Induction of androgen receptor formation by epithelium–mesenchyme interaction in embryonic mouse mammary gland

(development of hormone responsiveness/[3H]testosterone autoradiography/steroid receptors)

BARBARA HEUBERGER, ILSE FITZKA, GERTRAUD WASNER, AND KLAUS KROTOCHWIL

Institute for Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria

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ABSTRACT The role of tissue interaction in the development of hormone responsiveness was studied in the embryonic mammary gland of the mouse, which becomes sensitive to testosterone on day 14. Previously, the mesenchyme had been identified as the sole target tissue for the hormone, although it was also demonstrated that its response to testosterone required the presence of mammary epithelium. Using autoradiography, we now show that [3H]testosterone or [3H]5α-dihydrotestosterone is bound only by those mesenchymal cells closest to the epithelial mammary bud. When mammary epithelia were experimentally associated with mesenchyme of the mammary region and cultured together for 3 days in vitro, they also became surrounded by several layers of [3H]testosterone-binding mesenchymal cells. Correspondingly, this tissue association was accompanied by a substantial increase in androgen-binding sites in the explants. No hormone-binding mesenchymal cells were seen in combinations with epidermis or pancreas epithelium; only salivary epithelium showed a weak positive effect. From these results we conclude that mammary epithelium induces the formation of androgen receptors in adjacent mesenchyme and thereby controls the development of androgen responsiveness in this tissue.

The ability to respond to hormones is a differentiative property of certain tissues and organs acquired during ontogenesis. Hormone responsiveness is known to depend on the presence of specific hormone receptors, and the appearance of receptors in embryonic and postnatal life has been described for several organs (1–4). However, the developmental processes responsible for the initiation of receptor synthesis are still unknown. Because hormone action requires preexisting receptors, it follows that the hormone itself cannot be the inducer, although it may later regulate receptor level or induce the formation of receptors for another hormone (5). The development of hormone sensitivity is more likely dependent on the same processes that govern embryonic organ development in general, the most important of these being short-range tissue interactions (6, 7).

For several reasons, the embryonic mouse mammary gland is ideally suited for studies of the development of hormone responsiveness. In 14-day male fetuses, testicular androgens cause the partial or complete destruction of the mammary buds (8) by direct action on the organ anlage (9). The stage when the gland becomes responsive to testosterone has been precisely defined (10), and the associated mesenchyme has been identified as the target tissue for the hormone (11, 12). Thus, the epithelium of the gland is destroyed by testosterone-activated mesenchymal cells rather than by direct hormone action.

Although the mesenchyme is the sole target for testosterone, its response to the hormone still depends on the presence of the gland epithelium. No reaction was seen in isolated mesenchyme or when mammary epithelium was replaced by epithelia of other organ rudiments in experimental tissue combinations. In association with mammary epithelia of other species (rat, rabbit), however, the usual mesenchymal reaction to testosterone did take place (13). Such an organ specificity but no species specificity is a characteristic feature of embryonic tissue interactions (6).

The requirement for specific tissue association for hormone responsiveness suggested that the epithelium may influence the development of testosterone sensitivity in the mesenchyme. Indeed, our earlier results showed that only those mesenchymal cells closest to the epithelial gland bud, at least from day 12 onwards, take part in the testosterone response on day 14 (13). Using experimental combinations of mammalian epithelium with mesenchyme, it was also found that the tissues must be associated no later than on day 12 to produce a hormone response on day 14 (unpublished data). Both results suggested the possibility that the mesenchyme must be preassociated with epithelium to become reactive to testosterone. By analyzing the appearance and distribution (with respect to the epithelium) of [3H]testosterone-binding cells, both in the gland in situ and in experimental epithelium–mesenchyme combinations, we have now tested directly whether mammary epithelium in fact induces development of testosterone receptors, and thereby hormone sensitivity, in the mesenchyme.

MATERIALS AND METHODS

Tissue Combination and Culture Procedures. All tissues were from (BALB/c × C3Hf) hybrid mouse embryos, the day of detection of a vaginal plug being taken as day 0 of pregnancy. Tissues were taken from female embryos only, to avoid interference of [3H]testosterone binding by endogenous testicular androgens. The standard combination experiments were done with mammary tissues of 12-day embryos. The epidermis with the mammary buds was cleanly separated from the underlying mesenchyme as described (11). For recombination cultures, only those parts of the mesenchyme that had not been in contact with a mammary bud before tissue separation were used. Ten individual mammary epithelia were placed on one piece of mesenchyme. Trypsin-isolated (14) epithelia of the 13-day salivary gland and the 11-day pancreas were associated with mammmary mesenchyme in the same way. During subsequent culture of these combination explants at the medium–gas interface (see ref. 11), the epithelia became completely surrounded by the mesenchyme.

