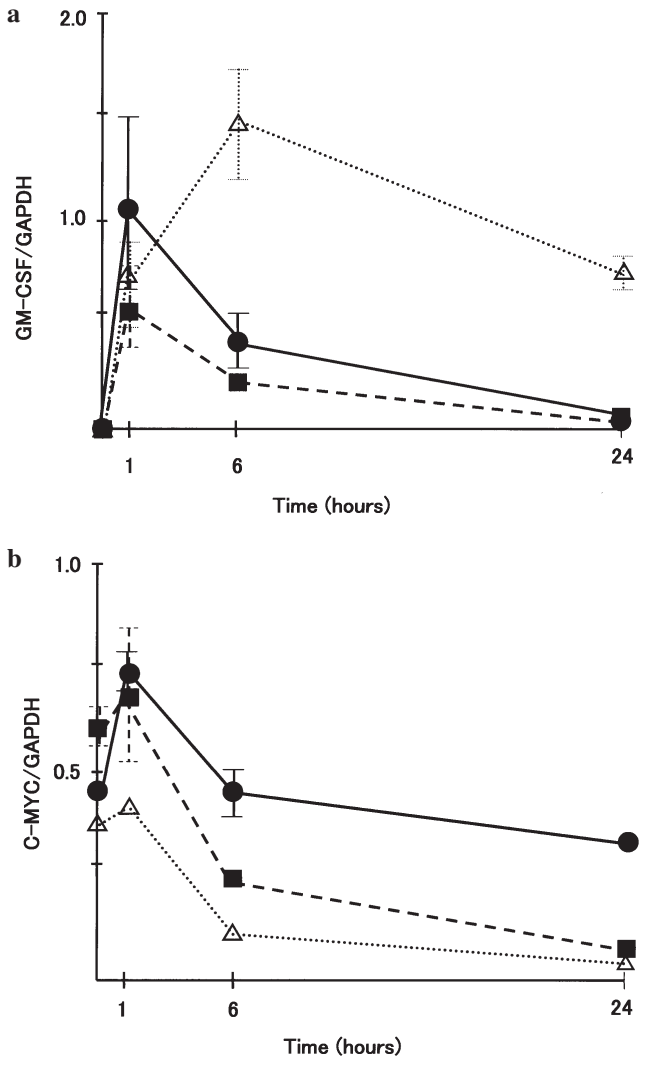

Figure 5. Expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) and c-myc mRNAs in Jurkat T cells transduced with the tristetraprolin (TTP) gene or its tandem zinc finger domain (TZF) gene. (a) Expression of GM-CSF mRNA in TTP- and TZF-transduced Jurkat T cells. Cells were stimulated with phytohemagglutinin (PHA) (5 μg/ml), and harvested at the indicated time points (see Materials and methods for details). Results are representative of four independent experiments and are the mean ± SD values of triplicate measurements. Closed circles, TTP gene-transduced Jurkat T cells; closed squares, TZF gene-transduced Jurkat T cells; open triangles, mock-transduced Jurkat T cells. (b) Expression of c-myc mRNA in TTP- and TZF-transduced Jurkat T cells. Cells were stimulated with PHA (5 μg/ml), and harvested at the indicated time points (see Materials and methods for details). Results are representative of four independent experiments and are the mean ± SD of triplicate measurements. Closed circles, TTP gene-transduced Jurkat T cells; closed squares, TZF gene-transduced Jurkat T cells; open triangles, mock-transduced Jurkat T cells.

become a possible therapeutic target for RA in the future. The main purpose of this study was to investigate whether TTP and TZF can reduce the production of TNF-α by Jurkat T cells.

In this study, we used retrovirus vectors inserted with the EGFP gene as a tool of gene transduction into Jurkat T cells. Retrovirus vectors have been used in human gene therapies for adenosine deaminase deficiency (29,30), and improvements have been made to reduce the toxicity and increase the efficacy of gene transduction (31,32). The addition of EGFP enabled us to easily assess the efficacy of gene transduction by flow cytometry or by fluorescent microscopy. Establishment of gene transferred cell clones was also confirmed using fluorescent microscopy. This enabled us to clearly evaluate the effect of TTP/TZF gene introduction into Jurkat T cells.

We first examined the effect of induction of the TTP gene into Jurkat T cells on cell growth. Johnson and Blackwell (24) reported that TTP protein induces the apoptosis of 3T3 cells, while the TZF protein destabilizes ARE-containing mRNAs, but has no effect on apoptosis. If this observation is applicable to human cells, introduction of the entire TTP gene into human cells may induce apoptosis of these cells. By directly counting the number of cells daily for 5 days, we found that both TTP- and TZF-transduced Jurkat T cells showed a slower growth rate compared to mock-transduced cells (Fig. 3). The cause of growth retardation in TTP- and TZF-overexpressing cells is not clear, but may reflect some ongoing apoptosis of these cells. To verify this assumption, we examined the ratio of cells undergoing apoptosis by flow cytometry, using an anti-annexin V antibody. When stimulated with camptothecin, the ratio of cells undergoing apoptosis was higher in TTP-transduced cells, compared with TZF- and mock-transduced cells. These results suggest that the introduction of the entire TTP molecule may render the cells prone to stimuli that enhance apoptosis. This conclusion is in agreement with the findings of Johnson and Blackwell (24) who reported that multiple TTP sequence domains are required to induce apoptosis.

