Androgen Receptor Expression in the Preputial Gland and Its Sebocytes

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As in other sebaceous glands, preputial gland sebocytes are stimulated to proliferate and produce lipid by androgen. As a necessary step in understanding the role of androgen in sebaceous gland growth and development, we have undertaken studies to determine the relationship between androgen receptor gene expression and sebocyte differentiation. Sebocytes throughout the preputial gland, with the exception of some basal sebocytes, stain intensely for androgen receptor. Quantitative assessment of androgen receptor mRNA by RNase protection assay confirms that androgen receptor mRNA content is similar in sebaceous and prostate epithelial cells, but is tenfold less in epidermal cells. When sebocytes were separated according to their state of differentiation by gradient density centrifugation, sebocytes in the 1.080 density fraction contrasted with the more buoyant fractions in that they immunostained weakly for androgen receptors. The 1.080 density fraction consists of approximately 50% immature (undifferentiated and early differentiated) sebocytes, whereas more mature sebocytes predominate in the other fractions. Androgen receptor mRNA quantity was found by RNase protection assay to be half as great in the 1.080 density fraction as in the fractions in which more mature sebocytes predominate. In primary monolayer culture androgen receptor mRNA content was significantly higher in sebaceous epithelial cells than in epidermal cells and similar to that in the 1.080 fraction of freshly dispersed sebocytes. These results suggest that there is little if any androgen receptor gene expression in undifferentiated preputial sebocytes and that androgen receptor gene expression increases as sebocytes begin to differentiate. Because androgen receptor expression seems to approach its maximum as sebocytes attain mid-differentiation, the stage at which sebocytes switch from a proliferative mode to commence their specialized holocrine function, androgen is postulated to play a direct role in regulating these aspects of sebocyte development. J Investig Dermatol 103:721-725, 1994

The preputial gland is a model murine sebaceous gland [1], and preputial glands and its cells resemble the human sebaceous gland and sebaceous epithelial cells (sebocytes) in many ways [2–7]. Like other sebaceous glands, it is stimulated to grow by androgen in vivo [8–10]. Testosterone administration is known to stimulate sebocyte proliferation [9] as well as lipid production [9,10]. These processes are known to be mediated by the androgen receptor (AR) [9,11,12]. Nuclei of human sebocytes in skin stain for AR as do those of parabasal epidermal cells and dermal papilla cells, more clearly than those of hair matrix cells and to a greater extent than those of fibroblasts [13–15]. This is particularly interesting because sebaceous glands and hair follicles are differentially regulated by androgen although comprising a single pilosebaceous unit; the biological factors that mediate this disparate pattern of responsiveness to androgen are unknown. Sebaceous glands resemble other skin appendages in that proliferation of their specialized epithelial cells occurs primarily in the undifferentiated and early differentiated cells located in the basal and parabasal layers [16–18]. However, subsequent differentiation of sebocytes is characterized by the holocrine accumulation of lipid in the cytoplasm rather than by the secretion of lipid organelles into the intercellular space in favor of the accumulation of keratin packets, as is the case with the epidermis [19,20]. To further understand the role of androgen in sebocyte growth and development, we have determined the pattern of distribution of AR in the preputial sebaceous gland and the relationship between AR gene expression and sebocyte differentiation in vivo and in vitro.