Binding Assays and Autoradiographic Procedures. Three batches of [3H]-labeled androgens were used: [1,2,6,7-3H]testosterone with a specific activity of 80 Ci/mmole (Radiochemical Centre, Amersham, England), [1,2,6,7,16,17-3H]testosterone

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with a specific activity of 152 Ci/mmol, and [1,2,4,5,6,7,16,17-\textsuperscript{3}H] 5a-dihydrotestosterone (17\textbeta-hydroxy-5a-androstan-3-one) with a specific activity of 200 Ci/mmol (both from New England Nuclear) (1 Ci = 3.7 × 10\textsuperscript{10} becquerels). Unlabeled hormones for competition experiments were from Sigma, the antiandrogenic compound cyproterone acetate (1,2a-methylene-6-chloro-5,6\textalpha-phenylindole-17\alpha-acetate) was a gift from Schering, Berlin.

Recombination cultures as well as freshly dissected organs were all incubated with the labeled hormone in vitro to obtain a satisfactory control of ligand concentration and diffusion. Standard [\textsuperscript{3}H]hormone concentration was 0.6 nM, applied in Eagle’s medium supplemented with 10% horse serum (Flow Laboratories, Irvine, Scotland). Before use, serum was shaken with the sediment of a suspension of 0.5% charcoal (Norit A, Serva, Heidelberg, FRG) and 0.05% dextran (Serva), prepared in Tris-HCl buffer, pH 7.5, to remove endogenous steroids. Unlabeled hormones in competition experiments were applied simultaneously at 100-fold excess (i.e., 60 nM). Incubation time was 60 min at 37°C, followed by two washes (20 min each) at room temperature in large volumes of medium. For scintillation counting of [\textsuperscript{3}H]-labeled ligand bound, the tissues were taken up in 200 \mu l of distilled water and homogenized by brief sonication, and the radioactivities of 100-\mu l aliquots were determined in a toluene-based scintillation cocktail. Counting efficiency was 45–50%. For comparison of binding capacity of various tissues, counts were related to DNA content of the homogenate, determined by a fluorometric method employing 3,5-diaminobenzoic acid (Aldrich) (15) or 4',6-diamidino-2-phenylindole (Sigma) (16). For autoradiography, incubation was followed by two washes at room temperature in medium containing 100 nM unlabeled testosterone to further reduce unspecific binding of the [\textsuperscript{3}H]-labeled ligand. Frozen 5-\mu m sections were placed on emulsion-coated slides (Ilford K5) that were subsequently exposed for 3–7 months at 4°C (17). After photographic processing the sections were stained with methyl green/pyronin.

**RESULTS**

**Presence of an Androgen-Binding Site in the Embryonic Mammary Gland.** The mammary gland is sensitive to androgens during day 14 of gestation. Therefore, its capacity to bind [\textsuperscript{3}H]testosterone was compared with that of other organs of 14-day (female) embryos: with adjacent skin taken from the area between mammary buds ("intermammary skin") or from the shoulder region ("dorsal skin"), with two organs known to be androgens-responsive in the adult mouse [salivary gland (18) and kidney (19)], with three presumed nontarget organs (lung, stomach, and pancreas), and with a classical target for testosterone-mediated embryonic sexual differentiation [urogenital sinus (20)]. All tissues were incubated under identical conditions, immediately after dissection. Ligand binding capacity was expressed as pmol of testosterone retained per mg of DNA after incubation in 0.6 nM [\textsuperscript{3}H]testosterone (Fig. 1).

On a per cell basis, mammary glands bound almost 5 times more hormone than did urogenital sinus, and 7 to 10 times more than did adjacent skin. No binding was detected in other organs, with the possible exception of the salivary gland. Virtually all (95%) of the [\textsuperscript{3}H]testosterone bound by mammary and urogenital tissues, and also a substantial portion of that bound by skin, was displaced by a 100-fold excess of unlabeled testosterone. In contrast, the minimal binding exhibited by other organs was not blocked by competing hormone and therefore is probably due to nonspecific sites. The relative effectiveness of various unlabeled hormones to compete for [\textsuperscript{3}H]testosterone binding by 14-day mammary glands indicates a binding site with characteristics of an androgen receptor (Table 1). A Scatchard (23) analysis revealed a single class of 50–55 × 10\textsuperscript{6} high-affinity binding sites per ruminant with an apparent \( K_d \) of 0.5 nM (unpublished data). A more detailed characterization of the mammary gland androgen-binding site will appear elsewhere.

