CLINICAL STUDY

Isoenzyme type 1 of 5alpha-reductase is abundantly transcribed in normal human genital skin fibroblasts and may play an important role in masculinization of 5alpha-reductase type 2 deficient males

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Abstract

Objective: 5alpha-reductase enzymes reduce testosterone (T) to the most potent androgen dihydrotestosterone (DHT). Two isoenzymes are known to day. While the type 2-enzyme (5RII) is predominantly expressed in male genital tissues and mutations are known to cause a severe virilization disorder in genetic males, the role of the type 1-enzyme (5RI) in normal male androgen physiology is unclear. We investigated whether 5RI is transcribed in normal male genital skin fibroblasts (GSFs) and if the transcription is regulated by age or by androgens themselves.

Methods: GSF from 14 normally virilized males of different ages, ranging from 8 months to 72 years, obtained at circumcision were cultured. Total RNA was isolated after incubation for 48 h with 100 nM T or without androgens. Each sample was amplified in triplicate by real-time PCR with porphobilinogen desaminase as a housekeeping gene used for semiquantification. Selected cultures were analyzed after incubation with 10 and 100 nM T and 1 and 100 nM DHT for 24, 48 and 120 h.

Results: 5RI was transcribed in all investigated samples with a 4.5-fold variability in the mRNA concentration of different individuals. However, neither age-related regulation nor significant influence of T or DHT on the transcription rate was discovered.

Conclusion: Since 5RI is abundantly transcribed in GSFs, we hypothesize that this isoenzyme may play important roles in the androgen physiology of normally virilized males and may contribute to masculinization in 5RII-deficient males at the time of puberty.

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Introduction

Conversion of testosterone (T) to dihydrotestosterone (DHT), the most potent known androgen in humans, is performed by two isoenzymes of 5alpha-reductase. 5alpha-reductase type 2 (5RII) is predominantly expressed in male genital tissues and is indispensable for normal virilization of the external male genitalia during embryonic life. Mutations in the corresponding gene, SRD5A2, lead to a severe virilization deficit in genetic males termed 5alpha-reductase type 2 deficiency (1–4).

In contrast, 5RI is not expressed in human genital skin fibroblasts (GSFs) during embryogenesis, and natural mutations in the corresponding gene, SRD5A1, have not been described in human disorders of somato-sexual differentiation (5). Therefore it was concluded that 5RI does not play an important role in early male sexual development. However, there is distinct

evidence for the possible importance of 5RI for post-natal sexual differentiation: individuals with mutations in SRD5A2 and resulting 5alpha-reductase type 2 deficiency show regular, clear, but unexplained signs of virilization during puberty even in cases of homozygous disruptive mutations (6). This can occur to such an extent that affected individuals change their sex of rearing from female to male (1, 3, 7, 8). Furthermore, these individuals can show normal DHT-concentrations (1) and normal T/DHT-ratios (4, 9) in serum. Additionally, testosterone treatment can lead to an increase of DHT-levels up to normal values (1, 10). The conversion of T to DHT with following virilization could be catalysed by 5RI in these individuals. On the basis of these observations, we hypothesized that DHT-synthesis in GSFs is possible due to the expression of both isoenzymes. Evidence for this is given by earlier biochemical studies in GSFs. These have demonstrated the enzymatic activity of 5RII with a pH optimum at

5.5, but also an additional broad shoulder of activity extending over a more alkaline range, reflecting the activity of 5RI (2, 5).

We focused our study on the transcription pattern of 5RI in GSFs, which represent a well established stromal-derived cell culture model for male sexual differentiation research. Using semiquantitative real-time-PCR, we investigated whether this isoenzyme is transcribed in all age groups in GSF-cultures of normal virilized males, and if there is an age-related transcription pattern. Furthermore we wanted to find out if the RNA-expression of 5RI is regulated by its substrate T or its product DHT.

Material and methods

Cell cultures

Foreskins from 14 normally virilized males of different ages ranging from 8 months to 72 years were obtained at circumcision, dissected mechanically, and incubated in medium (DMEM-F12, Gibco, 5% CO₂, 10% charcoal-stripped fetal calf serum, streptomycin and penicillin) at 37 °C to culture GSFs, and subsequently stored in liquid nitrogen. For further analysis, cells of the second passage were used. After thawing, GSFs were first grown to approximately 80% confluence and then pre-incubated in medium without androgens for 48 h. Cells were afterwards incubated in medium containing either 100 nM T or no hormone for 48 h. Additional cultures of the fourth passage from 8 month, 12 year and 42 year old males were incubated with 10 nM and 100 nM T, and 1 nM and 100 nM DHT for 24, 48 and 120 h respectively. To create standard curves for real time PCR, blood of a normal virilized adult male was obtained with the PAXgene Blood RNA Kit (PreAnalytiX, Switzerland).

