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Association of immunolocalization of matrix metalloproteinase 1 with ovulation in hCG-treated rabbit ovary

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Matrix metalloproteinase 1, MMP-1, which was previously called interstitial collagenase, is necessary for extracellular matrix reconstruction. The immunolocalization of the latent form of MMP-1 (proMMP-1) was examined in the ovulatory process in hCG-treated (100 iu per animal) rabbit ovaries. Immunoreactive products of proMMP-1 were identified by the avidin–biotin peroxidase complex formation using an anti-rabbit proMMP-1 polyclonal antibody. ProMMP-1 was distributed in the cytoplasm of theca interna cells, theca externa cells, interstitial glands and germinal epithelium throughout ovulation. However, at 9 or 10 h after hCG treatment, this enzyme was identified in several capillary lumina around the apex of preovulatory follicles. In addition, the staining density of immunoreactive products apparently increased in granulosa cells and theca interna cells around the orifice of the ruptured follicle 10 h after the stimulation. These results indicate that the spatiotemporal appearance of proMMP-1 in ovulation may be closely associated with the initiation of rupture of the follicle.

Introduction

Rabbits are useful experimental models for investigating the mechanisms of ovulation, because they do not have spontaneous oestrous cycles and ovulation can be induced by hCG treatment or coitus. We have studied the mechanisms of ovulation using hCG-treated rabbits both biochemically and morphologically. A wide range of sizes of follicles on ovaries represent various stages of their maturation. After stimulation by hCG, a mature follicle exhibits dynamic histological changes (Okuda *et al.*, 1980; Kanzaki *et al.*, 1982; Okuda *et al.*, 1983a,b). Histological examination of follicles in the course of ovulation shows dilated and hyperaemic blood vessels and oedematous intervascular areas at the basal walls of preovulatory follicles. At their apical walls, however, capillaries are compressed and it is difficult to observe the capillary lumina. The apex shows the local change in colour and the translucency of the wall. This particular region is called the *macula pellucida*. The germinal epithelium overlying the *macula pellucida* becomes discontinuous and the intervening stroma greatly thinned out. Thereafter, the *macula pellucida* bulges outward as a clear vesicle or cone with a thin layer of dense connective tissue. Eventually, the cone ruptures and releases the ovum about 11 h after hCG stimulation. Woessner *et al.* (1989) revealed that the thin layer of dense connective tissue of *macula pellucida* is rich in interstitial collagens, including type I and III in tunica albuginea and theca layer, and type IV in the basement membrane. Large bundles of type I and III collagen are degraded in the tunica albuginea and theca layer of mature swollen follicles just before rupture (Okamura *et al.*, 1980; Okamura, 1989). It is therefore possible that the process of ovulation requires the activation of interstitial collagenase, which can digest the type I and III interstitial collagens (Matrician, 1990; Nagase *et al.*, 1992), at the apex of the preovulatory follicle. We demonstrated that there is the peak of collagenase activity 9 h after hCG treatment in rabbit ovaries (Kawamura *et al.*, 1981). This peak occurs just before the onset of ovulation. However, Palotie *et al.* (1987) reported increased activity of type IV collagenase in preovulatory follicles after hCG stimulation.

Recently, a considerable amount of information has been obtained regarding the metabolism of the various extracellular matrix components in normal and pathological conditions. In addition, many specific proteases have been identified and characterized as matrix metalloproteinases that degrade at least one component of the extracellular matrix. Interstitial collagenase is the first member of the matrix metalloproteinase family and therefore renamed matrix metalloproteinase 1 (MMP-1) (Matrician, 1990; Nagase *et al.*, 1992). Recent investigations revealed that this enzyme is produced and secreted by fibroblasts in a latent form, pro-matrix metalloproteinase 1 (proMMP-1), and is then activated *in vivo* by a pathway that involves plasmin (He *et al.*, 1989; Matrician, 1990).

In this study, we report the spatiotemporal immunolocalization of proMMP-1 associated with ovulation in the hCG-treated rabbit ovaries.