We next investigated the expression of TNF-α mRNA in each transduced Jurkat T cell clone by using the TaqMan real-time PCR method. Upon stimulation with PHA, TTP-EGFP- and TZF-EGFP-transduced Jurkat T cells expressed less TNF-α mRNA and produced less protein than mock-transduced cells, as expected (Fig. 4). This result suggests that, similar to its mouse counterpart, the TZF region of human TTP can also destabilize TNF-α mRNA. There was no significant difference between TTP- and TZF-transduced cells in TNF-α protein production. Considered with the findings of previous reports on the functions of TTP, these results strongly imply that the TZF region of the TTP protein is sufficient to bind to the 3' ARE region of TNF-α production in Jurkat T cells. The suppressive effect of TTP- and TZF-overexpression on TNF-α production was observed without any serious toxic effects to the cells.

We also investigated the effects of TZF- and TTP-over-expression on other genes that encode ARE-containing mRNAs. AREs are categorized into three classes and the effects on gene regulation by ARE binding protein vary among classes (18,28). The ARE of TNF-α belongs to class II, TTP binds and exerts its function mainly on class II ARE (18,33). Other molecules that contain class II AREs in their mRNA include GM-CSF and interleukin-3 (IL-3) (28,34). On the other hand, molecules such as c-myc, and c-fos have class I AREs (28). To assess the specificity of the effect of TTP- and TZF-overexpression, we analyzed the expression of GM-CSF and c-myc mRNA expression upon PHA stimulation, and compared them with the expression of TNF-α mRNA. Similar to the results of TNF-α mRNA, PHA stimulation reduced the increment of GM-CSF mRNA in TTP- and TZF-transduced cells compared with mock-transduced cells. However, such an effect was delayed compared with that of TNF-α. A previous study indicated that the half-life of GM-

CSF mRNA expressed in T cells was extended when the cells were stimulated by 12-O-tetradecanoyl-phorbol-13 acetate (35). These differences in the time course of mRNA expression may be due to the contribution of other ARE binding proteins such as HuR. HuR also binds to the AREs present in mRNA of a number of genes including TNF-α and GM-CSF, but has a stabilizing effect on these mRNAs (36). A balance of stabilizing and destabilizing ARE binding proteins critically influences the expression of mRNA at the post-transcriptional level (28,37). In contrast, ARE in the mRNA of c-myc belongs to the class I family of AREs. The level of c-myc mRNA expression was not significantly different in TTP-, TZF- and mock-transduced Jurkat T cells. These results indicated that TTP could select the type of AREs to bind, in agreement with a previous report (22), and also that, in addition to the entire TTP molecule, the TZF region of the TTP molecule can exert this function as well. Unlike other studies that used cells with artificially introduced class II ARE-containing mRNAs, our study was performed using Jurkat T cells, which naturally produce TNF-α and GM-CSF. Thus, our data are obtained from a more physiological condition, and will aid in the understanding of the functions and role of post-transcriptional regulation of TNF-α production in humans.

Currently, there are several therapeutic options for RA (38). Biological drugs have been introduced into clinical practice in recent years and have shown dramatic effects in many patients who do not respond to conventional therapies. However, there are still limitations in the usage and effects for those drugs (39). Therefore, new strategies for the treatment of RA must be developed in the future, and we consider that TTP is a potential target in such new therapies. Introducing genes encoding TTP or TZF into synovial tissues, or other methods that induce the expression of the TTP gene may be considered as a possible therapeutic option in the future.

To date, there are few reports on gene transduction in monocytes, the primary source of TNF-α in RA, using retroviruses. Our preliminary studies showed that the transduction efficacy of our retrovirus vectors to a macrophagic cell line was not very high (data not shown). It is necessary to improve our methods to overcome this problem.

One problem for considering TTP as a target for therapy against RA is that, although it selectively destabilizes mRNA containing class II AREs in their 3' region, the mRNA of molecules other than TNF-α, such as GM-CSF and IL-3 also have class II ARE motifs (34). TTP or TZF overexpression may affect the production of these molecules as well as TNF-α, and cause undesirable adverse effects. To clarify the general effects of TTP and TZF over-production, the development of TTP or TZF transgenic animals would be helpful. It is also important to determine the effect and the fate of locally injected retrovirus vectors, since injection of those vectors directly into inflamed joints may enable us to attain the desirable effects with little adverse effects.

In conclusion, we have shown that the transduction of genes encoding TTP or TZF in a Jurkat T cells resulted in the reduction of TNF-α mRNA expression and, hence, reduction of TNF-α production by these cells. The importance of TNF-α in the pathogenesis of RA, and as a therapeutic target for RA, is now well established. Thus, TTP may also be considered as a possible target molecule in the future. The finding that the zinc finger region of the TTP molecule can exert its functions is also of importance, as the use of only the essential portion of TTP may aid in avoiding unwanted adverse reactions.

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