Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system

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Abstract

In males, androgens are essential in maintaining the integrity of the prostate. Androgen-ablation induces apoptosis of the prostatic epithelium. In females, ovariectomy induces apoptosis in uterine epithelium while progesterone inhibits this process. The objective of this study was to determine whether androgen and progesterone inhibit apoptosis, respectively, in mouse prostatic and uterine epithelia via steroid receptors in the epithelium or in the stroma. To address this question, prostatic tissue recombinants were prepared with rat urogenital sinus mesenchyme plus bladder epithelium from wild-type or testicular feminization mutant (Tfm) mice. Thus, prostatic tissue was generated having androgen receptor (AR) in both epithelium and stroma or in the stroma only. Castration of hosts induced apoptosis in the AR-negative Tfm prostatic epithelium with an epithelial apoptotic index virtually identical to prostatic tissue recombinants containing wild-type epithelium. Moreover, this castration-induced prostatic epithelial apoptosis was blocked by testosterone and dihydrotestosterone in both wild-type and Tfm prostatic tissue recombinants. Likewise, uterine tissue recombinants were prepared in which epithelium and/or stroma was devoid of progesterone receptor (PR) by using uterine epithelium and stroma of wild-type and PR knockout mice. Progesterone inhibited uterine epithelial apoptosis only in tissue recombinants prepared with PR-positive stroma. The PR status of the epithelium did not affect epithelial apoptotic index. Therefore, the apoptosis in prostatic and uterine epithelia is regulated by androgen and progesterone via stromal AR and PR, respectively. In both cases, epithelial AR or PR is not required for hormonal regulation of epithelial apoptosis in prostatic and uterine epithelium.
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Introduction

Both male and female reproductive tissues show dramatic growth and regression during reproductive cycles. Steroid hormones, androgen in males and 17-β estradiol (E_2) and progesterone (P_4) in females, are key regulators of these growth/regression cycles. Studies of epithelial apoptosis in male[1] and female,[2,3] reproductive tracts have demonstrated that epithelial apoptosis in steroid target organs is under the control of steroid hormones. In the male, the prostate is dependent on androgens for growth, function and maintenance of tissue architecture.[4] Androgen deprivation elicited by physiological or chemical castration induces apoptosis in prostatic epithelium of rats and mice. In the rodent ventral prostate, epithelial apoptosis reaches its maximum 3 days after castration.[4,5] In females, apoptosis plays an important role in regulating functions in the reproductive tract, and is regulated by two ovarian steroid hormones, E_2 and P_4. E_2 is mitogenic for uterine epithelium, while P_4 inhibits proliferation and apoptosis of uterine epithelium.[6,7] Increased systemic levels of E_2 at the proestrus/estrus-phases induces growth of uterine tissue. The integrity of the uterus is maintained in part by P_4, which prevents cell death. If pregnancy is not established, the level of P_4 drops, and uterine epithelial tissue regresses via apoptosis.[8] This prolifertive/apoptotic cycle in uterine epithelium can be mimicked in ovariectomized mice by a hormone treatment protocol with P_4 and E_2.[6,7]

Steroid hormone action is mediated via nuclear receptor proteins, which function as ligand modulated transcription factors.[9] Androgen and P_4 signals are mediated through the androgen receptor (AR) and progesterone receptor (PR), respectively. Many genes are regulated by steroid hormones, and it has been tacitly assumed that hormonal effects on a cell are mediated through receptors in the responding cell. However, hormone target organs such as the prostate and uterus express steroid receptors in both epithelial and stromal cells.[10,11] The importance of paracrine mechanisms in steroid hormone action in male and female reproductive tracts has been well established. For example, proliferation of normal epithelial cells induced by steroids in male and female reproductive tracts appears to be regulated via paracrine mediators produced by stromal cells. The idea that epithelial proliferation is

regulated *in vivo* by paracrine mechanisms is based upon several lines of evidence: (a) E_2 and testosterone (T) are not mitogenic when applied to primary cultures of normal target epithelial cells.[12,13] (b) Mitogenicity of E_2 for uterine and vaginal epithelia is reconstituted when cultured epithelial cells are recombined with stroma, and the tissue recombinants are grafted *in vivo*.[14] (c) Androgen stimulates proliferation of Tfm prostatic epithelium, which is devoid of functional AR, in prostatic tissue recombinants made with Tfm epithelium plus wild-type stroma.[15] (d) Uterine and vaginal tissue recombinants composed of ERα-negative epithelium derived from ERα knockout mice and ERα-positive wild-type stroma demonstrate conclusively that mitogenicity of estrogen in normal uterine and vaginal epithelia is dependent upon ERα in the stroma *in vivo*.[16,17] Epithelial ERα is neither necessary or sufficient for the mitogenicity of estrogen in normal target epithelial cells *in vivo*. Similar evidence has been demonstrated for anti-mitogenic effect of P_4 for uterine epithelium.[18] P_4 inhibits E_2-induced uterine epithelial DNA-synthesis in PR-negative epithelium from PRKO mice when the epithelial cells are combined with PR-positive uterine stroma from wild-type mice. In contrast, P_4 does not block E_2-induced DNA-synthesis in PR-positive uterine epithelium combined with PR-negative uterine stroma.[18] Thus, in all cases epithelial steroid receptors are not required for the regulation of proliferation of normal epithelial cells *in vivo*. Paracrine mechanisms are also involved in the regulation of lactoferrin expression by E_2[19] and P_4[20] in uterine epithelium, and regulation of uterine epithelial PR by E_2[21] and P_4.[20] These facts raise the possibility that the apoptosis of epithelial cells in male and female reproductive tracts may also be regulated by steroid hormones through paracrine mechanisms.