MATERIALS AND METHODS

Dispersed Epithelial Cell Preparations Young adult male Sprague-Dawley rats weighing approximately 220 g were killed by CO₂ narcosis and rapid cervical dislocation. Handling of rats was in compliance with NIH guidelines for the care and use of laboratory animals. Single-cell suspensions were prepared from the preputial glands and shaved skin as previously reported [21]. In brief, filleted glands were incubated with 2.5% Dispase (bacterial neutral protease, type II, Boehringer) in Dulbecco's modified Eagle's medium (DMEM) for 18 h at 4°C, after which they were successively washed with phosphate-buffered saline (PBS)/0.02% ethylenediaminetetraacetic acid (EDTA). The preputial gland was then incubated sequentially with 0.25% trypsin/0.02% EDTA at 37°C for 40 min and 0.25% soybean trypsin inhibitor for 5 min; the cells were then gently stripped from the capsule into DMEM/10% fetal bovine serum (FBS) with a rubber policeman. The epidermis was peeled from the dermis in PBS, minced, incubated as above for 30 min, and then vortexed for 5 min. Cells were filtered through 52-μm nylon mesh, gently vortexed, and centrifuged at 1000 × g before and after washing in PBS. They were then taken up into DMEM/FBS and dispersed by a Pasteur pipette. Prostate cells were isolated.
Figure 1. Androgen receptor in the preputial gland. a) Immunohistochemistry of the preputial gland (left panel) and prostate gland (right panel) incubated with polyclonal antibody to AR. In contrast to prostate, differentiating sebocytes with increasing lipid vacuolization are pushed toward the central collecting system by proliferating immature cells arising in the basal layer. Occasional basal sebocytes stain weakly if at all for AR (arrowhead), but all differentiating sebocytes stain for AR (thick arrow). AR staining is particularly prominent in sebocyte nuclei. Interstitial fibroblasts stain for AR, too (thin arrow). Incubation with control IgG (center panel) shows no AR staining. Bar, 50 μm. b) Immunocytochemistry of dispersed single-cell suspensions of preputial cells isolated by gradient density centrifugation. Cells from fraction 1.065 (left panel) stain strongly for AR (arrow) and are representative of more buoyant fractions as well. Some cells from fraction 1.030 (right panel) show weak brown staining for AR in the cytoplasm (thick arrow); these we presume to be mid-differentiated sebocytes. Others, generally smaller cells, showed no discernable staining (thin arrow). Cells from fraction 1.065 incubated with control rabbit IgG (center panel) do not stain for AR. Denuded nuclei stain dark purple. Bar, 25 μm. c) Immunocytochemistry of preputial and epidermal epithelial cells in primary culture. AR staining is seen throughout the cell cytoplasm, but is much more prominent in nuclei. Preputial cells (left panel) stained more strongly than epidermal cells (right panel), although the latter showed positive staining. Preputial control (center panel) incubated with rabbit IgG shows no brown staining for AR. Bar, 50 μm.
similarly. Cell counts were performed using a hemocytometer, and cell viability was determined by the exclusion of trypan blue dye.

Epithelial Monolayer Cell Culture Cell culture was carried out as previously reported on a J2 3T3 cell feeder layer (20,000/cm²) pretreated with mitomycin-C 8 µg/ml for 2 h [21]. Epithelial cell culture medium (ECM) consisted of DMEM containing 10% FBS supplemented with cholera toxin 10⁻5 M and cortisol 10⁻7 M. A single large batch of ECM was used for all growth experiments. Twenty-five to 50,000 epithelial cells were dispensed into 35-mm plastic tissue culture dishes (Costar). Penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin (0.25 µg/ml) were added. Medium was changed at 2- to 3-d intervals. The cells were then grown in primary culture for up to 2 weeks in 95% air/5% CO₂ at 37°C. Growth in monolayer was monitored by phase microscopy and stopped prior to confluency of the most dense plates from each experiment, typically at 10–14 d.

One-step isokinetic discontinuous density gradient centrifugation through Percoll (Sigma) was carried out as reported previously [21]. Five Percoll fractions with densities of 1.020–1.080 were prepared according to the manufacturer's instructions and buffered with pH 7.4 PBS. Aliquots of samples were applied in the 1.065 fraction. The gradient was then centrifuged at 400 X g at 4°C for 20 min. Cells from each fraction were then resuspended in the appropriate medium for further studies.

Flow Cytometry After Percoll gradient centrifugation, cells were fixed in ethanol, then stained in a mixture of propidium iodide (150 µg/ml) and RNase A (2.5 units/ml) for analysis on a FACScan flow cytometer (Becton Dickinson, Mountainview, CA). They were excited at 488 nm with an argon laser and emission was detected at 620–700 nm. Cell cycle analysis was performed using Cell FIT Cell-Cycle Analysis, version 2.01.2 (Becton Dickinson) [22–24].

