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Potential anti-androgenic activity of roxithromycin in skin

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Abstract

Since acne formation is a multistep process accelerated by androgens, we examined whether a new anti-acne antibiotic roxithromycin (RXM) may act as anti-androgen using transient transfection assays in human skin fibroblasts. The result showed no significant effect of 0.5, 1 and 5 μg/ml RXM on 10^{-9} M R1881-induced androgen receptor (AR) transcriptional activity. While the cotransfection of exogenous ARA55, a novel AR coactivator, increased AR transactivation up to 2.59-fold, this increase was attenuated by 5 μg/ml RXM to 64.7%. Semiquantitative RT-PCR results showed that 0.1 mM H_2O_2 treatment increased ARA55 mRNA expression level, indicating that reactive oxygen species increase the expression of ARA55 in skin. These results suggest that RXM may serve as anti-androgen only in the hypersensitive state to androgen, but not in the physiological state, through modulating end-organ hypersensitive condition to androgen possibly involving the pathway from reactive oxygen species to ARA55. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Acne; Anti-androgen; ARA55

1. Introduction

Acne formation is a multistep process, involving increased sebum production by androgens [1,2], colonization of *Propionibacterium acnes*, and resultant inflammation. The antibiotics against *P. acnes*, such as tetracyclines and macrolides, have been used as anti-acne drugs. These antibiotics have been revealed to have not only anti-bacterial but also anti-inflammatory properties [3]. However, so far it has not been examined whether anti-acne antibiotics may act as anti-androgen.

Recently we have cloned out a novel androgen receptor (AR) co-activator ARA55 from human prostate cDNA library using a yeast two hybrid assay system [4]. ARA55 enhances AR transactivation by around five-fold in the prostate cancer cell line, DU145 cells, indicating that ARA55 may play important roles in the progression of prostate

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cancer by the modulation of AR activity [4]. We examine here whether ARA55 can exert its function in human dermal fibroblasts and then overexpress ARA55 in dermal fibroblasts to recapitulate hypersensitive state to androgens in the skin. Moreover, we evaluate the potential anti-androgenic activity of roxithromycin (RXM), a new macrolide antibiotic, which have been reported to be effective in the treatment of acne [5].

2. Materials and methods

2.1. Cell culture

Human dermal fibroblasts were isolated from skin specimen obtained from plastic surgery operation and then maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

2.2. Reagents

RXM ($C_{41}H_{76}N_2O_{15}$, molecular weight = 837.06) was supplied by Roussel-Uclaf (Paris, France). Its stock solution was prepared in dimethyl sulfoxide (DMSO).