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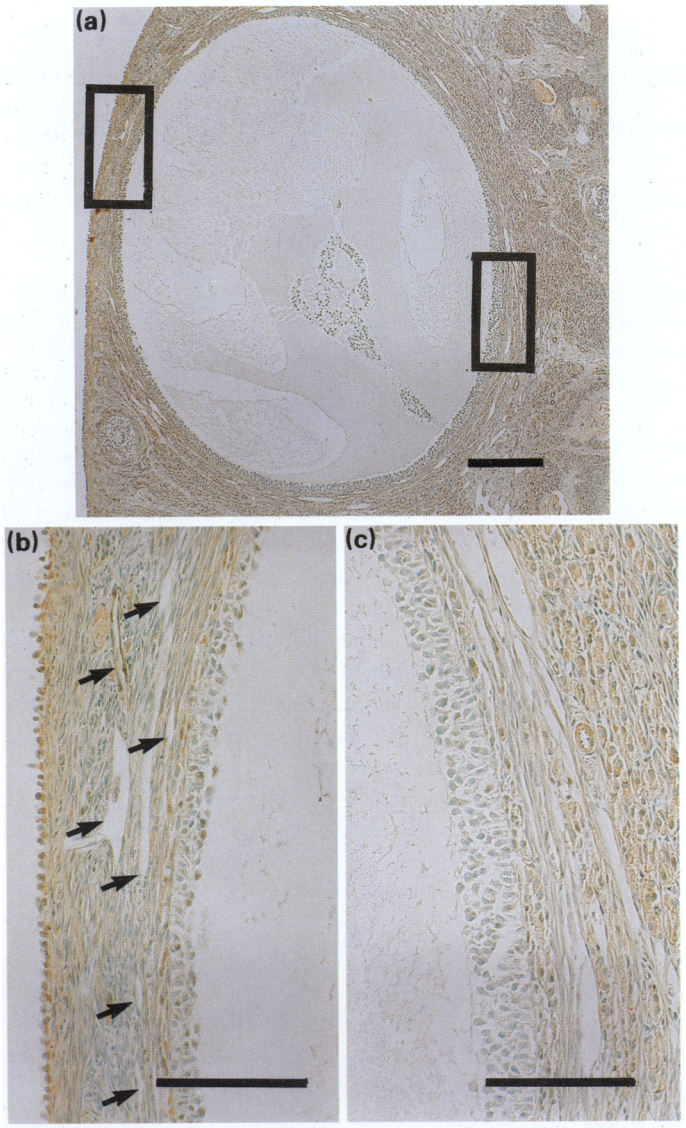

Fig. 1. Immunostaining of preovulatory follicle of a rabbit ovary 0 h after hCG injection using the anti-proMMP-1 IgG. (a) A large preovulatory follicle is surrounded by theca interna cells and theca externa cells with immunoreactivity for proMMP-1. (b) High magnification of the apex (left boxed area in (a)) showing positive staining of germinal epithelium, theca interna cells and theca externa cells for proMMP-1. Many granulosa cells are weakly stained. No immunoreactive product is noted in capillary lumina (arrows). (c) High magnification of the base (right boxed area in (a)) showing positive staining in the interstitial glands, theca interna cells and theca externa cells. Vascular lumina show no positive staining. Bars: a = 200 μm; b, c = 40 μm.

Materials and Methods

Animals and tissues

Ovaries were removed from adult female Japanese white rabbits (3.0–3.5 kg body weight) at 0, 4, 8, 9 and 10 h after hCG (HCG Mochida: Mochida Pharmaceutical Co., Ltd, Tokyo) i.v. (100 iu per animal) under pentobarbital (Nembutal Sodium Solution: Abbot Laboratories, North Chicago) anaesthesia (10 mg per animal, i.v.). In each experiment at any time, at least three rabbits were examined. The ovaries were fixed with 4% paraformaldehyde (Paraformaldehyde EM: TAAB Lab., Berks) in 0.01 mol phosphate-buffered saline l^{-1} (PBS), pH 7.4, at room temperature for 1 h, washed three times in PBS for 5 min each, dehydrated in a graded series of ethanol and then embedded in paraffin wax. Sections including several enlarged follicles were cut at 5 μm and processed for haematoxylin and eosin staining or immunohistochemistry. The stages of follicles were classified histologically according to the definition of Speroff *et al.* (1989).

