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Relative Roles of the Epithelial and Stromal Tissue Compartment(s) in Mediating the Actions of Relaxin and Estrogen on Cell Proliferation and Apoptosis in the Mouse Lower Reproductive Tract

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Abstract

Relaxin and estrogen are secreted by the ovary during the second half of pregnancy in rats and mice. Relaxin promotes marked growth of the lower reproductive tract in both species. Relaxin promotes accumulation of epithelial and stromal cells in the cervix and vagina by both stimulating cell proliferation and inhibiting apoptosis. Estrogen acting through estrogen receptor α (ER α) plays an essential permissive role in relaxin's actions. A fundamental step toward understanding the actions of relaxin and estrogen is to identify the tissue compartments that initiate their effects. Limited studies using either antibodies to human relaxin receptor (LGR7, RXFP1) or an IRES-LacZ reporter cassette in the LGR7 gene revealed relaxin receptors in subepithelial stroma cells and smooth muscle cells but not in epithelial cells in rodent vaginal and/or cervical tissues. ER α has been reported in both stromal and epithelial compartments in rodent reproductive tract. This chapter describes ongoing studies that use relaxin bioactivity as a means of identifying the tissue compartment(s) that initiates the actions of relaxin and estrogen on the lower reproductive tract. Specifically, a tissue separation/recombination methodology in combination with LGR7-knockout mice was initially used to obtain functional evidence that stromal LGR7 is both necessary and sufficient to promote proliferation and inhibit apoptosis in both stromal and epithelial cells in mouse cervix and vagina. The tissue separation/recombination method is currently being used in conjunction with ER α -knockout mice to determine if the obligatory permissive effect of estrogen on relaxin-induced cell proliferation occurs through stromal and/or epithelial ER α .

Keywords

relaxin receptor; tissue separation/recombination; cervix; vagina; paracrine

INTRODUCTION

Relaxin is secreted by the corpora lutea throughout the second half of pregnancy in rats and mice.¹ Relaxin enables rapid and safe delivery of the fetuses, and does so by promoting growth and softening of the lower reproductive tract.¹ Relaxin induces more than a 40% increase in the wet weight of the cervix and vagina by term.^{2,3} Relaxin-dependent growth of the cervix and vagina in pregnant rats is accompanied by a marked increase in both epithelial and stromal cells.^{4,5} Moreover, we found that relaxin brings about the accumulation of cells in both tissue compartments by promoting cell proliferation and inhibiting apoptosis.^{6,7}

Estrogen is secreted in increasing amounts by developing follicles as pregnancy progresses in rats and mice.^{8,9} An understanding of relaxin's growth-promoting actions on the lower reproductive tract in these species requires knowledge of the effects of estrogen, because relaxin-induced growth of the cervix and vagina is obligatorily estrogen dependent.¹⁰

Cellular and molecular mechanisms associated with the combined actions of relaxin and estrogen on the cervix and vagina during pregnancy are poorly understood. Fundamental steps toward understanding these mechanisms are to identify both the tissue component(s) that contains the relaxin receptor-bearing cells that initiate relaxin's effects and the tissue component(s) that contains the estrogen receptor-bearing cells that bring about estrogen's permissive effects. Progress has been made toward localization of relaxin receptors. In 2002 the orphan receptor LGR7 was identified as the relaxin receptor.¹¹ This discovery made possible several approaches toward the identification of cells that contain relaxin receptors, now also designated RXFP1.¹² Limited studies utilizing antibodies to human LGR7,¹² *in situ* hybridization of LGR7 transcripts¹³ and staining for β -galactosidase activity driven by an IRES-LacZ reporter cassette in the LGR7 gene¹⁴ reported putative relaxin receptors in subepithelial stroma cells and in smooth muscle layers in rodent vaginal and/or cervical tissues. Relaxin receptors were not observed in cervical and/or vaginal epithelial cells.^{11,13,14} In the mouse cervix and vagina, estrogen receptor- α (ER α) are found in both epithelial and stromal cells.^{15–17} Whereas both ER α and ER β are present, extremely low levels of ER β are found in the mouse reproductive tract.^{18,19} Additionally, studies with ER α knockout (ER α KO) mice indicate that ER α is required to mediate characteristic estrogen-induced responses including uterine and vaginal epithelial cell proliferation and vaginal cornification.²⁰ Consistent with these findings, growth-promoting effects of relaxin on the mouse cervix and vagina occur in ER β KO mice, but not in ER α KO mice (Cooke, P.S., D. LoTurco & O.D. Sherwood, unpublished observation).

