Gonadotropin-releasing hormone agonist has the ability to induce increased matrix metalloproteinase (MMP)-2 and membrane type 1-MMP expression in corpora lutea, and structural luteolysis in rats

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Abstract

Gonadotropin-releasing hormone (GnRH) and its agonist analog (GnRHa) are well known to have luteolytic effects. We previously reported that prolactin (PRL) stimulated matrix metalloproteinase (MMP)-2 activity to degrade collagen type IV as a mechanism of structural luteolysis. The effects of GnRHa treatment on developed corpora lutea are unknown. In this study we assessed the effect of GnRH on MMP expression and induction of structural involution of developed corpora lutea of superovulated rats using GnRHa.

Pregnant mare serum gonadotropin–human chorionic gonadotropin (hCG)-synchronized ovulation and luteinization were induced in immature female rats, followed by daily treatment with GnRHa from 5 days after hCG treatment. GnRHa-induced involution of corpora lutea was evident 3 days after the treatment, as shown by their markedly smaller size (60% of the control weight). Nine days after hCG injection, serum progesterone and 20α-dihydroprogesterone concentrations were as low as those associated with structural luteolysis. These findings revealed that GnRHa has the ability to induce structural luteolysis in superovulated rats in the same way that PRL does. To gain information on mechanisms of luteal involution induced by GnRHa, we performed gelatin zymography. This showed a significant increase in the active form of MMP-2 in the luteal extract of GnRHa-treated rats (more than twofold that of the control). Activation of pro-MMP-2 by membrane type-MMP (MT-MMP) is reported to be a rate-limiting step for catalytic function. Another function of MT-MMP is to degrade collagen types I and III. The plasma membrane fraction of corpora lutea of GnRHa-treated rats activated pro-MMP-2 of fetal calf serum, resulting in a marked shift of the 68-kDa band to the 62-kDa band in the zymogram. A Northern hybridization study also revealed simultaneous significant increases in expression of MMP-2 mRNA and MT1-MMP mRNA in corpora lutea of GnRHa-treated rats (more than threefold the control level).

In summary, hormonal and histological features of corpora lutea of GnRHa-treated superovulated rats correspond to those of structural luteolysis. GnRHa stimulated the expression of MMP-2 and MT1-MMP in developed corpora lutea associated with involution. These findings support the conclusion that MMP-2, activated by MT1-MMP, and MT1-MMP itself, remodel the extracellular matrix during structural luteolysis induced by GnRHa.

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Introduction

Gonadotropin-releasing hormone (GnRH) and its agonist analog (GnRHa) exert extrapituitary effects in several species. Chronic treatment with high doses of GnRH or GnRHa can initially cause a transient increase in serum gonadotropin, but leads to a significant reduction in serum luteinizing hormone (LH) concentrations thereafter (Sharpe *et al.* 1979). It has also been reported that GnRHa blocks the luteinization process of superovulated rats (Harwood *et al.* 1980*b*). However, it is not clear what the effects of GnRHa are on developed corpora lutea of superovulated rats.

Structural luteolysis is defined as involution of the corpus luteum, which is characterized grossly as a loss of tissue. There is sound evidence in species such as the rat that structural luteolysis is an event distinctly separate from functional luteolysis (Malven 1969). We previously reported the co-ordinated induction and activation of metalloproteinase and ascorbate depletion in prolactin (PRL)-induced structural luteolysis in rats. During this structural luteolysis in rats, matrix metalloproteinase-2

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(MMP-2) degrades type IV collagen, remodeling the extracellular matrix (Endo *et al.* 1993). One of us, with others, reported that membrane-type (MT)-MMP had the ability to activate pro-MMP (Sato *et al.* 1994), and MT1-MMP has also been reported to degrade collagens type I and III (d'Ortho *et al.* 1997). It is not known if any hormones other than PRL induce structural involution of rat corpus luteum, nor is the mechanism of processing of pro-MMP-2 in rat corpus luteum understood.

In this study, an attempt was made to determine whether GnRH can induce structural involution in rat corpora lutea using GnRHa, and to assess the relationship between GnRHa treatment and MMP activity as its mechanisms. We also attempted, using excess human chorionic gonadotropin (hCG) or excess ovine prolactin (PRL), to assess if the effects of GnRHa were caused by alterations in the serum concentrations of LH or PRL.

Materials and Methods

Hormones and drugs

Pregnant mare serum gonadotropin (PMSG) and hCG were obtained from Teikoku Hormone Manufactory Co. (Tokyo, Japan). The GnRH agonist analog, Leuprolide ((D-Leu^6,des-Gly^{10} ethylamide)GnRH), was a gift from Takeda Chemical Industries, Ltd (Osaka, Japan). Ovine prolactin, 20α-dihydroprogesterone (20a-DHP), 1,10-phenanthroline and *p*-aminophenylmercuric acetate (APMA) and bromocryptine mesylate (bromocryptine) were purchased from Sigma Chemical Co. (St Louis, MO, USA). ^{3}H-Labeled 20a-DHP was purchased from Dupont/NEN Research Products (Boston, MA, USA). Fetal calf serum (FCS) was purchased from Gibco (Grand Island, NY, USA).

