An Antiprogesterone, Onapristone, Enhances the Gene Expression of Promatrix Metalloproteinase 3/Prostromelysin-1 in the Uterine Cervix of Pregnant Rabbit

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Using a progestosterone receptor antagonist, onapristone/ZK 98.299, we examined the in-vivo effects of progesterone on the function of uterine cervix during pregnancy. Onapristone was intravenously administered to pregnant rabbits on day 20 post coitum. After 24 h, the antiprogesterone increased the wet weight of the uterine cervix and decreased the DNA concentration in the cervix. In-situ hybridization also indicated that antiprogesterone augmented the expression of matrix metalloproteinase (MMP)-3/prostromelysin-1 mRNA in the uterine cervix. These changes are very similar to those observed and reported thus far in ripened and dilated uterine cervix. These results suggest that during pregnancy, progesterone closely participates in the maintenance of the function of uterine cervix by preventing the production of MMPs and thereby destruction of extracellular matrix, and thus add support to the theory that antiprogesterone has the potential to accelerate for the uterine cervical ripening and dilatation.

Key words uterine cervical ripening; prostromelysin-1; promatrix metalloproteinase-3; antiprogesterone; onapristone/ZK 98.299

The uterine cervix is a typical connective tissue consisting of collagens, elastin, proteoglycans and hyaluronate.1,2) Throughout pregnancy this tissue must maintain its full tensile strength. Degradation of the extracellular matrix is very important in uterine cervical ripening and dilatation at term pregnancy.3) Matrix metalloproteinases (MMPs) and their endogenous inhibitors called TIMPs are considered to play essential roles in these processes.2,3) In fact, we have reported that rabbit uterine cervix at term pregnancy contains a great quantity of MMPs including MMP-1/collagenase 1 and MMP-9/gelatinase B in comparison to non-pregnant cervix.4,5) In addition, high levels of inflammatory cytokines such as interleukin (IL)-1α and neutrophil chemotactic factor IL-87) are found in rabbit uterine cervix at term pregnancy. Collagenolytic activity in human uterine cervix is also reported to increase along with the progress of pregnancy.2,3) Prostaglandins (PGs) are believed to closely participate in the initiation of labor and/or premature rupture of fetal membranes because they induce contraction in the myometrium and fetal membranes.8) Enhanced expression of cyclooxygenase (COX)-2 is also reported in ovine myometrium and amnion at term pregnancy.9)

In general, the production of proMMPs and TIMPs is known to be regulated by various cytokines, growth factors and hormones, but the principal regulators for the production of MMPs, cytokines and PGs in the reproductive tract are not understood well. From this point of view, it is of interest that plasma levels of progesterone change along with the progress of pregnancy,10) i.e., after implantation, plasma progesterone gradually increases and reaches its maximal level in the middle of pregnancy. This high level of progesterone is retained until just before parturition, and then suddenly decreases. Progesterone is, therefore, very likely to participate in the maintenance of function of the reproductive tract, which contains progesterone receptors.

We examined in-vivo effects of the antiprogesterone, onapristone/ZK 98.29912) on the expression of proMMP-3/ prostromelysin-1 in the uterine cervix of pregnant rabbit on day 20 post coitum, and confirmed the in-vivo participation of progesterone in the expression of MMP-3, which is identical to our previous in-vitro observations.13)

MATERIALS AND METHODS

Animals and Administration of Onapristone Japanese white female rabbits were obtained from Tokyo Experimental Animals Co. (Tokyo, Japan), and were housed in air-conditioned quarters illuminated from 07:00 to 19:00 h and were fed pelleted food and water ad libitum. The parturition of rabbit normally occurs on day 30 after mating.

Pregnant rabbits on day 20 post coitum were intravenously injected with onapristone once (3 mg/kg of body weight; Schering AG, Berlin, Germany). Onapristone was dissolved at 20 mg/ml in 50% (v/v) ethanol as a stock solution and the vehicle (0.15 ml/kg) was also administered to the control animals. After 24 h, the cervix was harvested from each animal and used for DNA determination and in-situ hybridization. The experimental procedures complied with the Guide for Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee of Tokyo University of Pharmacy and Life Science. It is known in New Zealand white rabbits, plasma progesterone during pregnancy gradually increases after mating and high levels (12—16 ng/ml) of progesterone are retained from 6 to 28 d after gestation.11) Another antiprogestosterone, mifepristone/RU486, effectively interferes with the progesterone-mediated suppression of IL-8 production when rabbit uterine cervical cells were co-treated with mifepristone and progesterone.
(1 μm each). Thus, the above dose of onapristone may have caused apparent plasma levels to be about 1000-times in excess of that of progesterone levels and also effectively interfered with action of endogenous progesterone.