Table 1. Immunoreactivities of matrix metalloproteinase 1 in hCG-treated rabbit ovarian follicles

Follicle	Time after hCG injection (h) 0	4	8	9	10
Primordial					
Granulosa cell	±	±	±	±	±
Preantral					
Granulosa cell	±	±	±	±	±
Thecal cell	+	+	+	+	+
Antral					
Granulosa cell	±	±	±	±	±
Theca interna cell	+	+	+	+	+
Theca externa cell	+	+	+	+	+
Preovulatory					
Granulosa cell	±	±	±	±	±
Theca interna cell	+	+	+	+	+
Theca externa cell	+	+	+	+	+
Capillary (apex)	−	−	−	++	++
Capillary (base)	−	−	−	−	−
Ruptured					
Granulosa cell (periorifice)					++
Granulosa cell (base)					+
Theca interna cell (periorifice)					++
Theca interna cell (base)					+
Theca externa cell (periorifice)					++
Theca externa cell (base)					+
Capillary (base)					−

Immunostaining was evaluated by two pathologists and the degree of staining was graded arbitrarily: ++ strong, + positive, ± weak and − negative. Increased immunostaining was noted from 9 to 10 h after hCG stimulation. This table is intended to show the trend of change rather than the quantity of staining. (See Figs 1–3).

Antibody to proMMP-1

The preparation and properties of the polyclonal antibody to proMMP-1 used in this study were reported by Vater *et al.* (1981). ProMMP-1 was produced by monolayer cultures of rabbit synovial fibroblasts. Antiserum to the pure proMMP-1 raised in sheep (provided by H. Nagase, University of Kansas Medical Center, Department of Biochemistry and Molecular Biology) was isolated by protein A affinity column. The anti-proMMP-1 IgG was used for immunohistochemistry.

Immunohistochemical examination of proMMP-1

After deparaffinization by heat at 60°C for 1 h in xylene, and rinsing in ethanol, sections were treated with 1% hydrogen peroxide in methanol for 30 min to minimize endogenous peroxidase activity in the tissue and were then washed in PBS. The slides were then placed in a moist chamber, and sections were covered with 5% normal rabbit serum in PBS for 30 min. The excess serum was removed by blotting and sections were covered with the primary antibody (anti-proMMP-1 IgG) solution and incubated overnight at 4°C. This antibody was used at a dilution of 1:50 with PBS containing 1% normal rabbit serum. Sections were washed in PBS and then covered with biotinylated anti-sheep IgG (1:200 dilution in PBS containing 1% normal rabbit serum) for 1 h at room temperature. Immunoreaction was performed by the avidin–biotin complex (Vector Laboratories, Burlingame, California) method of Hsu *et al.* (1981). The sections were rinsed in PBS, covered with avidin-biotinylated horseradish peroxidase complex (1:50 dilution in PBS), for 1 h at room temperature and then rinsed in PBS. Antigenic sites were demonstrated by reacting the sections with a mixture of 0.05% 3,3′ diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd, Tokyo) in 0.05 mol Tris–HCl buffer l^{-1}, pH 7.6, and 0.01% hydrogen peroxide for 7 min. The sections were counterstained with methyl green, dehydrated in ethanol, cleared in xylene, mounted in Entellan neu (Merck, Darmstadt, Germany) and examined with an Olympus photomicroscope. Immunostaining was evaluated by two pathologists. Negative controls for immunostaining were performed by replacing the primary antibody with non-immune sheep IgG. As positive controls, sections of involuting rabbit uterus indicating high contents of proMMP-1 in smooth muscle cells were prepared in each experiment.

Results

ProMMP-1 in the unstimulated follicle walls

In the preovulatory follicle walls of rabbit ovaries that had just been injected with hCG, that is in an unstimulated state, proMMP-1 was detected in theca interna and theca externa cells (Fig. 1a). The granulosa cells in the basal follicular wall were very weakly stained at all follicular stages (Fig. 1c, Table 1). Germinal epithelium showed immunoreactivity for proMMP-1 (Fig. 1b). Capillary lumina in the interstitial tissue of both apical and basal follicular walls showed no immunostaining for proMMP-1 (Fig. 1b; Table 1).