In this study we report a paracrine mechanism for regulation of apoptosis in prostatic and uterine epithelia. To study the respective roles of epithelial versus stromal steroid receptors in the anti-apoptotic action of androgen on prostatic epithelium and P_4 on uterine epithelium, tissue recombination techniques were utilized. Since stromal AR

is essential for the development of prostate,[22] we could not make prostatic tissue recombinants with AR-negative stroma. To demonstrate whether epithelial AR is required for the regulation of prostatic epithelial apoptosis, bladder epithelium (BLE) was combined with urogenital sinus mesenchyme (UGM) which result in prostatic induction.[23] If the BLE is derived from wild-type mice, the resultant induced prostatic epithelium expresses many markers indicative normal prostatic epithelium including prostatic specific secretory proteins,[24] Nkx3.1[25] and AR.[23] If Tfm bladder is combined with wild-type UGM prostatic ducts also form and the induced Tfm prostatic epithelium expresses Nkx3.1[25] and proliferates in response to androgen even though the Tfm prostatic epithelium does not express prostatic secretory proteins due to lack of epithelial AR.[15,24] To determine the role of stromal *versus* epithelial AR in prostatic epithelial apoptosis, prostatic tissue recombinants were prepared with BLE of Tfm and wt mice combined with rat UGM. The induced prostatic tissue expressed AR in both its epithelium (E) and stroma (S) in wt-S+wt-E tissue recombinants, or only in stroma but not the epithelium in wt-S+Tfm-E tissue recombinants. Likewise, to determine whether P_4 inhibits uterine epithelial apoptosis via epithelial *versus* stromal PR, 4 types of tissue recombinants were prepared with uterine stroma (S) and epithelium (E) from wild-type (wt) and PR knockout (PRKO) mice: wt-S+wt-E, wt-S+PRKO-E, PRKO-S+wt-E and PRKO-S+PRKO-E.

Results

Prostatic epithelial apoptosis induced by androgen-withdrawal in androgen receptor null prostatic epithelium

In intact male mice, very few apoptotic cells were observed in the epithelium of the ventral (Figure 1c) and dorsolateral lobes of prostate, and the apoptotic index (derived from terminal deoxynucleotidyltransferase dUTP nick end labeling [TUNEL] stained specimens) was low in the ventral prostate (VP) and
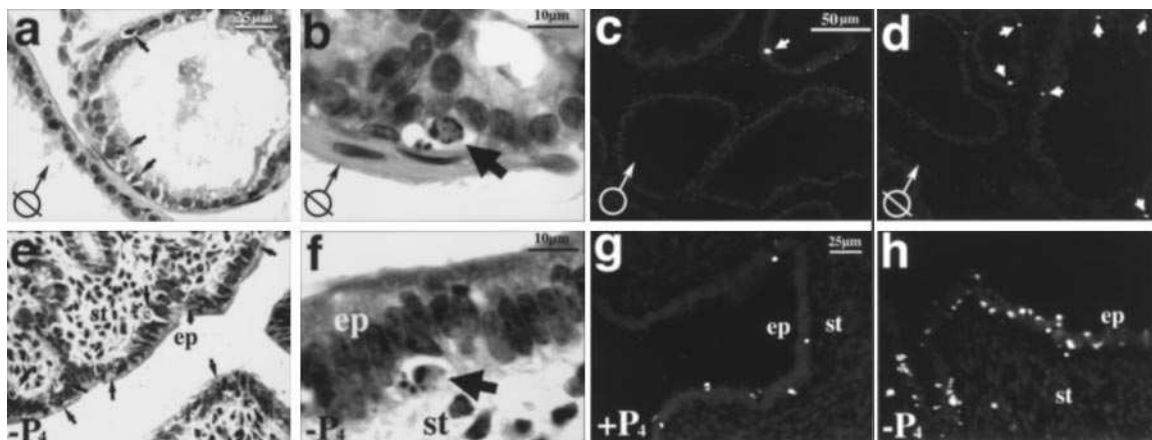
Figure 1 Apoptosis in mouse prostate and uterus. Ventral prostate (**a – d**) of castrated (**a,b,d**) and intact (**c**) mice. Uterus (**e – h**) of P_4-treated (**g**) and oil-treated (**d,e,f**) mice. Apoptotic cells (arrows) were detected by H&E staining (**a,b,e,f**) and TUNEL staining (**c,d,g,h**). ep, epithelium; st, stroma

dorsolateral prostate (DLP) (Table 1). Castration induced apoptosis of prostatic epithelium in both VP (Figure 1a,b,d) and DLP (not shown). At 3 days after castration, the epithelial apoptotic index significantly increased from that of intact mice in both VP and DLP with epithelial apoptotic index being significantly higher in VP than in DLP (Table 1). Rat VP is widely used as a model system for studying mechanisms of epithelial apoptosis. Epithelial apoptotic index in rat and mouse VP and DLP was also compared by counting positive cells for TUNEL staining. In both rat and mouse, epithelial apoptotic index was exceedingly low in intact animals, increased at 3 days after castration, and was significantly higher in VP than DLP following castration. In both VP and DLP, epithelial apoptotic index at 3 days after castration was significantly higher in rat than mouse (Table 1).

