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SP1-regulated *p27/Kip1* gene expression is involved in terbinafine-induced human A431 cancer cell differentiation: An *in vitro* and *in vivo* study

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ABSTRACT

In this study, the differentiation-promoting effects of terbinafine (Lamisil®, TB) were investigated in human epithelioid squamous carcinoma (A431) cells. The polyhydroxyethylmethacrylate (poly-HEMA)- and type-I collagen-coated culture plate models were adapted to harvest the TB-induced differentiated cells by agitation of the suspension medium. We demonstrated that p27/Kip1, p21/Cip1 and the keratinocyte differentiation marker, human involucrin (hINV), were induced (>25 μM) in TB-induced differentiated A431 cells. Animal studies demonstrated that administration of TB (10 mg/kg body weight) inhibited A431-xenografted tumor growth through differentiation processes as evidenced by expression of pancytokeratin in tumor tissues. Immunocytochemical staining analysis showed that p27/Kip1, but not p21/Cip1, positive-stained cells were detected in the early-differentiated cells of TB-treated tumor tissues. SP1, which regulates p27/Kip1 expression, was induced by TB (>10 μM) in A431 cells. The TB-induced promoter activity and protein expression levels of p27/Kip1 were significantly attenuated by pretreatment with mithramycin A, a SP1 specific inhibitor. We also demonstrated that TB-induced differentiated A431 cells sorted from the poly-HEMA-coated culture plates were arrested in the G1 phase. TB-induced G1 arrest in the suspension-cultured cells was attenuated by mithramycin A pretreatment. Such results

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Abbreviations: BM, basement membrane; ChIP, chromatin immunoprecipitation analysis; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ECM, extracellular matrix; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FIL, filaggrin; hINV, human involucrin; LD, lower differentiated region; LOR, loricrin; NBT, nitro blue tetrazolium; NHEKs, Normal human epidermal keratinocytes; poly-HEMA, polyhydroxyethylmethacrylate; RT-PCR, reverse transcriptase-polymerase chain reaction; SC, stratum corneum; TB, terbinafine; TGase-1, tissue transglutaminase-1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WD, well differentiated region.

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suggest that SP1 plays a critical role in the p27/Kip1 gene transcriptional activation that may be subsequently involved in the TB-induced A431 cancer cell differentiation process.

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1. Introduction

Terbinafine (Lamisil®, TB) is a newly synthesized oral antimycotic drug in the allylamines class: a fungicidal agent that inhibits ergosterol synthesis at the stage of squalene epoxidation [1]. The cream and oral tablet forms of TB have been approved for clinical use in the United States [2]. The oral formulation has been on the market in various countries for more than ten years and as of 1997, more than 7.5 million individuals had been treated with this drug [3]. It shows a good safety profile and relatively few drug interactions [4]. An *in vivo* study demonstrated that TB have potential as an anti-cancer agent by arresting human cancer cell growth at the G1 phase [5]. In this study, experiments using a human squamous tumorigenic cell line (A431) were performed to determine if this TB-induced anti-tumor effect was capable of inducing a full program of differentiation. In this context, it is important to note that human skin cancer may be retarded by either consumption or topical application of TB.

In human squamous skin cancer cells, drug-induced differentiation is a multi-step process in which irreversible growth arrest is an early event followed by the sequential expression of differentiation-associated genes, including keratin 1 and 3, human involucrin (hINV), loricrin (LOR), filaggrin (FIL), and transglutaminase-1 (TGase-1) [6]. Agents that inhibit cancer cell proliferation and enhance the conversion of malignant cells to differentiated cells are expected to terminate cancer development.

Consumption of dietary agents that reduce keratinocyte proliferation and enhance the conversion of premalignant cells to differentiated cells is expected to reduce cancer development. For example, recent studies have demonstrated that a bioactive polyphenol from green tea, (−)-epigallocatechin-3-gallate (EGCG), acts to increase hINV gene expression, suggesting that EGCG treatment enhances normal human keratinocyte differentiation but not apoptosis [7,8]. On the other hand, the cyclin-dependent kinase inhibitor (p21/Cip1) was induced by curcumin, which participates in the cell differentiation process in normal human keratinocytes [8]. In addition, it has been demonstrated that quercetin arrests primary human foreskin keratinocytes in G1 through p27/Kip1 induction [9]. The roles of increased p21/Cip1 or p27/Kip1 in skin tumor carcinogenesis have been investigated *in vivo* by utilizing the two-stage skin carcinogenesis model on p27/Kip1 and p21/Cip1 knockout mice. The results demonstrated that p27/Kip1 deficient mice displayed a more rapid clonal expansion of initiated cells during promotion. In contrast, p21/Cip1 deficient mice mainly displayed a higher grade of undifferentiated tumors [10]. The p21/Cip1 is considered to function as a specific inhibitor of tumor cell growth. Previous paper have demonstrated that p21/Cip1 inhibits tumor cell proliferation by participating in the activation of tumor cell differentiation as evidenced by a higher expression profile of p21/Cip1 during all-trans retinoic acid-induced differentiation in various types of human cancer cells [11]. Thus, the most important issue is to identify specific agents with dual mechanisms of action in human skin cancer cells that both decrease proliferation and increase differentiation.

In this study, the expression of cell cycle and differentiation regulatory proteins were determined during commitment of TB-induced squamous cancer cell differentiation. For example, increased levels of expression of p27/Kip1, p21/Cip1 and keratinocyte differentiation markers (such as TGase-1) were observed simultaneously, suggesting a close link between cell growth arrest and differentiation. Moreover, according to previous studies, a SP1 binding sequence appears in the *p27/Kip1* gene promoter [12]. In addition, differentiation-associated markers such as TGase-1 [13,14] and hINV [15] which are expressed in the suprabasal layers of the human epidermis, were also transcriptionally regulated by SP1. Interestingly, our results demonstrate that SP1 protein levels and its transcriptional activity were induced by TB treatment in A431 cells. The experimental findings reported below highlight the molecular mechanisms underlying TB-induced cell growth arrest and differentiation activity in human squamous carcinoma cells.

2. Materials and methods

2.1. Terbinafine (TB)

TB is manufactured by Patheon Whitby Inc. Whitby, Ontario, Canada L1N 5Z5. In this study, TB was purchased from Novartis Pharmaceuticals Corporation, East Hanover, New Jersey 07936. Chemically, terbinafine hydrochloride is (*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphthalene-methanamine hydrochloride. Terbinafine hydrochloride is a white to off-white fine crystalline powder with 99.5% purity. It is freely soluble in DMSO, methanol and ethanol, and is slightly soluble in water.

2.2. Determination of the cell growth curve

Normal human epidermal keratinocytes (NHEKs) from neonatal foreskin were first cultured for maintenance in EpiLife® medium containing 0.06 mM $CaCl_2$ and EpiLife® Defined Growth Supplement (Cascade Biologics, Portland, OR) and used as normal cell control. The A431 human epitheloid squamous carcinoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum at 37 °C in an incubator containing 5% CO_2. Media with and without TB were changed daily until cell counting.

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