Effects of RU486 on the Interstitial Collagenase in the Process of Cervical Ripening in the Pregnant Rat

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Abstract. Changes in interstitial collagenase activity in the rat uterine cervix during ripening were clarified in a time-dependent manner. Premature delivery was induced by an antiprogesterone agent, RU486, for rats in late pregnancy. The presence of interstitial collagenase in the extract from the rat cervical tissue was demonstrated, by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using the natural and unaffected collagen as a substrate. The collagenase activity was determined as the release of digested peptides from the radio-labeled collagen. Our experiments with RU486 were performed in rats on the 18th day of pregnancy. A single administration of RU486 (15 mg/kg) resulted in the premature delivery of all treated rats within 30 h after the injection (average time was 23.9 h). The marked increase in cervical wet weight was observed up to the time to premature delivery along with a significant acceleration from 18 h after the administration of RU486. In this state, the cervical collagenase activity was enhanced, the highest levels being recorded at 21 h after the administration. The interstitial collagenase in the uterine cervix appears to play a significant role in the regulation mechanisms of cervical ripening in late pregnant rats.

Key words: Interstitial collagenase, Cervical ripening, Premature delivery, Rat.

(*Endocrinol Japon* 38: 491–496, 1991)

THE UTERINE cervix in most species of animals is a firm and compact fibrous structure in the non-pregnant state. During pregnancy, the cervix tends to soften as a result of the morphologic and biochemical changes in the extracellular framework [1]. At term, with the appearance of some potent signals as triggers of the onset of labor and uterine contractions, progressive remodeling takes place rapidly in the uterine cervix.

There are numerous reports to date showing the effects of sex steroids [2, 3], prostaglandins [4, 5], relaxin [6] and other factors [7] on the mechanisms of cervical softening and dilatation

being induced with drastic physical and/or biochemical changes in the extracellular structure [8, 9]. On the other hand, there is evidence of a proteinase cascade on the mammalian collagenase as a key modulator of the degradation of connective tissue components in various conditions [10, 11].

Although a number of previous studies have shown that collagen breakdown in the cervical connective tissue plays a principal role in the process of cervical ripening and dilatation [12, 13], chronological observation has not yet been performed on the changes in cervical collagenase actively all through the period of cervical ripening and dilatation. Moreover, the existence of a true collagenase, which is able to cleave interstitial collagens into three quarter and one quarter fragments, was only recently demonstrated for the first time in the direct extract from the human

Received: February 26, 1991

Accepted: August 20, 1991

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cervical tissue during parturition [14].

The purpose of this study, therefore, is to show the presence of interstitial collagenase in the uterine cervix and to clarify the changes in the collagenase activity all through the progress of planned premature delivery in late pregnant rats. To induce premature labor, we used the antiprogesterone compound, RU486, the synthetic 19-norsteroid which produces pronounced contraceptive and abortive effects in rodents.

Materials and Methods

Preparation of the dilated cervixes

Virgin Wistar rats (9–12 w of age), weighing 190–230 g, were kept under a daily 12-h light schedule (07:00–19:00 h) with free access to food and water in a room at a constant temperature. Females and males were placed together on the night of proestrus. On the morning of Day 18 of pregnancy (Day 1 was defined as the day on which sperm was found in the vaginal smear), the rats were injected subcutaneously with either 70% ethanol vehicle or RU486 (17-hydroxy-11-[4-dimethylaminophenyl]-17 [1-propynyl]oestra-4,9-dien-3-one: Mifepristone: Roussel-Uclaf, Paris, France), (15 mg/kg) dissolved in vehicle. The time of premature delivery after RU486 administration was monitored. The onset of premature delivery was defined as the finding of the first pup in the vaginal inlet. RU486 of 15 mg/kg induced premature delivery 18 to 30 h after the injection (Fig. 1). However, a lesser amount (10 mg/kg) resulted in delivery about 30 h afterwards and a larger amount (30 mg/kg) yielded results similar to that of 15 mg/kg. Therefore, 15 mg/kg was exclusively used in the present experiment. The rats were sacrificed by dislocation of cervical vertebrae at 0, 8, 12, 16, 18, 21 h and intrapartum after RU486 treatment, and at 0, 8, 12, 16, 18, 21 and 24 h after vehicle injection. Because the average time from the RU administration to the onset of the premature delivery was 23.9 h, the control experiment was performed for 24 h. The cervixes were removed at the bifurcation of the uterine horns, weighed and then immediately frozen at -20°C in assay buffer (pH 7.5).

