Potentiation of glucocorticoid receptor (GR)-mediated signaling by the immunosuppressant tacrolimus in rheumatoid synoviocytes


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Objective
The immunosuppressant tacrolimus is known to enhance many aspects of glucocorticoid. In this study, we investigated the effects of tacrolimus on glucocorticoid receptor (GR) signaling using rheumatoid fibroblast-like synoviocytes (RA-FLS).

Methods
The nuclear translocation of GR was analyzed by immunocytochemistry. The DNA binding activity of p65 was assayed by a functional ELISA kit using nuclear extracts. GR-associated FK506-binding protein-51 (FKBP-51) was analyzed by Western blotting following immunoprecipitation of glucocorticoid receptor (GR) complexes.

Results
High concentrations (10^{-7}M) of Dexamethasone (Dex) induced GR translocation to the nucleus in RA-FLS. However, the nuclear GR translocation did not occur with low concentrations of Dex (10^{-9}M). Tacrolimus treatment of RA-FLS results in potentiation of GR translocation to the nucleus even in the presence of a low concentration of Dex (10^{-9}M). GR-associated FKBP-51 decreased after tacrolimus treatment. Furthermore, tacrolimus also decreased the IL-1β-induced DNA binding activity of p65, a subunit of NF-κB, in the presence of 10^{-9} M of Dex.

Conclusion
These data suggest that tacrolimus exerts anti-inflammatory properties by potentiating the GR signaling through the GR-immunosuppressant-binding proteins (immunophilins) interaction and its nuclear transport in rheumatoid synovium.

Key words
FK506-binding protein, glucocorticoid receptor, nuclear factor-kappa B, rheumatoid arthritis, synovium, tacrolimus.
Introduction
Tacrolimus (FK506) is an immunosuppressant primarily by interfering with the T cell activation process (1). Recent clinical studies have demonstrated the efficacy of tacrolimus in the treatment of autoimmune diseases, including rheumatoid arthritis (RA) (2), in which T cells play a pivotal role in pathogenesis. Tacrolimus binds to an intracellular protein termed immunophilin, FK506 binding protein (FKBP) (3). The drug-immunophilin complexes inhibit calcineurin (4), an enzyme involved in the activation of the nuclear factor of activated T cells (NF-AT), a transcription factor required for the expression of cytokines genes in T cells (5). Previous report suggested the interaction between tacrolimus and glucocorticoid receptor (GR) (6). GR is a hormone-activated transcription factor that requires movement from the cytoplasm to the nucleus, normally resides in the cytoplasm in a heterocomplex with heat-shock proteins (hsp) and immunophilins (7). Once glucocorticoid binding occurs, GR releases these chaperone complexes and translocates to the nucleus to associate with a specific DNA sequence (8). FKBP51 and FKBP52 are FK506-binding immunophilins that possess the binding site on hsp 90, that associated with GR (9). FKBP51 appears to be predominant immunophilin in hormone-free GR complexes, and binding of glucocorticoid causes exchange of FKBP51 for FKBP52. Following this exchange, FKBP52 serves to promote translocation of GR to the nucleus due to its interaction with motor proteins (10). These findings suggest that tacrolimus modulate the steroid-mediated biological action by affecting the GR-associated immunophilins. In the present study, we examined the effects of tacrolimus on steroid-mediated GR trafficking using RA-FLS.

Method
Preparation of RA-FLS
Synovial tissue samples were obtained from five patients with RA during synovectomy. All RA patients fulfilled the American College of Rheumatology criteria for RA. Synovial fibroblasts were isolated from the synovial tissues by enzymatic digestion. The isolated synovial fibroblasts were used at the third or fourth passages for subsequent experiments. All experiments were performed with the protocol approved by the ethics committee of Nagasaki Medical Center.

Immunocytochemistry
Subcellular locations of GR and NF-κB in RA-FLS were detected by immunocytochemistry. Cells grown in 8-chamber culture slides (Lab Tech, Rockville, MD, USA) were stimulated with Dex or IL-1β and then washed in PBS. Cells were fixed in cold acetone (-20 °C) for 15 min, and blocked in 0.01% H2O2 for 10 minutes. Incubation with rabbit polyclonal anti-NF-κB p65, GR (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phospho-GR (1:50, Cell Signaling, Boston, MA, USA) were performed for 1 hr then with HRP-conjugated anti-Rabbit IgG (Dako Japan, Kyoto, Japan) for 30 minutes and ENVISON plus (Dako) was used for detection. The slides were washed in PBS, then mounted and assessed by microscopy.

