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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 non-melanoma 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 term activate nuclear factor κB (NFκB) and p38 MAP kinase. Transcriptional activation of NFκB is evidenced by IKKβ-mediated phosphorylation-dependent degradation of IκB 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 NFκB and p38 MAP kinase provide mitogenic stimuli to augment the growth of SCCs.

1. 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 non-melanoma skin cancer (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

Abbreviations: CsA, cyclosporine A; TAK1, tumor growth factor β-activated kinase1; TAB1, TAK binding protein 1; NFκB, nuclear factor κB; NMSC, non-melanoma 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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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 κB (NFκB) 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 NFκB and p38 MAP kinase. NFκB activation occurs through the TAK1/TAB1-mediated activation of IκB kinase β (IKKβ) which allows faster degradation of Inhibitory κB (IκB) 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.

2. Materials and methods

2.1. 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 μg/ml of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Animals

Female nude mice (Athymic NCr-nu/nu, 3–5 weeks, 25–30 g) 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.

2.3. Antibodies

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

2.4. 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 h. The immunocomplex was detected with chemiluminescent substrate (Pierce) and was exposed to HyBlot CL autoradiography film (Denville Scientific Incorporated, Metuchen, NJ, USA).

2.5. Immunofluorescence analysis

Sections of tumor tissues (5 μm) 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 min 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).

3. Results

3.1. CsA regulates crosstalk between TGF1 and NFB 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 C). Consistent with the increased nuclear p65, the expression of its downstream transcriptional targets Cyclin D1 and Bcl2 is also significantly enhanced (Fig. 1D).

3.2. 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 and 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.