Animal treatments and tissue collection

Immature (25-day-old) Sprague–Dawley female rats were purchased from Hokudo Co. (Sapporo, Japan). Rats were superovulated by injection of 50 IU PMSG followed by injection of 25 IU hCG 56 h later. GnRHa (Leuprolide) was injected from day 5 to day 9 (day 0: injection of hCG) twice a day (0900 and 1800 h), in a dose of 100 μg/kg/day (in saline). PRL-induced structural luteolysis was achieved by a 3-day PRL treatment (1 mg/day) following a 3-day bromocryptine treatment (400 μg/day) from 4 days after hCG injection in superovulated rats (Endo *et al.* 1993). Rats were usually killed by decapitation in the evening of day 9. The ovaries were dissected, trimmed, weighed, flash-frozen on dry ice, and stored at −80 °C. Some ovaries were stained with hematoxylin and eosin. To obtain time-course data, five rats were killed on days 5, 6, 8 and 9 after hCG injection. During the first day (5 days after hCG), blood samples were collected 0, 1, 4, and 8 h after GnRHa injection (1100 h). To investigate if the luteolytic effects of GnRHa were due to alterations in the serum LH concentration, five rats were injected with 10 IU hCG in saline in the morning on days 5, 7 and 9, with daily injection of GnRHa, and five received 10 IU hCG without GnRHa. Five rats were also given 1 mg ovine PRL in saline from day 5 to day 9 with GnRHa and five received it without GnRHa, to examine whether the PRL surge corresponded to the luteolytic effects of GnRHa. Gonadotropin-primed immature rats were hypophysectomized on day 5; five animals were injected with GnRHa and five did not receive it (day 5 to day 9). After the surgery, serum PRL and LH were undetectable; serum LH was undetectable in the hypophysectomized rats even after GnRHa stimulation. Control rats were injected with the vehicle (saline) only. All rats were killed on day 9, except those used in the time-course study.

Progestins, LH and PRL assays

Serum progesterone and 20a-DHP concentrations were determined by RIA. Progesterone was assayed using Progesterone RIA kit from DPC Co. (Los Angeles, CA, USA) and 20a-DHP was assayed as described previously (Endo *et al.* 1993). Anti 20a-DHP antibody was a kind gift to us by Dr Michio Takahashi (Tokyo University). Serum rat LH and prolactin concentrations were assayed using RIA kits from Amersham (Amersham, Buckinghamshire, England).

Gelatin zymography and Western blot analysis

Ovaries were homogenized (100 mg wet weight/ml) in PBS (10 mM sodium phosphate and 150 mM sodium chloride, pH 7·8) containing 0·2% Triton X-100 with a Teflon glass tissue grinder on ice (15 strokes). Homogenates were centrifuged (12 000 ***g*** at 4 °C for 20 min) and the supernatant fractions were collected for protein assay and gelatin zymography as previously described (Endo *et al.* 1993). Luteal extracts (40 μg protein) were subjected to electrophoresis in 10% polyacrylamide gels containing 1 mg/ml gelatin. Samples were diluted in non-reducing sample buffer (final concentration, 1% SDS and 5% glycerol) and electrophoresed for 1–2 h at 10 mA. The gels were washed for 2 h in 2·5% Triton X-100 to remove SDS, rinsed three times with distilled water, and then incubated for 24 h in 50 mM Tris–HCl at pH 8·0 with 5 mM $CaCl_2$. After Coomassie blue staining, the bands were soon detected.

Luteal extract (20 μg protein) was separated by 12·5% SDS–PAGE under reducing conditions and transferred to nitrocellulose membranes using an electroblotting apparatus. Non-specific binding sites were blocked by

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immersing the membrane, overnight at room temperature, in PBS containing 0·05% Tween 20 (TPBS) and 5% skim milk, on an orbital shaker, and washed five times for 5 min in TPBS. Rabbit serum of anti-rat MMP-2 was obtained from Torrey Pines Biolabs (San Diego, CA, USA). The membrane was incubated with primary antibody (1:1000 diluted with PBS–BSA) for 45 min at room temperature in a humidfied chamber and washed five times for 5 min in TPBS. The membrane was then incubated with horse-radish peroxidase (HRP)-conjugated anti-rabbit IgG diluted 1:1000 in PBS–BSA, followed by addition of streptavidin–HRP conjugate at 1:1500 dilution for 45 min at room temperature in a humidfied chamber, and washed five times for 5 min in TPBS. The membrane was then incubated with Amersham enhanced chemiluminescence reagent for 1 min and exposed to X-ray film for 15 s in a dark room. The density of bands was measured by NIH Image (Version 1·61).

Activity of MMP-2 processing by membrane fraction

The mechanism of pro-MMP-2 activation under physiological conditions has been unclear. It has been demonstrated that the plasma membrane fraction prepared from the cells can activate pro-MMP-2, and that the reaction is sensitive to chelating reagents and tissue inhibitor of metalloproteinase-2 (Ward *et al.* 1991, Strongin *et al.* 1993).

Isolation of the crude luteal membrane fraction was carried out as reported previously (Luborsky *et al.* 1984). To obtain sufficient tissue to extract the membrane fraction, two to four ovaries, depending on ovarian size, were homogenized in 2 ml Tris buffer containing 0·25 M sucrose, using a Teflon glass tissue grinder at 400 r.p.m. on ice. Homogenates were filtered through nylon mesh (42 µm) supported by a funnel into a centrifuge tube and centrifuged at 120 ***g*** for 10 min. The supernatant fraction was centrifuged (10 000 ***g*** at 4 °C for 30 min) and pellets were resuspended in 500 µl 0·25 M sucrose–Tris buffer and stored at − 80 °C until required for use.

