Cyclosporine A Inhibits the mRNA Expressions of IL-2, IL-4 and IFN-γ, but not TNF-α, in Canine Mononuclear Cells

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ABSTRACT. The effects of the calcineurin inhibitors cyclosporine A (CsA) and FK506 on the mRNA expressions of various cytokines were evaluated in dogs to determine whether the effects of CsA and FK506 in dogs were similar to those in humans. The mRNA expression levels of the cytokines IL-2, IL-4, IFN-γ and TNF-α were measured in PHA-stimulated canine PBMC using real-time RT-PCR after incubation with CsA or FK506 for 5 hr. Both reagents inhibited IL-2, IL-4 and IFN-γ mRNA expressions in a dose-dependent manner. However, CsA hardly inhibited the mRNA expression of TNF-α. These findings are important for assessing the indications of CsA treatment in dogs.

KEY WORDS: atopic dermatitis, canine, cyclosporine A, cytokines, TNF-α.

Cyclosporine A (CsA), a neutral lipophilic cyclic undecapeptide isolated from the fungus Hypocladium inflatum gams, has been used as an effective immunosuppressive agent in human organ transplantation [4]. The immunosuppressive properties of CsA allow its utilization in multiple inflammatory diseases of presumed autoimmune origin, such as rheumatoid arthritis and inflammatory bowel disease (IBD) [5, 7]. In human dermatology, a large amount of evidence has been gathered to establish the efficacy and safety of CsA in the treatment of psoriasis and atopic dermatitis (AD) [11]. In veterinary dermatology, CsA has become a drug of choice in the treatment of canine AD [12, 19]. Several clinical trials have been performed to evaluate the efficacy of CsA for the treatment of dogs with AD [23, 30, 32], and demonstrated that CsA was as effective as glucocorticoids with minimal adverse effects.

The mechanism of action of CsA has been extensively investigated in humans. CsA permeates into cells and forms a complex with cyclophilin. The complex binds to calcineurin and inhibits its activation. Calcineurin activates the transcription factor nuclear factor of activated T cells (NFAT) by dephosphorylation, which in turn regulates the transcription of a number of cytokine genes including IL-2, IL-4 and IFN-γ [26]. By preventing NFAT dephosphorylation, CsA blocks the expressions of these cytokine genes [20, 28]. It is widely accepted that the immunosuppressive properties of CsA are mainly mediated via inhibition of IL-2, which is a major activation factor for T cells in numerous immunological processes. In dogs, CsA has been shown to inhibit T-cell activation and IL-2 production, similar to the case in humans [24, 35]. However, the effects of CsA on the mRNA expressions of cytokines have not been investigated in dogs.

FK506 (Tacrolimus) is another calcineurin inhibitor currently used in human AD patients [2]. The mechanism of action of FK506 is similar to that of CsA. FK506 binds to FK-binding protein in the cytoplasm, and the resulting complex inhibits the activity of calcineurin. Although FK506 was reported to be a safe alternative topical agent for dogs with AD [18], its effects on cytokine production have not been evaluated in dogs.

TNF-α plays key roles in the pathogenesis of many chronic inflammatory and rheumatic diseases, especially Crohn’s disease, rheumatoid arthritis and psoriatic arthritis, in humans [21]. CsA inhibits the mRNA expressions of not only IL-2 and IL-4, but also TNF-α, in human activated T cells [9]. A number of clinical trials have produced evidence to support the use of CsA for IBD and rheumatoid arthritis in humans [8, 25]. In dogs, TNF-α has been indicated to play an important role in the pathogenesis of IBD and immune-mediated polyarthritis [13, 27]. However, the effect of CsA on TNF-α mRNA expression has not been evaluated in dogs.

In the present study, we evaluated the effects of the calcineurin inhibitors CsA and FK506 on the mRNA expressions of various cytokines in dogs. The aim of this study was to determine whether the modes of action of CsA and FK506 in dogs are similar to those in humans.

