Minoxidil Acts as an Antiandrogen: A Study of 5α-reductase Type 2 Gene Expression in a Human Keratinocyte Cell Line

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ABSTRACT Although more than three decades have passed since the first use of minoxidil in androgenetic alopecia (AGA), its mechanisms of action have still not been comprehensively understood. 5α-reductase (5α-R) has an active role as the predominant enzyme in both AGA and female pattern hair loss (FPHL), which are also the main therapeutic indications of topical minoxidil. But there is insufficient literature data regarding the interaction of minoxidil and the enzyme 5α-R. Herein, we studied the in vitro expression levels of 5α-R type 2 (5α-R2) in a minoxidil-treated human keratinocyte cell line (HaCaT) in order to elucidate the relation of these two parameters. Cell proliferation assay was performed by a Xtt reagent (a yellow tetrazolium salt). After determination of non-cytotoxic concentration, HaCaT cells were treated with minoxidil. Ribonucleic acid (RNA) isolations were carried out from both non-treated and treated cell groups using a TRI reagent (an RNA, DNA, and protein isolation reagent). Gene expressions of 5α-R2 as study material and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control were determined by real time-quantitative polymerase chain reaction (RT-qPCR) analysis. Results were represented as 5α-R2 / GAPDH fold change. Minoxidil treatment resulted in a 0.22 fold change for 5α-R2 (p < 0.0001). This antiandrogenic effect of minoxidil, shown by significant downregulation of 5α-R2 gene expression in HaCaT cells, may be one of its mechanisms of action in alopecia.

KEY WORDS: minoxidil, 5α-R, mechanism of action

INTRODUCTION

Oral minoxidil has been used to treat hypertension since 1960s. Hypertrichosis as a consequence of minoxidil treatment was observed shortly thereafter, and these observations led to the development of topical minoxidil as a treatment for hair loss. Although it is being used for the treatment of male androgenetic alopecia (AGA) and female pattern hair loss (FPHL) for approximately three decades, but our understanding of its mechanisms of action on the hair follicle is still very limited (1,2). Due to the blood pressure lowering effect of oral minoxidil through relaxing the vascular smooth muscle by the action of its sulphated metabolite, as an opener of sarcolemmal adenosine triphosphate sensitive potassium channels (K₅,ATP), it is postulated that its stimulatory effect on hair growth is also related with the opening of potassium channels (1,3,4). Cutaneous blood flow was observed to increase 10-15 minutes after the application of topical minoxidil (5). A number of in vitro effects of minoxidil have been described in monocultures of various skin and hair follicle cell types including stimulation of cell proliferation, slowing the senescence of...
keratinocytes, inhibition of collagen synthesis, stimulation of vascular endothelial growth factor (VEGF), and prostaglandin synthesis (1,6-8). Some or all of these effects may be relevant to hair growth, but the application of results obtained in cell culture studies to the complex biology of the hair follicle is uncertain (1).

Although polygenic heredity is assumed to be the primary cause, androgens play an important role in both AGA and FPHL, seemingly independent of genetic predisposition (9). Androgen-dependent processes are predominantly due to the binding of dihydrotestosterone (DHT) to the androgen receptor (AR). The predisposed scalp exhibits high levels of DHT and increased expression of AR. DHT-related cell functions depend on the availability of weak androgens, i.e. their conversion to more potent androgens via the action of 5α-reductase (5α-R), low enzymatic activity of androgen inactivating enzymes, and functionally active ARs present in high numbers (10).

Although 5α-R has an active role as the predominant enzyme in both AGA and FPHL, which are also the main therapeutic indications of topical minoxidil, there is insufficient literature data about the interaction of the two. Herein, we studied in vitro expression levels of 5α-R type 2 (5α-R2) in a minoxidil-treated human keratinocyte cell line (HaCat) in order to elucidate the relation of these two parameters.

