Role of androgens in proliferation and differentiation of mouse mammary epithelial cell line HC11

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Abstract

Androgens have been found in mammary epithelium and in milk throughout the cycle of the mammary gland *in vivo*. The aim of this study was to investigate the possible role of these substances in mammary epithelial growth and differentiation in the mouse HC11 cell line. Cells were stimulated with testosterone, dihydrotestosterone, androstenedione and 5 α -androstane-3 α ,17 β -diol at concentrations ranging between 0.3 nM and 30 nM. Cyproterone acetate or flutamide, androgen receptor antagonists, (3 μ M) were used to block specific androgen effects. Proliferative effects were measured by an MTT (tetrazolium blue) conversion test and [³H]thymidine uptake. HC11 cells were transfected with p β cCAT, a chimeric rat β -casein gene promoter–chloramphenicol acetyl transferase (CAT) gene construct and CAT ELISA

was used to determine gene expression. RT-PCR was performed to detect androgen receptor expression. After 24, 48 and 72 h androgens significantly ($P < 0.05$) increased proliferation. Androgen antagonists significantly ($P < 0.05$) reduced the proliferative effects. Furthermore androgens potentiated the lactogenic effect of prolactin, insulin and dexamethasone ($P < 0.05$). Finally, the androgen receptor gene was expressed in both proliferating and differentiated HC11 cells. These observations lead us to hypothesize an activity of this class of steroids in mammary physiology. In particular, androgens stimulate cell proliferation and β -casein gene expression; this influence appears to be mediated by androgen receptors.

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Introduction

Development of the mammary gland may be divided into several stages that differ in morphology, function and hormonal responsiveness. Coordinated development, cyclical regulation of mammary gland morphogenesis and function are in large part dependent upon the ability of specific mammary cell types to respond to ovarian steroid hormones, namely estrogen and progesterone. The direct actions of steroid hormones on different mammary tissues are dependent upon the presence of their specific receptors (Hoshiai *et al.* 1982, Humphreys *et al.* 1997). However, the presence of steroid receptors may not be sufficient for initiation of a biological response at all developmental stages (Haslam 1987, Sutherland *et al.* 1998); in fact an important role in mammary function is exerted by pituitary hormones such as prolactin (PRL) (Topper & Freeman 1980), in particular for terminal differentiation and synthesis of milk proteins.

The role of another class of steroids, androgens, has been extensively investigated in breast cancer in human and

mouse (Labrie *et al.* 1992, Pasqualini 1993, Sato *et al.* 1993, Recchione *et al.* 1995, Birrel *et al.* 1998) and their specific stimulatory and/or inhibitory actions on growth have been described for several breast cancer cell lines (Poulin *et al.* 1988, Tanaka *et al.* 1992, Birrel *et al.* 1995).

Recent studies have provided substantial evidence that bioactive androgens can be synthesized not only by the classic steroidogenic structures (gonads, adrenal and placenta) but also by peripheral organs such as stomach and duodenum (Belvedere *et al.* 1993, Dalla Valle *et al.* 1995, Le Goascogne *et al.* 1995). Furthermore, androstenedione, testosterone, 5 α -androstane-3,17-dione and enzymes involved in biosynthesis or activation of androgens, are found in the mammary tissue at different stages of development (Belvedere *et al.* 1996). Moreover, 5 α -androstane-3,17-dione has been detected in cows' milk (1–5 ng/ml) suggesting that this hormone can be produced, at least in part, in the mammary gland and indicating a possible role of 5 α steroids in the hormonal control of lactogenesis (Darling *et al.* 1974).

The presence of androgens in the normal mammary gland may be explained as an effect of *in situ* progesterone or dehydroepiandrosterone (DHEA) metabolism, and led us to investigate whether they may exert a paracrine or autocrine role in the development and differentiation of the epithelium. *In vitro* methods to study mammary epithelial cell proliferation and synthesis of milk constituents need a physiological and reproducible model. This study was conducted using the mouse mammary epithelial cell line HC11. This cell line was derived from midpregnant BALB/c mouse mammary tissue and is considered to retain important characteristics of normal mammary epithelial cells such as the ability to produce milk protein in response to lactogenic hormones without cultivation on exogenous extracellular matrix or cocultivation with adipocytes or fibroblasts (Doppler *et al.* 1989). Furthermore, expression of c-myc in HC11, a gene commonly overexpressed in breast cancer cell lines, parallels expression in the mammary gland; in fact, c-myc is expressed in growing but is repressed in confluent and differentiated cells (Grolli *et al.* 1997). In this paper, the direct effects of androgens on HC11 proliferation are reported; furthermore, the influence of androgens on β -casein gene expression and the detection of specific androgen receptor (AR) mRNA are shown.

Materials and Methods

Materials

All reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise specified.

Cell culture and transfection

HC11 mouse mammary epithelial cells were grown in RPMI 1640 medium containing 10%, charcoal-stripped, heat-inactivated fetal calf serum (FCS) (Gibco BRL, Milan, Italy), 5 μ g/ml insulin, 10 ng/ml epidermal growth factor, 50 μ g/ml gentamicin (growth medium). The medium was changed every 2 days. For cell transfection, approximately 3×10^5 cells per 75 cm² flask were co-transfected with 1 μ g pSV2 neo and 4 μ g p β cCAT (Doppler *et al.* 1989) using Escort Transfection Reagent. After 14 days of selection in growth medium containing 200 μ g/ml geneticin (G418), colonies were trypsinized, expanded as pools of colonies and then used. To induce milk protein gene expression, cells were grown to confluence, cultured for a further 2 days in growth medium and subsequently cultured in 3% FCS medium with 5 μ g/ml insulin, 0.1–25 μ g/ml ovine PRL (oPRL) (32 IU/mg) and 1 μ M dexamethasone (DIP medium).

Treatments

Androgens and 17 β -estradiol were added at concentrations ranging from 0.3 to 30 nM; flutamide (FLU), cyproterone

acetate (CYP) and androsten-4-ol-3,17-dione acetate (AI) were added alone or in association with androgens at the concentration of 3 μ M. Proliferation was evaluated at 24, 48 and 72 h. Androgens at the concentration of 0.3 μ M and anti-androgens at the concentration of 3 μ M were added for 72 h for the experiments on β -casein induction.

Proliferation tests

Proliferation was measured by an MTT (tetrazolium blue) conversion test and tritiated thymidine uptake. Briefly, at the end of treatments, 20 μ l MTT (5 mg/ml) were added to the culture for 4 h. Then, after addition of 100 μ l of solubilization solution (10% SDS in 0.01 M HCl) cells were incubated at 37 °C overnight. Specific optical density was measured at 540 nm, using 690 nm as the reference wavelength in a microreader (SLT-Lab., Salzburg, Austria). To measure DNA synthesis, 0.5 μ Ci/well [methyl-³H]thymidine (Amersham International, Amersham, Bucks, UK) was added to each well for 8 h. At the end of incubation, cells were washed with cold PBS, dissociated in 0.25% trypsin solution for 10 min at 37 °C and transferred to Multiscreen plates with durapore filters (Millipore Co., Bedford, MA, USA). These plates were vacuum filtered and the cells were washed twice with 200 μ l PBS. To permeabilize the cells and to precipitate DNA, 200 μ l ethanol were added to the wells and allowed to stand for 10 min before vacuum filtering; the wells were then washed three times with 200 μ l ethanol. Discs from the Multiscreen plate were punched into 7 ml scintillation vials containing 0.5 ml sodium hypochlorite (0.42%) and the vials shaken for 30 min on a rotary table. Then, 5 ml scintillation cocktail (Packard Instrument Co, Meriden, CT, USA) were added to each vial and the radioactivity

Figure 1 Proliferative effects of testosterone (T), androstenedione (A), DHT and 3 α -diol (30 nM) in HC11 cells after 24, 48 and 72 h of treatment as measured by the MTT test. Value are mean $\pm$ S.E.M. for three independent experiments ($n=8$ per treatment). Data (optical density) were converted to percent of control cells cultured at each incubation time. Different letters show significantly different results ($P<0.05$).

Figure 2 Proliferative effect of testosterone, DHT, androstenedione and 3 α -diol measured by the MTT test after 24 h of treatment. Each androgen has been tested alone (open bars) or in association (hatched bars) with anti-androgens CYP (3 μ M) or FLU (3 μ M). Data are the absolute value $\pm$ S.E.M. for three independent experiments ($n=8$ per treatment). Different letters show significantly different results ($P<0.05$).

incorporated by filters was quantified in a β -counter (Tri-Carb 1600 TR; Packard Instruments).

p β CAT expression analysis

CAT (chloramphenicol acetyl transferase) expression was measured in triplicate using the CAT ELISA assay system (Boehringer Mannheim, Mannheim, Germany) as recommended by the supplier. The amount of protein present in cell extracts was determined using the Biorad protein assay (Bio-Rad Lab., Hercules, CA, USA). Values of CAT are reported as ng CAT/mg protein.

RT-PCR

Total RNA from HC11 proliferating or differentiated cells was extracted using Tri-Reagent. Mouse specific primer for AR were: downstream primer, 5'-AGTCATCCCTGCTTCATAAC, utilized both in reverse transcription and PCR steps; upstream primer, 5'-ATCCTGGTGGAGTTGTGAAC, utilized only in the PCR step. Reverse transcription was performed with 1 μ g total RNA at 42 °C for 45 min, the reverse transcriptase was inactivated at

95 °C for 5 min; PCR parameters were 95 °C 60 s, 42 °C 60 s, 72 °C 60 s for 35 cycles. PCR products were separated on 2% agarose gel and transferred to nylon membrane for Southern blotting. The membrane was hybridized by a radioactive internal oligoprobe (5'-TGA TCTGTGGAGATGAAGCT) (mAR.i). Labeling reactions were performed on the free 5'-end of the oligoprimers using T4 DNA polynucleotide kinase and [γ - 32 P]dATP. The cDNA on the membrane was then incubated at 45 °C for 4 h with the prehybridizing solution and then added with the 5' 32 P-end-labeled oligonucleotide probes (1×10^6 c.p.m./ml) specific for the mAR, cDNAs were added and hybridized at 45 °C overnight. After washing, the membranes were exposed to X-ray films. RT-PCR was also performed on total RNA (1 μ g) extracted from mouse testis and GT1-1, a mouse androgen-sensitive neuronal cell line (Poletti *et al.* 1994) as positive controls. RT-PCR blanks were performed omitting RNA from the reaction and simultaneously subjected to RT-PCR/Southern blotting with the same reagents and conditions described above; a specific signal was not obtained in any of the experiments performed thus indicating that no contamination of any reagents occurred in these studies.

Figure 3 Effects of testosterone, DHT, androstenedione and 3 α -diol on DNA synthesis measured by [3 H]thymidine uptake in HC11 cells after 24 h of treatment. Each androgen has been tested alone (open bars) or in association (hatched bars) with CYP (3 μ M). Data are the absolute value $\pm$ S.E.M. for three independent experiments ($n=8$ per treatment). Different letters show significantly different results ($P<0.05$).

Statistical analysis

For the proliferation studies, each experiment was repeated three times and each treatment was performed with eight replicates. Experimental data from CAT analysis were obtained in triplicate and each experiment was repeated three times. Statistical differences among treatments and interactions were calculated with multifactorial ANOVA using the STATgraphics package (STSC, Rockville, MD, USA). When significant differences were found, means were compared by the LSD test.

Results

Proliferative effects in HC11 cell line

Testosterone, androstenedione, dihydrotestosterone (DHT) and 5 α -androstane-3 α ,17 β -diol (3 α -diol) enhanced ($P<0.05$) cell proliferation at all tested incubation times with an increasing effect at 72 h (Fig. 1). The positive effect of each androgen was inhibited significantly ($P<0.05$) by FLU or CYP (Fig. 2). The positive effect on DNA synthesis at 24 h and the relative specific anti-androgen inhibition were measured also by [3 H]thymidine uptake (Fig. 3).

The proliferative effects of testosterone and androstenedione were not blocked by the aromatase inhibitor AI (Fig. 4). 17 β -Estradiol (0.3, 3, and 30 nM) did not induce any effect on proliferation (Fig. 4).