MATERIALS AND METHODS

Reagents: Culture medium comprising RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS (ICN Biomedicals Inc., Aurora, OH), 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 µg/ml amphotericin B (Gibco Invitrogen Co., Rockville, MD) was used for cell cultures. PHA (Sigma) was dissolved in PBS as a stock solution of 1 mg/ml. CsA (Sigma) was dissolved...
in ethanol as a stock solution of 100 mg/ml. FK506 (Sigma) was dissolved in DMSO as a stock solution of 100 µg/ml. The stock solutions of CsA and FK506 were diluted in culture medium to create appropriate drug concentrations prior to experiments. The final concentrations of ethanol and DMSO in the experiments were less than 1% (v/v).

Cell culture: PBMC were isolated from heparinized peripheral blood samples obtained from three healthy beagles by density gradient centrifugation with Histopaque®-1077 (Sigma) as previously described [34]. Briefly, 10 ml of heparinized blood was layered onto 10 ml of Histopaque®-1077, and the overlaid solution was centrifuged at 340 × g for 30 min at room temperature. Mononuclear cells at the interface were collected and washed twice with PBS by centrifugation at 300 × g for 5 min. The isolated PBMC were treated with lysis buffer (7.5 g/l ammonium chloride and 0.02 M Tris-HCl pH 7.2) and rocked gently for 5 min to remove any residual red blood cells. Subsequently, the PBMC were washed with culture medium and suspended in culture medium at 2 × 10^6 cells/ml.

Cytotoxicity assay: Isolated PBMC were incubated with CsA (10, 90 or 810 µg/ml) or FK506 (0.1, 10 or 1,000 ng/ml) in the presence of 10 mg/ml PHA in duplicate for 24 hr at 37°C in a humidified 5% CO2 atmosphere. After the culture, the cytotoxicities were evaluated by determining the total cellular metabolic activity using the Celltiter 96® AQueous One Solution Cell Proliferation Assay system (Promega, Madison, WI). The absorbances at 490 nm were analyzed by using the Celltiter 96® AQueous One Solution Cell Proliferation Assay system (Promega, Madison, WI). The absorbances at 490 nm were measured using an ELISA plate reader (Multiskan JX; Thermo Electron Corporation, Waltham, MA).

Two-step real-time RT-PCR assay: Isolated PBMC were incubated with CsA or FK506 in the presence of 10 mg/ml PHA for 5 hr. After the culture, the cells were collected and total RNA was extracted using an RNeasy® Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The extracted RNA was stored at –70°C until analysis.

Oligonucleotide primers for amplifying the IL-2, IL-4, IFN-γ, TNF-α and GAPDH genes were designed using the Primer Express® software (Applied Biosystems, Foster City, CA) based on the reported nucleotide sequences of the canine genes: IL-2 (GenBank accession number: U28141), IL-4 (AF239917), IFN-γ (AF126247), TNF-α (DQ923808) and GAPDH (AB038240). The nucleotide sequences of the primers used for each cytokine are shown in Table 1.

First-strand complementary DNA (cDNA) synthesis and real-time PCR were performed using a SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen Corp., Carlsbad, CA). The RT reaction mixture contained 20 µl of total RNA, 40 µl of 2 × RT Reaction Mix and 8 µl of RT Enzyme Mix. After incubations at 25°C for 10 min, 42°C for 50 min and 85°C for 5 min, 1 µl of Escherichia coli RNase H supplied in the kit was added to the reaction mixture. After incubation at 37°C for 20 min, the resulting cDNAs were stored at –20°C until use. Real-time PCR was performed using a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) in aliquots of 50 µl total volume containing 3 µl of cDNA solution from the first-strand cDNA synthesis reaction, 25 µl of Platinum® SYBR® Green qPCR SuperMix-UDG, 1 µl of ROX Reference Dye and 10 µM of forward and reverse primers. All PCR reactions were performed in duplicate at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min during which fluorescence data were collected. The absence of genomic contamination of the isolated RNA was confirmed by performing GAPDH PCR reactions on the purified RNA samples.