2.3. Transfection and reporter gene assays

Human dermal fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. At 50–70% confluency in a 24-well plate, the cells were transfected using lipofectamine plusTM (Gibco-BRL) according to the manufacturer's instruction. For luciferase assays, we transfected 0.3 μg of the reporter plasmids, mouse mammary tumor virus luciferase (MMTV-Luc) [6]. In some samples, the expression plasmids for ARA55 and AR, pSG5-ARA55 [4] and pSG5-AR [6], were co-transfected to the cells. The pRL-CMV vector, the *Renilla* luciferase control reporter vector driven by the CMV immediate-early enhancer/promoter, was co-transfected as an internal control. At 24 h after transfection, we put fresh medium with methyltrienolone (R1881, a synthetic androgen) or ethanol as a mock. We also treated the cells with RXM or ethanol as a mock in some experiments. At 48 h after transfection, the cells were harvested for luciferase assays. Luciferase activities were measured by the luminometer using the Dual-LuciferaseTM reporter assay system (Promega). The results were summarized from three independent sets of transfection and presented as mean ± SE. In the preliminary study, we confirmed that the cotransfection of ARA55 has no significant effect on the basal reporter activity without the ligands.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Cultured human dermal fibroblasts at the subconfluency were treated with 0.1 mM H_2O_2 for 2, 4, 8, 12, and 24 h. Then the cells were harvested and total RNA was isolated by acid–guanidinium thiocyanate–phenol–chloroform method. The recovery and purity of the RNA was calculated from the optical densities at 260 and 280 nm. One microgram of total RNA of each sample was reverse-transcribed using 2.5 μmol/l random hexamer, 5 mmol/l, $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris–HCl, pH 8.3, 1 mmol/l each of deoxynucleotide triphosphate (dNTP), 1.0 U/μl Rnase inhibitor (Takara, Tokyo, Japan), and 2.5 U/μl M-MLV Reverse Transcriptase (Gibco-BRL). The mixture was incubated at 25 °C for 10 min, followed by 42 °C for 45 min, 99 °C for 5 min, then 4 °C for 5 min. The resultant DNA was amplified using a thermal cycler (Astec, Fukuoka, Japan) in a final volume of 25 μl containing 2 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris–HCl, pH 8.3, 0.025 U/μl recombinant *Taq* DNA polymerase (Takara), and 0.5 μmol/l of each primer. The following sense and antisense primers were used: sense primer, 5′-GCACTTCGTTTGCGGAGGC-3′; antisense primer, 5′-CCGAAGAGCTTCAGGAAGC-3′. This combination of primers amplifies 633 bp of the C-terminal region of ARA55 [4]. After an initial denaturation at 95 °C for 9 min, 27 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min, and extension at 74 °C for 1 min) were followed by a terminal extension at 74 °C for 1 min. These ARA55 primers are the same as we have used previously and amplify 633 bp of the

C-terminal region of ARA55 [4]. PCR amplification was performed for 22 cycles (1 min at 94 °C, 45 s at 60 °C, and 45 s at 72 °C) for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as an internal control to demonstrate comparable RNA amounts and quality among samples. The products were analyzed by 2% agarose gel electrophoresis.

3. Results

3.1. Effect of RXM on the R1881-induced transcriptional activity in dermal fibroblasts

To examine whether RXM can suppress androgen activity in terms of transcriptional regulation, we performed luciferase assays using dermal fibroblasts transiently transfected with 0.3 μg of pSG5-AR. We tested the concentrations from 0.5 to 5.0 μg/ml, because 5.0 μg/ml of RXM is consistent with the maximal concentration in the serum after taking 150 mg of RXM per os [7]. The result showed no significant effect of 0.5, 1 and 5 μg/ml RXM on 10^{-9} M R1881-induced AR transcriptional activity (Fig. 1). From this result, RXM does not seem to have anti-androgenic effect in skin dermal fibroblast.

3.2. Effect of co-transfection of ARA55 with AR on R1881-induced transactivation in dermal fibroblasts

It has been demonstrated that ARA55 potentiates AR transcriptional activity by around five-fold in DU145 cells [4]. To ascertain whether ARA55 can enhance AR transcriptional activity in dermal fibroblast, we examined the effect of exogenous ARA55 overexpression on R1881-induced AR transcriptional activity. pSG5-AR (0.3 μg) was transiently transfected into all samples. The co-transfection of 0.3 μg or 0.6 μg pSG5-ARA55 showed 2.35- or 2.59-fold increase in R1881-induced AR transcriptional activity (Fig. 2). Although, compared with the ARA55 effect in prostate cancer cells [4], its effect in dermal fibroblasts is relatively weak, it was confirmed that ARA55 can potentiate AR transcriptional activity in dermal fibroblasts.