Induction of β -casein gene expression

Figure 5 shows the dose-response curve of β -casein promoter induction with increasing concentrations of oPRL (0.1–25 μ g/ml). When testosterone or DHT was added to the cultures without oPRL but with insulin and dexamethasone, no effects were observed at any concentration of steroids. On the contrary, when oPRL (0.5, 1 and 5 μ g/ml) was coincubated, testosterone and DHT significantly ($P<0.05$) increased β -casein gene expression. Maximum response was obtained at the lowest oPRL concentration (0.5 μ g/ml). Addition of CYP to DHT-treated cells reduced expression of the β -casein gene to levels not significantly different from control (Table 1).

AR expression

Figure 6 shows the RT-PCR and Southern blot of total RNA from proliferating and differentiated HC11 cells.

Figure 4 Effects on proliferation of testosterone and androstenedione alone (open bars) or in association (hatched bars) with AI or 17β-estradiol after 24 h treatment. Data are the absolute value $\pm$ S.E.M. for three independent experiments ($n=8$ per treatment).

RT-PCR followed by electrophoresis resulted in one band. The size of the amplified RNA product was 394 bp, corresponding to the expected size from AR mRNA. Southern blotting and hybridization of the amplified products with an AR-specific probe demonstrated the specificity of the primer pairs and the RT-PCR protocol. Positive controls, the GT1-1 cell line and mouse testis, showed a comparable signal when RT-PCR and Southern blot were performed under the same conditions.

Figure 5 Dose-response curve for induction of β-casein promoter with increasing concentrations of oPRL. HC11 cells were grown to and maintained at confluency for 48 h in growth medium and then treated with increasing concentrations of oPRL (0.1–25 μg/ml) in the presence of insulin (5 μg/ml) and dexamethasone (1 μM). Data are the mean of three replicates. Error bars are not shown because they are smaller than the symbols.

Table 1 Confluent HC11 cells stably transfected with pβcCAT construct were treated for 48 h with DIP medium (with three different oPRL concentrations) containing testosterone (0.3 μM), DHT (0.3 μM) or DHT+CYP. Data from four independent experiments ($n=3$ per treatment). Fold induction represents the ratio between CAT values of treated cells and cells maintained in the absence of lactogenic hormones (no DIP)

	CAT (ng/mg protein)	Fold induction	Stimulation (% $\pm$ S.D.)
No DIP	0.2	—	—
0.5 μg PRL			
DIP	1.41	7	100
Testosterone	1.97	10	140 $\pm$ 8*
DHT	2.01	10	143 $\pm$ 11*
DHT+CYP	1.38	7	98 $\pm$ 3
1 μg PRL			
DIP	7.39	37	100
Testosterone	8.14	41	111 $\pm$ 3
DHT	8.75	44	120 $\pm$ 5*
DHT+CYP	6.94	35	94 $\pm$ 6
5 μg PRL			
DIP	22.15	111	100
Testosterone	24.14	121	109 $\pm$ 1*
DHT	25.21	126	114 $\pm$ 2*
DHT+CYP	21.92	110	99 $\pm$ 3

* $P<0.05$ vs control value.

Figure 6 Ethidium bromide-stained agarose gel (A) and Southern blot analysis of HC11 RNA RT-PCR (B) showing the hybridization of PCR products (394 bp) with a radioactive internal oligoprobe (mAR.i). The free 5'-end of the oligoprobe was labeled using T4 DNA polynucleotide kinase, and [γ - 32 P]dATP. After hybridization, one specifically labeled band was observed. Lanes: (MW) molecular weight standards; (1) and (2) proliferating HC11 cells; (3) and (4) HC11 differentiated cells; (5) reverse transcriptase-negative control performed omitting HC11 RNA; (6) GT1-1 cells; (7) mouse testis; (8) RNA positive control RT-PCR reaction done with the mRNA and primers provided by the Gene Amp Kit (Perkin-Elmer Inc., Foster City, CA, USA) for reverse transcription.

Discussion

In this study we have shown effects of some androgens on the mouse epithelial mammary cell line HC11. This line is considered a valuable model to study the role of factors and hormones involved in the growth and differentiation of mammary epithelial cells (Marte *et al.* 1995). HC11 cells retain the ability to express milk proteins if cultured to confluence and treated with the appropriate hormones and have been extensively used for analysis of regulation of milk protein gene expression by lactogenic hormones using gene transfer methods (Doppler *et al.* 1989).

