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Procarcinogenic Effects of Cyclosporine A are Mediated through the Activation of TAK1/TAB1 Signaling Pathway

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Abstract

Cyclosporine A (CsA) is an immunosuppressive drug commonly used for maintaining chronic immune suppression in organ transplant recipients. It is known that patients receiving CsA manifest increased growth of aggressive nonmelanoma skin cancers. However, the underlying mechanism by which CsA augments tumor growth is not fully understood. Here, we show that CsA augments the growth of A431 epidermoid carcinoma xenograft tumors by activating tumor growth factor β -activated kinase1 (TAK1). The activation of TAK1 by CsA occurs at multiple levels by kinases ZMP, AMPK and IRAK. TAK1 forms heterodimeric complexes with TAK binding protein 1 and 2 (TAB1/TAB2) which in turn activate nuclear factor κ B (NF κ B) and p38 MAP kinase. Transcriptional activation of NF κ B is evidenced by IKK β -mediated phosphorylation-dependent degradation of I κ B and consequent nuclear translocation of p65. This also leads to enhancement in the expression of its transcriptional target genes cyclin D1, Bcl2 and COX-2. Similarly, activation of p38 leads to enhanced inflammation-related signaling shown by increased phosphorylation of MAPKAPK2 and which in turn phosphorylates its substrate HSP27. Activation of both NF κ B and p38 MAP kinase provide mitogenic stimuli to augment the growth of SCCs.

Keywords

Cyclosporine A; Nonmelanoma skin cancer; Immune suppression; TAK1 signaling; Nuclear Factor Kappa B

Introduction

Skin cancers exceed all other types of neoplasm with about one-third of all human cancers occurring in the skin. The lifetime risk for development of skin cancer in the US population is estimated to be 1 in 5. More than 1.2 million new cases of nonmelanoma skin cancer

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(NMSC) including both squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are reported annually in the US alone. Ultraviolet B (UVB) is considered to be the major etiologic factor for NMSCs. The risk for NMSCs is further augmented by up to 250 fold in chronically immune-suppressed organ transplant recipients (OTRs). In addition, compared to the general population, OTRs develop NMSCs at a relatively young age with an increased risk of local recurrence, regional and distant metastasis and significant morbidity and mortality [1;2]. Although, the aggressive phenotypes of NMSCs in chronically immune-suppressed populations have been well-described, their exact underlying mechanism(s) remains elusive. It is believed that immunosuppressive medications lead to impairments of immune surveillance and -dependent eradication of precancerous lesions [3]. In addition to these predictive mechanisms, direct carcinogenic effects of these agents also occur.

Cyclosporine A (CsA) is a common immunosuppressive drug used in OTRs to reduce rejection risk. CsA is a cyclic non-ribosomal peptide of 11 amino acids produced by the fungus *Tolypocladium inflatum*, and contains D-amino acids. It is known to act by binding to a cytoplasmic protein, cyclophilin (immunophilin) in lymphocytes, particularly in T-lymphocytes, that ultimately inhibits IL-2 production, leading to an impairment in effector T-cell functioning [4]. In addition, to its effects on T-cells, it alters mitochondria-dependent cellular functions and blocks the mitochondrial permeability pore (MPP) opening, which alters the ability of cells to undergo apoptotic cell death [5]. We and others have shown that CsA-pretreated skin carcinoma cells do not respond to agents that induce apoptosis by inhibiting mitochondrial cytochrome c release, a potent pro-apoptotic stimulation factor [6]. Recently, we showed that xenograft squamous cell tumors developed in nude mice by A431 epidermoid carcinoma cells grow much faster and become much larger in size following treatment with CsA. We also showed that CsA-tumors manifested enhanced cellular proliferation and tumor vascularity with high expression of vascular endothelial growth factor (VEGF). In addition, these tumors manifested increased expression of mesenchymal and other tumor progression markers such as fibronectin, α -SMA, vimentin, N-cadherin, MMP-9/-2, snail, slug, and twist with a concomitant decrease in the epithelial polarity marker E-cadherin [7].