We examined the activity of MMP-2 processing of this fraction as described before (Cao *et al.* 1995). Crude luteal membrane fractions (20 µg) were mixed with 1 µl of FCS as a source of pro-MMP-2, and incubated for 2 h at 37 °C. The reaction was terminated by the addition of sample buffer, and the mixture was analyzed by gelatin zymography.

Northern blot hybridization

Total RNA (10 µg) of corpus luteum was extracted by the guanidine–phenol method using Ultraspec RNA from Biotex Laboratories, Inc. (Houston, Texas, USA). RNA was electrophoresed on 1% agarose–formaldehyde gel (100 V for 2 h) and transferred onto nylon membranes (Nytran-Plus; Schleicher & Schuell, Keene, NH, USA) in 20 × SSC (3 M sodium chloride, 0·3 M trisodium citrate) overnight, and then fixed with a u.v. linker. Filters were prehybridized for 4 h and then hybridized overnight at 42 °C with a radiolabeled cDNA probe. Human cloned MMP-2 and MT1-MMP cDNA probes (Sato *et al.* 1992, 1994) were radiolabeled with [^{32}P]dCPT from Amersham using Prime-It II random primer labeling kits from Stratagen (La Jolla, CA, USA). Human MMP-2 and MT1-MMP sequences show more than 85% homology with rat MMP-2 and MT-MMP sequences, respectively (Marti *et al.* 1993, Okada *et al.* 1995). Filters were washed in 2 × SSC containing 0·1% SDS at room temperature twice for 15 min, and twice with 0·2 × SSC containing 0·1% SDS at 65 °C for 15 min. Filters were exposed to Fuji RX X-ray film at − 70 °C for 1–2 days. For quantitative analysis, the radioactivity of the specific mRNA band was measured with a BAS 2000 Bio-Imaging Analyzer (Fujix, Tokyo, Japan). Radioactivity was adjusted to that of L38 (ribosomal protein) RNA.

Statistics

Treatment effects were evaluated by one-way ANOVA, followed by Scheffe's F test *post hoc* analysis, and by the unpaired Student's *t*-test. A difference of $P<0·05$ was considered significant.

Results

Effects of GnRHa on corpora lutea and serum progestins, LH and PRL concentrations

As shown in Table 1, luteal weight and serum progesterone of GnRHa-treated rats were significantly reduced compared with those of control rats at day 9 after hCG treatment. Ovaries were occupied with by a large amount of corpora lutea of superovulated rats. Histology revealed that the diameter of the corpora lutea decreased and stromal elements increased as did areas of vacuolization in the corpus luteum (Fig. 1), similar to PRL-induced structural luteolysis (Malven & Sawyer 1966). Time-dependent changes evoked by GnRHa are shown in Fig. 2*a*. Luteal weight was not reduced within 48 h of GnRHa treatment (day 7 after hCG), but was evident after 72 h (day 8). Near maximal reduction of tissue weight (60% of initial weight) was seen within 3–4 days of GnRHa treatment (day 8 and day 9). Reduction of the serum progesterone concentration began from 4 h to 8 h after GnRHa treatment started, and showed a progressive decline from 8 h after GnRHa treatment, throughout 3–4 days of treatment. In contrast, the serum 20a-DHP concentration of the rats peaked from 4 h to 8 h after GnRHa treatment started, and was as low as that of control rats from day 6 to day 9 (Fig. 2*a*).

The serum LH concentration was 1·38 ± 0·05 ng/ml in the morning of day 5. GnRHa injection caused an increase

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Table 1 Effects of hCG and PRL on ovarian weight and progestins of GnRHa-treated rats. Values are means ± S.E.M., with numbers of animals in parentheses. GnRHa was administered continuously from day 5 to day 9 after hCG treatment. Additional hCG was injected on days 5, 7 and 9 after PMSG–hCG treatment. PRL was injected continuously from day 5 to day 9 after hCG treatment (see Materials and Methods)

	Ovarian weight (mg)	**P4** (ng/ml)	**20a-DHP** (ng/ml)
Control	194·6 ± 10·3 (36)	755·6 ± 42·8 (20)	319·6 ± 52·3 (20)
GnRHa	125·8 ± 8·9** (28)	8·81 ± 0·86** (14)	288·4 ± 48·1 (14)
hCG/GnRHa	129·0 ± 9·7* (6)	10·9 ± 3·25* (6)	291·6 ± 20·9 (6)
hCG	218·8 ± 15·4 (6)	738·6 ± 51·0 (6)	258·2 ± 59·0 (6)
PRL/GnRHa	117·0 ± 5·9* (6)	12·5 ± 2·09* (6)	281·6 ± 37·1 (6)
PRL	201·8 ± 9·7 (6)	697·6 ± 28·3 (6)	309·0 ± 32·2 (6)

P4, progesterone. *P<0·05 compared with control, hCG and PRL groups; **P<0·01 compared with control rats.

in the serum LH concentration (42·2 ± 4·52 ng/ml) 1 h after the treatment, but the concentration returned to the initial value within several hours. Chronic treatment with GnRHa did not reduce the serum LH concentrations to less than the initial level in this study (Fig. 2*b*). The treatment seemed not to cause desensitization of the pituitary gland, although changes in bioactive LH concentrations were not determined. However, a study using GnRHa revealed that densensitization of the pituitary actually occurred because pituitary concentrations of LH and FSH were suppressed, and additional treatment with GnRHa did not stimulate gonadotropin release from the already stimulated pituitary (Sudo *et al.* 1990).