Prostatic tissue recombinants were prepared with rat urogenital sinus mesenchyme (rUGM) plus mouse bladder epithelium (mBLE) from wild-type (wt) or Tfm mice. The rUGM+mBLE tissue recombinants were grown under the kidney capsule of male athymic nude mice for 1 month (see Figure 6A for treatment protocol). After 1 month, both types of prostatic tissue recombinants developed normally and showed comparable prostatic histology as reported previously[23] (Figure 2a,b). AR was detected in the wild-type rat stroma whether wt-mBLE or Tfm-mBLE was used to prepare the tissue recombinants (Figure 2a,b, arrows). AR was detected in the epithelium of rUGM+wt-mBLE tissue recombinants (Figure 2a) but not in the epithelium of rUGM+Tfm-mBLE tissue recombinants (Figure 2b). In intact hosts, epithelial apoptotic indices of both rUGM+wt-mBLE and rUGM+Tfm-mBLE prostatic tissue recombinants were equally low; there was no significant difference in epithelial apoptotic index in rUGM+wt-mBLE and rUGM+Tfm-mBLE tissue recombinants (Figure 4A,a). Three days after castration, many epithelial cells positive for TUNEL staining were detected in epithelium of both wt-mBLE and Tfm-mBLE prostatic tissue recombinants (Figure 3a,b). In castrated hosts the epithelial apoptotic indices of both rUGM+wt-mBLE and rUGM+Tfm-mBLE prostatic tissue recombinants (Figure 4A,b) increased significantly relative to tissue recombinants in intact hosts (Figure 4a). In castrated hosts, the difference in epithelial apoptotic index between rUGM+wt-mBLE and rUGM+Tfm-mBLE prostatic tissue recombinants was not significant. In addition to the androgen, orchiectomy could change levels of other hormones, such as estrogen. To confirm that the castration induced epithelial apoptosis in the

prostatic tissue recombinants was due to a decrease in the androgen level, the host nude mice were given an androgen pellet (20 mg of T or DHT), and then castrated on 3 days after the pellet implantation. Both T and DHT blocked castration-induced epithelial apoptosis in both rUGM+wt-mBLE and rUGM+Tfm-mBLE prostatic tissue recombinants (Figure 3 and 4). Thus, differences in apoptotic indices were not statistically different in T-pellet/castration, DHT-pellet/castration and intact groups in both rUGM+wt-mBLE and rUGM+Tfm-mBLE tissue recombinants (Figure 4a). These results clearly showed that androgen maintains integrity of prostatic epithelial tissue via stromal AR. Epithelial AR is not involved in this process. Since DHT cannot be aromatized to estrogenic compounds, a role of estrogens in this process can be excluded.

Inhibition of apoptosis by P_4 in PR null uterine luminal epithelial apoptosis

Uterine tissue recombinants (wt-S+wt-E, wt-S+PRKO-E, PRKO-S+wt-E and PRKO-S+PRKO-E) were grafted under the renal capsule of adult female athymic nude mice, and then all hosts received hormone treatments on the schedule shown in Figure 6B. To establish a baseline, we first examined uterine epithelial apoptosis in the host's uteri. Twenty-four hours after the last injection, many apoptotic cells were detected in uterine luminal epithelium of oil-treated hosts as judged by both apoptotic bodies observed in H&E stained slides (Figure 1e,f) and by TUNEL staining for in situ DNA fragmentation (Figure 1h). In contrast, very few apoptotic bodies and TUNEL stained epithelial cells were detected in uterine luminal epithelium of P_4-treated hosts (Figure 1g). P_4-treatment significantly reduced the apoptotic index in uterine luminal epithelium of host mice from $15.91 \pm 0.572\%$ to $4.46 \pm 0.951\%$ ($P < 0.001$). Thus, for the uteri of host nude mice, P_4 was anti-apoptotic as described previously.[26]

In all uterine tissue recombinants, the tissue types (PR-positive (wild-type) or PR-negative (PRKO)) were confirmed with PR-immunohistochemistry. Only tissues of wild-type mouse origin stained for PR. This is particularly evident in heterotypic tissue recombinants (wt-S+PRKO-E and PRKO-S+wt-E) (stroma in Figure 2d and epithelium in Figure 2e). In all four types of uterine tissue recombinants, apoptotic index of oil-treated ($-P_4$) group was high ($14-18\%$) and similar to that of the host's uterine epithelium (Figure 4B). P_4-treatment inhibited uterine epithelial apoptosis only in tissue recombinants prepared with wt-S (wt-S+wt-E and wt-S+PRKO-E, Figure 3e,f, respectively). Thus, in wt-S+wt-E and wt-S+PRKO-E tissue recombinants, epithelial apoptotic index was significantly lower in the P_4-treated group ($+P_4$) than that of the oil-treated group ($-P_4$) (Figure 4Bc). The presence or absence of PR in the epithelium did not affect its response to P_4. In contrast, P_4-treatment did not inhibit apoptosis in tissue recombinants lacking PR in the stroma (PRKO-S+wt-E and PRKO-S+PRKO-E, Figure 3g,h, respectively). Thus, in response to P_4 epithelial apoptotic index in PRKO-S+wt-E and PRKO-S+PRKO-E tissue recombinants was significantly higher (Figure 4Bd) than that of wt-S+wt-E and wt-S+PRKO-E uterine tissue recombinants in the P_4-treated host (Figure 4Bc).