PATIENTS AND METHODS

Cell Culture

HaCat was cultured in Dulbecco's Modified Eagle's medium with high glucose, supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL gentamicin. The cells were maintained at 37°C in a humidified atmosphere at 5% CO₂ in a New Brunswick incubator. All supplements and media were purchased from Sigma Aldrich.

Preparation of Minoxidil Solution

We dissolved 522.5 mg minoxidil in 25 mL distilled water – 25 mL ethanol mixture to get a 5 mM minoxidil solution. This solution was used as a 100% sample, and other concentrations (10.0%, 5.0%, 3.0%, 1.0%, and 0.2%) of the solution were prepared by dilution with distilled water.

Table 1. Primers (5′ – 3′) of the genes studied

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>5α-R2</td>
<td>CGCTTCACGAGTAGCCAG</td>
<td>AATTAAGCCGATGCCCGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ATGGGTTGGAACCATGAGA</td>
<td>GTGCTAAGCAGTGTTGGTG</td>
</tr>
</tbody>
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5α-R2: 5α-reductase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase used as control

Cell Proliferation Assay

HaCat cells were seeded into 96-well plates (1×10⁴ cells/well) and were subjected to different concentrations of minoxidil solution to assess the cell proliferation. An XTT reagent (a yellow tetrazolium salt), was added to the plates after a 72-hour incubation period according to the manufacturer’s (Roche) instructions. Cells were then incubated at 37°C for 4 hours in order to reduce the XTT reagent to an orange formazan compound. The optical density of the soluble formazan compound was measured at 495 nm by a microplate reader (Bio-Rad).

Ribonucleic Acid (RNA) Isolation and Reverse Transcription

Total RNA was extracted from cells treated with minoxidil solution and from untreated cells using a TRI reagent (an RNA, DNA, and protein isolation reagent), according to the manufacturer’s (Sigma Aldrich) instructions. The concentration and purity of isolated RNA samples were determined by measuring optical densities at 260 nm and 280 nm using BioSpec-nano. The Transcriptor First Strand cDNA Synthesis Kit (Roche) was used for reverse transcription. Complementary deoxyribonucleic acid (cDNA) synthesis was performed with 500 ng total RNA; 2 µM of each final concentration of gene-specific primers of 5α-R2 as study material and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control (Integrated DNA Technologies); 10 U of Transcriptor Reverse Transcriptase; 20 U of Protector RNase Inhibitor; 1 mM each of dNTP mix and Transcriptor Reverse Transcription Buffer (5X) according to the manufacturer’s (Roche) instructions. Primer sequences (5′ – 3′) are presented in Table 1.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was carried out in Light Cycler 96 (Roche). Amplification of products was detected via intercalation of the SYBR green fluorescent dye (Fast Start DNA Green Master Kit, Roche). Briefly, total volume of reaction mix was 20 µl; containing 10 µl SYBR Green Master Mix (×2), 0.5 µM of reverse and forward primers, 2.5 ng cDNA, and the appropriate amount of nuclease free water. All samples were run as triplicates in
each run, including a non-template control and four standards (1:1, 1:10, 1:100, 1:1000). The RT-qPCR parameters were determined separately for each target according to melting and annealing temperatures of primers. Each parameter included a pre-incubation step for 10 min at 95°C and followed by 45 cycles of three amplification and melting steps. Melting curve analysis was performed to verify specificity. For quantitation of RT-qPCR results, the ΔΔCt method was used (2-ΔΔCt).

**Statistical Analysis**

All data are representative of three repetitions (n=3) and expressed as mean ± standard error of the means (SEM). Statistical evaluation was performed by an unpaired t-test, using Graph Pad Prism 5 Software (USA); results with a P value less than 0.05 were accepted as significant.

**RESULTS**

**Cytotoxicity Analysis (Cell Proliferation Assay)**

Based on cell proliferation ratios of treated cells with respect to the control cells, cytotoxicity levels of the minoxidil solution were determined. Higher concentrations were found to be cytotoxic for HaCat cells. For the subsequent analysis, the possible highest concentration was determined as 1%, and HaCat cells were incubated with 1% concentration of minoxidil solution before total RNA isolation (Figure 1).