In this study, we investigated the mechanism by which CsA enhances growth of human SCCs. We provide evidence that CsA mediates activation of both nuclear factor κ B (NF κ B) and p38 MAP kinase by activating tumor growth factor β -activated kinase1 (TAK1). The activation of TAK1 by CsA occurs at multiple levels by the upstream kinases ZMP, AMPK and IRAK. TAK1 forms heterodimeric complexes with TAB1/TAK1 and regulates the activation of NF κ B and p38 MAP kinase. NF κ B activation occurs through the TAK1/TAB1-mediated activation of I κ B kinase β (IKK β) which allows faster degradation of Inhibitory κ B (I κ B) by its phosphorylation. To the best of our knowledge, this study provides the first demonstration of activation of the TAK1/TAB1 axis by CsA during the pathogenesis and progression of aggressive SCCs.

Materials and Methods

Cells

Human epidermoid carcinoma A431 (CRL-2592) cells were obtained from the American Type Culture Corporation (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO₂.

Animals

Female nude mice (Athymic NCr-nu/nu, 3-5 weeks, 25-30g) were purchased from NCI-Frederick Animal Production Program (Frederick, MD, USA). All experiments were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Antibodies

List of primary antibodies used in this study is provided as supplementary Table S1.

Western blotting

Briefly, 50 µg of total protein from tumor cell lysate was electrophoresed on 10% polyacrylamide gel (BioRad, Hercules, CA, USA). The protein was transferred, via electrotransfer, to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) and then the membranes were incubated with primary antibody overnight at 4°C. After washing with TBST the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) for 1 hour. The immunocomplex was detected with chemiluminescent substrate (Pierce) and was exposed to HyBlot CL autoradiography film (Denville Scientific Incorporated, Metuchen, NJ, USA).

Immunofluorescence analysis

Sections of tumor tissues (5µm) were cut, deparaffinized, rehydrated and then processed as follows: Tissue sections were first treated with Vector Antigen Unmasking solution according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). The nonspecific binding sites were blocked with 2% bovine serum albumin (BSA) (Sigma) in PBS for 30 minutes at 37°C. Tissues were then incubated at 4°C overnight with primary antibody, washed and positive cells were detected by an Alexa Fluor 594 (Invitrogen, Carlsbad, CA, USA), Dylight 488 (Pierce) or Fluorescein (Pierce)-coupled secondary antibody. Sections were mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories). Results were evaluated and pictures were taken microscopically using an Olympus BX51 microscope with an Olympus DP71 digital camera using software from the manufacturer (Olympus).

Results

CsA regulates crosstalk between TGFβ1 and NFκB by enhancing the expression of TAK1

CsA induces TGFβ production in various cell types. TGFβ is known to regulate NFκB through TAK-1 [8;9]. TAK-1 is a key mediator of stress and pro-inflammatory signals. It is well-established that both stress and pro-inflammatory signals are regulated at least in part by NFκB. Recently, it has been shown in malignant cells that TAK-1 by binding to its partner proteins TAB1 and TAB2, forms heterodimeric protein complexes which phosphorylate IKKβ. IKKβ is then activated and phosphorylates IκBα of IκBα-NFκB-p65/p52 and IκBα-NFκB-p65/p50 heterotrimeric complexes in the cytoplasm. This leads to the release of IκBα for its proteolytic degradation and consequently transcriptionally active protein complexes p65/p52 and p65/p50 migrate to the nucleus [10]. Transcriptional activity of NFκB is required for the activation of multiple genes involved in cell cycle and proliferation regulation and apoptosis induction. Following CsA treatment, we observed a significant increase in p-TAK1 levels while non-phosphorylated protein is not significantly altered (Fig. 1A). In addition, we observed substantial enhancement in the expression of both TAB1 and TAB2. However, CsA promoted complex formation substantially with TAB1 while TAK1/TAB2 complexes were less frequently visible. We also observed nuclear

NFκB-p65 staining in the CsA-treated tumors (Fig. 1B and 1C). Consistent with the increased nuclear p65, the expression of its downstream transcriptional targets Cyclin D1 and Bcl2 is also significantly enhanced (Fig. 1D)