3.3. RXM effect on the R1881-induced transcriptional activity enhanced by ARA55 in dermal fibroblasts

Although RXM does not appear to have an anti-androgenic function in dermal fibroblasts without exogenous ARA55 transfection (Fig. 1), RXM may modulate the AR transactivation enhanced by ARA55 overexpression. Thus, we transfected 0.3 μg of pSG5-AR and 0.6 μg of pSG5-ARA55 and examined the effect of 1 and 5 μg/ml RXM on R1881-induced transactivation in dermal fibroblasts. The co-transfection of ARA55 increased MMTV-luciferase activity by around 2.5-fold (Fig. 3, lane 3). Although 1 μg/ml RXM did not show any significant effect on this enhanced induction (Fig. 3, lane 4), RXM at the concentration of 5 μg/ml suppressed the AR transactivation to 64.7% induction (Fig. 3, lane 5). These results suggest that RXM has an anti-androgenic activity under the overexpression of ARA55.

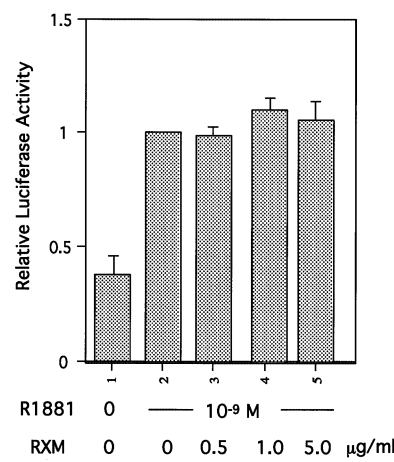

Fig. 1. RXM effect on the R1881-induced transcriptional activity in dermal fibroblasts without transfection of ARA55. We transfected 0.3 μg of pSG5-AR and 0.3 μg of MMTV-Luc into the cell cultured at 50–70% confluency in a 24-well plate using lipofectamine plusTM (Gibco-BRL) and treated with 0.5 (lane 3), 1 (lane 4) or 5 μg/ml (lane 5) of RXM and 10^{-9} M R1881 (lanes 2-5) or ethanol as a mock (lane 1). The relative reporter gene activities were compared with the luciferase activity in the presence of R1881 and the absence of RXM (lane 2).

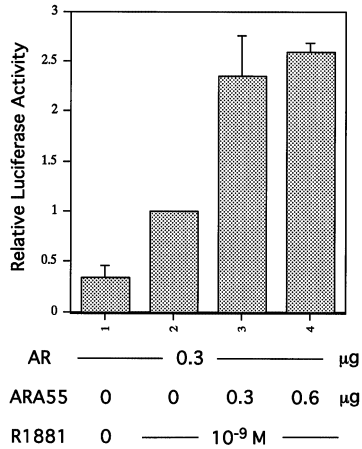

Fig. 2. Exogenous ARA55 effect on the R1881-induced transcriptional activity in dermal fibroblasts. We transfected 0.3 μg of pSG5-AR, 0.3 μg of MMTV-Luc (lanes 1–4), and 0.3 (lane 3) or 0.6 μg (lane 4) of pSG5-ARA55 into the cells cultured at 50–70% confluency in a 24-well plate using lipofectamine plusTM (Gibco-BRL), and treated with 10^{-9} M R1881 (lanes 2–4) or ethanol as a mock (lane 1). The relative reporter gene activities were compared with the luciferase activity in the presence of R1881 and the absence of ARA55 (lane 2).

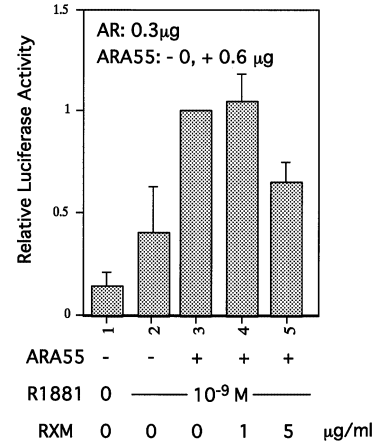

Fig. 3. RXM effect on the R1881-induced transcriptional activity in dermal fibroblasts with transfection of ARA55. We transfected 0.3 μg of pSG5-AR, 0.3 μg of MMTV-Luc (lanes 1–5), or 0.6 μg of pSG5-ARA55 (lanes 3–5) into the cells cultured at 50–70% confluency in a 24-well plate using lipofectamine plusTM (Gibco-BRL), and treated with 10^{-9} M R1881 (lanes 2–5) or ethanol as a mock (lane 1). The relative reporter gene activities were compared with the luciferase activity in the presence of R1881 and ARA55 and the absence of RXM (lane 3).