RNA analysis and reverse transcription

Total RNA from GSFs and blood was isolated using Rneasy columns according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA was quantified photometrically by measuring the absorbency at 260 nm in a DNA/RNA calculator (Pharmacia, Freiburg, Germany). To determine the quality and integrity of RNA, 5 µl aliquots were electrophoresed on formaldehyde-denaturing 1% agarose gels.

Reverse transcription was achieved by random primer (Invitrogen, Mannheim, Germany). 1 µg of whole RNA from each GSF sample was used. RNA, 20 pmol of random primer and DEPC-H₂O were added to a final volume of 10 µl. Mixtures were incubated at 70 °C for 5 min. Then 1x first-strand buffer, 1 mM dNTPs, 10 mM DTT and 10 U Rnase inhibitor (Boehringer-Mannheim, Mannheim, Germany) were added to a final volume of 20 µl. Reverse transcription was performed with 40 U Superscript II RT (Gibco BRL,

EGgenstein-Leopoldshafen, Germany) at 37 °C for 1 h using a thermocycler (PTC 200 DNA-engine, Biozym, Germany).

Real-time RT-PCR and melting curve analysis

SYBR Green (LightCycler-DNA Master SYBR Green I, Roche, Mannheim, Germany) was used as fluorescent signal in Real-Time PCR employing the LightCycler (Light-Cycler System, Software 3.0, Roche, Mannheim, Germany). For standardisation we used porphobilinogen desaminase (PBGD) as a control.

To synthesize standard curves for target and standard, we performed a dilution from 1:1 to 1:10 000 from the blood-RNA. The PCR-solution contained 10 µl of cDNA in different dilutions, 20 pmol of 5RI sense primer (5'-GGT-TTT-GGC-TTG-TGG-TTA-ACA-3') and 20 pmol of 5RI antisense primer (5'-CTC-TTC-AAA-TTT-CCG-GAG-GTA -C-3'), 20 pmol of PBGD sense primer (5'-AGA-GTG-ATT-CGC-GTG-GGT-ACC-3') and of PBGD antisense primer (5'-GGC-TCC-GAT-GGT-GAA-GCC-3') (TIB MOLBIOL, Berlin, Germany); 4 mM MgCl₂ and 2 µl FastStart DNA Master SYBR Green I. Mixtures were produced according to the manufacturers instructions (Roche, Mannheim, Germany). Cycling conditions were: 10 min denaturation at 95 °C to activate the polymerase, followed by amplification cycles: 15 s denaturation at 95 °C, 10 s primer annealing at 65 °C, 30 s primer extension at 72 °C and 5 s measuring of fluorescence at 81 °C for 40 cycles. Each dilution was amplified three times in PCR, calculated by Light-Cycler Software 3.0 (Roche, Mannheim, Germany) and subsequently shown on a logarithmic scale as standard curves.

RNA obtained from GSFs was amplified under the same conditions. To load the standard curves, we used two calibrators for each PCR. All samples were included in duplicate and each experiment was repeated three times (11). Due to the reliable results, cultures which were incubated with different concentrations of T and DHT were amplified only once.

To confirm the PCR product identity, each PCR was followed by a melting curve-analysis. After the final cycle, the products were denaturated at 95 °C, annealed at 70 °C, and slowly heated from 70 °C to 95 °C. During the slow heating process, fluorescence was measured. The fluorescence of the SYBR Green dye bound to the double-stranded amplicon dropped sharply as the fragment degraded. Specific identity of the PCR-products was confirmed by direct sequencing (SeqLab, Mannheim, Germany).

Results

5RI mRNA as well as PBGD was successfully detected in all samples by Real-Time PCR. The standard deviation did not show significant variations between the

different PCRs. Furthermore, we could show a constant transcription level of PBGD under androgen incubation compared with hormone-free conditions by evaluation of the crossing points (results not shown). We confirmed the specific identity of both PCR products by direct sequencing, revealing the published sequence of 5RI and PBGD (<http://www.ncbi.nlm.nih.gov>).