Table I. AR Gene Expression in Preputial and Comparison Cells

<table>
<thead>
<tr>
<th></th>
<th>Prostate</th>
<th>Preputial</th>
<th>Epidermal</th>
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<tr>
<td>Fresh</td>
<td>0.34 ± 0.03</td>
<td>0.30 ± 0.06</td>
<td>0.03 ± 0.01</td>
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<tr>
<td>(n = 6)</td>
<td>(n = 6)</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>0.14 ± 0.04*</td>
<td>0.03 ± 0.01</td>
<td></td>
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<tr>
<td>(n = 5)</td>
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* Ratio AR/18S RNA by RNase protection assay (mean ± SD).

Lipid Staining Cells were stained by a modification of the method of Greenspan et al. [25]. Stock solutions of Nile red dye 10 µg/ml (Kodak) were prepared in methanol and stored protected from light at 4°C. Live cells (0.5–1.0 X 10⁶ cells/ml) were centrifuged at 2000 X g, washed in PBS, and dye stock solution diluted 1:100 in PBS was added. After a 10-min incubation, a drop of cell suspension on a microscope slide was observed under a fluorescence microscope (625X) using a 450- to 500-nm band pass exciter filter by light emission of >528 nm.

Seboocyte differentiation was classified according to lipid content using Nile Red fluorescence according to a modification of the method of Tosti [21]: a) intracytoplasmic lipid absent (undifferentiated), b) diffuse, faint cytoplasmic lipid staining (early differentiated), c) discrete lipid droplets scattered throughout cytoplasm of cells with cytoplasmic: nuclear ratio ≤ 1.0 (mid-differentiated), d) lipid droplets throughout cytoplasms of cells with cytoplasmic: nuclear ratio > 1.0 (late differentiated), and e) cytoplasm dispersed by fat (terminally differentiated).

Immunohisto/cytochemistry Cells grown on glass slides or smears of cells were fixed in Zamboni's solution [26] for 15 min at 20°C. They were then immersed in methanol for 5 min and in acetone for 3 min, both at −20°C. Immunostaining was carried out essentially as described in the Vectastain ABC kit (Vector). Briefly, slides were rinsed in PBS and then incubated with goat normal serum for 20 min at 20°C in a humidified chamber. They were then incubated for 1 h at 4°C with rabbit polyclonal primary anti-AR immunoglobulin (IgG) (2 µg/ml) [27] or control rabbit IgG (5 µg/ml), washed 2 times with PBS, and subsequently incubated for 45 min at 20°C with biotinylated goat anti-rabbit IgG (Vector Laboratories) [27]. After washing in PBS, the slides were incubated in avidin-HRP reagent for 30 min at 20°C, then stained with diaminobenzidine solution (DAB) and counterstained with hematoxylin. Sections were dehydrated with increasing concentrations of ethanol, then cleared with xylene and mounted in Permapl (Alban Scientific Co.).

RNA Probes Rat AR cDNA was cut with Tag I-Pst I (354 bp from 2472-2826 in exon 6–8) [28] and inserted into the ampicillin-resistant plasmid pGEM-4Z (Promega) [29]. A radiolabeled probe (1 X 10⁶ cpm) was made by linearizing the plasmid with Hind III and transcribing with SP6 RNA polymerase in the presence of [35S]-cytosine triphosphate (CTP) and three other nucleotide triphosphates (NTPS). An internal control probe was similarly prepared by linearizing pT7 18S RNA, which contains 109 bp (Ambion) and transcribing with T7 polymerase [30].

RNase Protection Assay [31] Primary cultures grown on 100-mm plates were washed with 0.02% EDTA to remove the 3T3 feeder layer. Total RNA (1 µg) isolated by the guanidinium thiocyanate method [32] and AR or control riboprobes were mixed and heated to 95°C for 5 min and hybridization was carried out for 16 h at 45°C using a RPA II kit (Ambion). Unprotected RNA was digested with RNase for 30 min at 37°C. The protected RNA hybrids were recovered and electrophoresed on a 5% polyacrylamide/8 M urea gel. The gel was dried and exposed to X-ray film (Kodak). Electrophoretic bands were quantified using an AMBS image acquisition and analysis system.