ProMMP-1 in the follicular walls after hCG stimulation

At 4 and 8 h after hCG stimulation, proMMP-1 was detected in theca interna cells and externa cells with immunoreactivities similar to that of unstimulated ovaries (Table 1).

At 9 and 10 h after hCG treatment, changes of the immunoreactivity were identified especially in the preovulatory follicles. The other follicles at less mature stages showed the same immunostaining as those in the unstimulated ovaries. The bulging preovulatory follicle clearly demonstrated proMMP-1 immunoreactivity in the compressed capillary lumina at the thinned apical wall (Fig. 2a,b). However, immunoreactivity of proMMP-1 was not identified in the dilated blood vessels containing red blood cells at the base of the follicle (Fig. 2c).

In the wall of the ruptured follicles of the ovaries that were removed at 10 h after hCG treatment, the granulosa cells and theca interna cells around the orifice showed the strong immunostaining of proMMP-1 (Fig. 3a,b). In contrast, those in the basal region were weakly stained (Fig. 3c).

Immunohistochemical findings of the various components of the follicle indicated the following points (Table 1). First, no apparent changes of the immunoreactivity were observed in the primordial, preantral and antral follicles at all time points examined. Second, irrespective of time after hCG stimulation, proMMP-1 was observed in theca interna and theca externa

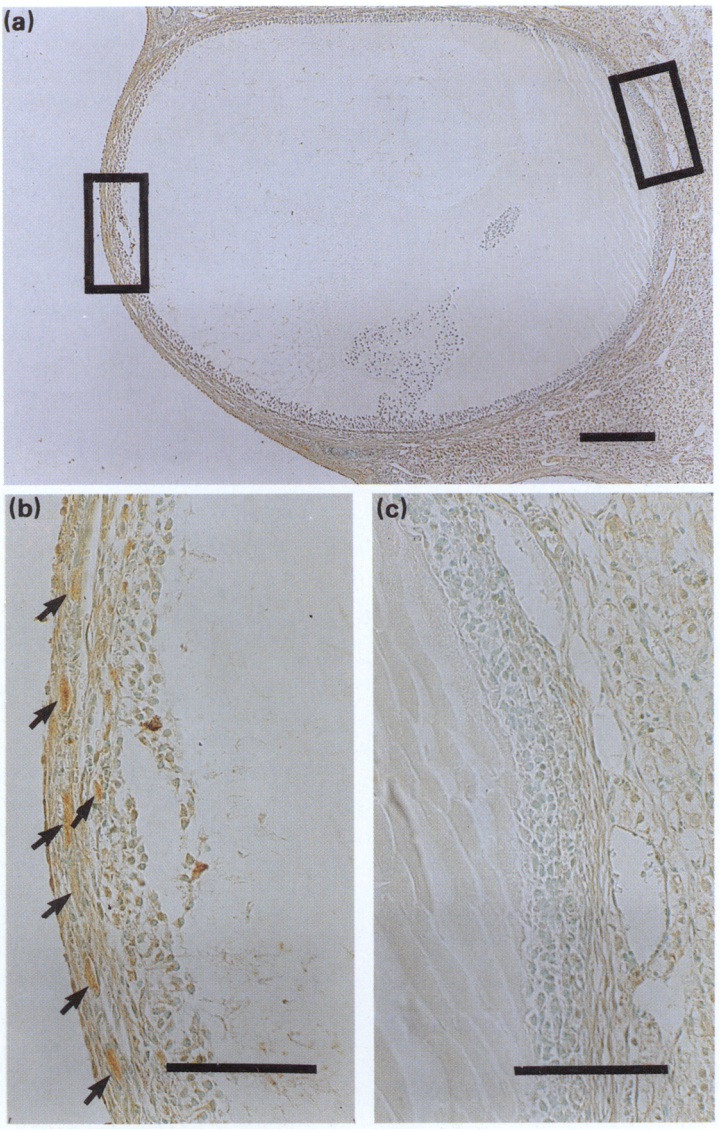

Fig. 2. (a) Immunostaining of an enlarged preovulatory follicle of a rabbit ovary 9 h after hCG injection. (b) High magnification of the apex (left boxed area in (a)). Elongated germinal epithelium show positive staining for proMMP-1. Note capillary lumina containing immunoreactive products for proMMP-1 (arrows). Granulosa cells are stained weakly. (c) High magnification of the base (right boxed area in (a)). No immunoreactive product is visible in the dilated capillaries. Theca interna cells, theca externa cells and interstitial glands show positive staining. Bars: a = 200 μm; b, c = 40 μm.