**Distribution of [\textsuperscript{3}H]Testosterone-Binding Cells in the Mammary Gland In Situ.** Because the competition experiments had shown that 95% of [\textsuperscript{3}H]testosterone, when applied at 0.6 nM, was bound by specific sites, we assumed that this concentration can be safely used for the autoradiographic demonstration of androgen receptors. Sections from more than 200 glands, taken

![Fig. 1. Relative [\textsuperscript{3}H]testosterone-binding capacity of various organs of 14-day female mouse embryos: mammary glands (MGL), intermammary skin (IM), dorsal skin of the shoulder region (DO), salivary gland (SAL), kidney (K), lung (L), stomach (ST), pancreas (P), and urogenital sinus (UGS). The shaded part of each bar represents the portion of binding that is displaced by competition with a 100-fold excess of unlabeled testosterone. Note that the hormone was applied at a nonsaturating concentration of 0.6 nM; the values shown do not, therefore, represent the number of binding sites per mg of DNA. All measurements were done in triplicate, except for skin (IM and DO), eight determinations each. Error bars are SD.](image-url)

**Table 1. Ligand specificity of the mammary gland androgen-binding site as determined by competition assays**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Exps.</th>
<th>Glands</th>
<th>[\textsuperscript{3}H]Testosterone bound, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9</td>
<td>450</td>
<td>100</td>
</tr>
<tr>
<td>Testosterone</td>
<td>9</td>
<td>450</td>
<td>4.5 ± 1.9</td>
</tr>
<tr>
<td>5a-Dihydrotestosterone</td>
<td>5</td>
<td>216</td>
<td>4.7 ± 2.2</td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>4</td>
<td>194</td>
<td>47.0 ± 7.8</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>4</td>
<td>190</td>
<td>51.1 ± 3.6</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>3</td>
<td>121</td>
<td>98.0 ± 11.4</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>3</td>
<td>165</td>
<td>96.1 ± 3.0</td>
</tr>
</tbody>
</table>

A large number of mammary glands were dissected from 14-day female embryos, pooled, and then divided into groups of 40–65 each. The intact glands were incubated in medium (0.5 ml per group) with 0.6 nM [\textsuperscript{3}H]testosterone; competitors were present at a 100-fold excess (except for diethylstilbestrol, which was applied at 0.6 \mu M). Ligand binding was expressed as cpm bound per gland. Because it was impossible to dissect enough glands to do all the assays simultaneously, competition was related to the no-competitor group (100% was 7.45 ± 1.01 cpm per gland) of each experiment. Results are mean ± SD. Cyproterone acetate is an anti-androgen (21) and estradiol-17β is known to have some affinity for the androgen receptor (22).
from day 12 (bud formation) to day 19 (birth), were examined. Fig. 2 shows the distribution of hormone-binding cells in a 14-day gland—i.e., at the responsive stage. The gland epithelium is surrounded by a distinct population of mesenchymal cells retaining substantial amounts of the labeled steroid. Neither mammary epithelium nor epidermis, nor the more distant mesenchyme, shows more than background label. In all sections studied (of a total of 50 glands), hormone binding was strictly confined to mesenchymal cells in the immediate vicinity of a mammary bud, especially around the "stalk" connecting the gland epithelium to the epidermis. We estimate that each 14-day mammary rudiment contains about 3,000 to 3,500 testosterone-binding mesenchymal cells. The lower level of androgen binding found in adjacent skin by scintillation counting (Fig. 1) remained undetectable in standard autoradiographs. In one experiment with a ligand of highest specific activity ([3H]dihydrotestosterone with 200 Ci/mmole) and an exposure time of 7 months, some label was seen over dermal cells, especially in the uppermost layers immediately beneath the epidermis.

The first [3H]testosterone-binding cells were already detectable in the mesenchyme of 12.5-day glands. At this early stage, very few cells were labeled, only those closest to the epithelium (not shown). After day 14, this characteristic population of hormone-binding mesenchymal cells persisted, and its location remained the same during the outgrowth of the epithelial sprout into the deeper mesenchyme (Fig. 3). In newborn animals, testosterone-binding mesenchymal cells are confined to the nipple area, with only a few of them situated along the invading duct (not shown). We have not observed a reduction in the number of hormone-binding cells or reduced labeling of individual cells after the end of the androgen-responsive phase (early day 15) in the development of the gland.