Extraction of collagenase

Collagenase was directly extracted from the

uterine cervix by the method of Kishi & Hayakawa [15] with a slight modification. The cervixes from 9 to 19 rats were homogenized in 4 vol. of 0.25 M sucrose. The homogenate was centrifuged at 9,000 g for 30 min at 4°C , and the supernatant was collected [sucrose fraction 1]. The pellet was resuspended in 4 vol. of urea buffer (4M urea in 50 mM Tris-HCl, pH 7.5) and centrifuged immediately at 9,000 g for 30 min at 4°C and the supernatant was again collected [urea fraction 1]. The urea extraction was then repeated twice in the same manner except for the difference in the length of the extraction period, that is 2 h and overnight, respectively [urea fractions 2 and 3]. Each fraction was dialyzed overnight against 3M KSCN in 40 mM Tris-HCl, containing 10 mM CaCl_2 , pH 7.6 and subsequently dialyzed overnight against the assay buffer (50 mM Tris-HCl, 5 mM CaCl_2 , 0.2 M NaCl and 0.02% NaN_3 , pH 7.5). The dialysate was centrifuged at 10,000 g for 30 min at 4°C to remove insoluble materials. Each supernatant was assayed individually for collagenase activity.

Collagenase assay

In vitro-labeled rat skin acid soluble type I collagen was prepared as the substrate according to the method of Cawston & Barrett [16] with [$1\text{-}^{14}\text{C}$] acetic anhydride (sp. act. 10mCi/mmol; ICN Radiochemicals, Irvine, CALIF, USA). Collagenase activity was measured by the method of Terato *et al.* [17] and was determined as the release of ^{14}C -labeled peptide from the substrate in the solution (7.0×10^4 cpm/200 μg labeled collagen/tube) after incubating for 24 h at 30°C . Activation of latent collagenase with trypsin (Sigma Chem. Co., St Louis, MO, USA) (50 $\mu\text{g}/\text{ml}$) was performed at 37°C for 10 min and then the mixture was treated with an excess of soybean trypsin inhibitor (Sigma Chem. CO., St Louis, MO, USA) (200 $\mu\text{g}/\text{ml}$; one mg inhibits approximately 1.8 mg trypsin) for 20 min at room temperature. One unit of collagenase was defined as the activity by which 1 $\mu\text{g}/\text{min}$ of *in vitro*-labeled collagen was hydrolyzed at 30°C . In this study, collagenase activity was expressed as the sum of active, latent and total collagenase activity in each four fraction, and was described as the absolute activity per whole cervix.

Electrophoresis

To assess the collagen fragments digested by the

collagenase, the enzyme solutions collected from each fraction were taken to 60% saturation with ammonium sulfate and fractionated on Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (1.6 × 45 cm) equilibrated and eluted with an assay buffer at a flow rate of 4 ml/h. Partially purified enzyme fractions were combined and concentrated by ultrafiltration (Collodion Bags) threefold to fourfold. Intact collagen (600 µg) was digested for several days at 24°C with this concentrated sample. Subsequently the reaction mixtures were subjected to the SDS-polyacrylamide disc gel electrophoresis system of Hayashi *et al.* [18] with 7.5% acrylamide gels, and staining was performed with 0.25% Coomassie Brilliant blue.

Statistical analysis

Results are shown as the mean ± SD. Statistical comparisons were made by an unpaired Student's *t*-test.

Results

Antiprogesterone treatment of late pregnant rats

Figure 1 shows the distribution of the number of rats initiating premature delivery after RU486 treatment on Day 18 of pregnancy.

Premature delivery was induced in all rats from 18 to 30 h after the injection of RU486 (15 mg/kg s.c.). The average time up to onset after the treatment was 23.9 h. Between 21 and 24 h, the highest number of rats went into premature delivery (40%), and thereafter the number decreased. In our preliminary studies, the lesser amount of RU486 (10 mg/kg) had a tendency to delay initiation of premature delivery, which was induced in about 30 h, and the larger amount (30 mg/kg) provoked delivery within a similar time interval to that with 15 mg/kg (data not shown).

Figure 2 demonstrates the time-dependent changes in cervical wet weight in the pregnant rats after RU486 or vehicle treatment.

The wet weight of the cervix on Day 18 of pregnancy increased greatly in comparison with that of the nonpregnant cervix ($p < 0.005$). There were no significant changes in the control group at 24 h after vehicle injection. In the rats treated with RU486, however, the cervical wet weight increased in a time dependent fashion, especially with a marked acceleration from 18 h after the adminis-

Fig. 1. The distribution of the number of rats corresponding to the onset time of premature delivery after a single subcutaneous injection of RU486 (15 mg/kg) on Day 18 of pregnancy.

Fig. 2. Changes in wet weight of cervix in the control (open circle) and RU486-treated (closed circle) groups. Uterine cervixes were collected at intervals as described in "Materials and Methods". Open and closed squares indicate the cervical wet weight of nonpregnant and spontaneous delivery groups, respectively. *: $p < 0.05$, **: $p < 0.005$ compared with the corresponding groups.

