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Apigenin Inhibits UVB-Induced Skin Carcinogenesis: The Role of Thrombospondin-1 as an Anti-Inflammatory Factor

Salida Mirzoeva[*,1], Xin Tong[†,1], Bryan B. Bridgeman[‡], Michael P. Plebanek[§] and Olga V. Volpert[¶]

[*]Department of Dermatology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; [†]Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; [‡]Division of Hematology and Oncology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; [§]Department of Urology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; [¶]Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77054

Abstract

We have previously demonstrated that apigenin promotes the expression of antiangiogenic protein thrombospondin-1 (TSP1) *via* a mechanism driven by mRNA-binding protein HuR. Here, we generated a novel mouse model with whole-body *THBS*-1 gene knockout on SKH-1 genetic background, which allows studies of UVB-induced acute skin damage and carcinogenesis and tests TSP1 involvement in apigenin's anticancer effects. Apigenin significantly inhibited UVB-induced carcinogenesis in the wild-type (WT) animals but not in TSP1 KO (TKO) mice, suggesting that TSP1 is a critical component of apigenin's chemopreventive function in UVB-induced skin cancer. Importantly, TKO mice presented with the elevated cutaneous inflammation at baseline, which was manifested by increased inflammatory infiltrates (neutrophils and macrophages) and elevated levels of the two key inflammatory cytokines, IL-6 and IL-12. In agreement, maintaining normal TSP1 expression in the UVB-irradiated skin of WT mice using topical apigenin application caused a marked decrease of circulating inflammatory cytokines. Finally, TKO mice showed an altered population dynamics of the bone marrow myeloid progenitor cells ($CD11b^+$), with dramatic expansion of the population of neutrophil progenitors ($Ly6C^{low}Ly6G^{high}$) compared to the WT control. Our results indicate that the cutaneous tumor suppressor TSP1 is a critical mediator of the *in vivo* anticancer effect of apigenin in skin, specifically of its anti-inflammatory action.

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Introduction

There are more cases of skin cancer in the United States population than all other cancers combined. These cancers are on the rise and represent a significant health and economic problem. Nonmelanoma skin cancer (NMSC) occurs in all races worldwide, and more than two million new cases are diagnosed annually in the USA alone. Extensive epidemiologic, clinical, and biological studies have proven that ultraviolet B (UVB) radiation is the major cause of NMSC [1–5]. Despite significant risk mitigation by the application of sunscreens, a substantial part of the population remains subjected to increasing UVB exposure due to occupational hazards, recreational activities, and climate changes, causing a continued rise in the NMSC incidence [6].

Abbreviations: BM, bone marrow; COX-2, cyclooxygenase 2; IL, interleukin; MPO, myeloperoxidase; NHEKs, normal human epidermal keratinocytes; NMSC, nonmelanoma skin cancer; TSP1, thrombospondin 1; UVB, ultraviolet B; VEGF, vascular endothelial growth factor.

Address all correspondence to: Xin Tong, Department of Pathology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave, Chicago, IL 60611.
E-mail: x-tong1@northwestern.edu

[1]These two authors contributed equally to the manuscript.

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Skin is a critical barrier organ, which maintains organismal defenses against environmental insults. A proper inflammatory milieu in skin relies on reciprocal communications between the epidermal keratinocytes and other cell types [7–9]. Acute inflammation due to exposure to UVB radiation or xenobiotics involves massive production of cytokines and chemokines, often underpinned by cyclooxygenase (COX)-2 expression [10–12]. The resultant recruitment of neutrophils [13], monocytes, and macrophages [14] to the site of irradiation and further dynamic interactions between newly recruited inflammatory cells, which continuously produce inflammatory cytokines and matrix-degrading enzymes, further amplify the inflammatory responses, leading to acute responses, such as edema, or chronic changes including inflammation, fibrosis, and cancer [10,11].

In addition, UVB irradiation can induce potent angiogenic response, vascular leakage, and edema caused by the elevated vascular endothelial growth factor (VEGF) secretion by epidermal keratinocytes and fibroblasts, and exacerbated by attenuated production of an angiogenesis inhibitor thrombospondin-1 (TSP1) [15–17]. TSP1, a 450-kDa trimeric matricellular protein and the first identified endogenous angiogenesis inhibitor [18,19] is expressed at high levels in normal skin, especially in epidermal keratinocytes. On the cellular level, TSP1 blocks angiogenesis by causing endothelial cell apoptosis [20]. It impedes angiogenesis by blocking endothelial cell proliferation, chemotaxis [21], and through depletion of circulating endothelial cell progenitors, as was shown using TSP1 peptide mimetics [22]. TSP1 expression is severely downregulated in mouse and human epidermis following UVB irradiation and throughout progressive steps of skin carcinogenesis [15,23–27]. In contrast, overexpression of TSP1 in murine epidermis curtails angiogenesis and reduces the incidence of squamous cell carcinoma in a two-stage chemical model of skin carcinogenesis [25].