CsA enhances stress and MAP Kinase signaling pathways

TAK1 is also known to phosphorylate several members of the MAPK family including MAPK-p38. Therefore we assessed the expression of total and phosphorylated MAPK-p38 in tumors excised from CsA- and vehicle-treatment groups. We also assessed both phosphorylated and total ERK levels. A significant increase in the expression of total and p-ERK (Fig. 2A&B), as well as in MAPK-p38 levels in CsA-treated tumors was observed. Then, we tested whether p38 transduces downstream signals through the phosphorylation-dependent activation of MAPKAPK-2. A striking increase in MAPKAPK-2 expression as well as in the phosphorylation of its substrate, heat shock protein-27 (Hsp27), was noticed. The co-localization of high levels of p-Hsp27 with MAPKAPK2 suggests the involvement of p38-dependent pro-inflammatory signaling in CsA-mediated tumor progression. This is further confirmed by the enhanced expression of COX-2 in these tumors.

CsA activates ZMP, AMP kinase and IRAK

We also investigated the expression of upstream kinases ZMP, AMP-activated protein kinase (AMPK) and interleukin-1 receptor associated kinase (IRAK). ZMP is a known activator of AMPK, a key controlling element in the mammalian target of rapamycin (mTOR) pathway [11]. We observed an increase in both ZMP and AMPK following CsA treatment as shown in Fig. 3A & B. In addition, we observed an increase in IRAK. IRAK associates with an E3 ubiquitin ligase, TRAF6, ultimately leading to activation of TAK1/TAB1 and TAK1/TAB2 complexes, which subsequently enhance IKKβ activation. CsA-mediated enhancement in the expression of these kinases suggests their role in the pathogenesis of aggressive neoplasm via TAK1/TAB1 pathway (Fig. 3A&B).

Discussion

OTRs are highly susceptible to early cancer development in multiple organs. In this population the risk of NMSCs is particularly enhanced by many fold as compared to normal cohorts [1]. Interestingly, the early molecular changes underlying the development of NMSCs in OTRs are identical to those which occur in immune competent populations [3]. However, we and others have demonstrated that immune suppressive drugs manifest direct effects on tumor cells. Some of these molecular alterations appear to be important in mediating a highly aggressive and invasive tumor phenotype [7,12]. In this study, we further defined the molecular mechanism by which skin cancer cells achieve an invasive phenotype in OTRs.

It has been shown that CsA enhances the expression of TGFβ in multiple human carcinoma cells [13]. We confirmed these earlier reports, and also found a significant induction of TGFβ1 in human epidermoid carcinoma A431 cells [7]. In addition, in these studies we showed that TGFβ-dependent signaling proteins including its receptors TGFβRI and TGFβRII and their target proteins, Smads, are increased in CsA-treated tumors [7]. Similar results were reported for the skin SCCs excised from OTRs [14]. Further strength for the notion that TGFβ1 is involved in the pathogenesis of CsA-mediated invasive neoplasm is provided by studies in which SCID-beige mice carrying lung and bladder xenograft tumors manifested increased pulmonary metastatic lesions following treatment with CsA, whereas metastatic tumor growth was significantly reduced in mice receiving treatments with anti-TGFβ antibodies [13]. We also demonstrated that A431 xenograft tumors from mice treated with CsA overexpress EMT markers and manifest enhanced migration and invasion [7].

TAK1 is an upstream member of the MAP kinase super-family which functions as a pivotal integrator of membrane-bound signals elicited by cytokines, particularly TGF β 1 [15]. The mechanism by which TAK1 functions involves formation of its complexes with TAB1 and TAB2. The observations in this study that CsA enhances phosphorylation of TAK1 and promotes binding of TAK1 with TAB1 and TAB2 suggest the involvement of this pathway in CsA tumorigenesis. Furthermore, Smad7 has been shown to bind with TAB2 and TAB3 to block recruitment of TAK1 [16]. In this regard, in earlier studies, we observed a reduced expression of Smad7 following CsA treatment [17;18]. Downstream signaling of TGF β that involves TAK1 and TABs has been shown to activate NF κ B transcriptional functions [10;19]. Our observations that CsA enhances nuclear localization of transcriptionally active NF κ B protein p65, with a concomitant enhancement in the expression of its transcriptional targets, cyclin D1, Bcl2, and COX2, suggest that TAK1 mediates enhanced proliferation and reduced apoptosis through CsA-dependent NF κ B. CsA in non-tumor cells inhibits COX-2 expression and –dependent angiogenesis. However, in OTRs chronic use of calcineurin inhibitors enhances tumor angiogenesis (microvessel density) and COX-2 expression in NMSCs [20]. Our results confirm these observations in OTRs and provide an explanation for increased VEGF and COX-2 in NMSCs.