A comparative threshold cycle (Ct) method was applied for the quantification of each cytokine. For each sample, the Ct values for the target and endogenous reference (GAPDH) genes were determined to calculate the relative transcription of the target mRNA against GAPDH mRNA. Subsequently, ∆Ct was calculated to normalize the amount of sample mRNA using the following formula:

\[ \Delta Ct = Ct \text{ value of the target cytokine} - Ct \text{ value of GAPDH} \]

All samples were examined in duplicate and the mean value of ΔCt was used for further analysis. The amount of mRNA for each cytokine was calculated by 2^{−\Delta Ct}. The mRNA expression of each cytokine was expressed as the percent cytokine mRNA expression using the following for-
mula:

\[
\text{Percent cytokine mRNA expression} = \left( 2^{-\Delta Ct} \right) \text{of sample with reagents} / \left( 2^{-\Delta Ct} \right) \text{of sample without reagents} \times 100.
\]

RESULTS

Cytotoxicities of CsA and FK506: The cytotoxicities of CsA and FK506 toward canine PBMC were evaluated using the MTS assay. The percent viable cell numbers were measured after incubation with CsA or FK506 for 24 hr. The viable cell numbers in the presence of either CsA or FK506 were not decreased at any of the concentrations tested. These results indicate that the cytotoxic effects of CsA and FK506 toward canine PBMC were negligible at concentrations from 10–810 ng/ml for CsA and from 0.1–1000 ng/ml for FK506 (Fig. 1).

Effects of CsA and FK506 on PHA-stimulated PBMC assessed by real-time RT-PCR: The effects of CsA and FK506 on the cytokine mRNA expressions in PHA-stimulated canine PBMC were determined using real-time RT-PCR. CsA and FK506 inhibited the mRNA expressions of IL-2, IL-4 and IFN-\(\gamma\) in a dose-dependent manner (Fig. 2). Specifically, 90 ng/ml CsA reduced the mRNA expression levels of IL-2 to 4.6%, IL-4 to 7.0% and IFN to 22.1%, while 10 ng/ml FK506 reduced the mRNA expression levels of IL-2 to 2.3%, IL-4 to 7.2% and IFN-\(\gamma\) to 15.7%. FK506 at 0.1–10 ng/ml inhibited the mRNA expression of TNF-\(\alpha\) in a dose-dependent manner, with the level of TNF-\(\alpha\) mRNA expression reduced to 44.5% at 10 ng/ml FK506. CsA also inhibited mRNA expression of TNF-\(\alpha\) at 10–90 ng/ml. However, at a higher concentration, CsA augmented the TNF-\(\alpha\) mRNA expression. The level of TNF-\(\alpha\) mRNA expression was increased to approximately 150% at 810 ng/ml CsA. Results those we obtained from further experiments of three other dogs also showed an increase of TNF-\(\alpha\) mRNA expression at 810 ng/ml CsA (Fig. 3).

DISCUSSION

CsA and FK506 are among the most potent immunosuppressive drugs available at the present time. Despite their lack of structural similarity, the modes of actions of these two drugs are similar, since they inhibit the phosphate activity of calcineurin, which regulates nuclear translocation and subsequent activation of the NFAT transcription factor. NFAT is thought to regulate a large number of cytokine genes, including IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, IFN-\(\gamma\), TNF-\(\alpha\) and GM-CSF [14]. These NFAT target cytokines are inhibited by CsA or FK506 in human activated T cells [26]. In the present study, the calcineurin inhibitors CsA and FK506 were found to inhibit the mRNA expressions of various cytokines, especially IL-2, in canine PBMC in a dose-dependent manner, similar to the case in humans. IL-2 plays important roles in the activation and proliferation of T cells. We have demonstrated that CsA markedly inhibited IL-2 mRNA expression within the therapeutic blood concentration in dogs, since the maximum blood CsA concentration was reported to reach more than 1,000 ng/ml in dogs treated with a therapeutic dosage of CsA (5 mg/kg) [31]. Lesional skin in human AD patients is histologically characterized by inflammatory T-cell infiltration, suggesting a key role for T cells in the generation of AD [10, 15]. In canine AD, CD3-positive T lymphocytes represent a major component of the mononuclear cell infiltrate in the skin lesions [29]. Therefore, a key role for CsA in the treatment of canine AD may be inhibition of T-cell activation by decreasing IL-2 production.