**Quantitative Analysis for DNA in Rabbit Uterine Cervix** The determination of DNA in the rabbit uterine cervixes was carried out by the method of Burton. DNA in the tissue was extracted twice with 5% (w/v) trichloroacetic acid at 90°C for 10 min. A portion of the DNA extract (1.0 ml) was mixed with 2.0 ml of diphenylamine reagent and incubated at 37°C for 18 h. The resultant coloration was measured at 600 nm.

**Human MMP-3 cDNA** XbaI and XhoI fragment, excised from the cDNA of the human MMP-3 mRNA, was inserted in pBluescript KS, giving a plasmid (phMMP-3) containing a 1434-bp open reading frame from human MMP-3. PhMMP-3 was then cut with XhoI or XbaI to generate a sense or an antisense probe for in-situ hybridization, respectively. The homology of nucleotide sequence between rabbit and human MMP-3 is 84.2% (DDBL/EMBL GeneBank database: X05232 for human MMP-3 and M25664 for rabbit MMP-3, respectively), and thus the human probe should be suitable to in-situ hybridize with rabbit MMP-3 mRNA.

**In-Situ Hybridization** This was carried out following the method described by Nomura et al. Rabbit uterine cervixes were fixed in 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight, and the tissues were dehydrated and embedded in low-melting-point paraffin wax. Sections (5 μm) of the tissue were mounted on silicone-coated glass slides and dried at 37°C. After removal of the wax with alcohol, the sections were treated with 20 mg/ml of proteinase K, postfixed in 4% (v/v) paraformaldehyde in PBS, treated with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine, and dehydrated again. Digoxigenin (DIG)-labeled, single-stranded sense and antisense RNA probes were synthesized by T3 or T7 RNA polymerase after linearization of the human MMP-3/stromelysin 1 with XhoI or with XbaI (DIG-RNA labeling kit SP6/T7, Roche Diagnostics, Tokyo, Japan). Then the RNA probe was treated with an alkaline solution containing 40 mM NaHCO3 and 60 mM Na2CO3 at pH 10.2 and 60°C for 55 min. This treatment resulted in fragmentation of the probe into 100—200-bp sections, which were added to the hybridization solution [50% (v/v) formamide, 10 mM Tris–HCl (pH 7.6), 1 mM ethylenediaminetetraacetic acid, 600 mM NaCl, 1X Denhardt’s solution, 0.25% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) dextran sulfate, 200 mg/ml of yeast tRNA] at a final concentration of 1 mg/ml. After hybridization at 50°C overnight, a washing procedure including RNAase treatment (20 mg/ml) was performed. After incubation with 100 mM Tris–HCl (pH 7.5) and 150 mM NaCl (DIG buffer), the sections were incubated with DIG buffer containing alkaline phosphatase-conjugated anti-DIG antibody and blocking reagent, and then were further incubated with 100 mM Tris–HCl (pH 9.5)/100 mM NaCl/50 mM MgCl2/0.28 mM nitro blue tetrazolium/0.7 mM 5-bromo-4-chloro-3-indolyl phosphate to indirectly visualize MMP-3 mRNA.

## RESULTS

**Effect of Onapristone on the DNA Concentration and Wet Weight of Pregnant Uterine Cervix** When onapristone was intravenously injected into pregnant rabbits at 20 d gestation, the wet weight of the uterine cervix at 24 h after the injection in the antiprogestosterone-treated group was significantly higher than that in the untreated (p<0.01) as shown in Table 1. The DNA concentration in the uterine cervix was also lowered by the onapristone treatment. It is well recognized that an increase in wet weight of the uterine cervix and a decrease in DNA concentration are observed in dilated rat uterine cervix. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of rabbits</th>
<th>Wet weight (g/cervix)</th>
<th>DNA concentration (μg/100 mg of wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>1.00±0.18</td>
<td>173.3±12.0</td>
</tr>
<tr>
<td>Onapristone</td>
<td>3</td>
<td>1.79±0.22**</td>
<td>146.1±29.7</td>
</tr>
</tbody>
</table>

Onapristone (3 mg/kg of body weight) in 50% (v/v) ethanol was intravenously administered to pregnant rabbits at day 20 post coitum. Control rabbits were injected with the same volume of vehicle alone. After 24 h, the uterine cervix was removed and its wet weight and DNA concentration were determined as described in the text. **, Significantly different from the control (p<0.01).