3.4. H_2O_2 increases ARA55 mRNA expression in dermal fibroblasts

Reactive oxygen species (ROS), generated from infiltrating neutrophils around the lesions of acne, has been known to play important roles in the acne formation mainly by progressing inflammation [3,8]. We assumed that ROS may modulate androgen sensitivity in the skin, possibly through upregulating the expression of ARA55. To test this possibility, we treated cultured dermal fibroblasts with 0.1 mM H_2O_2 and examined the change of ARA55 mRNA expression level by semiquantitative RT-PCR. As shown in Fig. 4, ARA55 mRNA was gradually increased by the H_2O_2 treatment, suggesting that ROS increase the expression of ARA55 and consequently modulate androgen sensitivity in the skin.

4. Discussion

The previous study showed the striking variability in the relationship between plasma free testosterone and acne severity in women and proposed a model in which variation in skin sensitivity to androgen and the level of androgen equally contribute to the pathogenesis of acne [9]. Very recently it has been reported no positive correla-

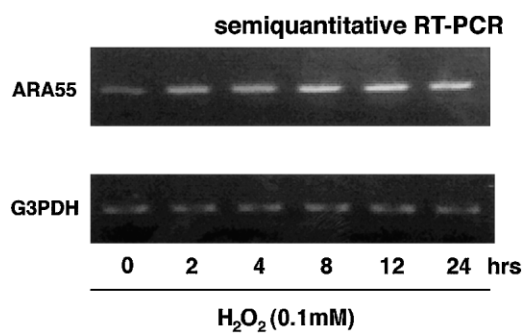

Fig. 4. H_2O_2 effect on ARA55 mRNA expression in dermal fibroblasts. Semi-quantitative RT-PCR was performed using the same amount of cDNA reverse-transcribed from total RNA isolated from cultured human dermal fibroblasts treated with 0.1 mM H_2O_2 for 0, 2, 4, 8, 12, and 24 h. The upper panel shows the 633 bp products of ARA55 mRNA, and the lower shows G3PDH mRNA as the internal controls.

tion between the grade of acne severity and the clinical or laboratory markers of androgenicity tested [10]. From these observations, the end-organ sensitivity may be a very important factor in the pathogenesis of acne. In the present study, we recapitulated the hypersensitive state to androgen by overexpression of exogenous ARA55 in vitro. Since RXM showed anti-androgenic activity only under overexpression of ARA55, we suggest that RXM serves as anti-androgen only in the state of androgen hypersensitivity, but not in the physiological state. We showed here that H_2O_2 treatment increases the expression of ARA55, suggesting that ROS may modulate androgen sensitivity through ARA55 in the skin. Therefore, RXM may exert the potential anti-androgenic activity in the hypersensitive state to androgen such as acne, which has been reported to involve ROS production by neutrophils in the inflammatory process [3,8].

So far, the concentration of H_2O_2 in the tissue of acne in vivo is unknown. Previously it was reported that H_2O_2 at higher concentrations than 0.2 mM triggered apoptosis of dermal fibroblasts, but not at 0.05 and 0.1 mM [11]. Therefore, we determined for the biological relevance that the concentration of H_2O_2 should be 0.1 mM in this study. Since we used skin dermal fibroblasts from normal human, but not sebocytes from acne patients, it is questionable whether our experimental system can reflect the pathological condition of acne inflammation. Further studies using cultured human sebocytes are necessary to conclude our hypothesis. However, we report here for the first time that anti-acne antibiotics may have anti-androgenic activity in the skin.

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