**Gene Expression Analysis (RT-qPCR)**

Results were represented as Target / GAPDH Fold Change. Results of gene expression analysis via RT-qPCR showed that minoxidil solution caused statistically significant downregulation of 5α-R2 gene expressions, compared with untreated control cells. Minoxidil treatment resulted in 0.22 fold changes (P<0.0001) for 5α-R2 (Figure 2).

**DISCUSSION**

The pilosebaceous unit is the site of numerous cell interactions, and although the histology and structure of the follicle is well-known today, the molecular events controlling the hair cycle remain obscure. The development of *in vitro* and *in vivo* models, however, have provided some insights on the role of growth factors such as insulin like growth factor-1 (IGF-1), transforming growth factor-α (TGF-α), and VEGF, as well as androgens such as DHT and cytokines such as interleukin-1 (IL-1) (7,11).

Although the basic etiopathogenesis is not thoroughly understood, besides the aspect of genetic predisposition, the role of androgens in AGA is well established (2,10,12,13). The concentrations of DHT, 5α-R, and ARs are increased in a balding scalp. The higher the concentration of androgens and ARs, the greater the effect on the expression of genes which control follicular cycling (14). The finding indicate Xq12, the X chromosome region which encodes the AR, may represent a common genetic factor underlying both AGA and FPHL (15-18). Although the X chromosomal location of the AR gene indicates that the maternal line is the major inheritance of AGA in men, family studies showing resemblance of hair loss between fathers and sons suggest that some autosomal genes might also contribute to the phenotype (2,16,19). In a study on the identification of new susceptibility genes, strong evidence was found for an alopecia susceptibility locus on chromosome 3q26 (19). In another study, significant association was found with AGA on chromosome 20p11, suggesting that the 20p11 locus has a role in a yet-to-be-identified androgen independent pathway (20). It was later reported that although this locus is responsible from AGA, it has no association with FPHL (17,18).

On the other hand, contradicting results have been reported on the interaction of minoxidil and...
its androgen-dependent mechanisms of action: In a study on the effect of minoxidil on testosterone metabolism through cultured dermal papilla cells of balding or non-balding scalp and dermal fibroblasts, 5α-R activity was slightly increased in dermal papilla cells of a balding scalp, while there was no increase in other groups of cells. In the same study, the increase of 17β-hydroxysteroid dehydrogenase activity was much higher with minoxidil in dermal papilla cells of a balding scalp (21). In another study, minoxidil was found to be a weak inhibitor of human hair follicle 5α-R (22). Although one study found no antiandrogenic potential of minoxidil on androgen-dependent cutaneous structures in an animal model (23), it was contradicted by another group because female animals were used in the trial, and testosterone which can be converted to estradiol in hair follicles, rather than DHT, was chosen. The later study analyzing the antiandrogenic potential of minoxidil claimed that minoxidil suppresses AR-related functions by decreasing AR transcriptional activity and reducing the expression of AR targets at the protein level (24).

The significant suppression of 5α-R2 in HaCaT cells by minoxidil in our study, although not at the receptor level, supports the thesis of minoxidil’s antiandrogenic mechanism of action. This thesis, together with the literature data on the X chromosome-linked AR pathway and the autosomal chromosome-linked androgen independent pathway in the etiopathogenesis of alopecia, it may provide a better explanation of why some patients do not respond well to minoxidil therapy. Although further studies are needed, this thesis may also allow the exclusion of poor responders to minoxidil therapy and avoid waste of time in clinical practice by identifying probable androgen-independent alopecia patients to some extent.

CONCLUSION

The antiandrogenic effect of minoxidil, demonstrated by significant downregulation of 5α-R2 gene expression in HaCaT cells in our study, may be one of its mechanisms of action in AGA and FPHL, which is not being emphasized well in the dermatology literature.

References:


