FK506 Enhances Triptolide-Induced Down-regulation of Cyclooxygenase-2, Inducible Nitric Oxide Synthase as Well as Their Products PGE2 and NO in TNF-α-stimulated Synovial Fibroblasts from Rheumatoid Arthritic Patients*

H. Yao1, J. Zhou1, D. Li1, N. Wu1, A. Bader2, S. Höxtermann2, P. Altmeyer2, N. H. Brockmeyer2

1National Key Laboratory of Infectious Diseases, Institute of Infectious Diseases, First Affiliated Hospital, School of Medicine, Zhejiang University, P.R.China
2Department of Dermatology and Allergology, Ruhr-University Bochum, St. Josep-Hospital, Bochum, Germany

Abstract

Objective: To explore the effects of FK506 on the inhibition of cell proliferation and the expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and their products PGE2 and NO in TNF-α-stimulated human rheumatoid arthritis synovial fibroblasts (RASF) treated with triptolide (TP), and to study the mechanisms involved when combining FK506 and TP in RA therapy.

Materials and Methods: RASF used in the experiments were obtained from the synovial tissue of patients with RA before being cultured. RASF were pretreated with FK506 (10 ~ 1000 nM) for 2 hours before being stimulated with TNF-α (20 ng/ml) in the presence or absence of TP (10 ng/ml). RASF proliferation was determined by [3H]-TdR incorporation. Production of PGE2 and NO in culture supernatants of RASF was detected by competitive ELISA and enzymatic reduction of nitrate, respectively. Expressions of COX-2 and iNOS mRNA in RASF were analyzed by semi-quantitative RT-PCR. Expressions of COX-2 and iNOS protein were estimated by Western-blot and a cellular enzyme immunoassay. NFκB activity in whole-cell extract of treated RASF was also measured using an ELISA-based method.

Results: Neither FK506 nor TP at a lower concentration (10 ng/ml) affected TNF-α-induced COX-2 and iNOS expressions or PGE2 and NO productions in synovial cells. Combined treatment of FK506 and a lower concentration of TP (10 ng/ml) reduced both COX-2 and iNOS mRNA and protein expression, and correspondingly reduced PGE2 and NO produced by synovial fibroblasts. This effect was highly correlated with FK506 concentration (10 ~ 1000 nM). NFκB activity in TNF-α-stimulated synovial cells was suppressed more profoundly by FK506 plus TP (10 ng/ml) than by TP (10 ng/ml) alone. However, no change was observed regarding the inhibition of synovial cell proliferation after combined treatment of FK506 and TP.

Conclusion: FK506 enhanced TP-mediated down-regulation of COX-2 and iNOS as well as their products PGE2 and NO in human TNF-α-stimulated RASF by more profoundly suppressing the activity of NFκB.

Key words: FK506; Triptolide; Arthritis, rheumatoid; Synovial fibroblasts; Cyclooxygenase-2; Inducible nitric oxide synthase

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disease involving polyarticular synovitis that leads to the formation of rheumatoid pannus and a subsequent erosion of articular cartilage and bone. Although a small percentage of patients go into natural remission, the untreated disease progresses to cause disability, morbidity and early mortality. In the last decade there has been much successful research on cytokine expression and regulation [1-3]. It has become clear that pro- and anti-inflammatory cytokines, derived predominantly from cells of a macrophage lineage, play a major role in the initiation and perpetuation of the chronic inflammatory process in the RA synovial membrane [4-7].

Prostaglandins (PGs) found at elevated levels in synovial fluid and synovial membranes are considered to play a pivotal role in the development of vasodilatation, fluid extravasation and pain in synovial tissues [8, 9]. Moreover, there is increasing evidence that PGs (especially prostaglandin E2) are mediators involved in complex interactions that lead to articular cartilage and juxta-articular bone erosion [10]. Cyclooxygenase is an enzyme that plays a crucial role in PG production [11,12]. Although two forms of cyclooxygenase (COX), i.e. COX-1 and COX-2, exist [13, 14], more and more experiments are suggesting that synovial tissues from patients with RA contain COX-2 to a greater extent than COX-1 [15-17]. Our understanding of the critical role of COX-2 in synovial inflammation has made this enzyme a target for arthritis therapy.

Triptolide (TP), the major component of the diterpenoids of the Chinese herbal remedy Tripterygium

* The first two authors contributed equally to this study.
wilfordii Hook.f. (TWHF), has been reported to be therapeutically effective in patients with RA in China, although its mechanism of action still remains unclear [18-20]. FK506 is an immunosuppressant used to prevent allograft rejection after organ transplantation or as a substitute for typical chemotherapeutics [21]. Some clinical studies found that FK506 delays clinical progression and improves symptoms in patients with RA [22-24]. However, the use of TP and FK506 has been tempered by the development of side effects at therapeutic doses. No data are available at present about using combined FK506 and TP to treat RA. In this study we investigated the effect of TP and/or FK506 on the expression of COX-2 and iNOS in human synovial fibroblasts, and found that FK506 in the presence of a low concentration of TP strongly inhibited TNF-α-stimulated NFκB activation, COX-2/iNOS expression and PGE₂/iNOS NO generation.

MATERIALS AND METHODS

PREPARATION AND CULTURE OF SYNOVIAL FIBROBLAST CELL LINES

Synovial tissue samples were obtained from 5 patients with RA satisfying American College of Rheumatology criteria, who had undergone total knee replacement surgery or synovectomy. Fresh synovial tissues were minced aseptically, then dissociated with 4mg/ml collagenase (Sigma, Louisiana, USA) for 4 hours in RPMI-1640 medium at 37°C. The dissociated cells were plated on culture dishes and allowed to adhere in RPMI-1640 (Gibco BRL, N.Y., USA) supplemented with 10% fetal bovine serum (Hyclone, Utah, USA) at 37°C in a humidified atmosphere containing 5% CO₂. After removal of non-adherent cells, the plated cells were further incubated for 18 hours, then thoroughly washed with D-Hank’s solution. The adherent synovial cells were harvested using trypsin(0.25% w/v)-EDTA(0.02% w/v) followed by washing with D-Hank’s solution containing 5% CO₂. The collected synovial cells whose homogeneity was more than 98% were plated on culture dishes and allowed to adhere in RPMI-1640 (Gibco BRL, N.Y., USA) supplemented with 10% fetal bovine serum (Hyclone, Utah, USA) at 37°C in a humidified atmosphere containing 5% CO₂. The isolated synovial cells were plated on culture dishes and allowed to adhere in RPMI-1640 (Gibco BRL, N.Y., USA) supplemented with 10% fetal bovine serum (Hyclone, Utah, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

RNA ISOLATION AND RT-PCR

Total cellular RNA in the treated RASFs was isolated using Trizol Reagent (Gibco BRL, N.Y., USA) in accordance with the manufacturer’s instructions. Complementary DNA (cDNA) was prepared by reverse transcription (RT) of 2µg total RNA using oligo dT₁₈ and 200U superscriptII reverse transcriptase (Invitrogen, California, USA) at 42°C for 70min according to the manufacturer’s instructions. cDNA was amplified using Taq polymerase buffer containing 1.5mM MgCl₂ and 200 µM of each dNTP. The oligonucleotide primers used were as follows: for COX-2 (product size: 756 bp), sense: 5’-CAGCACCTCACGATCATGT-3’, antisense: 5’-CTCGTGCTAAAAGTCTGGTC-3’; for iNOS (product size: 237 bp), sense: 5’-TCTGGTGCTAAAGTCTGGTC-3’, antisense: 5’-GATTGGGCGACATCGTCTGG-3’, β-actin, (product size: 619 bp) sense: 5’-CAGCCGGGGTGTGCTAGCT-3’, antisense: 5’-GTTGCGGACATTCTCCGCT-3’. PCR primers were from Sangong (Shanghai, China).

ASSAY FOR PGE₂, NO PRODUCTION

Synovial fibroblasts were seeded at a density of 1x10⁶/well onto 6-well culture plates. When adherent cells became confluent, cells were stimulated with 20ng/ml TNF-α (R&D systems, Minnesota, USA) in the presence of different concentrations of TP (0-100ng/ml, Calbiochem, California, USA) for 20 hours. Where FK506 was applied, cells were incubated with FK506 (0-100ng/ml, Fujisawa Ireland Ltd, Japan) for 2 hours before 20ng/ml TNF-α and TP (10ng/ml) were added and incubation was allowed to proceed for a further 18 hours. The culture supernatants were collected and kept at -20°C.

The levels of PGE₂ in culture supernatants were determined using a competitive ELISA kit (R&D systems, Minnesota, USA) according to the manufacturer’s instructions. The lower limit of detection was 36.2 pg/ml.

Nitric oxide (NO) levels in culture supernatants were measured as its oxidized product nitrate. The kits were purchased from R&D systems, the lower limit of detection was 1.35µmol/L.

ASSAY FOR COX-2, iNOS PRODUCTION

Synovial cells were plated at a density of 5x10⁴/well in 96-well plates for detecting COX-2 and iNOS protein in cells by Cell-ELISA. At the end of culture, cells were fixed in 4% paraformaldehyde (w/v in PBS) and blocked with defatted milk. Then the cells were incubated with rabbit antiserum against human COX-2- or iNOS-specific antibody (Santa Cruz Biotechnology, California, USA) for 2 hours at 37°C, with biotinylated goat anti-rabbit IgG for 1 hour, and with diluted HRP-conjugated streptavidin for 30 minutes at room temperature. The plates were washed three times at each step. Add 100µl of substrate solution (Tetramethylbenzidine, TMB) to each well and incubate for 20 minutes at room before development was stopped with 0.5mol/L H₂SO₄ (50µl/well), and absorbance was measured in a spectrophotometer (Biotek-Elx800) at 450nm.
Cell lysates were prepared for western blot analysis of iNOS and COX-2 by using whole cellular protein extraction kits (Active Motif, California, USA). The concentration of protein in each cell lysate was determined using a BCA-protein assay kit (Pierce, Illinois, USA) with bovine serum albumin (BSA) as the standard. An identical amount of protein (40µg) from each sample was loaded onto a 10% SDS-PAGE gel which was then run at 200 volts. Proteins were then transferred to nitrocellulose membranes (0.45µm, Millipore, Massachusetts, USA) that were blocked with 5% BSA (Sigma, Louisiana, USA) in TBS (25mM Tris-HCl, 150mM sodium chloride, pH 7.2) for 1 hour at room temperature. Blots were incubated with anti-COX-2 or anti-iNOS or anti-β-actin specific rabbit polyclonal IgG primary antibody (Santa Crutz, California, USA) in a 1:500 dilution at 37°C for 2 hours. Blots were washed three times and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:2000 dilution) for 2 hours at room temperature. All blots were developed using enhanced chemiluminescence (ECL) reagents (Super-Signal Dura Kit, Pierce, Illinois, USA) following the manufacturer’s instructions. Blots were scanned and analyzed for the measurement of the band intensities. Results were calculated as the relative ratio between the band of interest and the β-actin band.

Activity of NFκB in Synovial Fibroblasts

NFκB p65/ NFκB p50 transcription factor assay kits (Active Motif, California, USA) which combine all the benefits of fast, sensitive and specific assays were used to monitor NFκB activation. 5 µg of cell extracts/well were added to a 96-well plate coated with an oligonucleotide probe containing the site for NFκB binding. Each well was then incubated with primary antibody specific for the active form of the bound transcription factor. Wells were then incubated with an HRP-conjugated secondary antibody. The plate was washed three times at each step, and 100µl of standard developing solution (TMB) was added to each well. The absorbance results were read using a spectrophotometer (Biotek-Elx800) at 450nm with a reference wavelength of 655nm. Both positive control wells and blank wells were set for this assay. Specificity of binding was determined by prior addition of a 20-fold excess of unlabeled competitor consensus oligonucleotide.

[^H]-Thymidine Incorporation Assays

In order to check the proliferation of synovial fibroblasts in response to the treatments described above, 1 x 10^4 cells per well were plated on 96-well plates in RPMI-1640 medium supplemented with 10% FBS. [^H]-Thymidine (1µCi/well) was added for 18 hours after 54 hours of culture. The plates were then washed three times with PBS before being fixed in 5% trichloroacetic acid at 4°C for 20 minutes, washed three times in trichloroacetic acid and solubilized in 0.5N NaOH (0.15ml). The resulting solution was neutralized using 0.5N HCl (0.15ml) and radioactivity was assessed by liquid scintillation counting.

Results

Effects of TP and FK506 on PGE2 and NO Production

Treatment of RASF with TNF-α (20ng/ml) in the presence of TP reduced the PGE2 and NO production seen upon TNF-α-stimulation alone. The response was dose-dependent (r = 0.847, 0.787 for PGE2 and NO, respectively, P<0.05) and was significant at TP concentrations above 20ng/ml (Table 1A).

To determine the effect of FK506 combined with TP, 10ng/ml TP was chosen for its only minor, statistically undetectable effect on TNF-α-stimulated PGE2 and NO as shown above. Co-application of FK506 (10-1000ng/ml) and TP (10ng/ml) resulted in a significant reduction of PGE2 and NO whereas FK506 alone induced only slight decreases even at a high concentration (1000ng/ml) which did not achieve statistical significance (Table 1B).

Effects of TP and FK506 on COX-2 and iNOS mRNA Expression

As described previously, PGE2 is synthesized by COX-2 and NO by iNOS. The results of the specific RT-PCR analysis corresponded well with the levels of PGE2 and NO in the supernatants. TP greatly inhibited COX-2 and iNOS mRNA expression at a concentration above 20ng/ml. The additive inhibitory effect of FK506 was evident at the low concentration of TP (10ng/ml). FK506 pre-treatment alone did not inhibit the COX-2 and iNOS mRNA expression (Figs. 1, 2).

COX-2 and iNOS Protein Expression in RASF Treated Cells

As expected, the COX-2 and iNOS protein expression in synovial cells paralleled the mRNA expressions with the different treatments. Treatment of RASF with FK506 in the presence of the TP (10ng/ml) abrogated TNF-α-stimulated COX-2 and iNOS production, and the inhibition effect was positively correlated with the concentrations of FK506 (Fig. 3). Neither FK506 nor lower concentrations of TP alone affected TNF-α-stimulated COX-2 and iNOS production.

Effects of TP and FK506 on NFκB Activity in Synovial Cells

The promoter region of COX-2 contains an NFκB element that is known to be an induction target for COX-2. To examine the molecular action of TP and/or FK506 on COX-2 expression, ELISA based NFκB p65/p50 transcription factor assay kits (non-radioactive) for detecting oligonucleotide probes were used to measure NFκB activation.

As shown in Figure 4, NFκB activation was consistent with the corresponding COX-2 and iNOS expression. TNF-α-induced NFκB activities were significantly reduced by combining FK506 with TP, an
Table 1A. Effect of TP on TNF-α-stimulated PGE₂, NO production.

<table>
<thead>
<tr>
<th>Group</th>
<th>PGE₂(µg/L)</th>
<th>NO(µmol/L)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.56 ± 0.23</td>
<td>22.02 ± 10.77</td>
</tr>
<tr>
<td>TNF-α</td>
<td>27.43 ± 5.29#</td>
<td>140.32 ± 24.06#</td>
</tr>
<tr>
<td>TNF-α + TP (5ng/ml)</td>
<td>26.28 ± 6.44</td>
<td>134.26 ± 19.38</td>
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<tr>
<td>TNF-α + TP (10ng/ml)</td>
<td>24.48 ± 3.86</td>
<td>126.28 ± 20.34</td>
</tr>
<tr>
<td>TNF-α + TP (20ng/ml)</td>
<td>15.18 ± 3.12*</td>
<td>81.60 ± 16.82*</td>
</tr>
<tr>
<td>TNF-α + TP (50ng/ml)</td>
<td>4.02 ± 1.76Δ</td>
<td>36.52 ± 13.24Δ</td>
</tr>
<tr>
<td>TNF-α + TP (100ng/ml)</td>
<td>3.24 ± 1.28Δ</td>
<td>32.26 ± 11.58Δ</td>
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</table>

Table 1B. Effect of TP and FK506 on TNF-α-stimulated PGE₂ and NO production.

<table>
<thead>
<tr>
<th>Group</th>
<th>PGE₂(µg/L)</th>
<th>NO(µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.56 ± 0.23</td>
<td>22.02 ± 10.77</td>
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<tr>
<td>TNF-α</td>
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<tr>
<td>TNF-α + TP</td>
<td>24.48 ± 3.86</td>
<td>126.28 ± 20.34</td>
</tr>
<tr>
<td>TNF-α + FK506(1000ng/ml)</td>
<td>21.22 ± 2.68</td>
<td>125.58 ± 17.34</td>
</tr>
<tr>
<td>TNF-α + TP + FK506(10ng/ml)</td>
<td>8.46 ± 2.38*</td>
<td>68.60 ± 13.62*</td>
</tr>
<tr>
<td>TNF-α + TP + FK506(100ng/ml)</td>
<td>2.86 ± 0.62*#</td>
<td>9.52 ± 10.84*#</td>
</tr>
<tr>
<td>TNF-α + TP + FK506(1000ng/ml)</td>
<td>1.91 ± 0.34*#</td>
<td>22.96 ± 13.58*#</td>
</tr>
</tbody>
</table>

PGE₂ and NO production were measured in the supernatants of the culture.
(A) Rheumatoid synovial fibroblasts cultured for 20 hours with increasing concentrations of TP (0-100ng/ml). #, P<0.01 relative to unstimulated control. *, P<0.05 relative to low concentration of TP (0-10ng/ml). ∆, P<0.01 relative to 0-20ng/ml TP.
(B) Rheumatoid synovial fibroblasts were pretreated with FK506 for 2 hours and then with TNF-α and TP (10ng/ml) for 18 hours. *, P<0.01 relative to non-FK506.  #, P<0.01 relative to low concentration of FK506 (100ng/ml).

Fig. 1. RT-PCR analysis of mRNA for COX-2 and iNOS in synovial fibroblasts. Cells were stimulated with TNF-α (20ng/ml) in the presence of different concentrations of TP (0-100ng/ml) for 20 hours (Fig 1A). If FK506 was applied, the cells were incubated with FK506 (0-1000ng/ml, Fujisawa Ireland Ltd) for 2 hours before 20ng/ml TNF-α and TP (10ng/ml) were added and incubation was continued for 18 hours (Fig1B). COX-2 and iNOS mRNA expression was examined with RT-PCR. PCR products were separated on 1.5% agarose gels and analyzed after ethidium bromide staining. β-actin was used as a housekeeping gene for densitometric analysis.

Fig. 2. COX-2 and iNOS expression in relation to β-actin mRNA expression. Densitometric analysis was performed on an ethidium bromide-stained agarose gel (Fig. 1) using the software program Kodak Digital Science. The net intensity of each band was compared with that of the housekeeping gene β-actin and their ratio is reported here. Bars show the mean ± SD.
effect which disappeared when FK506 or low concentrations of TP were applied alone. The decrease was positively correlated with the concentration of FK506.

**[H]-THYMIDINE INCORPORATION**

To investigate whether the reduced COX-2, iNOS, PGE2, and NO resulted from the inhibition of DNA synthesis, [H]-thymidine incorporation was assayed. No significant changes were found in synovial cells treated either with FK506 or TP at any concentration.

**DISCUSSION**

Triptolide, an active component of the Chinese herbal remedy TWHF, has been reported to be therapeutically effective in patients with RA. Because of this beneficial clinical impact, an understanding of how this preparation exerts its therapeutic activity is necessary. Previous studies have demonstrated that TP effectively suppresses inflammatory and reactive processes induced by a variety of stimuli, such effects include the down-regulating of the gene expression and production of COX-2 and iNOS, the inhibition of PGE2 and NO production[25, 26], and the induction of T lymphocyte apoptosis [27]. Lin et al demonstrated that TP exerts a chondroprotective effect via a direct suppression of pro-MMP 1 and 3 production and a simultaneous up-regulation of TMPs in IL1-treated synovial fibroblasts [18]. Another study showed that TP, with an IC50 of 20-50ng/ml, selectively inhibits epithelial cell expression of inflammatory cytokines and chemokines stimulated by PMA and TNF-α or IL-1β via the suppression of NFκB activity [28]. Furthermore, TP also inhibits the transcriptional activation of NFκB in various human cell types [29, 30]. In this study we confirmed those findings and found that TP suppressed NFκB activity in a dose-dependent manner with an IC50 of about 35ng/ml. The low concentration of TP (<10ng/ml) did not affect NFκB activity or the expression of COX-2 and iNOS. Based on these results, the low concentration of 10ng/ml TP was the concentration chosen for subsequent study.

FK506, an antifungal natural macrolide that suppresses the immune system by blocking T-cell activation, and which is mainly used in organ transplantation as an immunosuppressive agent [31, 32], can also be used to treat patients with autoimmune diseases including RA[33-35]. One of the therapeutic mechanisms appears to be an FK506-induced reduction of the high activation of NFκB that plays a pivotal role in synovial inflammation in patients with RA [36]. Recent studies have shown that FK506-binding protein binds to the glucocorticoid receptors that activate signal transduction pathways [37, 38]. The association may explain FK506 influences on steroid function and throws light on how drugs might be combined for the purposes of treatment.

The use of TP is common in patients with RA. However, because of side effects at therapeutic doses [19], combinations of lower doses with synergistically acting safer drugs might serve to preserve the therapeutic effect while preventing any serious complications from arising. In order to pursue this line of thinking we investigated the possibility of combining low concentrations of TP with FK506 using an in vitro rheumatoid synovial fibroblast model. No marked alterations in synovial cells were induced by FK506
alone regarding NFkB activation, COX-2 and iNOS gene expression, or PGE\(_2\) and NO production at any tested concentration. However, in the presence of TP an inhibitory effect could be revealed. These results suggest that TP enhances the sensitivity of RASF towards FK506.

To extend our results further, we addressed the mechanism underlying the down-regulation of COX-2 and iNOS, and assessed whether this was the result of an inhibitory effect could be revealed. These results tested concentration. However, in the presence of TP we assayed \([3H]\)-TdR incorporation. No effect was observed on the inhibition of synovial cell proliferation by TNF-\(\alpha\) when either TP or FK506 were used alone or in combination.

In conclusion, treatment with TP and FK506 in combination exerts a significantly higher inhibitory effect than TP or FK506 alone regarding NFkB activation, COX-2 and iNOS gene expression, as well as PGE\(_2\) and NO generation in rheumatoid synovial fibroblasts. This inhibitory effect is most likely mediated through a suppression of NFkB activity. These in vitro results shed light on an important approach as to how RA may be theraped in future.

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Address for correspondence:
Prof. Dr. med. Norbert H. Brockmeyer
Department of Dermatology and Allergology
Ruhr-University Bochum
Gudrunstr. 56
D-44791 Bochum, Germany
e-mail: n.brockmeyer@derma.de