Table 1 Epithelial apoptotic index in mouse and rat VP and DLP

| Species	Lobe	Apoptotic index (%)	
		Intact	Castrated
Rat	VP	0.43 ± 0.23^a	4.47 ± 0.66^b
	DLP	0.45 ± 0.25^a	2.32 ± 0.49^c
Mouse	VP	0.49 ± 0.28^a	2.49 ± 0.49^c
	DLP	0.07 ± 0.05^d	0.23 ± 0.15^a

Mature adult male rats and mice (approximately 3 month old) were castrated. Three days after castration, VP and DLP were collected. Positive and negative cells for TUNEL staining were counted to determine epithelial apoptotic indices. Value with different letter designation ([a–d]) are all significantly different ($P < 0.05$) in following order, $^d < ^a < ^c < ^b$

prostatic tissue recombinants
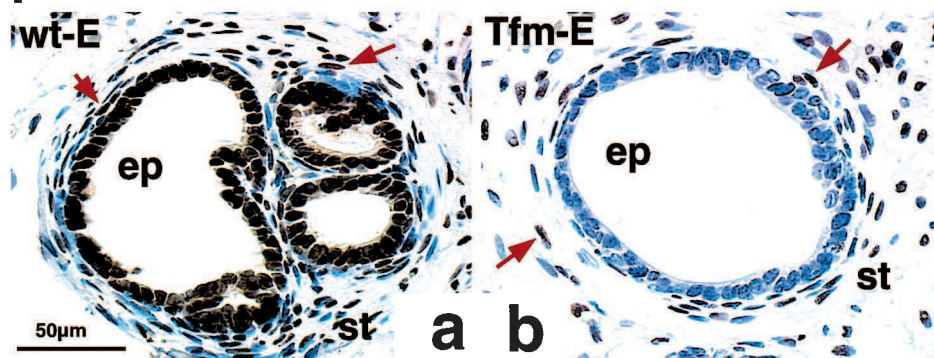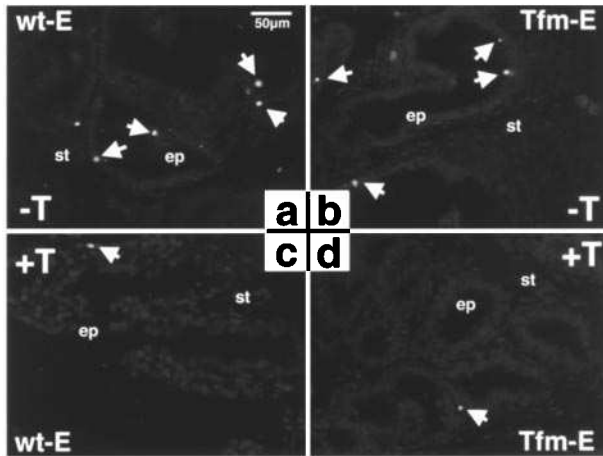

uterine tissue recombinant
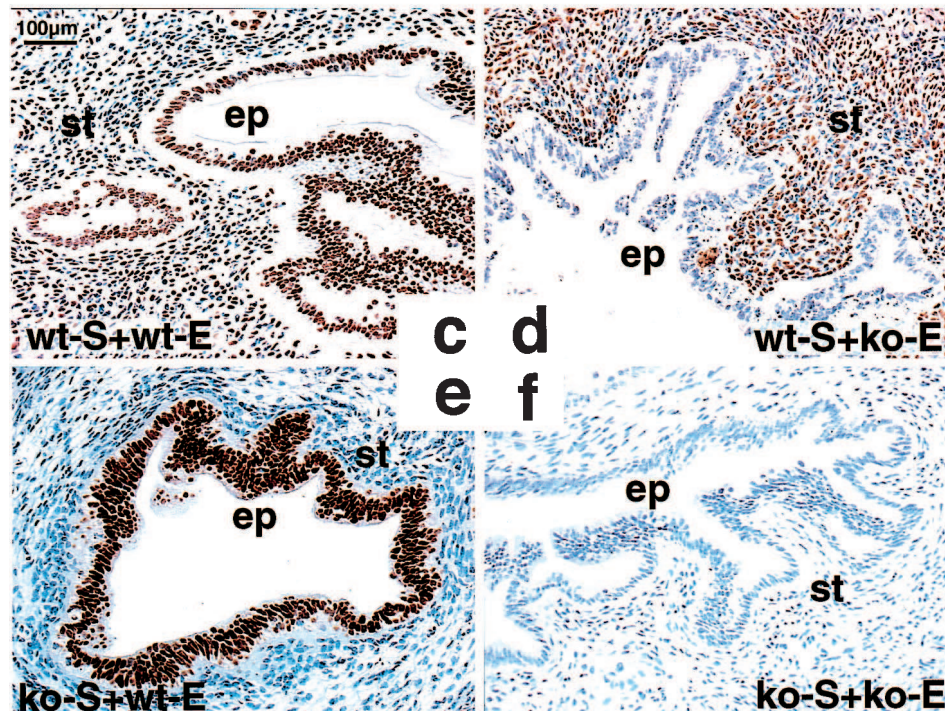
Figure 2 Immunohistochemistry for AR and PR in uterine and prostatic tissue recombinants. ep, epithelium; st, stroma. AR-immunohistochemistry in prostatic tissue recombinants (**a,b**). Tissue recombinants were prepared with rat urogenital sinus mesenchyme (rUGM) and bladder epithelium (BLE) of wild-type (wt) or Tfm mice (m). In rUGM+wt-mBLE recombinants (**a**), AR-positive cells were detected in both epithelium and stroma (arrows). In contrast, AR-positive cells were detected only in stroma (arrows) of rUGM+Tfm-mBLE tissue recombinants (**b**). PR-immunohistochemistry in uterine tissue recombinants prepared with wild-type and PRKO tissues (**c–f**). Tissue recombinants were prepared with uterine epithelium (E) and stroma (S) from wild-type (wt) and PR knockout (ko) mice. In wt-S+wt-E tissue recombinants (**c**), positive nuclear staining for PR was detected in both epithelium and stroma. In contrast, both epithelium and stroma were negative for PR in ko-S+ko-E tissue recombinants (**f**). In wt-S+ko-E (**d**) and ko-S+wt-E (**e**) tissue recombinants, only wt-S or wt-E is positive for PR, respectively