The overall objective of our ongoing research is to use bioactivity as a means to identify the tissue compartment(s) that contain not only LGR7-bearing cells but also the ER α -bearing cells required for relaxin's actions. The biological end points used are cell proliferation and/or apoptosis in the mouse lower reproductive tract. To accomplish the objective, a tissue separation/recombination methodology using wild-type and either LGR7 knockout (*Lgr7*^{-/-}) or ER α KO mice is being employed. Specific aim 1, to identify the tissue compartment(s) containing the LGR7-bearing cells that initiate relaxin's actions, was recently accomplished and published.²¹ Specific Aim 2, to identify the tissue compartment(s) containing the ER α -bearing cells that mediate the permissive effects of estrogen on relaxin's actions, is underway. This chapter describes the recently published functional evidence that LGR7-expressing cells in the stroma are the sole mediator of relaxin's actions on proliferation and apoptosis in both stromal and epithelial cells of mouse cervix and vagina.²¹

METHODS

Tissue Separation/Recombination and Grafting

FIGURE 1 illustrates the procedure used for separation and recombination of epithelium and stroma from neonatal mouse cervix and vagina. The procedure closely follows that previously used to determine respective roles of stromal and epithelial ER α in estrogen-induced epithelial cell proliferation in mouse uterine and vaginal tissues.²⁰ LGR7-deficient mice (*Lgr7*^{-/-}) on a C57BL/6 background²² were used. Briefly, cervixes and vaginas from neonatal wild-type (wt) and *Lgr7*^{-/-} littermate mice (3- to 5-days-old) were removed and cut into small pieces. The pieces were enzymatically dissociated by placing them in a 1% trypsin solution in calcium- and magnesium-free Hank's Balanced Salt Solution for 90 min at 4 C. After trypsin digestion, stroma (St) and epithelium (Ep) were physically separated using fine surgical instruments. For both cervix and vagina, four tissue recombinants were prepared: wt-St+wt-Ep, wt-St+ko-Ep, ko-St+wt-Ep, ko-St+ko-Ep. Stroma and epithelium were recombined and allowed to adhere overnight. Tissue recombinants were then grafted under the renal capsules of 70- to 90-day-old syngeneic wt female C57BL/6J mice.

Animal Treatment

Approximately three weeks after grafting, all hosts were ovariectomized and received hormone treatments as previously described.²¹ Briefly, mice were ovariectomized (O) and fitted subcutaneously (sc) with silicone rubber tubing containing progesterone (P) and estradiol-17 β (E) in quantities that provide physiological levels in mice. Beginning at 0600 h on day 8 and terminating at 0600 h on day 10 of treatment, animals were injected sc at 6-h intervals with either porcine relaxin (R)²³ at a dose of 4 μ g in 0.2 ml PBS (group OPER) or R vehicle (group OPE). Ten hours before tissue collection, mice were injected sc with 5-bromo-2'-deoxyuridine (BrdU).

Tissue Collection and Processing

At 1000 h on day 10 of treatment, grafts of the four tissue recombinants (n=6 for each type of recombination) were collected from the OPE and OPER groups as previously described.⁵ and serially sectioned (5 μ m).

Morphometric Analysis of Relaxin's Effects on Cell Proliferation and Apoptosis

Cell proliferation was determined by quantitating BrdU incorporation, using a mouse monoclonal antibody and a Vectastain Elite ABC kit as described.^{5,24} Apoptosis was determined by examining terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine 5'-triphosphate nick end labeling (TUNEL) immunostaining using a commercial kit.^{6,25}

Morphometric analysis was conducted to determine both the cellular proliferation and apoptosis rates. Labeling index was determined by dividing number of immunostained cells by total number of cells analyzed per section and multiplying by 100. Epithelial cells and stromal cells were analyzed independently. Data were obtained from four sections per tissue (cervix, vagina, or tissue recombinant), and at least 500 epithelial cells and 500 stromal cells were analyzed per section. Thus, at least 2000 epithelial cells and 2000 stromal cells were analyzed for each tissue sample.