Immunoprecipitation and Immunoblotting
Cellular extracts were prepared from a subconfluent 10 cm plate of RA-FLS treated with tacrolimus for 4 hr. Following treatment with these drugs, cells were placed on ice, washed twice with phosphate-buffer saline (PBS), and lysed in 200 μl of lysis buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1% Triton X-100, 10% glycerol, and additional protease and phosphatase inhibitors: 1 mM PMSF, 20 mM β-glycerophosphate, 8 mM sodium pyrophosphate, 1 μg/ml leupeptin, 1mg/ml leupeptin, 1 μg/ml aprotinin (Sigma). Cellular lysates were pre-cleared with protein-G-Sepharose 4B (Pharmacia, Uppsala, Sweden) for 30 min and centrifuged at 6000 g for 5 min. The pre-cleared supernatants were incubated with anti-GR antibodies (Santa Cruz Biotechnology) for 1 hr. Collection of immunocomplex was performed using protein-G Sepharose 4B (Pharmacia) for 30 min. Lemmli sample buffer (2%SDS
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10% glycerol 100 mM DTT 60 mM Tris[PH6.8]) was added and boiled for 10 min. Immunoprecipitates containing GR were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis using the primary antibodies against FKBK51 (Affinity BioReagent, Golden, CO, USA), and horseradish-conjugated anti-rabbit IgG (TrueBlot, eBioscience, San Diego, CA, USA) was performed with an ECL Western blotting kit (Amer- sham, Little Chalfont, UK).

Preparation of nuclear extracts
The cells were washed with cold phosphate buffered saline (PBS), harvest- ed, and pelleted, then resuspended in buffer A (10 mM Heps, pH 7.9, 10 mM KC1, 0.6% Nonidet P-40, 1 mM EDTA, 1 mM PMSF and 1 mM DTT) and incubated on ice for 15 min. Af- ter centrifugation at 14,000 rpm for 15 min, the supernatant cytoplasmic protein was collected. The remnants were resuspended in buffer B (20 mM PMSF and 1 mM DTT) and incubated on ice for 30 min, and stirred dis- continuously every 10 min, and then were centrifuged at 14,000 rpm for 15 min, and the supernatant nuclear extract was collected. All proteins were stored at -80°C. The protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

p65 activity detection
The p65 subunit activity was detected using a Trans AM™ NF-κB p65 Kit (Active Motif, Cambridge, Mass., USA). The kit contained a 96-well plate on which had been immobilized an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3'). The active form of NF-κB contained in the nuclear extract specifically binds to this oligonucleotide. The primary anti- bodies used to detect NF-κB recognize an epitope on p65 that was accessible only when NF-κB was activated and bound to its target DNA. An HRP-con- jungated secondary antibody provided a sensitive colorimetric readout that was easily quantified by spectrophotometry. The nuclear extracts (1μg/well) iso- lated from IL-1β-treated RA-FLS were analyzed using this kit.

A. RA-FLS were treated with various concentrations of Dex (10⁻⁷–10⁻⁹M) for 1 hr. Cells were fixed and the subcellular localization of GR was examined by immunocytochemistry using anti-GR antibodies.

B. RA-FLS were pretreated with the indicated concentrations of tacrolimus or ethanol (vehicle) for 4 hr followed by further treatment with Dex (10⁻⁹M, 1 hr). Tacrolimus induced the nuclear translocation of GR in the presence of 10⁻⁹M of Dex.

Statistical analysis
Differences between groups were ex- amined for statistical significance using analysis of variance (ANOVA) with Fisher’s PLSD test. Statistical signifi- cance was defined as p<0.05.
Results
Tacrolimus augmented GR nuclear translocation in the presence of low concentrations of dexamethasone
To ask whether the GR translocation to the nucleus is modulated by tacrolimus, we analyzed the intracellular localization of GR in RA-FLS after treatment with dexamethasone (Dex). High concentrations of Dex (>10^-7M) treatment induced the nuclear translocation of GR. In the treatment with a low concentration of Dex (10^-9M), which is close to the physiological levels, the nuclear translocation of GR was obscure (Fig. 1A). However, the pretreatment of tacrolimus induced the nuclear translocation of GR in this condition (Fig. 1B). In contrast, tacrolimus alone did not induce the nuclear translocation of GR (data not shown).