cells at all stages of the follicular phase. Third, at 9 and 10 h after treatment, at the apices of preovulatory follicles, a high immunoreactivity for proMMP-1 was clearly demonstrated in the capillary lumina. The granulosa cells and theca interna cells showed strong staining for proMMP-1 around the orifice of the ruptured follicles at 10 h after hCG treatment.

Discussion

Several electron microscope studies have revealed the disappearance of the collagen bundles in the thinned apical wall of the preovulatory follicles after ovulatory stimulation (Espey, 1967; Espey *et al.*, 1981; Okamura, 1989). In addition, the collagen content of dissected follicles from rat ovaries significantly decreases after induction of ovulation (Morales *et al.*, 1983). These reports indicate that MMP-1 is a key enzyme for interstitial collagenolysis and that it may play an important role in these dynamic morphological and biochemical changes in the preovulatory follicles. Increased collagenolytic activity in the preovulatory follicles of rabbit ovaries was demonstrated (Kawamura *et al.*, 1981). Curry *et al.* (1985) also reported the preovulatory increase of MMP-1 activity in the rat ovary. Moreover, many investigators showed that the administration

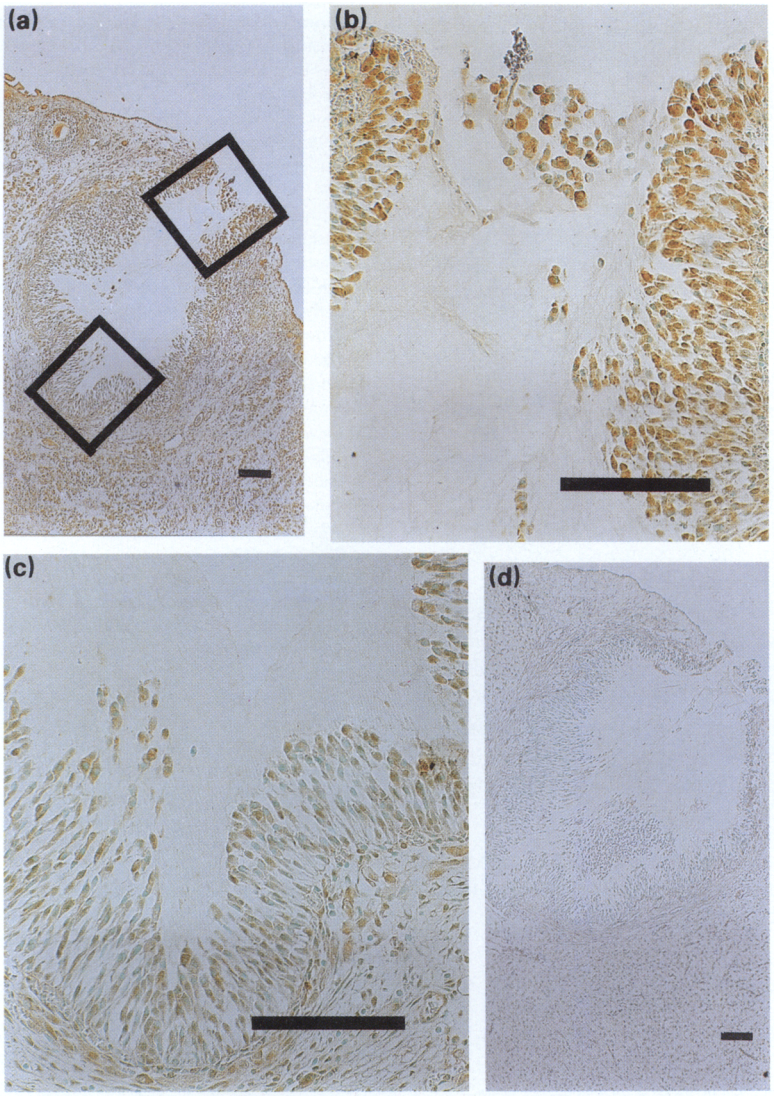

Fig. 3. (a) A ruptured follicle of a rabbit ovary 10 h after hCG injection. (b) High magnification of the ruptured region (upper boxed area in (a)). Granulosa cells and theca interna cells around the orifice show increased immunoreactivities. (c) High magnification of the base (lower boxed area in (a)). Granulosa cells, theca interna cells and theca externa cells show less staining than those around the orifice. (d) Ruptured follicle of a rabbit ovary 10 h after hCG injection stained with non-immune sheep IgG as a negative control. Bars: a, d = 200 μm; b, c = 80 μm.

of inhibitors of metalloproteinases suppresses the ovulation in rats and hamsters (Ichikawa *et al.*, 1983; Reich *et al.*, 1985; Brännström *et al.*, 1988). These reports emphasize the involvement of MMP-1 in ovulation.

In the study reported here the association of immunolocalization of proMMP-1 with ovulation was demonstrated in rabbit preovulatory follicles. Immunoreactivities for proMMP-1 in the immature follicles are stable. At 9 and 10 h after the stimulation, immunoreactive products for proMMP-1 were apparently identified in the capillary lumina at the apices of preovulatory follicles. In the ruptured follicles at 10 h after hCG treatment, increased immunoreactivities for proMMP-1 were observed in the granulosa cells. In particular, granulosa and thecal cells in the periorifice region exhibited intensive stainings. Electron microscope studies have shown many thrombi in the capillary lumina at the apices of preovulatory follicles just before ovulation (Okuda *et al.