Appearance of [3H]Testosterone-Binding Cells in Experimental Epithelium—Mesenchyme Combinations. The characteristic apposition of testosterone-binding cells to the epithelium of intact glands suggested to us either that mammary epithelium attracts preexisting mesenchymal cells with receptors or that it induces the formation of testosterone receptors in the surrounding mesenchyme. These possibilities were tested in recombination cultures in which pieces of mesenchyme of the mammary region (not previously in contact with a mammary bud) were associated with trypsin-isolated mamo-

mary epithelia. Both tissues were taken from 12-day embryos, grown in combination for 3 days in vitro, then exposed to [3H]testosterone and processed for autoradiography or scintillation counting.

Autoradiographs of 18 such combination explants revealed the same distribution of hormone-binding mesenchymal cells as observed in the gland in situ: Labeled cells were found exclusively around mammary epithelia, and virtually all gland epithelia were surrounded by labeled mesenchyme, whereas pieces of epidermis, inadvertently included with some mammary buds, lacked such an envelope of testosterone-binding cells (Fig. 4).

Variable results were obtained when epithelia of the salivary gland were placed on the same type of mesenchyme (18 explants, each with four to five epithelia). In most cases, no hormone binding was seen in the mesenchyme. About one-third of the epithelia, however, was lined by mesenchymal cells that retained the steroid. These cells were distinctly associated with salivary epithelia (Fig. 5). In contrast, pancreatic epithelia did not induce the appearance of testosterone-binding mesenchymal cells in eight combination explants.

When the total amount of [3H]testosterone bound by combination explants was measured by scintillation counting it was seen that, after 3 days' culture, pieces of mesenchyme combined with 10 mammary epithelia each on the average bound 11 times more hormone than did mesenchyme grown under identical conditions but without epithelium (0.42 ± 0.07 fmol per combination explant vs. 0.037 ± 0.019 fmol per mesenchyme alone; see Fig. 6). This total increase in the number of binding sites per explant indicates that the appearance of receptor-rich cells around mammary epithelia is not the result of cellular segregation within the mesenchyme.

DISCUSSION

For three reasons we believe that the label seen in our autoradiographs represents genuine androgen receptors. First, low ligand concentrations allow only high-affinity binding sites to retain sufficient amounts of label for autoradiographic demonstration, and our competition experiments have in fact shown that 95% of [3H]testosterone, applied at 0.6 nM (as used for autoradiography) is bound by specific sites. Second, competi-
tion by various compounds revealed a binding specificity characteristic for a typical androgen receptor. Finally, the localization of labeled cells in the autoradiographs agrees with our earlier finding, obtained in tissue combination experiments utilizing the androgen-insensitive Tfm mutant (24), that the mesenchyme is the target tissue for testosterone (11).

It was further shown by the autoradiographs that androgen receptors are not present at the same level throughout the mesenchyme of the entire mammary region but are restricted to, or at least greatly increased within, a well-defined subpopulation of cells surrounding each gland bud. This suggests that mammary epithelium induces the formation of androgen receptors in adjacent mesenchymal cells, and the results obtained with epithelium-mesenchyme recombination cultures have substantiated this conclusion. The alternative explanation for the characteristic distribution of androgen-binding cells—i.e., that epithelium attracts preexisting receptor-rich cells—can be excluded because association with mammary epithelia also resulted in a substantial increase in the total number of binding sites in mesenchymal explants. Earlier experiments of a different design, using androgen-insensitive (Tfm) tissues for combination cultures (13), have already demonstrated that the response of the 14-day mammary gland does not depend on migration of mesenchymal cells to the bud, at least not after day 12. Induction of mesenchymal androgen receptor formation by mammary epithelium explains both the autonomous development of androgen responsiveness in explanted intact glands (10) and the dependence of the mesenchymal response on close association with a mammary bud (13). The short range of this inductive influence ensures that the action of the hormone remains limited to the few mesenchymal cells around each gland.