Serum PRL concentrations were slightly increased, from 15·6 ± 1·0 to 32·1 ± 6·4 ng/ml, 24 h after GnRH treatment. Serum PRL returned to the initial value 1 day later (Fig. 2*b*).

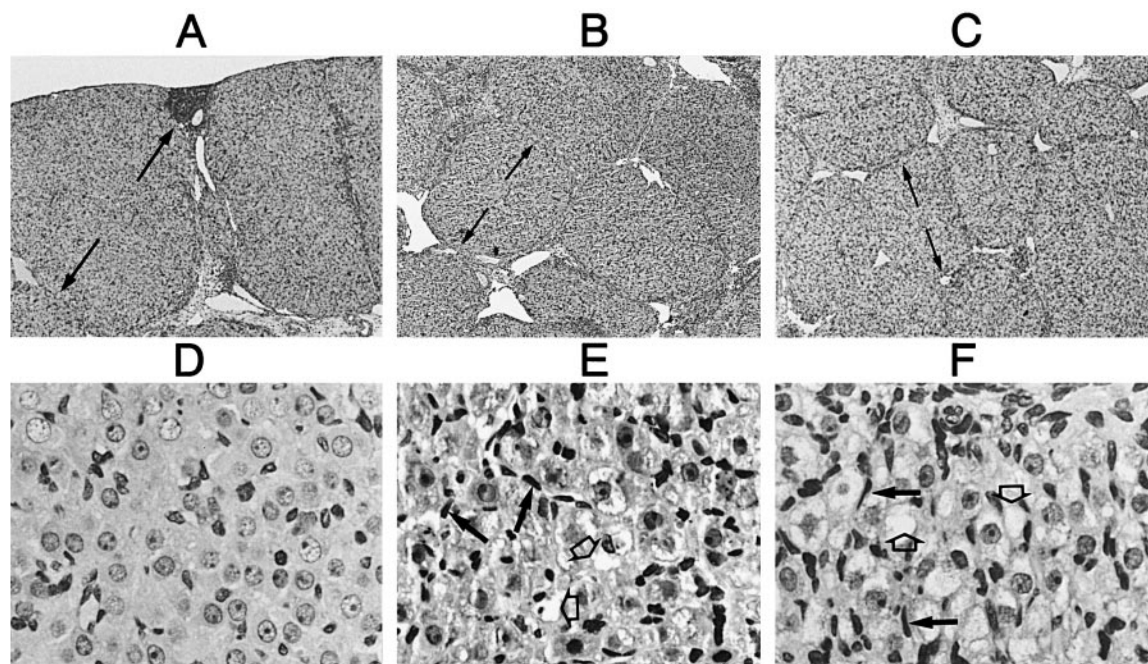

Figure 1 The histological features of sectioned ovaries of superovulated rats, stained with hematoxylin and eosin. For details of techniques, see Materials and Methods. (A) Low (× 40) and (D) high (× 200) magnification of an ovary of a superovulated rat with vehicle. (B) Low magnification of structural luteolysis of PRL-treated rat. (C) Low magnification (× 40) of an ovary of a GnRHa-treated rat. The diameters (←/→) of the corpora lutea of PRL- (B) or GnRHa- (C) treated rats are decreased compared with those of the control (A). (E, F) High magnification (× 200) of an ovary of prolactin- and GnRHa-treated rats, respectively. Stromal elements (thick arrows) are greatly increased, and many vacuoles (open arrows) are also present.

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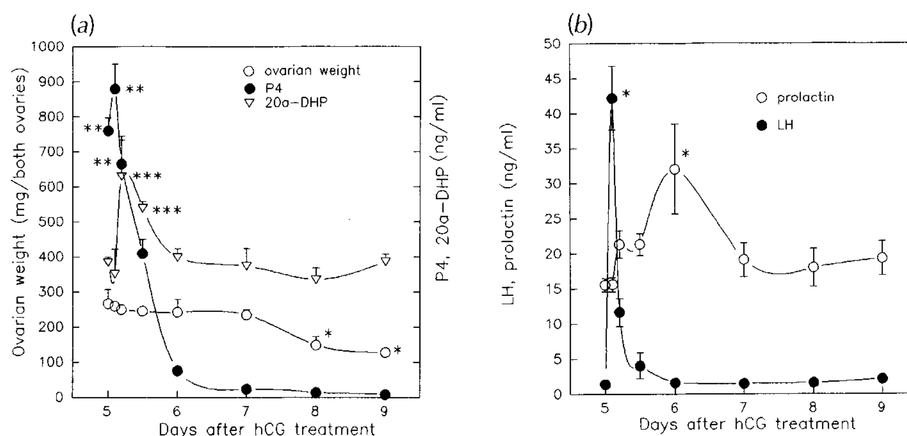

Figure 2 (*a*) Time-dependent effects of GnRHa on ovarian weight and progestins in superovulated rats. Values are the means ± S.E.M. (n=5 per group). P4, serum progesterone; 20a-DHP, 20α-dihydroprogesterone. GnRHa was given to superovulated rats from day 5 to day 9 after hCG injection (see Materials and Methods). *P<0·05 compared with total ovarian weight of other groups except * groups; **P<0·05 compared with serum progesterone of other groups except ** groups; ***P<0·05 compared with serum 20a-DHP of other groups except *** groups. (*b*) Time-dependent effects of GnRHa on serum LH and PRL in superovulated rats. Values are the means ± S.E.M. (n=5 per group). GnRHa was administered to superovulated rats from day 5 to day 9 after hCG injection (see Materials and Methods). *P<0·05 compared with serum PRL, LH of other groups except * groups.