In addition to its role in NF κ B activation, TAK1-TAB1/TAB2-dependent pathways may enhance MAPK-p38 expression and –dependent signaling pathway [10]. CsA in mesangial cells increases p38 signaling by augmenting the binding of TGF β to TGF β RII [21]. However, its effects on p38 pathway in tumor cells in OTRs remain so far unknown. Our studies showing an increased expression of p38 in CsA-treated tumors and an enhancement in its downstream signaling proteins, MAPKAPK-2 and p-Hsp27, suggest that CsA augments p38-dependent pathway during the progression of skin carcinogenesis. Furthermore, enhancement in ERK signaling by CsA in this xenograft model suggests that CsA acts at multiple targets in tumor cells to augment tumor growth and invasion.

This study also provides insight into the upstream signaling pathway that activates TAK1/TAB1/TAB2 pathway. In this regard, our observations that CsA enhances the accumulation of ZMP with a concomitant activation of AMPK and IRAK suggest novel molecular targets involved in the pathogenesis of CsA-mediated tumor growth in OTRs. In conclusion, CsA directly affects SCC pathogenesis under the predisposed conditions of immune suppression by promoting tumor growth and invasion invoking TAK1/TAB1/TAB2 pathway that blocks apoptosis and enhances cell survival. These effects are mediated through aberrantly activated p38, MAPK and NF κ B pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviation

CsA	Cyclosporine A
TAK1	Tumor growth factor β -activated kinase 1
TAB1	TAK binding protein 1

NFκB	Nuclear factor κB
NMSC	Nonmelanoma skin cancer
SCC	Squamous cell carcinoma
BCC	Basal cell carcinoma
UVB	Ultraviolet B
OTRs	Organ transplant recipients
MPP	Mitochondrial permeability pore
VEGF	Vascular endothelial growth factor
IκB	Inhibitory κB
IKKβ	IκB kinase β
IRAK	Interleukin-1 receptor associated kinase
AMPK	AMP-activated protein kinase

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Figure 1. CsA mediates activation of TAK1/TABs signaling

(A) Western blot analysis showing an increased expression of TAK1 and phosphorylated TAK1 proteins in CsA-treated tumors when compared to vehicle-treated control tumors. (B) Immunofluorescence staining showing co-localization and accumulation of TAK1/TAB1 and TAK1/TAB2 protein complexes in CsA-treated tumors (20X). (C) Immunofluorescence staining for NF- κ B. (D) Western blot analysis of cyclin D1 and Bcl2.

Figure 2. CsA enhances stress kinase and mitogen kinase signaling pathways in SCCs
 (A) Western blot analysis of ERK/p-ERK, p-p38, HAP27/p-HSP27 and COX-2 proteins in vehicle and CsA-treated tumors. (B) Immunofluorescence staining illustrating the expression of pERK, p-p38, MAPKAPK-2, pHSP27 in vehicle and CsA-treated tumors.

Figure 3. CsA activates AMP kinase and Inhibitory kappa kinase β (IKK β) in cutaneous SCCs
(A) Western blot analysis of proteins of ZMP, AMPK, IRAK1, IKK β , p-IKK β , I κ B in vehicle and CsA-treated tumors. (B) Western blot analysis of p-I κ B proteins in vehicle and CsA-treated tumors.

Figure 4. CsA activates TGFβ activated kinase 1 (TAK1) signaling pathway

Summary of the cascades of events triggered by CsA. CsA enhances TAK1 by activating TGF β -dependent signaling. CsA also augments the expression of upstream kinases ZMP and AMPK in addition to IRAK. The enhancement in the expression/activity of these signaling proteins contributes to MAP kinase and NF κ B activation, which together triggers proliferation, reduced apoptosis and enhanced angiogenesis.