The antiangiogenic properties of TSP1 are mediated *via* two cell surface receptors, CD36 and CD47 [28], whereby TSP1 binding to CD36 causes the induction of Fas-Fas ligand [20] or TRAIL–TRAIL-R dependent death [29] predominantly in the endothelial cells. CD47-dependent events entail suppression of endothelial nitric oxide synthase (NOS) activity and NO production [30,31]. The decreased TSP1 levels have been linked to inflammation in the gut, whereby mice with whole body TSP1 knockout display aggravated symptoms of DSS-induced colitis, an experimental model of inflammatory bowel disease, and this condition can be ameliorated by administration of TSP1 peptide mimetic ABT-510 [32–34]. However, in patients with atopic dermatitis, circulating TSP1 levels show reverse trend (positive correlation with disease progression) [35], and the exact mechanisms by which TSP1 controls inflammatory responses are not clearly understood. In an earlier study [36], we have confirmed findings by others where UVB irradiation blocks TSP1 expression in skin keratinocytes *in vitro* and *in vivo*. Importantly, we demonstrated for the first time that normally high TSP1 expression in UVB-irradiated keratinocytes and skin can be restored by application of a natural bioflavonoid apigenin, which is known for its anti-inflammatory and antiangiogenic properties.

Apigenin (5,7,4′-trihydroxyflavone) is present in a wide variety of food sources including sweet pepper, parsley, thyme, celery, onions, and tea [37]. Topical application of apigenin reduces the incidence and the size of the tumors in both chemical and UVB-induced mouse models of skin carcinogenesis [38,39]. Apigenin has multiple properties of a successful chemopreventive agent. It inhibits angiogenesis *via* multiple pathways, including suppression of proangiogenic transcription factor HIF-1α and downstream major angiogenic growth factor VEGF [40–42] as well as COX-2/prostaglandin E2 related pathways [36,43,44]. Several *in vitro* studies also indicate the inhibition of NOS and IL6/STAT pathways by apigenin [45,46]. Apigenin also can stabilize and enhance the expression of tumor suppressive p53 [47,48] and therefore cause cell cycle arrest [49,50] and apoptosis [51–54]. In addition, apigenin displays potent anti-inflammatory activity in skin and other tissues [55]. Notably, apigenin inhibits UVB-induced expression of proinflammatory COX-2 in skin through multiple mechanisms [56,57]. Importantly, we have demonstrated that apigenin, *via* a mechanism driven by mRNA-binding protein HuR, restores the expression of antiangiogenic TSP1 and that proliferative effects of apigenin as well as its ability to block HIF-1α and COX-2 are, at least in part, mediated through TSP1. Here, we generated a novel mouse model with whole-body *THBS*-1 gene knockdown on SKH-1 genetic background, which allows studies of UVB-induced acute skin damage and carcinogenesis as well as therapeutic interventions, unhampered by pigmented skin and the coat of hair. Hairless mice with genetic ablation of TSP1 mounted an exacerbated cutaneous response to UVB, both in a short-term model and in a long-term (carcinogenesis) study. Topical application of chemopreventive agent apigenin significantly ameliorated UVB-induced carcinogenesis in the wild-type (WT) animals but not in TSP1 KO (TKO) mice, suggesting that TSP1 is a critical component of apigenin's anticancer and/or chemopreventive action. Importantly, TKO mice presented with the elevated cutaneous inflammation at baseline, which was manifested by increased inflammatory infiltrates (neutrophils and macrophages) and elevated levels of the two key inflammatory cytokines, IL-6 and IL-12. In addition, TKO mice showed a different population dynamics of the bone marrow (BM) myeloid progenitor cells ($CD11b^{+}$), with dramatic expansion of the population of neutrophil progenitors ($Ly6C^{low}Ly6G^{high}$) compared to WT control. Our data suggest that, in skin, TSP1 is a critical molecule that suppresses cutaneous carcinogenesis through its antiangiogenic and especially anti-inflammatory action.

Materials and Methods

All chemicals were from Sigma-Aldrich (St Louis, MO) unless otherwise specified. Apigenin was prepared as 50 mM stock in DMSO and kept at −20°C in aliquots.

Generation of TSP1-Null Mice on SKH-1 Background

All housing and experimental procedures were approved by the Animal Care and Use Committee at Northwestern University and performed in a strict adherence with guidelines provided by the National Institutes of Health. Homozygous *THBS1* −/− mice on C56BL/6 background (Jackson Labs, Bar Harbor, MN) were back-crossed onto hairless SKH-1 mice (Charles River, Houston, TX), and F5 hairless heterozygotes were selected and crossed onto each other to generate homozygous offspring, which was identified by genotyping (tail-snips). Tail DNA was prepared by proteinase K digestion at 55°C followed by isopropanol precipitation and used for PCR with reverse TSP1 primer (GAGTTTGCTTGTGGTGAACGCTCAG) and a forward TSP1 primer (AGGGCTATGTGGAATTAATATCGG). The expected size of PCR product is 700 bp for the wild type and 400 bp for the TSP null, respectively [58]. The pups homozygous for *THBS1* loss were subjected to further back-crossing (six generations) to ensure the gain of the new hairless trait together with the loss of