Effects of GnRHa on MMP activity in extracts of luteal tissues

Gelatinase activity in extracts of luteal tissue was examined by gelatin zymography (Fig. 3*a*). The major gelatinase activity in luteal extracts was present in bands of around 68 kDa, with less activity at around 62 kDa on the zymograms. Treatment with EDTA or phenanthroline eliminated gelatinase activity in luteal extracts when assessed by zymography (data not shown). Pretreatment of luteal extracts with organomercurial APMA, a procedure known to activate latent collagenase, resulted in a marked shift of the 68-kDa band to the 62-kDa band. Although not all MMPs can be detected by zymography, we suspected that the major MMP in luteal extracts was MMP-2, present predominantly as the latent (68-kDa) form, with minor levels of activated MMP-2 (62 kDa). A marked increase in the activity of the activated 62-kDa form of MMP-2 was consistently seen (more than twofold the control level), along with an increase in the level of latent 68-kDa activity (more than 1·5-fold that of the control), which was confirmed in 12 separate experiments using GnRHa-treated rats (Fig. 3*a*). From these studies, we concluded that structural luteolysis induced by GnRHa was associated with increased levels of activated MMP-2, emphasizing matrix dissolution as an important event in the process of luteal involution. Time-course studies showed that MMP-2 activity was already stimulated, 1 day after GnRHa treatment (day 6 after hCG treatment) and the increase in MMP-2 activity continued from day 6 to day 9 (Fig. 3*c*). Western blot analysis also revealed that these two bands corresponded to the latent form (72 kDa) and active form of MMP-2 (67 kDa) and that GnRHa treatment stimulated expression of both the latent and the active forms of MMP-2, which was confirmed in three separate experiments (Fig. 3*b*). Gelatinase activity was also detected at around 97 kDa; there was no gelatinase activity at around 88 kDa. APMA treatment showed that this gelatinase activity corresponded to the latent form of MMP-9, as the 97-kDa band shifted to a band around 88 kDa after APMA treatment (Fig. 3*a*). Thus MMP-9 did not have an important role during structural involution in GnRHa-treated rats.

Effects of GnRHa treatment on pro-MMP-2 processing activity in corpus luteum

Plasma membrane fractions of corpora lutea were mixed with FCS as a source of procollagenase, as reported before (Cao *et al.* 1995). Figure 4 shows that the crude plasma membrane-dependent activation of the MMP-2 proenzyme resulted in the appearance of enzyme forms around 62 kDa. The crude plasma membrane fraction from corpus luteum of GnRHa-treated rats had activity sufficient to generate twice the amount of active forms of MMP-2 compared with the control samples, which was confirmed in five separate experiments. This amount of the fraction itself had no gelatinase activity on zymography (data not shown).

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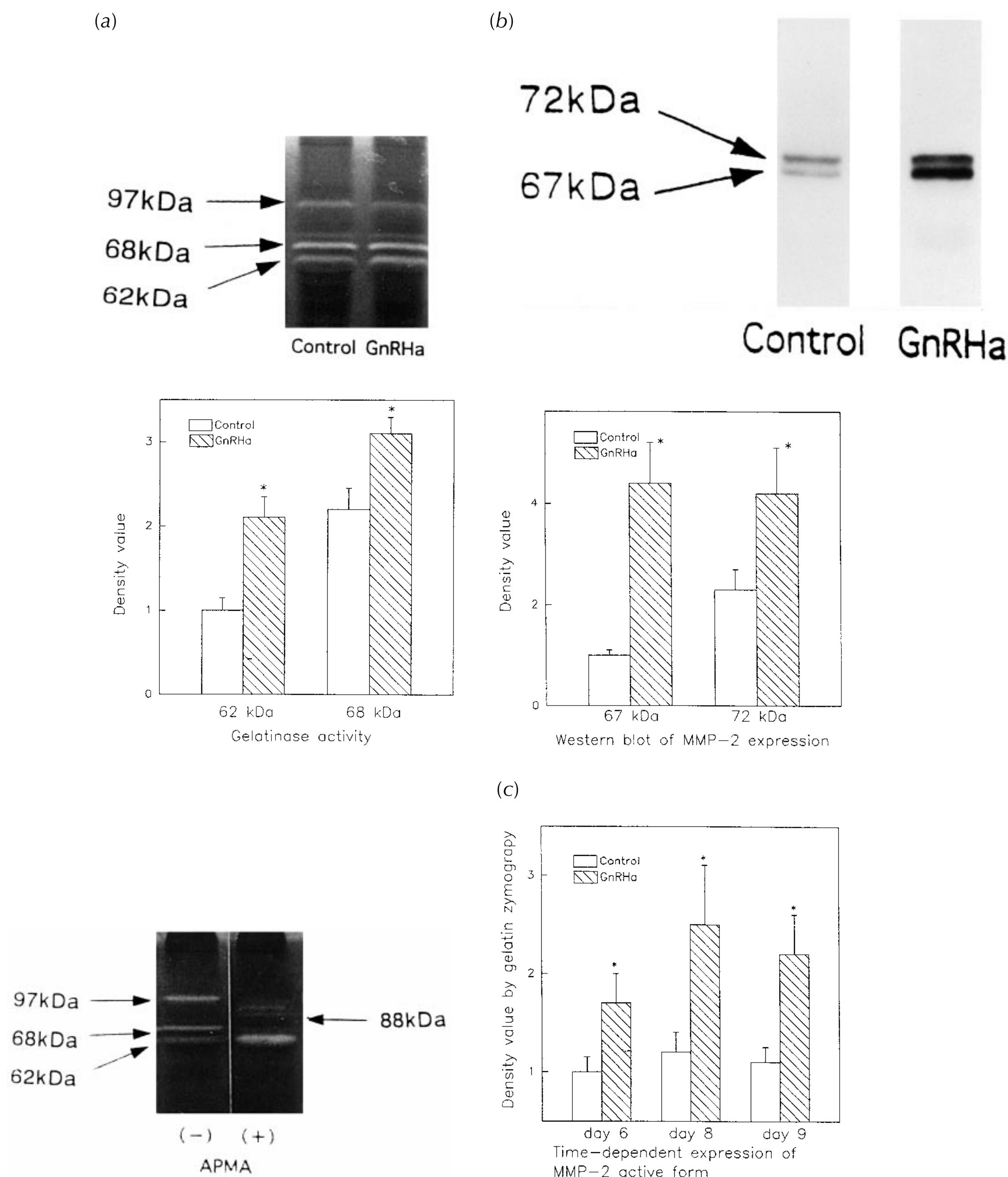