Discussion

This study clearly demonstrated that steroid hormones regulate survival of epithelial cells via stromal steroid receptors in both prostatic and uterine tissue. Thus, androgen and progesterone regulate apoptosis in prostatic and uterine epithelia through paracrine mechanisms mediated by hormone receptors in the stroma. Paracrine regulation of epithelial cell death has been proposed in various models of development. For example, regression of

embryonic mammary epithelial cells in male mice is induced by androgen through a paracrine mechanism mediated via AR in mammary mesenchyme.[27] Likewise, apoptosis of Müllerian epithelium is induced by Müllerian inhibitory substance presumably via receptors in surrounding mesenchyme.[28] In the case of mammary gland and Müllerian duct, the epithelial receptors for the hormones triggering epithelial apoptosis were not detected. In contrast, in prostate and uterus both the epithelium and stroma express receptors for the steroid hormones regulating epithelial apoptosis. Never-

prostatic tissue recombinant

uterine tissue recombinant
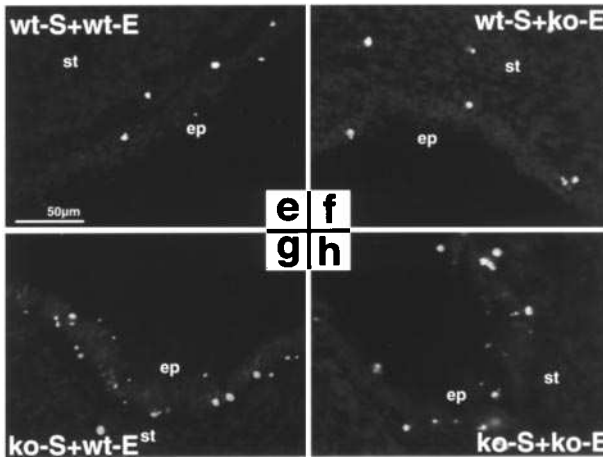
Figure 3 Detection of apoptotic cells by *in situ* DNA fragmentation in prostatic and uterine tissue recombinants. rUGM+wt-mBLE (wt-E) (**a,c**) and rUGM+Tfm-mBLE (Tfm-E) (**b,d**) prostatic tissue recombinants in hosts 3 days post castration with (**c,d**) and without (**a,b**) 20 mg T-pellet (see Figure 6A for treatment protocol). Uterine tissue recombinants prepared with wild-type (wt) and PRKO (ko) tissues growing in P_4-treated hosts. Wt-S+wt-E (**e**), wt-S+ko-E (**f**), ko-S+wt-E (**g**) and ko-S+ko-E (**h**). All hosts were received 2 mg of P_4 injection daily following E_2 treatment (see Figure 6B for treatment protocol)

theless, this study has clearly shown that epithelial steroid receptors are not involved in regulation of prostatic or uterine epithelial apoptosis induced by the withdrawal or the absence of androgen and progesterone, respectively. Hence, epithelial AR and PR are not required to directly regulate transcription of intracellular effectors for apoptosis in the mouse prostatic and uterine epithelial cells. This implies that some form of communication between the stroma and epithelium is required to elicit apoptosis in the epithelium.

There are two possible mechanisms by which stromal cells may control apoptosis of epithelial cells (Figure 5). In presence of steroid hormone, stroma may produce survival factors for epithelium (Figure 5A). Withdrawal of steroid hormones would down-regulate production of the survival factors and thus trigger apoptosis of epithelial cells (Figure 5A'). In some cells, cell death can be induced by

withdrawal of peptide survival factors, such as epidermal growth factor (EGF).[29] In prostate, androgens modify expression levels of members of EGF family ligand and receptors.[30–32] In uterus, E_2 and P_4 regulate expression of peptide growth factors, such as members of EGF family,[33,34] insulin-like growth factors (IGFs)[35–37] and fibroblast growth factors (FGFs).[38,39] Such peptide growth factors are good candidates for the survival factors for prostatic and uterine epithelium.

Alternatively, in response to decreased steroid levels stroma may produce death signals, which induce epithelial apoptosis (Figure 5B). In the prostate castration induces expression of several secreted molecules such as IGF binding proteins (IGFBPs).[40] In the uterus, levels of IGFBPs are modulated by E_2.[41] IGF signaling plays a role in growth and survival of prostatic[42] and uterine[43] epithelia, and IGFBPs antagonize IGF signaling.[44] Thus, IGFBPs are possible mediators of stromal-epithelial interactions in prostatic and uterine epithelial apoptosis. In prostate, castration increases mRNA levels of transforming growth factor (TGF)-βs.[45] TGF-βs are known to be produced by prostatic stromal cells, and their receptors are expressed in prostatic epithelium.[46,47] Transcripts for TGF-β-1,-2,-3 and their type-II receptors are upregulated in the mouse uterus after E_2 withdrawal, and administration of P_4 inhibits this up-regulation of mRNA for TGF-βs.[48] TGF-βs are known to induce cell death in many different cell types including prostatic and uterine epithelia.[49,50] Therefore, TGF-βs are also candidates as paracrine mediators of apoptotic (death) signaling. Epithelial apoptosis in uterus and prostate may be regulated by a balance of these two possible mechanisms (Figure 5A,B). A subtle change in the balance of survival/death signaling may determine the fate of epithelial cells.

Prostatic and uterine stromal tissue consists of many different cell types. The stromal cell types that are the direct target of androgen and progesterone in the regulation of epithelial apoptosis is not clear. Castration of mature male rats results in a rapid reduction of blood flow in VP coinciding with the appearance of TUNEL-positive vascular endothelial and stromal cells.[51] This reduction in the blood flow precedes prostatic epithelial apoptosis in VP.[51] P_4 also regulates proliferation of the spiral arteries in endometrial stroma of primates.[39] These studies suggest that action of androgen and P_4 on the prostate and uterus, respectively, may involve blood vessel integrity and thus, regulation of blood flow. Vascular endothelial growth factor (VEGF) may be a mediator of androgen action on prostate blood flow. Exogenous androgen given to long-term castrated rats increases the level of vascular endothelial growth factor (VEGF) in VP.[52]

It has been tacitly assumed that activation of the molecular machinery of apoptosis elicited by androgen deprivation is due to failure to maintain ligand occupancy of the AR within the epithelial cells themselves. By this reasoning it is presumed that the occupancy status of the epithelial AR plays a key role in regulating the molecular machinery required for epithelial apoptosis. Our results demonstrate definitely that this is not the case. Epithelial AR is not required to elicit prostatic epithelial apoptosis

A. prostatic tissue recombinant

B. uterine tissue recombinant
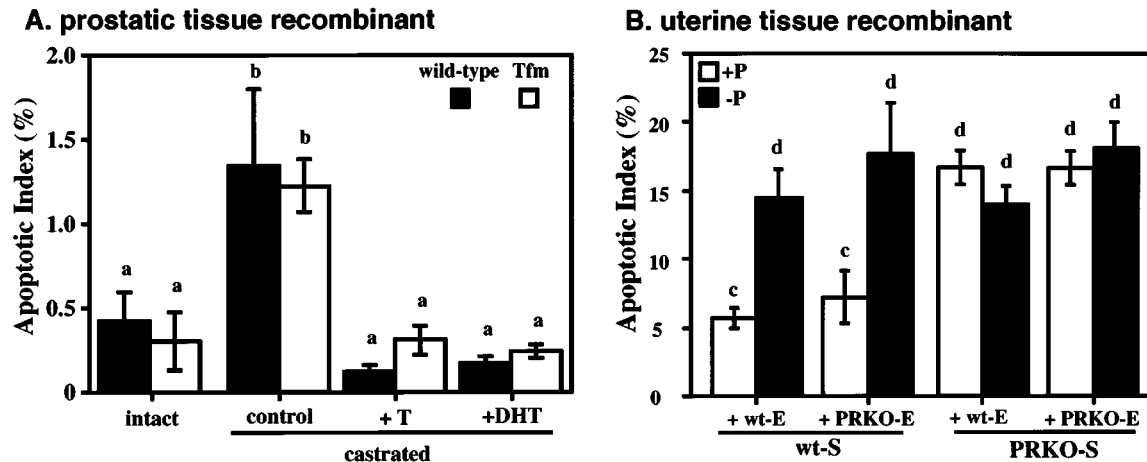
Figure 4 Epithelial apoptotic index in prostatic and uterine tissue recombinants. (**A**) Prostatic tissue recombinants. (b) is significantly higher than (a) ($P<0.05$). (**B**) uterine tissue recombinants. (d) is significantly higher than (c) ($P<0.05$)
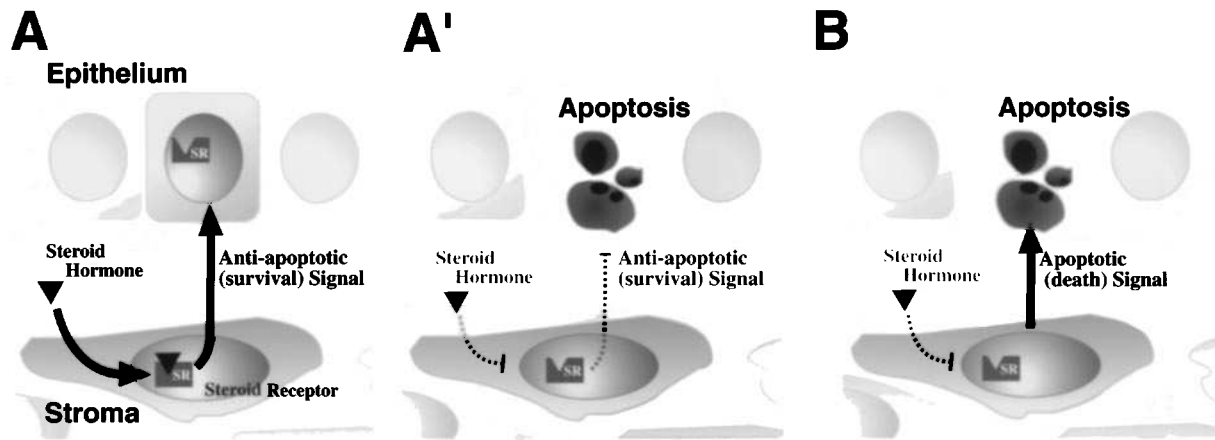
Figure 5 Models of paracrine mechanisms of uterine and prostatic epithelial apoptosis. (**A**) In presence of steroid hormones, steroid hormone binds to stromal steroid receptor, and the ligand occupied receptor activates signaling pathway to maintain epithelial cells. Even though epithelial steroid receptors are expressed, they are not involved in this signaling pathway. (**A'**) As the tissue steroid level is depleted, stromal production of the epithelial survival signal decreases, and epithelial cells undergo apoptosis (**B**) In response to a reduced steroid hormone level, stroma generates death signals which induce epithelial apoptosis