Statistical Analysis One-way analysis of variance followed by Scheffe's test or paired Student t tests of statistical significance were used, as appropriate [33].
RESULTS

Flow Cytometry  The percent of cells in cell cycle stages G2 + M decreased successively from 4.7% in density fraction 1.080 to 1.6% in density fraction 1.020 (r = 0.953, p < 0.02). This indicates that the gradient fraction containing the least mature cells has the highest rate of mitosis.

Immunohistochemistry  The preputial gland stained as strongly for AR as prostate (Fig 1a). Staining for AR could be visualized in most but not all basal sebocytes and appeared to be present in the nuclei of virtually all differentiated preputial sebocytes. Preputial fibroblasts were frequently stained, too.

Immunocytochemistry  Immunocytochemistry indicated that dispersed preputial cells contained greater amounts of AR than dispersed epidermal cells (not shown). Single-cell suspensions of preputial sebocytes were submitted to density gradient centrifugation in a Percoll gradient, which had previously been shown to separate sebocytes according to stage of cell differentiation [21], with undifferentiated and early differentiated sebocytes occupying the band with the greatest relative density (1.080) and fully mature sebocytes the band with the least relative density (1.020). Immunocytochemistry showed that virtually all cells in the more buoyant fractions (1.020–1.065) stained for AR (Fig 1b). However, cytoplasmic staining for AR was light in cells in the 1.080 density fraction; approximately 80% of such cells stained weakly and 15% had no discernable staining. By way of contrast, we estimated that in the 1.065 band approximately 70% of cells stained strongly for AR, 30% weakly. In monolayer culture preputial cells stained much more heavily for AR than epidermal cells, particularly in nuclear membranes of preputial cells (Fig 1c).

RNase Protection Assay  AR mRNA quantity was similar in freshly dispersed preputial cells to that in prostate and tenfold greater than in dispersed epidermal cells based on total RNA content (p < 0.01) (Fig 2, Table I). This assay showed that mRNA for AR was distributed similarly among mid-differentiated through mature sebocytes, but the quantity was half as great in the Percoll gradient fraction containing the least mature cells (1.080) as in all other fractions (p < 0.02) (Fig 3, Table II). This contrasts with the situation in epidermis where cells in the comparable basal fraction express more AR mRNA than cells from the fractions containing more mature cells (Table II).

The RNase protection assay also demonstrated that AR mRNA abundance in preputial cells in culture was significantly different from both that of freshly dispersed preputial cells and that of freshly dispersed or cultured epidermal cells (p < 0.01) (Table I). It was half as great as in freshly dispersed preputial cells and yet approximately fivefold greater than in epidermal cells in culture.

DISCUSSION

These findings indicate that AR is expressed to a similar extent in the sebaceous gland and the prostate gland, another classic site of androgen action [34,35]. As in rat ventral prostate, the site of AR expression is predominantly in epithelial cells. With the exception of some basal sebocytes, as is also the case in prostate [34], sebocytes throughout the preputial gland show intense immunostaining for AR. AR is also found scattered among interstitial fibroblasts within the preputial gland, again similar to the situation in prostate.

The preputial gland model has permitted exploration of the distribution of AR in isolated sebocytes to an extent impossible with the sebaceous glands of skin. Quantitative assessment of AR mRNA by RNase protection assay confirms the impression obtained by means of immunostaining, namely, that AR mRNA abundance is similar in sebaceous and prostate epithelial cells. Furthermore, in both these cell types the AR mRNA content was tenfold greater than in epithelial cells. When sebocytes were separated according to state of differentiation by gradient density centrifugation, the staining of sebocytes in the density fraction containing the least mature sebaceous epithelial cells (1.080) was clearly less than that in the other fractions. AR mRNA quantity by RNase protection assay was found to be half as great in the 1.080-density fraction as in the fractions in which more mature sebocytes predominate. These results are compatible with there being little if any AR gene expression in undifferentiated preputial sebocytes, which comprise about 8% of cells in the 1.080 fraction. Early expression of AR would seem to correspond approximately to early sebocyte differentiation, the stage at which cytoplasmic lipid synthesis begins, which is not unique to sebaceous epithelial cells [19,20]. AR gene expression then seems to approach its maximum at mid-differentiation, the stage at which lipid droplets begin to accumulate throughout the cytoplasm of sebocytes.