Figure 3 (*a*) Gelatinase activity of luteal extracts of superovulated rats. Top: Luteal extracts (40 µg protein) of control and GnRHa-treated rats were electrophoresed, and the gels were incubated with 5 mM $CaCl_2$ (see Materials and Methods). Middle: Band densities, shown as a bar graph. The density was normalized against the density of the 62-kDa band of the control group. *$P<0{\cdot}05$ compared with control group. Bottom: Aliquots (40 µg protein) of luteal extracts from GnRHa-treated rats were incubated in the absence or presence of APMA for 5 h and electrophoresed. The gels were incubated in the presence of 5 mM $CaCl_2$ (see Materials and Methods). (*b*) MMP-2 expression of luteal extracts of superovulated rats, by Western blot analysis (top). Luteal extracts (20 µg protein) of control and GnRHa-treated superovulated rats were separated by SDS–PAGE and transfered to nitrocellulose membrane. Expression of MMP-2 was determined by immunodetection using anti-rat MMP-2 antibody (see Materials and Methods). Bottom: Band densities, shown as a bar graph. The density was normalized against the density of the 67-kDa band of the control group. *$P<0{\cdot}05$ compared with control group. (*c*) Time-dependent gelatinase activity of luteal extracts in GnRHa-treated rats. Luteal extracts (40 µg protein) from control rats or GnRHa-treated rats were electrophoresed, and the gels were incubated with 5 mM $CaCl_2$ (see Materials and Methods). The density was normalized against the density of the 62-kDa band of the control group on day 6 after hCG injection. *$P<0{\cdot}05$ compared with respective control group.

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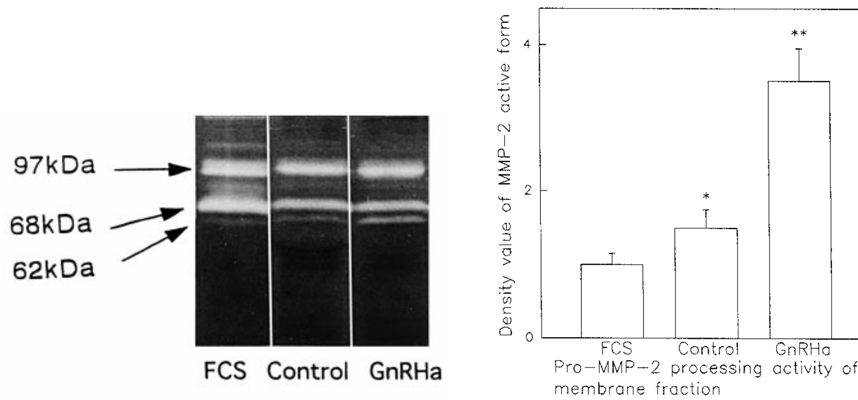

Figure 4 Pro-MMP-2 activation by the plasma membrane fraction of corpora lutea from superovulated rats. Left: FCS without or with membrane fractions of corpora lutea from control- and GnRHa-treated rats were incubated, and gelatinolytic activities were analyzed by gelatin zymography (see Materials and Methods). Right: Density of MMP-2 active form (62-kDa bands), shown as bar graphs. The density was normalized against the density of the 62-kDa band of the FCS-alone sample. *$P<0{\cdot}05$ compared with FCS alone; **$P<0{\cdot}01$ compared with FCS alone and control group.

Effects of the treatment with hCG or PRL on GnRHa-treated rats

Excess doses of hCG or PRL did not block the decrease in luteal weight of GnRHa-treated rats. GnRHa-treated rats still had low serum progesterone and 20a-DHP concentrations, which were characteristic of structural luteolysis, even after the additon of hCG or PRL (Table 1). These findings showed that the effects of GnRHa were not due to alterations in serum LH or PRL concentrations. Zymography showed that hCG or PRL treatment did not block the increases in MMP-2 activity and MT-MMP activity induced by GnRHa treatment (data not shown).

Northern blot hybridization of MMP

GnRHa treatment significantly stimulated MMP-2 (3·2 kb) and MT1-MMP (4·2 kb) mRNA expression in corpora lutea, to more than threefold the control levels, which was confirmed in four separate experiments (Fig. 5*a*). We also analyzed MMP mRNA expression of hypophysectomized rats. Expression of MMP-2 and MT1-MMP mRNA was increased in corpora lutea by GnRHa treatment, to more than threefold the control levels, which was confirmed in three separate experiments (Fig. 5*b*). These data revealed that GnRHa directly acted on corpus luteum, rather than via the hypothalamus.

Discussion

The present studies revealed that GnRHa simultaneously induced increased expression of MMP-2 and MT1-MMP in corpora lutea, which degraded collagens type IV, and types I and III, respectively, and remodeled the extracellular matrix, inducing structural luteolysis in superovulated rats.

The morphological characteristics of structural luteolysis include dissolution of the luteal capsule and invasion of the corpus luteum by stromal elements, which is most notable in the periphery of the gland (Malven & Sawyer 1966). The histological features of structural involution noted years ago are highly reminiscent of degeneration and remodeling of the extracellular matrix. In a previous paper, we suggested that loss of extracellular matrix is an early response to the action of PRL in the luteolytic process that appears to be mediated by increased expression and activation of MMP-2, which degrades collagen type IV (Endo *et al.* 1993). In the present study, we observed that the luteal extract of GnRHa-treated rats showed strikingly high activity of MMP-2 during structural involution, and that this was similar to PRL-induced structural luteolysis. Thus it is possible that the increase in MMP-2 activity is not specific to the action of PRL or GnRHa, but common to the process of structural luteolysis. It is possible that MMP-2 activation may cause remodeling in the corpus luteum, like the remodeling by MMP-2 during liver fibrosis in rats (Takahara *et al.* 1995), or it may assist stromal invasion of the corpus luteum. As GnRHa induced MMP-2 mRNA in corpora lutea of hypophysectomized rats, GnRHa appears to act directly on the corpus luteum via its GnRH receptor. An inactive precursor of pro-MMP-2 is secreted and activated in invasive tumor tissue as a result of proteolysis, which is mediated by a fraction of the tumor-cell membrane that is sensitive to metallo-proteinase inhibitors (Brown *et al.* 1993, Azzam *et al.* 1993). Activation of pro-MMP-2 by MT-MMP is a rate-limiting step for the catalytic function (Sato *et al.* 1992), and MT-MMP is also reported to degrade types I and III collagen (d'Ortho *et al.* 1997). We detected plasma membrane-dependent activation of the MMP-2

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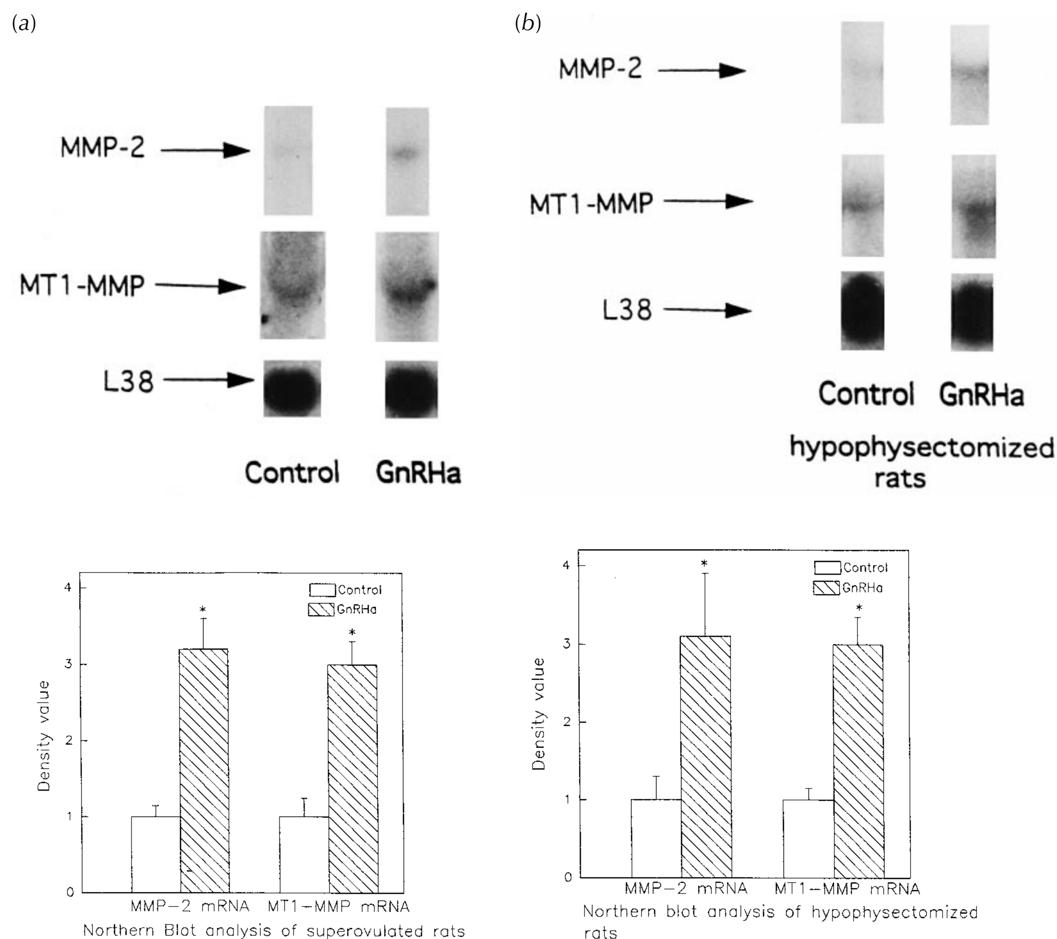