Localization of LGR7-bearing Cells

Because the tissue separation/recombination, grafting, ovariectomy and hormone treatments differ somewhat from normal physiological circumstances, tissue location of LGR7-bearing cells in cervix and vagina was determined during late pregnancy in mice by a more

physiological approach. In the development of the *Lgr7*^{-/-} mice used here²¹ an IRES-LacZ reporter cassette was inserted into the mutant allele in the correct orientation, which allows the expression of the reporter gene. For histological analysis of *LacZ* expression, cervical and vaginal tissues from day 17.5 pregnant wt and *Lgr7*^{+/-} mice were processed for β -galactosidase activity as described previously^{21,22,26}

RESULTS

Relaxin's effects on cervical and vaginal tissue recombinants

Relaxin increased epithelial and stromal cell proliferation to a dramatic and equivalent degree in wt-St+wt-Ep and wt-St+ko-Ep cervical tissue recombinants (FIGURE 2A and 2B). In contrast, relaxin had no effect on either epithelium or stroma of tissue recombinants lacking wild-type stroma (ko-St+wt-Ep and ko-St+ko-Ep). The epithelial origin (either wt or ko) had no effect on the relaxin response of either stromal or epithelial cells. FIGURE 3 contains representative micrographs of two cervical tissue recombinants from treatment group OPER stained to detect cell proliferation. In tissue recombinants that contained wt-St, relaxin induced substantial proliferation of basal epithelial cells as well as proliferation of cells throughout the stromal tissue.

Consistent with cell proliferation findings, relaxin decreased epithelial and stromal cell apoptosis markedly and equally in tissue recombinants wt-St+wt-Ep and wt-St+ko-Ep, but had no effect on tissue recombinants lacking wild-type stroma (FIGURE 2C and 2D). Similar to the proliferation results, epithelial origin (either wt or ko) had no effect on the anti-apoptotic relaxin response in either stroma or the epithelium.

Results from morphometric analysis of vaginal tissue recombinants were highly consistent with those obtained with cervical tissue recombinants.²¹ Relaxin markedly and equally increased proliferation and decreased apoptosis of epithelial and stromal cells in recombinants that contained wt stroma.

Localization of LGR7-bearing Cells

The β -galactosidase activity in cervical and vaginal tissue of *Lgr7*^{-/-} mice on day 17.5 of pregnancy was observed in stroma just beneath the basal layer of the epithelium (FIGURE 4). Moderate staining was associated with deeper smooth muscle bundles. Staining was not observed in the epithelium.

DISCUSSION

This study²¹ is the first to examine the relative roles of stromal and epithelial LGR7 in critical reproductive responses to relaxin. It demonstrated that stromal LGR7 is both necessary and sufficient for relaxin to promote proliferation and inhibit apoptosis in both stromal and epithelial cells in mouse cervix and vagina. There is no evidence that epithelial LGR7 contributes to either of these critical biological actions. Findings in this study are in agreement with the limited studies that reported relaxin receptors in stromal but not epithelial cells in rodent vaginal and/or cervical tissues.^{11,13,14}

Earlier studies have demonstrated that actions of steroid hormones on mouse stromal cell receptors are necessary and sufficient to bring about full hormonal responses in epithelia via paracrine action(s).^{20,27,28} For example, the stimulatory effects of androgens on prostatic epithelial proliferation are mediated through androgen receptors in the prostatic stroma, and following androgen stimulation the stromal cells induce epithelial proliferation by a paracrine process that is not well understood. Likewise, estrogen stimulation of epithelial proliferation in the uterus also occurs through estrogens acting through ER α in the stromal

cells, again triggering a paracrine interaction between the stromal and epithelial cells that induces epithelial proliferation. Critical progesterone effects also appear to be mediated through stromal-epithelial interactions. For example, the ability of progesterone to inhibit the mitogenic effect of estrogen on epithelial proliferation is mediated through stromal, rather than epithelial, progesterone receptors.

This is the first report of protein hormone acting through its stromal receptor to elicit epithelial responses in adult developed reproductive tract via a paracrine mechanism. It appears that relaxin's actions on stromal cells are also attributable, at least in part, to paracrine actions within the stromal compartment itself. Whereas the great majority of LGR7-containing stromal cells lie just beneath the epithelium (FIGURE 4), relaxin promotes proliferation and inhibits apoptosis of cells spread throughout the stroma (FIGURE 3).