GR was shown to be phosphorylated after it becomes activated and Ser211-phosphorylation is a biomarker for activated GR in the nucleus (11). Ser211-phosphorylated GR was demonstrated in the nucleus of RA-FLS treated with high concentrations of Dex (10^-7M) (Fig. 2A). In contrast, Ser211-phosphorylated GR was weakly induced in response to the low concentrations of Dex (10^-9M), however, tacrolimus pretreatment enhanced the Ser211-phosphorylation of GR in this condition (Fig. 2B). To elucidate whether this effect of tacrolimus on GR nuclear translocation was due to alterations in the GR hetero-complexes, we co-immunoprecipitated GR complexes and the levels of GR-associated FKBP51 were measured. As shown in Figure 3, the protein amount of GR-associated FKBP51 decreased by tacrolimus treatment.

Tacrolimus inhibited IL-1β-induced NF-κB activation in the presence of low concentrations of dexamethasone
The activation of NF-κB occurs by a wide variety of inflammatory stimuli (12). This allows NF-κB to translocate to the nucleus and bind to the promoter of its target gene (13). IL-1β is one of the important cytokines which is elevated in RA patients and linked with rheumatoid bone erosion. NF-κB translocation to the nucleus was induced in IL-1β-stimulated RA-FLS. This IL-1β-induced nuclear translocation of NF-κB was not affected by the presence of either tacrolimus or Dex (Fig. 4). Next, we investigated the DNA-binding activity of NF-κB in IL-1β-stimulated RA-FLS. Dex inhibited IL-1β-induced DNA-binding activity of p65, a subunit of NF-κB, in a dose-dependent manner, and its inhibitory effect was not clear in the low dose of Dex (10^-7M) (Fig. 5A). However, tacrolimus pretreatment inhibited the IL-1β-induced DNA binding activity of p65 in the presence of a low concentration Dex (Fig. 5B).

Discussion
FK506-FKBPs interactions may modulate the different target proteins which are responsible for the distinct immunosuppressive activities (15). This study was undertaken to clarify the effects of
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We found that tacrolimus enhanced the GR nuclear translocation in response to low concentrations of dexamethasone. However, this synergistic effect by tacrolimus was not observed on the GR nuclear translocation in response to high concentrations of dexamethasone (≥10⁻⁷M). In the presence of high concentrations of glucocorticoid, GR translocation could be completed and it may be difficult to detect the tacrolimus-mediated enhancing effects on GR nuclear translocation.

GR complexes contain several proteins that possess tetra-tricopeptide repeats (TPRs) (16). The TPRs proteins, that are associated with GR-hsp90 heterocomplexes, include FKBP51, FKBP52, CYP-40, and the serine/threonine phosphates, PP5 (17). Riggs et al. showed that FKBP52 selectively potentiates GR-mediated transactivation (18). In contrast, FKBP51 appears to be the predominant immunophilin in hormone free GR complexes (19). It was demonstrated that glucocorticoid resistance in the squirrel monkey partly results from the overexpression of FKBP51 that reduces GR’s ligand binding (20). In contrast, FKBP52 potentiates GR-dependent genomic responses (21). From the series of studies, the switching mechanism, in which GC causes the exchange of FKBP51 for FKBP52 in the GR complex, was proposed and this exchange leads to the co-recruitment of motor protein that translocates GR to the nucleus (22). Tacrolimus was shown to potentiate the GR-mediated transcriptional activity (6, 23), and the possible involvements of GR-associated immunophilins have been suggested. Davies et al. demonstrated that tacrolimus increases the GR hormone-binding affinity that was correlated with the loss of GR-associated FKBP51 and replacement of PP5 in mammalian L929 cells (24). Our data indicating that tacrolimus decreases the

Fig. 3. Tacrolimus causes the release of GR-associated FKBP51

RA-FLS were treated with various concentrations of tacrolimus for 4 hr. GR complex were immunoprecipitated and analyzed by anti-FKBP51 immunoblot. Three experiments were performed using different RA-FLS and each result (A, B, C) is shown.