triggered by androgen deprivation. One of the intracellular regulators of apoptosis is the bcl-2/bax ratio, which changes after castration in epithelium of rat ventral prostate.[53] Castration of mature male rats also induces expression of transglutaminase[54] and c-fos[55] in the epithelium of rat ventral prostate. In the prostate these intracellular regulators and effectors for epithelial apoptosis must be regulated by androgen via stromal AR through a paracrine mechanism.

We observed that the epithelial apoptotic index of ventral and dorsolateral prostates in the rat is significantly higher than that of the mouse at 3 days after castration. The epithelium in rUGM+wt-mBLE prostatic tissue recombinants mostly shows a dorsolateral prostatic phenotype as assessed by expression of secretory protein specific for DLP.[24] The prostatic tissue recombinants analyzed in this study were heterospecific (epithelium=mouse, mesenchyme=rat). Epithelial apoptotic index in rUGM+mBLE prostatic tissue recombinants was lower than that of rat DLP and

higher than that of mouse DLP. Thus, the level of prostatic epithelial apoptosis may be determined by the origin and phenotype of both stromal and epithelial tissues, even though the kinetic pattern of apoptosis is similar in both species.

Our tissue recombination studies with steroid receptor null mutants have revealed the importance of paracrine mechanisms in steroid actions that regulate growth and differentiation in reproductive organs.[56–58] This study emphasizes the importance of paracrine mechanisms in steroid hormone regulation of epithelial apoptosis in male and female reproductive tracts. In both male and female reproductive tracts (different systems) androgen and progesterone (different steroid hormones) elicit their anti-apoptotic effect via stromal receptors. These data, therefore, strongly suggest general mechanism, that for steroid target organs, hormones regulate epithelial apoptosis via paracrine mechanisms involving stromal-epithelial tissue interactions. Future studies on regulation of the machinery of apoptosis must take into account these paracrine mechanisms.

Materials and Methods

Animals

All animals were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all procedures described here were approved by the UCSF. Mice were maintained under controlled temperature and lighting conditions during the experiment, and were given food and water *ad libitum*. Adult athymic nude mice, adult male and time-pregnant female Sprague-Dawley rats were purchased from Charles River (Wilmington, MA, USA). AR null male mice (Tfm male) were bred in our laboratory in a colony from breeding pairs (purchased from Jackson Laboratory).[24] The affected Tfm males were identified by the presence of testis and female external genitalia. PRKO mice were produced by breeding heterozygous males and females, and genotypes of pups were determined by PCR as described before.[59]

Tissue separation, recombination and grafting

Details of prostatic[24] and uterine[18] tissue recombination have been described, previously. For prostatic tissue recombination, bladder epithelium from neonatal or adult wild-type and Tfm male mice was combined with urogenital sinus mesenchyme from gestation 18 day-old embryonic rats. For uterine tissue recombination, uteri from neonatal PRKO and wild-type litter-mate mice (days 3–10) were used. Female athymic mice were used as hosts for uterine tissue recombinants, and male athymic mice were used as hosts for prostatic tissue recombinant (Charles River, Wilmington, MA, USA).

Hormone treatment and castration

The experimental design to study prostatic epithelial apoptosis is illustrated in Figure 6A. Prostatic tissue recombinants were grown under the kidney capsule of male athymic nude mice for a month. After 1 month, some hosts were sacrificed to collect tissue recombinants at time zero as a control group, while others were castrated. Three days prior to the castration, 20 mg of T or DHT pellet were subcutaniously implanted into some of the hosts. Three days after castration tissue samples were collected. Pellets were made in our laboratory from T (Sigma) and DHT (Sigma) powder with a pellet press (Parr Instrument Co., Moline, IL, USA).

To study uterine epithelial apoptosis, a hormone treatment protocol described by Terada *et al*[7] was utilized with modifications. The experimental design is illustrated in Figure 6B. Approximately 4 weeks after grafting, all hosts were ovariectomized and kept for another 2 weeks to allow establishment of a hormone deprived state. Two weeks after ovariectomy, daily dosages of 1 μg E_2 (Sigma, St. Louis, MO, USA) was given to all hosts subcutaneously in 0.1 ml of vehicle for 3 days to stimulate uterine epithelial proliferation thus mimicking the E_2 surge at the proestrus/estrus. E_2 was dissolved in 0.9% NaCl, 0.4% Polysorbate 80 (Sigma), 0.5% carboxymethylecellose 80 (Sigma), and 0.9% benzyl alcohol 80 (Sigma) as described.[6,7] From days 4 to 6, 2 mg progesterone (P_4) (Steroids Inc., Wilton, NH, USA) in 0.1 ml peanut oil (Sigma), or 0.1 ml oil alone were given intraperitoneally. Twenty-four hours after the last hormone injection, host animals were sacrificed to harvest tissue samples.

Immunohistochemistry and apoptosis detection

Anti-human PR rabbit polyclonal antibody was purchased from DAKO (Carpenteria, CA). Anti-human AR was purchased from Affinity Bio Reagents (Golden, CO, USA). Tissues were fixed with 4% paraformaldehyde and processed into paraffin, and then sectioned
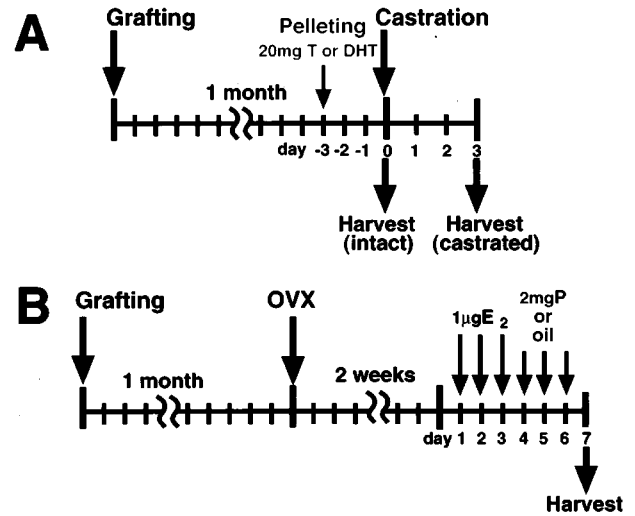
Figure 6 Time lines for experiment protocol. (**A**) Protocol for induction of prostatic epithelial apoptosis. Prostatic tissue recombinants were grafted under the renal capsule of adult male athymic nude mice. After 1 month, some hosts were sacrificed to collect tissue recombinants at time zero as a control group, while others were castrated. Three days prior to the castration, 20 mg T or DHT pellets were subcutaneously implanted into some hosts. Three days after castration tissue samples were collected. (**B**) Protocol for progesterone treatment for female mice. Uterine tissue recombinants were grafted under the renal capsule of adult female athymic nude mice. All hosts were ovariectomized at approximately 1 month after grafting, and 2 weeks after the ovariectomy, all hosts were given 1 μg E_2 daily for 3 days (days 1–3). After three injections of E_2, hosts were treated with 2 mg/day of progesterone (P_4) or oil for 3 days (days 4–6), and 24 h after the last hormone injection, tissue recombinants and host reproductive tracts were collected

at 6 μm. Slides were deparaffinized, re-hydrated and heated in 10 mM citrate buffer (pH 6.0) by microwave. Sections were incubated with primary antibody overnight. Immunoreactivity was developed by Vectorstain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). ApoTag kit (Oncor Co., Gaithersburg, MD, USA) was used to detect 3′-hydroxyl termini of DNA *in situ* (TUNEL assay).

Apoptotic index

For prostate and prostatic tissue recombinants, since counting apoptotic bodies in H&E stained sections was technically difficult due to small number of apoptotic cells, positive and negative cells for TUNEL staining were counted to determine epithelial apoptotic index. For the apoptotic index of prostatic epithelium, propidiumiodide-counter stained epithelial cells were counted under a fluorescent microscope in randomly selected fields. Cells positive for TUNEL staining were counted in the same field. At least 10 fields from five to 33 prostate or tissue recombinants from at least 3 independent experiments were analyzed (minimum 2000 cells/group). For uterus and uterine tissue recombinants, epithelial apoptotic index was determined by counting apoptotic bodies on images of H&E slides captured with a DC330 camera (Dage-MTI Inc., Michigan City, IN, USA), interfaced with a PowerBase 200 computer (Power Computing, Round Rock, TX, USA). At least 2000 epithelial cells in 4–33 uterine tissue recombinants from 3 to 5 independent experiments were counted for each group, and in total 351 images from 24 nude mouse hosts and 85 tissue recombinants were analyzed. The result is analyzed by the factorial-ANOVA and Fisher's protected least significant difference (PLSD) tests.

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