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Short communication

Ciclosporin modulates the responses of canine progenitor epidermal keratinocytes (CPEK) to toll-like receptor agonists

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ABSTRACT

Toll-like receptor (TLR) 2 dependent pathways have an important role in the antimicrobial defense of human keratinocytes, and various factors and compounds have been shown to affect those pathways. Investigating Toll-like receptor function in canine keratinocytes and the potential for their modulation is of similar relevance in dogs due to the frequency of staphylococcal skin infections in this species, particularly in the context of canine atopic dermatitis. This pilot study hypothesized that ciclosporin would have a modulatory effect on the cytokine and TLR mRNA expression of canine progenitor epidermal keratinocytes in response to TLR2 agonists. No detectable up-regulation of TLR2, TLR4, IL-8 and TNF-α mRNA was detected following exposure to FSL-1, Pam3CSK4 and staphylococcal peptidoglycan (PGN). Ciclosporin alone did not alter the expression levels of these transcripts but in the presence of ciclosporin, TNF-α mRNA expression was upregulated in response to all three agonists and both TNF-α and IL-8 transcript abundance was increased in response to Pam3CSK4. The enhanced responsiveness of canine keratinocytes to TLR2 agonists in response to ciclosporin may imply that administration of this drug might enhance the innate immune barrier of skin.

1. Introduction

Once considered an inert barrier to infection, the skin is now thought of as an important immunological organ actively participating in innate and adaptive immunity. Keratinocytes contribute to host defence by secreting a large number of cytokines with pro-inflammatory and chemo-attractant effects, as well as an array of antimicrobial peptides (Suter et al., 2009).

Toll-like receptors (TLRs) constitute a protein family of cellular pattern-recognition receptors that mediate recognition of microbial pathogens and subsequent innate and adaptive immune responses in vertebrates (Kawai and Akira, 2010). Conserved pathogen-associated molecular patterns exhibited by substances such as peptidoglycan (PGN), lipopolysaccharide (LPS), lipopeptide and lipoteichoic acid vary only to a limited extent between microbial species; these molecules are amongst those that serve as ligands for TLRs. Interaction of TLRs with their ligands activates signalling cascades leading to the translocation of NF-κB into the nucleus, resulting in the production of effector molecules such as cytokines, chemokines and antimicrobial peptides. The cell-surface TLRs 2 and 4 seem to be especially important for recognition of antimicrobial cell wall components (Kawai and Akira, 2010). In fact, TLR2-related pathways appear to be required for the antimicrobial activity of keratinocytes, and are important in the context of staphylococcal infections (Sumikawa et al., 2006). A link between TLR2 polymorphisms and human atopic dermatitis has also been reported, although its role in this context is unclear at present (Ahmad-Nejad et al., 2004; Niebuhr et al., 2008; Oh et al., 2009).

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Calcineurin is a calcium and calmodulin-dependent serine/threonine phosphatase that is involved in various biological processes and signal-transduction pathways. Calcineurin inhibitors such as ciclosporin are useful in preventing transplant rejection and in the management of inflammatory diseases, presumably because of their ability to suppress the transcription of pro-inflammatory cytokine genes in T-lymphocytes. More recent work has indicated that ciclosporin has the ability to regulate calcineurin activity and influence keratinocyte proliferation, differentiation and innate immune function (Aizu et al., 2008; Al-Daraji et al., 2002, 2009; Lauer et al., 2006; Smit et al., 2008). Furthermore, the calcineurin pathway has been shown to interact specifically with TLR2 and 4 signalling (Kang et al., 2007). For instance, the calicneurin inhibitor pimecrolimus has been shown to increase the inhibitory capacity of human keratinocytes against *Staphylococcus aureus*, potentially related to up-regulated antimicrobial peptide production associated with exposure to TLR2/6 ligands (Buchau et al., 2008).

These observations suggest that calcineurin inhibitors such as ciclosporin, tacrolimus, and pimecrolimus may have therapeutic benefits in inflammatory skin disease such as atopic dermatitis via previously un-recognized effects on the immune functions of keratinocytes.

We have previously observed using immunohistochemistry that a canine keratinocyte progenitor cell line CPEK (CELLnTEC, Bern, Switzerland) expresses keratin 10, keratin 14, and involucrin, a pattern seen in canine suprabasal epidermis, and that these cells express TLR1, TLR2, TLR 4 and TLR6 transcripts (unpublished observations). We hypothesized that TLR2/1 and TLR2/6 agonists (Pam3CSK, FSL-1) and a TRL2/Nod2 agonist (staphylococcal PGN) would activate CPEK as measured by IL-8 and TNF-α transcript expression, and that co-incubation with ciclosporin would have a modulatory effect on cytokine and TLR mRNA expression in response to TLR2 agonists.