The 5RI-transcript was found in all different age groups. We measured the highest concentrations of mRNA in cultures of the 42 and 51 year old males, but there were no significant differences in the mRNA-concentrations between children and adults. Additionally, no obvious association with age was discovered. However, we found an up to 4.5-fold variability in the 5RI mRNA-concentration of different individuals (see Fig. 1).

We investigated the identical 4 cell lines from 2, 7, 42 and 51 year old males in whom Hellwinkel *et al.* had previously reported an age-related down-regulation of the mRNA-concentration of 5RII (12). Due to different methods being used by Hellwinkel *et al.* and due to low sample number, a statistical analysis comparing the previous and the present results was not possible. However, on the basis of the obtained results, it may be postulated that the relation of transcription of type 1 to type 2 increases with age.

When values of cells incubated with T and without androgens were depicted, no characteristic difference was found (see Fig. 2). Moreover, most samples of pre-pubertal males showed a relatively similar mRNA-concentration. In contrast, cell cultures of the 42, 48 and 51 year old males showed a more pronounced variability. Also in these samples, no significant differences between hormone treated and untreated cells were present.

These findings were reproducible in further experiments employing either a shortened or a prolonged T exposure at concentrations of 10 nM and 100 nM using pre-pubertal (8 month old), juvenile (12 years

old) and post-pubertal (42 years old) GSF and incubation times of 24, 48 and 120 h respectively. In an attempt to analyze the effect of the more potent androgen DHT on 5RI transcription, we exposed the cell lines to 1 nM and 100 nM of DHT for increasing periods of time. Again, no change in transcript levels was observed, no matter which DHT concentration was used and no matter how long the incubation time was.

Discussion

We found the mRNA of 5alpha-reductase type 1 in all investigated GSF cultures. GSFs represent a well established stromal-derived cell-culture model for male sexual differentiation research. They originate from the external genitalia – the most obvious androgen target tissue during male sexual differentiation – and they express the androgen receptor as the mediator of androgen action (13–17). Furthermore, these cells have been used to demonstrate the activity of both 5alpha-reductases as well as the decreased 5RII-activity in patients affected by 5RII deficiency for diagnostic purposes (1, 5).

Our results are in accordance with further studies investigating the transcription of 5RI in other human genital tissues. 5RI is transcribed in different androgen-dependent organs like the epididymis (18), the testis (19) and the prostate (20, 21). The localisation of 5RI in the cell nucleus of human prostate cells (22) and the close spatial relationship to the androgen receptor in prostate, epididymis and testis reported by Aumüller *et al.*, also suggest a functional connection between this isoenzyme and cellular androgen metabolism (19). On the basis of these studies and our results, 5RI may have important roles in androgen physiology of normal virilized males.

Despite our clear demonstration of considerable 5RI transcription in GSFs, the exact function of this

Figure 1 The diagram shows the transcription pattern of 5RI in human genital skin fibroblasts (GSFs) of various age groups. The results are described as a relative ratio of the target 5RI and the housekeeping gene PBGD. To determine the ratio, concentrations of both PCR products were measured. Then the concentration level of 5RI was divided by concentration level of PBGD using a special quantification software. As each sample was amplified three times, the standard deviation of these investigations is also shown in the diagram. It shows the transcription of 5RI in all investigated age-groups. We found no specific influence by age, but a high variability in the transcription level of different individuals.

Figure 2 This figure shows the influence of testosterone (T) on the transcription level of 5RI in human genital skin fibroblast (GSF) cultures. The gray bars demonstrate the relative ratio of cell cultures that were incubated with 100 nM T for 48 hours, and the black bars depict the ratio of cultures incubated with androgen-free medium. Most samples incubated with or without T reveal similar mRNA concentration. Therefore, there is probably no long-term influence by T on the transcription rate of 5RI. We used the same method for measurement of the relative ratio as described in the legend of Figure 1.

enzyme in androgen metabolism of these cells remains speculative. Both enzymes are expressed in GSFs, but exhibit their activity at different pH values (5). There is some further evidence from transcription studies that in normally virilized human males DHT-synthesis in GSFs could be controlled by both isoenzymes. Hellwinkel *et al.* (12) investigated the transcription pattern of 5RII in human GSFs of different age groups. As DHT is crucial for the most androgen-dependent processes (23), and based on the assumption that 5RII is the important isoenzyme which produces DHT from T in GSE, it should be expected that it is transcribed at constant levels despite increasing age. Unexpectedly, Hellwinkel *et al.* reported a clear decrease of the mRNA-expression of isoenzyme type 2 in adult males below measurable values (12). This is in accordance with earlier studies demonstrating a decrease of enzyme activity with age in patients with virilization disorders and normal controls (24).

