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Effects of dihydrotestosterone on rat dermal papilla cells in vitro

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ABSTRACT

Androgenetic alopecia involves the action of dihydrotestosterone (DHT) on dermal papilla cells (DPCs) that line the base of the hair follicle. However, the mechanism of DHT action is not completely understood. The effects of DHT on DPCs, regulatory cells that function in follicle growth and the hair cycle, were examined in immortalized cells derived from rat vibrissa follicles. DHT did not affect the proliferation of immortalized DPCs. However, flow cytometry analysis revealed that DHT increased cell-cycle arrest in these cells, which was accompanied by an increase in the $p27^{kip1}$ level and by decreases in cyclin E, cyclin D1, and cyclin-dependent kinase 2 levels. DHT treatment resulted in the phosphorylation and nuclear translocation of Smad2/3, a mediator of the transforming growth factor-β (TGF-β) signaling pathway, which leads to the catagen phase of the hair cycle. DHT also induced the phosphorylation and nuclear translocation of heat shock protein 27 (HSP27). Moreover, DHT decreased the levels of total and nuclear β-catenin, an important regulator of hair growth and proliferation, while lithium chloride, a glycogen synthase kinase-3β inhibitor, attenuated the DHT-induced downregulation of the β-catenin level. On the other hand, DHT increased the phosphorylation of mammalian target of rapamycin (mTOR), a regulator of proliferation, in immortalized DPCs. These results illustrate that DHT could shorten the duration of the hair growth cycle by initiating cell-cycle arrest, downregulating the β-catenin level, and upregulating the TGF-β/Smad and HSP27 level, whereas activation of mTOR by DHT could attenuate the inhibition of hair growth cycle in immortalized DPCs.

1. Introduction

Androgenetic alopecia (AGA) is the most common type of hair loss in males (Sinclair, 1998). A previous study showed that AGA is caused by 5α-reductase (Kaufman, 1996), an enzyme involved in the conversion of testosterone to dihydrotestosterone (DHT). Finasteride is a drug for treatment of prostatic hyperplasia and is also known to repress the progression of AGA through inhibition of type II 5α-reductase (Kaufman, 1996). DHT, a potent androgen, is synthesized by specific cells of the prostate, testis, and hair follicle, and it has a higher affinity than testosterone for the androgen receptor (AR) (Carson and Rittmaster, 2003). AR is expressed by various organs, including the prostate and testis, as well as by cells of the hair follicle, and its expression is higher in dermal papilla from AGA patients than in control subjects (Gelmann, 2002 and Hibberts et al., 1998). Previous studies suggest that other mechanisms of DHT action might also underlie AGA. For example, DHT can induce the miniaturization of dermal papilla and hair follicles, which leads to the transition from anagen to catagen (Sinclair, 1998). DHT can also increase the levels of transforming growth factor-β1 and -β2 (TGF-β), major regulators of hair follicle morphogenesis and hair loss (Foitzik et al., 2000 and Soma et al., 2002), in dermal papilla cells (DPCs), which decreases epithelial cell proliferation (Inui et al., 2002). β-Catenin, a key molecule in the Wnt/β-catenin pathway, can regulate hair follicle development, differentiation (Huelsken et al., 2001), and hair growth (Ouji et al., 2008). Nuclear β-catenin level was found to be upregulated in dermal papilla as well as upper matrix of anagen hair follicles (Ridanpää et al., 2001). (2′Z,3′E)-6-Bromoindirubin-3′-oxime, a GSK-3β inhibitor, induces the nuclear translocation of

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β-catenin (Yamauchi and Kurosaka, 2009). Lastly, mammalian target of rapamycin (mTOR), a serine/threonine kinase, can regulate cell proliferation, cell cycle progression, and hair cycle (Fingar et al., 2004 and Kellenberger and Tauchi, 2013). Previous study showed that the hair cycle initiation was delayed by treatment with the mTOR inhibitor (Kellenberger and Tauchi, 2013).

DPCs are mesenchyme-derived cells that line the base of the hair follicle. They are important regulators of hair growth and hair follicle regeneration (Stenn and Paus, 2001). DPCs from AGA patients express a higher level of AR than DPCs from control subjects (Hibberts et al., 1998). In another study, the expression of heat shock protein 27 (HSP27) was also higher in DPCs from balding individuals than those from non-balding counterparts (Bahta et al., 2008), suggesting that DHT may regulate DPC function and repress hair growth.

While many studies have reported a relationship between AGA and DHT, it is not clear how DHT causes alopecia. The mechanism of DHT action on DPCs is not completely understood. In the present study, we investigated the effects of DHT on the levels of TGF-β/Smad, HSP27, mTOR and β-catenin in immortalized DPCs.