Fig. 4. Effects of Dex and tacrolimus on IL-1β-induced p65 nuclear translocation. RA-FLS were pretreated with tacrolimus for 4 hr and followed by Dex for additional 1 hr. Cells were stimulated with IL-1β (10 ng/ml) for 1 hr. Cells were fixed and the subcellular localization of p65 was examined by immunocytochemistry using anti-p65 antibodies. Three experiments were performed using different RA-FLS and a representative result is shown.
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amount of GR-associated FKBP51 are consistent with the notion that FKBP51 is dissociated from the GR complex by tacrolimus in vitro, and it is possible that these phenomenon are linked with the tacrolimus-mediated augmentation of GR nuclear translocation. We favor the idea that tacrolimus can enhance the GR nuclear translocation by causing removal of FKBP51 from the GR complex, however, as this effect of tacrolimus occurred at a higher concentration than physiological levels, further investigations to fully understand the molecular interactions between tacrolimus and GR-associated immunophilins are thus needed.

GR phosphorylation affects the trans-activational function of GR. It has been suggested that Ser211 phosphorylation of GR occurs after it becomes activated and acquires the ability to bind to target DNA (11). Our observations that tacrolimus increased the Ser211 phosphorylation of GR that was located in the nucleus in low-dose dexamethasone-treated RA-FLS suggest that tacrolimus also affects the GR phosphorylation by facilitating the GR nuclear translocation in the presence of low concentrations of dexamethasone.

NF-κB is an essential element in the inflammatory cytokine production (12). In the present study, we focused on the effects of dexamethasone and tacrolimus on NF-κB activation. It has been postulated that glucocorticoid enhances the expression of 1κB-α and a model has been proposed in which activation of NF-κB could be counteracted by inducing an inhibitory protein by glucocorticoid (25, 260. However, our results showed that the IL-1β-induced translocation of NF-κB was not affected by the presence of tacrolimus or dexamethasone. This discrepancy could be attributed to the different concentrations of dexamethasone used in the experiments. There is a report demonstrating that increased 1κB-α is neither required nor sufficient for GR inhibition of NF-κB (27). Therefore, it is thus concluded that the induction of 1κB-α which consequently decreased the NF-κB nuclear translocation, is one of many mechanisms by which GR modulates NF-κB activation and the following activation of the immune or inflammatory systems. We herein demonstrated that tacrolimus decreased the DNA binding activity of NF-κB in the presence of low concentrations of dexamethasone. Activated GR is associated with NF-κB and inhibits the binding to their DNA motif (28). Our data showed that tacrolimus suppressed the binding activity of the p65 to its appropriate DNA motif in the presence of low concentrations of dexamethasone. The tacrolimus-potentiated GR nuclear translocation could be implicated in this suppressive effect on IL-1β-induced NF-κB activation. Our data suggest that tacrolimus can augment steroid-mediated anti-inflammatory activities even in the presence of low concentrations of steroid.

In conclusion, we investigated the interaction between GR signaling and tacrolimus in the rheumatoid synovium. In the presence of physiological levels of steroid, the GR sequester in the cytoplasm, however, tacrolimus induced the GR translocation in this condition, possibly by modulating the interaction between GR and GR-associated immunophilins. These data suggest that tacrolimus can exert anti-inflammatory properties by affecting GR signaling.

Fig. 5. Effects of Dex and tacrolimus on IL-1β-induced p65 DNA binding activity
A. RA-FLS were preated with Dex (10⁻⁷–10⁻⁹M) for 1 hr and then stimulated with IL-1β (10 ng/ml) for additional 1 hr. The DNA binding activity of p65, a NF-κB subunit, was detected TransAM™ NF-κB p65 kit using nuclear extracts as described in materials and methods. Data are means of two different RA-FLS run in triplicate ± SD. B. RA-FLS were preated with tacrolimus (4 hr) followed by Dex for additional 1 hr. Cells were stimulated IL-1β (10 ng/ml) for 1 hr. The DNA binding activity of p65, NF-κB subunit was detected Trans AM™ NF-κB p65 kit using nuclear extracts as described in materials and methods. Data are means of two different RA-FLS run in triplicate ± SD.
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References