In contrast to the type 2 isoenzyme, our present results demonstrate that transcription of type 1 isoenzyme is not influenced by age. Rather, it is transcribed at constant rates at all investigated ages. Therefore, it is likely that the role for type 2 isoenzyme is mainly restricted to virilization of the external genitalia in the embryo (2). In contrast, the type 1 isoenzyme may

take over DHT production after puberty and thus contribute to a continuous synthesis of DHT after birth and in later life. The age-independent expression level of the androgen receptor in GSF (12) could indicate a low but continuous demand of androgens in these cells provided via 5RI actions with increasing age.

We found up to 4.5-fold differences in 5RI transcription levels between individual cell cultures. Based on well-standardized methods of our present study, we believe that this reflects true inter-individual variabilities of 5RI transcription levels, as is also true for the enzyme activity in fibroblasts (24). This is in accordance with other studies, which showed obvious variations in transcript concentrations of normally virilized males for 5RII in the prostate (25) and in GSFs (12). The detected individual differences in transcript concentrations could contribute to the known, inter-individual variability of normal male sex phenotype and also to explaining the lack of a strict genotype–phenotype correlation in individuals with virilization disorders (1, 2).

Our observations indicate that neither the substrate T nor the product DHT control 5RI transcription in cultured GSFs (Fig. 2). This observation was independent from the used cell line, the incubation time and androgen concentration. This is supported by previous studies e.g. in human genital skin melanocytes (26), which also point to an androgen-independent transcription of 5RI-transcription. Similar results were found in GSFs for 5RII (12). In contrast to these experimental observations, studies on other tissues showed either a down-regulation of 5RII by T, e.g. in human liver (27), testis of rats (28) and in brains of adult rats (29) or up-regulation by T and/or DHT in human prostate (22) suggesting that 5RII is androgen-regulated in some tissues.

For 5RI, there is no report to date in which androgen-dependence of its transcription has been experimentally shown. There are several possible interpretations for the absence of testosterone effects on 5RI transcription in our study: first, SRD5A1 may not be an androgen-regulated target gene and, secondly, investigations on cell cultures do not reflect circumstances *in vivo*. Therefore, it cannot be excluded that GSFs would be responsive to androgen with respect to 5RI transcription *in vivo*. In this respect, potential influences of androgens on the transcription rate could be mediated by substances *in vivo*, potentially synthesized in adjacent tissue under androgen influence, acting in a paracrine manner but these would be absent in the investigated cell cultures. This is supported by a previous study from our own group which failed to show a reproducible androgen response program of these cells using microarray analyses covering a genome-wide scale (17).

Some studies show that mRNA concentrations do not generally reflect the concentrations of the translated proteins or the resulting enzymatic activity of

5RI accurately (30, 31) suggesting potential importance of post-transcriptional or post-translational modifications for this isoenzyme. We have not done experimental studies on the 5RI protein level, however, a possible influence by T or DHT on post-transcriptional and/or post-translational level cannot be excluded. Interestingly, some studies demonstrated good correlations between mRNA concentration and activity of the 5RI isoenzyme (32) supporting in general the biological relevance of mRNA-transcription levels which may also hold true for 5RI.

Since we demonstrated that 5RI is transcribed in all cell cultures from genital tissue of all different postnatal age groups in contrast to 5RII (12), we hypothesize that this isoenzyme is likely to contribute to masculinization in 5RII-deficient males at the time of puberty. This is in contrast to existing hypotheses suggesting that only T itself in high doses rather than DHT in lower doses will lead to the masculinization during puberty (33) and that 5alpha-reduction would only be mandatory to amplify the signal. In this hypothesis, however, there is no good explanation why virilization does not occur properly in the 5RII-deficient embryo that is under strong influence of high doses of T. One could speculate that mutations in SRD5A2-gene leading to reduction of 5R2 enzymatic activity would only lead to genital undervirilization, because its isoenzyme 5RI is not sufficiently transcribed in the preliminary stage of external male genitalia during embryonic life (34).

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