Apparently, induction of hormone receptor formation starts

Fig. 4. Localization of [3H]testosterone-binding cells in an epithelium-mesenchyme recombination culture. Ten trypsin-isolated 12-day mammary epithelia were placed on one piece of 12-day mesenchyme of the mammary region and grown in vitro for 3 days. The culture was then incubated in medium with 0.6 nM [3H]testosterone (152 Ci/mmole) and processed for autoradiography (exposure time 3 months). This section shows six or seven pieces of mammary epithelium, each surrounded by several layers of hormone-binding mesenchymal cells. The epithelium in the upper center is a gland with an adhering piece of epidermis (top). Note that the steroid-binding mesenchymal cells are concentrated around the mammary part of this epithelium. The epithelium to the left of it, not surrounded by receptor-containing mesenchyme, is a piece of epidermis, identifiable by the lighter staining in its center (due to the onset of keratinization). Bar represents 100 µm.

Fig. 5. [3H]5α-Dihydrotestosterone autoradiograph of an explant of 12-day mesenchyme from the mammary region, grown in vitro for 3 days in association with salivary epithelium. In about one-third of such combination cultures, a few androgen-binding cells were found close to the epithelium, as seen in this figure (compare with the effect of mammary epithelium shown in Fig. 4). Ligand activity, 200 Ci/mmole; exposure time, 4 months. Bar represents 100 µm.

Fig. 6. Total [3H]testosterone-binding capacity in explants of 12-day intermammary mesenchyme grown in vitro for 3 days either alone (light bars) or in combination with 10 mammary epithelia each (shaded bars). Eight identical experiments with a total of 43 recombination cultures and 47 control explants are shown. The marked increase in binding capacity shows that the appearance of receptor-possessing cells around mammary epithelia in recombination cultures, as seen in Fig. 4, is not the result of their redistribution within the mesenchyme.
as soon as the primordial mammary bud has formed, because
the first effects were already seen in autoradiographs of 12.5-
day glands. It is worth noting that the more sensitive scintil-
ation measurements revealed androgen receptors in neigh-
boring skin, too, although at a much lower level and therefore
not detectable in standard autoradiographs. Presumably then,
the gland bud causes a substantial increase of a differentiative
property rather than its de novo appearance, a phenomenon not
uncommon in embryonic tissue interaction [e.g., in cartilage
induction (25)]. Mammary epithelium may also differ from other
epithelia only in the degree of its inductive capacity, as sug-
gested by the adepidermal localization of receptor-containing
dermal cells in long-exposure autoradiographs of skin, or by the
few but definite cases of androgen receptor induction by salivary
epithelia. Nevertheless, in normal development there can be
little doubt that the mammary bud is responsible for the dif-
ferentiation within the dermis of the "mammary mesenchyme,"
one characteristic of which is the possession of increased levels
of androgen receptors and, consequently, responsiveness to tes-
tosterone. A comparable case may be seen in a recent report
(26), which showed that bladder epithelium, induced to develop
prostatic acini by experimental association with mesenchyme
of the urogenital sinus, also forms androgen receptors. Disso-
ciation and reaggregation experiments in the embryonic chicken
retina led to the suggestion that maintenance of glucocorticoid
receptors in glia cells depends on their contact with neuronal
elements (27).

Hormone action in the embryonic mammary gland thus de-
pends on tissue interaction in at least two steps: First, as shown
here, the epithelium of the early gland induces the formation
of receptors, and thereby the development of androgen re-
sponsiveness, in the surrounding mesenchyme. In the second
step, initiated by testicular hormones on day 14, this responsive
mesenchyme mediates the effect of testosterone on the epithe-
lial gland bud and causes its destruction (11, 12). It seems pos-
sible that similar conditions might prevail in other organs, too.
Mesenchymal responsiveness to sex steroids, especially to and-
genins, was also demonstrated for the fetal urogenital sinus
(28) and is suggested by autoradiographs of the fetal genital
tract and accessory sex organs (29, 30), as well as of the chicken
bursa (31). It is certainly conspicuous and suggestive that during
the phase of sexual differentiation, when androgens control or-
gan morphogenesis rather than tissue function, they do so by
acting on the mesenchymal component, which is known to play
the dominant role in morphogenetic tissue interaction (e.g., see
refs. 32–34). The distribution of steroid-binding cells in some of
the autoradiographs published by others (29–31, 35) sug-
gests, in addition, an epithelial influence on mesenchymal hor-
mon binding similar to the one shown here for the mammary
gland. If organ-specific tissue interactions were found to regu-
late receptor synthesis in the adult organism as well, the rel-
evance for pathological processes would be obvious—e.g., for
the maintenance of hormone responsiveness in metastases of
hormone-sensitive tumors.

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