### DISCUSSION

Since synthetic anti-progesterones are known to exert anti-glucocorticoid activity, it is not clear whether the acceleration of uterine cervical ripening by onapristone re-
ported here results predominantly from its anti-progestin ac-
tivity. In this point of view, the observations that the anti-glu-
cocorticoid activity of onapristone is substantially lower than
that of mifepristone,12) and a pure progesterone agonist,
promegesterone/R5020, fully reversed the accelerative effects
of onapristone on the extensibility and inner circumference
of guinea pig uterine cervix19) are of interest and do eliminate
the above possibility.

The reason why onapristone primarily augmented the ex-
pression of MMP-3 mRNA in capillary vessels of the uterine
cervix is not clearly understood at present. It may be as-
sumed that onapristone disrupts the actions of progesterone
on the cervical stroma and/or epithelium and then the epithe-
lial and/or stromal cells influence blood-vessel MMP-3 ex-
pression. Otherwise, intravenous administration of onapris-
tone directly antagonizes the action of progesterone in blood
vessels since the presence of progesterone receptor in rabbit
aorta25) and rat vascular tissue26) has been reported. MMPs in
blood vessels very likely participate in tissue angiogenesis
including tumor tissue.27) The increase in vascularity and in-

Fig. 1. Effect of Progesterone Receptor Antagonist, Onapristone, on the Expression of MMP-3 mRNA in Pregnant Rabbit Uterine Cervix

Onapristone (3 mg/kg of body weight) or vehicle was intravenously administered to pregnant rabbits on day 20 post coitum, as described in the text. After 24 h, the uterine cervi-
cices were removed and fixed in 4% (v/v) formaldehyde for 48 h, and then embedded in paraffin wax. Tissue slices were subjected to in-situ hybridization using a digoxigenin-la-
beled antisense probe for human MMP-3 as described in the text. Panel [V-3], vehicle-treated control rabbit and [ZK-3], onapristone-treated rabbit. The original magnification is
×40, and the black bar indicates 50 μm. The white bars indicate stromal tissue layer. Three independent experiments using different pregnant rabbits were highly reproducible, and
typical data are shown. MMP-3 mRNA was predominantly observed in the stroma of the uterine cervix from the onapristone-treated rabbits. EP, epithelium; EG, endocervical
gland and BV, blood vessel.

Fig. 2. Onapristone Predominantly Increased the MMP-3 mRNA in Endothelial Cells and/or Perithelial Cells of Capillary Vessels in Pregnant Rabbit Uter-
ine Cervix

Except for the animals used, the experimental conditions were the same as in Fig. 1, and the stromal tissue in uterine cervix of onapristone treated rabbit was examined by the an-
tisense [AS] and sense [S] probes for MMP-3 at higher magnification (×200). The bar indicates 300 μm. MMP-3 mRNA was mainly located in endothelial cells, stromal cells
and/or pericytes, and the sense probe did not show any significant signal. BV, blood vessel; EP, epithelial cell and FB with arrow head, fibroblastic cell.
filtration of polymorphonuclear leukocytes are frequently observed in the ripened and dilated uterine cervix. Further studies are needed to clarify the primary expression of MMP-3 in endothelial cells and/or pericytes of blood vessels of onapristone-treated rabbit uterine cervix. Many in-vitro observations indicated that progesterone is a key hormone for maintaining the functions of the reproductive tract via the suppression of many biological activities, i.e., progesterone suppresses the production and gene expression of proMMPs including proMMPs-1 and -3, proMMP-9 and a chemotactic factor of IL-8, and up-regulates the TIMPs-1 and -2 production in cultured rabbit uterine cervical cells. Moreover, we have recently confirmed that the production of membrane type (MT)1-MMP, which degrades many components of the extracellular matrix and effectively activates progelatinase A/proMMP-2 is also down-regulated by progesterone (K. Imada and A. Ito, unpublished data).

In conclusion, these results indicate that our previous in-vitro observations with progesterone were highly likely to appear in our in-vivo experiments, and that progesterone closely participates in maintaining the function of the reproductive tract at early and middle pregnancy by suppressing the production of MMPs along with up-regulation of TIMPs. Therefore, these findings do support the theory that antiprogesterone has a potential to accelerate uterine cervical ripening and dilatation.

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REFERENCES

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