Androgens positively influenced HC11 mammary epithelial cell proliferation and β -casein gene induction. These effects appear to be mediated by AR since we observed AR expression both in growing and differentiated cells and the effects of androgens were blocked by AR antagonists, FLU and CYP.

A positive effect on growth of HC11 cells has been observed for both aromatizable (testosterone and androstenedione), and non-aromatizable (DHT and 3- α -diol) androgens and the effect was retained also in the presence of an aromatase inhibitor (AI). This observation is consistent with our hypothesis that androgens exert their effect

directly through ARs, independent of aromatase activity. In fact, although testosterone may be a precursor of 17 β -estradiol, a recent study suggests that the mammary gland is not a significant site for aromatization (Belvedere *et al.* 1996). Furthermore, 5 α -reductase activity, which converts testosterone to DHT, has been observed in normal murine mammary gland (Hahnel & Twaddle 1974, Belvedere *et al.* 1996). Very recently, HC11 cells have been reported to express a 17 β -hydroxysteroid dehydrogenase/17-ketosteroid reductase activity that modulates the biological activity of both estrogens and androgens (Nokelainen *et al.* 1998). Furthermore this study reports the ability of HC11 cells to convert androstenedione to testosterone; this may explain the effect of androstenedione, considered a precursor of active androgens, on cell growth. Our results show that also 3 α -diol has a positive effect on HC11 cell proliferation. Although this 5 α -reduced androgen is thought to be a breakdown product of androgen metabolism, it has been recently reported to have a biological activity through different mechanisms (Mahendroo *et al.* 1996, Ding *et al.* 1998).

Extensive studies on testosterone and DHT in breast cancer indicate a role for androgens in the regulation of cancer growth acting either as precursors of estrogens (Pasqualini 1993), directly through interaction with the AR (Labrie *et al.* 1992, Tanaka *et al.* 1992, Pasqualini 1993, Sato *et al.* 1993) or, at higher concentrations, as competitors for estrogen receptors (Hahnel & Twaddle 1974). Nevertheless, the role of androgens in normal mammary tissue is poorly understood. In androgen-secreting organs, the biosynthesis of these steroids results from the conversion of either pregnenolone or progesterone to DHEA or androstenedione respectively, which are then transformed into their final forms, testosterone and DHT, by the 17-hydroxysteroid dehydrogenase and 5 α -reductase enzymes. It has been recently demonstrated that mammary tissue possesses these activating enzymes for DHEA or androstenedione (Belvedere *et al.* 1996); thus, the mammary gland can be considered a site for the synthesis of active androgens. Our data on HC11 cell growth support the hypothesis that local intracrine formation of androgenic steroids from precursors may play a role in the regulation of growth and function of the mammary gland (Recchione *et al.* 1995, Turgeon *et al.* 1998). The ability to block the effect of androgens using AR antagonists provides further support that the effect is specific.

As for milk protein gene expression in HC11 cells, both aromatizable and non-aromatizable androgens appear to increase the induction of transfected rat β -casein gene promoter in the presence of the lactogenic hormones insulin, PRL and dexamethasone. As for cell proliferation, AR antagonists blocked the effect. To our knowledge, this is the first report on the stimulation of β -casein gene induction by androgens.

Although our study does not support any specific mechanism, we cannot exclude the possibility that these molecules may act through any of the mechanisms proposed for the other sex steroids progesterone and 17 β -estradiol. Progesterone inhibits PRL-mediated expression of milk protein in mammary epithelial cells by an interaction of progesterone receptor with the signal transducer and transcription activator STAT5 (Gass *et al.* 1998). 17 β -Estradiol has been reported to increase milk protein expression by increasing PRL receptors (Mizoguchi *et al.* 1997, Ormandy *et al.* 1992a, b).

In conclusion, since androgens and enzymes involved in their metabolism are present in normal mammary gland, our findings on their putative specific effect on HC11 cell line proliferation and β -casein gene expression suggest a paracrine/autocrine role during growth and differentiation of mammary epithelial cells. The HC11 cell line should provide a valuable model for further studies aimed at differentiating pharmacological from physiological effects of these androgens and at investigating their intracellular mechanisms of action and the relationship with other mammogenic and lactogenic hormones during growth, differentiation and involution of the mammary gland.

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