*, 1980). In addition, we have demonstrated that the injected anticoagulants reduced the number of the ovulated ova in PMSG–hCG treated immature rats (Iwamasa *et al.*, 1991). Thrombi produce platelet-derived growth factor (PDGF) and activate plasminogen. PDGF stimulates proMMP-1 production by aortic smooth muscle cells and skin fibroblasts (Lynch *et al.*, 1987; Yanagi *et al.*, 1991). It is therefore possible that PDGF produced by the apical thrombi stimulates perivascular cells and endothelial cells to produce proMMP-1 and to secrete it into the capillaries. It also suggests that, in the ruptured follicle, PDGF permeates from the capillaries, diffuses and stimulates proMMP-1 production by granulosa and thecal cells in the periorifice region. However, plasmin, which is the first activator of proMMP-1 (He *et al.*, 1989), may activate this enzyme at the

apical wall. If this is the case, the results reported here can explain the process of apical dissociation leading to the ovulation.

Another finding is that theca cells of follicles of all stages show similar immunoreactivities for proMMP-1. The follicles are surrounded by collagen-rich dense connective tissue and follicular enlargement requires remodelling of the extracellular matrix, such as collagenolysis. Nakano *et al.* (1977) reported that FSH rapidly stimulates follicular growth including proliferation of theca cells surrounding the follicle in the ovary from the hypophysectomized rat, although FSH receptors are not localized in theca cells. It is therefore possible that the multiplication of theca cells, which contain proMMP-1 and probably release this enzyme, is necessary for follicular enlargement. Puistola *et al.* (1986) reported that the activity of type IV collagenase increases in response to maturation of follicles before the LH surge. Because interstitial glands are derived from theca interna cells, they may also contain this enzyme. The physiological significance of the presence of proMMP-1 in interstitial glands and germinal epithelium is unclear.

This is the first immunohistochemical report showing high immunoreactivities for proMMP-1 around the *macula pellucida*. Reich *et al.* (1991) reported ovarian expression of MMP-1 mRNA just before ovulation by northern blot analysis. We are now investigating the expression of MMP-1 mRNA particularly around the *macula pellucida* using an *in situ* hybridization technique to determine the kinds of cells that produce proMMP-1 in association with ovulation.

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