In cultured epithelial cells, the histocytochemical stain for AR was particularly prominent in sebaceous cells; it was seen diffusely throughout the cytoplasm, but was particularly prominent in the nuclei and nuclear membranes. RNase protection assay confirmed that sebaceous epithelial cells express more AR mRNA than epithelial cells in culture, yet to only the extent of the 1.080 fraction of freshly dispersed preputial cells. This suggests that approximately half of the sebocytes in culture are undifferentiated or early differentiated, whereas half are more mature. This evidence of AR gene expression is compatible with this and a previous study [21], which together suggest that both undifferentiated and partially mature preputial epithelial cells are capable of proliferating in culture and, furthermore, that they undergo differentiation in a pathway unique to sebocytes in monolayer culture, arresting at a stage of differentiation prior to the expression of clear-cut holocrine lipogenesis [4,5]. Our present data extend these conclusions, indicating that sebaceous epithelial cells express fivefold more AR mRNA than epidermal cells in culture. In contrast to the expectations from the findings in freshly dispersed sebocytes, AR is abundant in cultured sebaceous epithelial cells that have not achieved morphologic evidence of mid-differentiation. This is compatible with the monolayer culture system being incompatible with substantial expression of lipogenesis in vitro.

The apparent increase in AR gene expression as sebocytes differentiate has implications for the role of androgen in sebocyte growth.

Table II. AR Gene Expression in Preputial Cells According to Stage of Differentiationa

<table>
<thead>
<tr>
<th>Percoll Gradient Density Fraction</th>
<th>Sebocytes</th>
<th>Epidermal Cells</th>
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<tbody>
<tr>
<td>1.020</td>
<td>1/4/11/18/66</td>
<td>AR/18S Ratio</td>
</tr>
<tr>
<td>1.035</td>
<td>1/7/19/26/49</td>
<td>0.32 ± 0.11</td>
</tr>
<tr>
<td>1.050</td>
<td>3/12/24/29/32</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>1.065</td>
<td>4/16/32/28/17</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>1.080</td>
<td>8/40/33/16/4</td>
<td>0.28 ± 0.01</td>
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* Ratio AR/18S RNA by RNase protection assay (mean ± SD) in preputial cells separated by Percoll density gradient centrifugation.

1 Mean percentage of undifferentiated/early/mid/late/terminally differentiated sebaceous cells in each fraction.

2 Mean percentage of cells with diffuse modest lipid staining, comparable to early differentiated sebocytes.

3 Mean percentage of cells with diffuse intense lipid staining, comparable to late/terminally differentiated sebocytes.

4 p < 0.05 versus other groups of sebocytes.

5 p < 0.05 versus 1.035 fraction of epidermal cells.
and development. Because immature sebocytes seem to be the major proliferating cohort of sebocytes, yet express little if any AR, our data imply that androgens exert little direct effect on sebaceous cell proliferation. If so, their effect on sebocyte proliferation would mainly be indirect, mediated through action on the surrounding stromal fibroblasts, analogous to the situation with hair growth [36,37]. On the other hand, AR seems to approach its maximal expression as sebocytes achieve mid-differentiation, the stage at which sebocytes switch from a proliferative mode to commence their specialized holocrine function, which includes lipogenesis. This suggests a role for androgen in directly affecting these latter aspects of sebocyte development. More precise methods will be required to directly relate AR gene expression to the stages of sebocyte differentiation.

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REFERENCES