2. Materials and methods

2.1. Keratinocyte culture

Cryo-preserved CPEK cells (CELLnTEC, Bern, Switzerland) were cultured in 75 cm^3 tissue culture flasks (Milian Nunc, Geneva, Switzerland) containing epidermal keratinocyte medium (CnT-09, CellnTec) supplemented with 5% foetal calf serum and 1% L-glutamine (CellnTEC) at 37 °C in an atmosphere of 5% CO_2. Cells were harvested for experiments between passages 5 and 7 at approximately 80% confluency using trypsin and suspended in culture medium at 1×10^6 cells/mL. Aliquots of the cell suspension (100 μL) were added to 900 μL of culture medium in 24 well tissue culture plates (Scientific Laboratory Supplies, East Yorkshire, UK) to yield a final concentration of 1×10^5 cells/mL. These cells were grown to 95% confluency over 44 h prior to stimulation with TLR agonists in the presence or absence of ciclosporin.

Previous experiments demonstrated under the same culture conditions that CPEK constitutionally express TLR1, TLR2 and TLR6 (as well as involucrin) transcripts on five time points between 24 h and 120 h after seeding. Indeed, immunohistochemistry of CPEK harvested as described above and stained 20 h after seeding showed keratin 10, keratin 14 and involucrin protein expression characteristic of canine suprabasal keratinocytes. The aim was specifically to investigate the response of suprabasal, rather than undifferentiated keratinocytes, as in vivo TLR2 expression was found to be most pronounced in the suprabasal human epidermis (Pivarcsi et al., 2003).

2.2. TLR stimulation in the presence or absence of ciclosporin

The TLR agonists were obtained from Invivogen (San Diego, USA) and included PGN, peptidoglycan from *S. aureus* and a TLR2 and Nod2 agonist; Pam3CSK4, a synthetic lipopeptide and TLR2/TLR1 agonist; and FSL-1 (Pam2CGDPKHPKSF), a synthetic lipoprotein and TLR2/TLR6 agonist. Stock solutions were prepared and stored at −20 °C prior to inoculation into culture wells to yield final culture concentrations of 10 μg/mL (PGN-SA), 300 ng/mL (Pam3CSK4) and 50 ng/mL (FSL-1). These concentrations have previously been shown to activate canine monocytes/macrophages to produce pro-inflammatory cytokines (House et al., 2008).

Stock solutions (1 mg/mL) of ciclosporin (Cyclosporin A) (Sigma–Aldrich, Gillingham, UK) were prepared in 200 proof ethanol and stored at −20 °C prior to inoculation into culture wells to yield final concentrations of 1000, 100 and 1 ng/mL.

Replicate cultures were treated with medium alone or with either of the three TLR agonists, in the presence of vehicle or 1000, 100 and 1 ng/mL of ciclosporin. After 8 h incubation, culture medium was discarded, lysis solution (Sigma L8285, triplicate wells pooled) added and the lysate stored at −80 °C.

2.3. RNA extraction and cDNA synthesis

RNA was extracted from 500 μL volumes of thawed lysate using the GenElute Mammalian Total RNA mini-prep kit (Sigma) according to the manufacturer's instructions. Each sample was processed in duplicate. Extracted RNA concentration and purity were assessed using a NanoDrop ND1000 (Thermo Scientific, Wilmington, USA). Residual DNA was then digested using TURBO DNase (Applied Biosystems, Warrington, UK) and the RNA stored in aliquots at −80 °C.

After thawing, RNA integrity was determined by agarose gel electrophoresis. The duplicate RNA samples were combined before performing reverse transcription in duplicate. cDNA was synthesized using 2 μg RNA, a mixture of 0.5 μg oligo dT primers (Promega Corporation, Southampton, UK) and random primers (Promega), 0.5 mM each dNTP, 5 mM $MgCl_2$, 20 U RNasin RNase inhibitor (Promega), and Improm-II reverse transcriptase (Promega). Controls were performed without reverse transcription. Genomic DNA (gDNA) contamination was assessed using GAPDH primers (Table 1) in quantitative real-time PCR (RT-qPCR). Conditions for this assay were initial denaturation, 95 °C for 10 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 20 s, elongation at 72 °C for 10 s, primer dimer melt at