Results of this study advance our basic understanding of how relaxin works and simultaneously raise fundamental questions. How does stroma communicate with epithelium? Speculation has centered on growth factors being secreted by stroma and acting in a paracrine manner in epithelium, but other means of communication are possible. If stroma does communicate with epithelium via paracrine factors, does the same paracrine factor(s) secreted by the subepithelial LGR7-bearing cells promote proliferation and inhibit apoptosis in both epithelial and stromal compartments? Is there a single paracrine factor that not only promotes proliferation but also inhibits apoptosis? Finally, the close proximity of the prominent stromal LGR7-bearing cells to the epithelium tempts the speculation that the epithelium plays a role in the development of these relaxin-responsive cells. There are precedents for such a phenomenon. Durnberger and Kratochwil²⁸ demonstrated that androgen responsiveness of male mouse mammary mesenchyme could only be initiated at the epithelial surface and only when mammary epithelium was present. Thus, it was concluded that mammary epithelium induced androgen receptor expression in adjacent mesenchymal cells.²⁸ Similarly, stromal-epithelial communication is reciprocal as has recently been demonstrated for progesterone responses in the uterus.²⁹ The similar findings in both cervical and vaginal recombinants support the view that relaxin promotes cell proliferation and inhibits apoptosis via similar mechanisms in both portions of the lower reproductive tract.

Finally, the tissue separation/recombination technique in conjunction with the subsequent hormonal treatments and cellular activity indicators offers a promising experimental method for additional efforts to determine cellular and molecular mechanisms associated with relaxin's actions in these sites. This is the case because relaxin's effects on cell proliferation and apoptosis in stroma and epithelium are both dramatic and precise. This tissue separation/recombination methodology is also applicable to the question of how the permissive actions of estrogen on the typical relaxin responses in the female reproductive tract are mediated. Previous work discussed above has indicated that estrogen actions on proliferation of reproductive tract epithelia are mediated indirectly through estrogen acting on ER α in the stroma and subsequent paracrine interactions between stroma and epithelium. This suggests the possibility that the critical permissive actions of estrogens for such classic relaxin responses as increased proliferation and decreased apoptosis of reproductive tract epithelium may be due to estrogen acting indirectly through stroma, rather than directly through the epithelial cells themselves. The tissue recombination methodology provides a powerful tool to test this hypothesis. These experiment results, in conjunction with recent data on how relaxin itself acts in the female tract, have the potential to give us a more complete understanding of how relaxin and estrogen in conjunction induce reproductive effects that are critical in preparing the pregnant animal for parturition.

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FIGURE 1.

Tissue separation/recombination procedure used in experiments to determine whether stromal and/or epithelial relaxin receptor (LGR7) is required for relaxin to promote cell proliferation and inhibit apoptosis of stromal and epithelial cells in the mouse cervix and vagina. (From Cooke *et al.* 20 Modified and reprinted with permission.)

FIGURE 2.

Influence of relaxin on labeling indices for proliferation (A, B) and apoptosis (C, D) in the cervical tissue recombinants. Four sections were analyzed per recombinant, and at least 500 of both epithelial and stromal cells were analyzed per section. Each column represents mean (+SEM) of six recombinants. The OPER recombinants wt-St+wt-Ep and wt-St+ko-Ep did not differ from each other, but differed from all other groups (**, = $P < 0.01$). (From Yao *et al.*²¹ Reprinted with permission.)

FIGURE 3.

Histology of wt-St+ko-Ep (A) and ko-St+wt-Ep (B) tissue recombinants from group OPER mice. Sections were stained for BrdU to identify proliferating cells and with hematoxylin to determine cells that were not proliferating following BrdU administration. Arrows indicate BrdU-positive cells. Bar in top micrograph = 200 μm. Both micrographs are the same magnification. Ep, epithelium; St, stroma. (From Yao *et al.*²¹ Reprinted with permission.) Figure 3 is a black and white reproduction; the online version is in color.

FIGURE 4.

LGR7 expression in cervical and vaginal stromal cells. β -galactosidase activity in cervix (A, B) and vagina (C, D) from mice on day 17.5 of pregnancy. In *Lgr7*^{+/-} mice (A, C), strong staining was observed in subepithelial stroma (large arrows). Modest staining was seen in smooth muscle bundles (small arrows). Staining was not observed in either cervix or vagina of wild type mice (B, D). Ep, epithelium; St, stroma. (From Yao *et al.*²¹ Reprinted with permission.) Figure 4 is a black and white reproduction; the online version is in color.