2. Materials and methods

2.1. Reagents

DHT was purchased from TCI (Tokyo, Japan). Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dithiothreitol (DTT), phenylmethylsulfonylfluoride (PMSF), and the GSK-3β inhibitor, lithium chloride (LiCl), were obtained from Sigma (St. Louis, MO, USA). The p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580, and the mitogen-activated protein kinase kinase (MEK) inhibitor, U0126, were purchased from Biosource (Camarillo, CA, USA). Antibodies against extracellular signal-regulated kinase (ERK)1/2, phosphorylated ERK1/2, p38, phosphorylated p38, and phosphorylated HSP27 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against β-catenin, cyclooxygenase-2 (COX-2), CDK2, cyclin E, $p27^{kip1}$, nuclear factor kappa-B (NF-κB p65), Smad2/3, HSP27, proliferating cell nuclear antigen (PCNA), and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against β-actin, cyclin D1, and lamin B1 were purchased from Sigma, BD Biosciences (San Diego, CA, USA), and Abcam (Cambridge, UK), respectively. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit, goat anti-mouse, and mouse anti-goat IgGs were purchased from Santa Cruz Biotechnology. Goat anti-rabbit Alexa Fluor® 594 and chicken anti-goat Alexa Fluor® 488 were obtained from Invitrogen (Carlsbad, CA, USA). Aprotinin and leupeptin were obtained from Calbiochem (San Diego, CA, USA). Nonidet™ P-40 (NP-40) was from purchased from Roche (Indianapolis, IN, USA). Western blotting detection reagent, West-zol enhanced chemilumin, was purchased from Intron Biotechnology (Sungnam, Korea).

2.2. Cell culture

The rat vibrissa immortalized DPC line (Filsell et al., 1994) was kindly provided by the Skin Research Institute, Amore Pacific Corporation R&D Center, South Korea. DPCs were cultured in Dulbecco's modification of Eagle's medium (DMEM) (Hyclone Inc., UT, USA) supplemented with 10% fetal bovine serum (v/v) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively) at 37 °C in a humidified atmosphere of 5% CO_2 (v/v).

2.3. Cell viability assay

The effect of DHT on the proliferation of immortalized DPCs was evaluated by measuring the metabolic activity of these cells by an MTT assay (Scudiero et al., 1988). Immortalized DPCs (1.0×10^4 cells/ml) were seeded onto 96-well plates, allowed to attach for 24 h, and then cultured for an additional 24 h in serum-free DMEM. Thereafter, cells were treated with 10, 100, or 1000 nM DHT for 4 days. Control cells were treated with vehicle (i.e., ethanol diluted 1:1000 in serum-free DMEM). Cells were then incubated with MTT dye (50 μl of a 2 mg/ml stock) and incubated at 37 °C for 4 h. The plates were centrifuged at 150*g* for 5 min at room temperature, and the medium was carefully aspirated. Thereafter, dimethyl sulfoxide (200 μl) was added to each well to dissolve the formazan crystals. Cell viability was determined by measuring the absorbance at 540 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). This experiment was performed at least three independent times.

2.4. Cell-cycle analysis

The effect of DHT on cell-cycle progression was determined by flow cytometry after staining the cells with PI (Fried et al., 1976). Immortalized DPCs (1×10^5 cells/ml in 100 mm dishes) were incubated with 10, 100, and 1000 nM DHT for 24 and 48 h. Cells were harvested, washed twice with PBS, and fixed overnight with 70% ethanol at −20 °C. Thereafter, cells were washed with PBS, incubated with 50 μg/ml RNase A at 37 °C for 30 min, and stained with 50 μg/ml PI solution for 15 min in the dark. Flow cytometry analysis was performed with a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). Histograms were analyzed with Cell Quest software (Becton-Dickinson).

2.5. Immunoblotting

Immortalized DPCs (1.0×10^5 cells/ml in 100 mm dishes) were incubated for 24 h under serum-free conditions, and the cells were then treated with different concentrations of DHT for 24 h. In some cases, the cells were pretreated with or without 20 μM SB203580 or 20 μM U0126 for 1 h and then treated with 100 nM DHT for 0, 5, 15, 30, and 60 min or for 24 h. Cells were washed twice with ice-cold PBS, lysed with lysis buffer (50 mM Tris–HCl pH 7.5, containing 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/ml aprotinin, 25 μg/ml leupeptin and 1% NP-40 [v/v]) to obtain total proteins, and kept on ice for 30 min. Cell lysates were centrifuged at 22,000*g* at 4 °C for 15 min, and supernatants were stored at −20 °C until analysis. To obtain nuclear and cytoplasmic fractions, cells were lysed with 1 ml of lysis buffer (1 mM Tris–HCl pH 7.9, containing 10 mM NaCl, 3 mM $MgCl_2$, and 1% NP-40 [v/v]) at 4 °C for 10 min. After centrifugation at 3000*g*, supernatants were harvested as cytoplasmic fractions. The pellets were suspended in extraction buffer (20 mM HEPES pH 7.9, containing 20% glycerol [v/v], 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF) and kept on ice for 1 h. The lysates were centrifuged at 14,000*g* for 15 min, and supernatants were harvested as nuclear fractions. Fractions were stored at −70 °C until analysis. The protein concentration was determined by the Bradford method (Bradford, 1976). Around 30 μg of nuclear and cytoplasmic protein used to examine subcellular expressions. In the others experiments, 20–30 μg of proteins used to investigate the total or phospho protein levels. Equal amounts of protein were separated on 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) with a glycine transfer buffer (25 mM Tris–HCl pH 8.8, containing 192 mM