Fig. 3. The time course of interstitial collagenase activity in the process of cervical ripening directly demonstrated from the rat uterine cervix after RU486 treatment on Day 18 of pregnancy. In each group at 0, 12, 21 h and intrapartum after the treatment, the experiment for induction of premature labor was repeated three or four times using the numbers of rats indicated in the figure. The bars represent the mean $\pm$ SD. a, $p < 0.01$; b, $p < 0.005$; c, $p < 0.05$; d, $p < 0.01$ compared with the corresponding groups.

tration, and was about 1.8-fold at the time of premature delivery. The wet weight of the cervix at the time of spontaneous delivery was similar to that of premature delivery.

Identification of interstitial collagenase in the mechanisms of cervical ripening

Figure 3 shows the chronological change in interstitial collagenase activity from the rat uterine cervix in relation to the induction of premature delivery.

Total collagenase activity in the rat uterine cervix on Day 18 of pregnancy was 1.5 times as high as that in the non-pregnant cervix. Twenty-one hours after the injection of RU486, both active and total collagenase activities reached their maximum values, i.e., 4.6- & 3.2-fold potent compared with those just prior to the treatment ($p < 0.01$ and

$p < 0.005$). There are two peaks in the active form of collagenase at 8 and 21 h after the injection: the activity of the active form decreased from 8 to 16 h, thereafter markedly increased to the highest level at 21 h. Both active and total collagenase activities at the time of premature delivery were seen to be lower than those at 21 h (not significant). There were no remarkable changes in the control group at 24 h after vehicle injection.

EDTA and 1,10-phenanthroline completely blocked this enzyme activity at the concentrations of 10 mM and 1 mM. That is, in the treated group, the activities decreased to 2.3% and 0%, respectively. Thus, the collagenolytic activity may belong to one of the metalloproteinases. Figure 4 shows the reaction products from enzymatic hydrolysis of collagen after incubation of collagen and the enzyme sample directly extracted from the rat

Fig. 4. SDS-polyacrylamide disc gel electrophoresis patterns of the digestion products of intact collagen molecules. The digestion of collagen and electrophoresis is described in "Materials and Methods". The major bands of collagen are β (dimers), and α (monomers). The typical digestion fragments are labelled with an A superscript. **A**, control, rat skin acid soluble type I collagen; **B**, enzyme alone; **C**, type I collagen plus the enzyme sample. E shows protein bands derived from the enzyme sample. F denotes a buffer front.

uterine cervix after RU486 treatment. The appearance of digested fragments (α^A and β^A) indicates the presence of interstitial collagenase in the enzyme sample.

Discussion

The present study showed that 15 mg/kg of RU486, a strong progesterone receptor antagonist, induced premature delivery within 30 h after the injection in all experimental rats (9–12 w of age) on Day 18 of pregnancy. We demonstrated, for the first time, the presence of interstitial collagenase from the rat cervical tissue by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of breakdown products of the substrate collagen. Moreover, for the first time, we chronologically observed the changes in collagenase activity in the rat cervix during its ripening.

It has been suggested that changes in the estrogen level, an increase in the estrogen-progesterone ratio or the appearance of other unknown factors in the cervix during pregnancy would trigger progressive cervical ripening and dilatation at term [19]. In the present study, the wet weight of the rat cervix increased rapidly from 18 h after RU486 treatment and was about 1.8-fold at the time of premature delivery (Fig. 2). With the changes in its wet weight, the interstitial collagenase activity in the cervix varied, recording the highest levels at 21 h after the treatment, shortly before the premature delivery. This finding appears to be compatible with the observation of Downing & Sherwood [20] which showed a rapid breakdown of collagen in the rat uterine cervix shortly before term parturition on Day 22 of pregnancy. Several conditions in the uterine cervix, such as changes in its wet weight, the levels of some sex hormones and the activity of the interstitial collagenase, are possibly important in rapid and sufficient dilatation of the softened cervix for the passage of the conceptus on the initiation of premature delivery.

In cultures of post-partum rat uterus, the collagenase activity is inhibited by progesterone [22]. It has been shown that the action of RU486 is mediated through the progesterone receptor on preterm birth in the rat [23]. Accordingly, in our study it was speculated that the collagenase activity was expressed through the antiprogesterone effect of RU486 at the receptor level. Estrogens [3] and prostaglandins [21] are also involved in the mechanism of cervical dilatation, as possible factors in regulating the collagenase activity. However, we must further investigate the action and correlation of the possible factors which must have an effect on the control of the collagenase activity in the process of cervical ripening in order to fully understand this phenomenon.

Summing up the findings of the present study, it has been shown that interstitial collagenase plays an important role in the cervical ripening mechanism on the induction of premature delivery with RU486 in pregnant rats. Further investigations designed to assess the nature of other factors which are involved in collagen metabolism, such as tissue inhibitor of metalloproteinase [24, 25] and plasminogen activator, will provide more information on the mechanism of cervical ripening in pregnant rats.

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