Studies in humans have suggested that IL-4 expression is a hallmark of AD, since IL-4 is known to induce isotype switching to IgE synthesis [16]. At the same time, other studies of AD in humans have indicated that IFN-\(\gamma\) plays a more important role than IL-4, especially in the development of chronic skin lesions [10, 16]. In dogs, significantly higher levels of IL-4 mRNA are expressed in atopic skin [22], and increased IFN-\(\gamma\) mRNA expression in atopic skin...

![Fig. 1. Cytotoxicities of CsA (A) and FK506 (B) toward canine PBMC. The viable cell numbers were measured by the MTS assay after incubation with CsA or FK506 for 24 hr. Each relative OD value is expressed as a percentage of the OD value without reagents. The results represent the mean ± SD of duplicate experiments.](image-url)
Fig. 2. Effects of CsA and FK506 on cytokine mRNA expressions in canine PBMC. The cytokine mRNA expression levels in PHA-stimulated canine PBMC were measured using real-time RT-PCR after incubation with CsA or FK506 for 5 hr. The level of mRNA expression for each cytokine was normalized to the corresponding GAPDH mRNA expression level. The results represent the mean ± SD of triplicate experiments. (A) IL-2 in the presence of CsA; (B) IL-2 in the presence of FK506; (C) IL-4 in the presence of CsA; (D) IL-4 in the presence of FK506; (E) IFN-γ in the presence of CsA; (F) IFN-γ in the presence of FK506; (G) TNF-α in the presence of CsA; (H) TNF-α in the presence of FK506.
cations and complications of CsA in dogs. These findings are important for assessing the mRNA expressions in dogs, similar to the case in humans.

Inhibitors CsA and FK506 inhibit IL-2, IL-4 and IFN-γ mRNA expressions with CsA is vomiting with a prevalence of 25% [33], and increased TNF-α-mediated diseases. TNF-α is known to cause nausea and vomiting via brain cell targets [6]. The results represent the mean ± SD of triplicate experiments.

On the other hand, the mRNA expression of TNF-α was not inhibited by CsA. In humans, CsA inhibits the mRNA expressions of not only IL-2, IL-4 and IFN-γ, but also TNF-α, in a dose-dependent manner [3]. Furthermore, our results showed that the mRNA expression of TNF-α tended to be increased by CsA at 810 ng/ml, which is within the therapeutic blood concentration in dogs treated with 5 mg/kg CsA [31]. It is therefore possible that TNF-α production is increased in dogs treated with CsA. TNF-α is a proinflammatory cytokine and considered to be an important effector molecule in the pathogenesis of IBD and immune-mediated polyarthritis [13, 27]. Although a previous clinical study reported that CsA appeared to be effective in dogs with IBD that were refractory to steroids [1], the results of the present study indicate that CsA should be used with care for the treatment of TNF-α-mediated diseases. TNF-α is known to cause nausea and vomiting via brain cell targets [6]. The most frequently observed adverse reaction in dogs treated with CsA is vomiting with a prevalence of 25% [33], and increased TNF-α production could be responsible for this vomiting observed in dogs after CsA treatment.

In conclusion, we have demonstrated that the calcineurin inhibitors CsA and FK506 inhibit IL-2, IL-4 and IFN-γ mRNA expressions in dogs, similar to the case in humans. However, TNF-α mRNA expression was hardly inhibited by CsA. These findings are important for assessing the indications and complications of CsA in dogs.

REFERENCES

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**Fig. 3. Effects of CsA on TNF-α mRNA expression in PHA-stimulated canine PBMC on further experiments.**