Figure 5 Effects of GnRHa treatment on MMP-2 or MT1-MMP mRNA expression of corpora lutea from superovulated rats or hypophysectomized superovulated rats by northern hybridization. (*a*) Top: Total RNA was extracted from corpora lutea of control or GnRHa-treated superovulated rats, and transferred onto nylon membranes and hybridized with ^{32}P-labeled cDNA probes of human MMP-2 and MT1-MMP, with L38 as a control (see Materials and Methods). (*b*) Top: Total RNA from control or GnRHa-treated superovulated, hypophysectomized rats was transferred onto nylon membranes and hybridized with ^{32}P-labeled cDNA probes of human MMP-2 and MT1-MMP, with L38 as a control. In the lower parts of (*a*) and (*b*), band densities are shown as bar graphs. The densities were normalized against the densities of the MMP-2 and MT1-MMP bands of the control group. All densities were previously normalized against the density of the L38 band. *$P<0{\cdot}05$ compared with control group.

proenzyme that was strikingly increased in GnRHa-treated rats, and there was more expression of MT1-MMP mRNA in corpora lutea of GnRHa-treated rats than in those of control rats. GnRHa also induced MT1-MMP mRNA expresson of corpora lutea in hypophysectomized rats. These novel findings are consistent with the conclusion that GnRHa induces luteal matrix degradation and remodeling of the extracellular matrix at the onset of structural involution of the corpus luteum.

We previously reported that functional luteolysis is a prerequisite for PRL-induced structural luteolysis (Endo *et al.* 1993). Functional luteolysis is hormonally defined as a decrease in serum progesterone and an increase in serum 20a-DHP . Whereas serum progesterone decreased several hours after GnRHa treatment, serum 20a-DHP peaked within 1 day, which can be called functional luteolysis, and thereafter continued to be low in the present study. Thus, it became clear that the depletion of serum progesterone was not due to the stimulatory effect of GnRH on 20α-hydroxysteroid dehydrogenase.

After GnRHa treatment had caused an LH surge, the LH concentration returned to the initial value several hours later, and did not become less than the initial concentration in our study. The treatment seemed not to cause desensitization of the pituitary gland, although the changes in bioactive LH concentrations were not

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determined. However, an earlier study using GnRHa revealed that desensitization of the pituitary actually occurred because pituitary concentrations of LH and FSH were suppressed and additional treatment with GnRHa did not stimulate gonadotropin release from the already stimulated pituitary (Sudo *et al.* 1990).

Curious effects of GnRHa on the PMSG–hCG-induced luteinization process of superovulated rats have been reported (Harwood *et al.* 1980*b*). GnRH inhibits the effects of PMSG and hCG on ovarian follicular development, and ovarian GnRH, LH, and PRL receptors are altered by the administration of GnRH. The effects of GnRHa might be due to an LH surge induced by the GnRHa treatment, which down-regulates LH receptors in superovulated rats. However, GnRHa could not have induced structural luteolysis in hypophysectomized rats by the same mechanisms as seen in non-hypophysectomized rats, because there were no pituitary hormones present after hypophysectomy. The same report also stated that GnRHa can act directly on the ovary: data revealed inhibitory effects of GnRHa on structural development. The mechanism of inhibition of the luteinization process seems to be different from the mechanism of structural luteolysis.

Corpus luteum formation of PMSG–hCG-treated immature female rats was complete around 5 days after hCG treatment. Both ovarian weight and serum progesterone concentrations were similar from day 5 to day 9, during which period the 20a-DHP concentration did not significantly change. This period can be regarded as the active luteal phase of superovulated rats (data not shown). We initiated GnRHa treatment on day 5 in order to investigate the effects of GnRHa on the developed corpus luteum, not to examine the effects on the process of corpus luteum formation.

Rat corpus luteum contains specific high-affinity receptors for GnRH, through which GnRH or GnRHa can inhibit the steroidogenic response to hormonal stimulation (Harwood *et al.* 1980*a*). GnRH-like protein has been reported to exist in the rat ovary (Aten *et al.* 1987) and GnRH mRNA expression in the rat ovary has been demonstrated (Oikawa *et al.* 1990, Goubau *et al.* 1992). These reports indicate that GnRH acts as a paracrine or autocrine hormone. Although a high pharmacological dose of GnRHa was injected into rats in the present study, it is possible that these actions of GnRHa may indicate the existence of physiological actions via a paracrine or autocrine mechanism. We speculate that GnRH may at least partially play a physiological role in causing structural involution of the corpus luteum, in addition to PRL as the main mediator.

In summary, the present study shows that the natural functional corpus luteum permits expression of GnRHa-induced structural luteolysis. This response was associated with marked simultaneous increases in MMP-2 and MT1-MMP activities to degrade collagens type IV, and type I and III, respectively, in the corpus luteum. We suggest that MMP activation is one of mechanisms for remodeling of the extracellular matrix in the GnRHa-induced luteolytic process.

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