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# Association of expression of mRNA encoding the $PGF_{2\alpha}$ receptor with luteal cell apoptosis in ovaries of pseudopregnant mice

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The relationship between the expression of mRNA encoding the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor and luteal cell apoptosis as determined by oligonucleosome formation was determined in mouse corpora lutea on days 2 (early phase), 6 (mid-phase), and 11 and 13 (late phase) of pseudopregnancy. No signals for mRNA encoding the $PGF_{2\alpha}$ receptor were detected in the ovary as shown by RNA blot analysis of pregnant mares' serum gonadotrophin (PMSG)-treated mice. The expression of the mRNA that could be detected in the corpora lutea on day 2 after hCG treatment was low, but it was increased on day 6 and reached a plateau on days 11 and 13. On days 11 and 13, *in situ* hybridization signals for mRNA encoding the $PGF_{2\alpha}$ receptor were localized to large luteal cells of the corpora lutea, especially the cells in the superficial layer. However, ethidium bromide staining revealed marked oligonucleosome formation in the corpora lutea on days 11 and 13. Similarly, positive signals of *in situ* nick-DNA-end labelling were also detected in the corpora lutea on days 11 and 13. Analysis of the adjacent sections of the corpora lutea on day 13 showed that both signals for DNA strand breaks and mRNA encoding the $PGF_{2\alpha}$ receptor were co-expressed in the corpus luteum. These results suggest that apoptosis during structural luteolysis closely associates with the increased expression of mRNA encoding the $PGF_{2\alpha}$ receptor in luteal cells of pseudopregnant mice.

## Introduction

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is one of the major arachidonate metabolites produced by the cyclooxygenase system in the mammalian female reproductive tissues (Coleman *et al.*, 1990; Boone *et al.*, 1993). Accumulating evidence has suggested that $PGF_{2\alpha}$ is of uterine or ovarian origin, depending on animal species, and that it is responsible for luteal regression in the ovary (Horton and Poyser, 1976; Rothchild, 1981; Hansel and Dowd, 1986; McCracken and Schramm, 1988). As an initiator of luteolysis, $PGF_{2\alpha}$ is known to inhibit steroidogenesis through $PGF_{2\alpha}$ receptors by increasing the intracellular $Ca^{2+}$ concentration or inhibiting cAMP synthesis. Recent evidence indicates that the disruption of the corpus luteum during luteolysis involves apoptosis on the basis of pharmacological experiments in rats and cattle (Zeleznik *et al.*, 1989; Sawyer *et al.*, 1990; Juengel *et al.*, 1993). However, little is known about on temporal relationship between luteal cell apoptosis and the expression of the $PGF_{2\alpha}$ receptor during the oestrous cycle. Recently, mouse cDNA clone for the $PGF_{2\alpha}$ receptor was isolated, and abundant expression of the receptor mRNA was demonstrated in the corpus luteum in the pregnant mouse ovary (Sugimoto *et al.*, 1994). The objective of this study was to investigate the possible association between the expression of mRNA encoding the $PGF_{2\alpha}$ receptor and apoptosis in corpora lutea in pseudopregnant mice.

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## Materials and Methods

### *Animals*

Immature 21-day-old female ddY mice were obtained from Shimizu Experimental Materials Co. (Kyoto). Animals were housed under standard conditions of light and temperature. Immature female mice were injected intraperitoneally with 5 iu pregnant mares' serum gonadotrophin (PMSG, Teikoku Hormone MGF, Tokyo), which was followed 48 h later by a injection of 5 iu hCG to obtain luteinized ovaries. These mice were then mated with castrated male mice. Ovaries ($n \geq 4$) were collected at specific time points, frozen in liquid nitrogen and stored at $-80°C$ until assayed. Ovaries were categorized as early (2 days after hCG treatment), mid- (6 days after hCG treatment) or late (11 and 13 days after hCG treatment) luteal phase.

### *RNA blot and* in situ *hybridization*

Total RNA was extracted from ovaries using the procedures of Chomczynski and Sacchi (1987). Total RNA (10 μg per lane)


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was separated by electrophoresis on a 1.2% (w/v) agarose-formaldehyde gel, transferred onto nylon membranes (Hybond-N, Amersham Corp.), and hybridized with a $^{32}$P-labelled *NotI*/*Hind*III fragment of MC205, which is a functional cDNA clone for mouse $PGF_{2\alpha}$ receptor (Sugimoto *et al.*, 1994). At least three independent experiments were carried out.

The linearized MC205 was used as template DNA and an antisense riboprobe was transcribed by T3 RNA polymerase (Stratagene, La Jolla) in the presence of [$^{35}$S]CTPαS. Unlabelled antisense riboprobe was synthesized similarly with unlabelled nucleotides. The resultant riboprobes were degraded to about 150 bases by alkaline hydrolysis. The ovaries were removed from each animal ($n \geq 4$) at each specific time phase. Three or four ovaries were frozen together in OCT compound (Miles, Elkhart, IN), cut out, and sections 10 μm in thickness were mounted on poly L-lysine-coated slides. The sections were fixed with 4% (w/v) formaldehyde, treated with 0.25% (v/v) acetic anhydride, and dehydrated. The slides were hybridized with a $^{35}$S-labelled antisense RNA probe at 57°C for 4 h in the presence or absence of a 100-fold excess of unlabelled probe (Sugimoto *et al.*, 1994). Hybridized sections were washed, treated with 20 μg RNase A $ml^{-1}$, and washed again. The slides were air-dried and dipped in NTB2 emulsion (Kodak) diluted 1:1 with distilled water. After exposure for 4 weeks at 4°C, the dipped slides were developed in Kodak D-19 developer, fixed and counter-stained with haematoxylin and eosin.

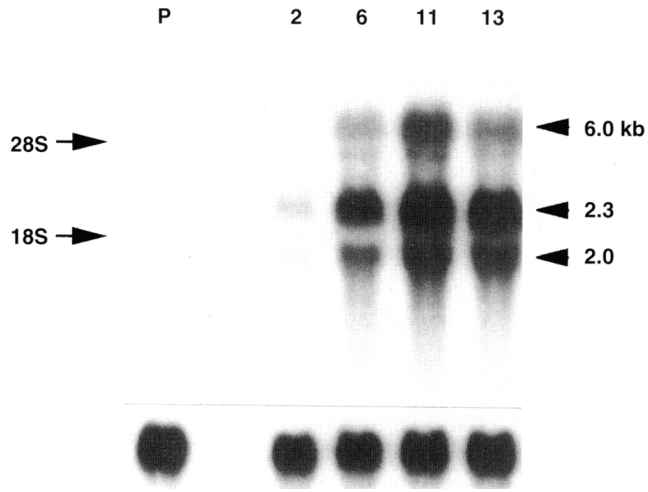



**Fig. 1.** RNA blot analysis of mRNA encoding the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor expressed in mouse ovaries during gonadotrophin-primed pseudopregnancy. Total RNA (10 μg of each sample) isolated from ovaries at different phases of the pseudopregnancy were separated, transferred onto a nylon membrane, and then hybridized with a $^{32}$P-labelled $PGF_{2\alpha}$ receptor-specific probe. Hybridization signals for mRNA encoding glyceraldehyde-3-phosphate dehydrogenase are shown in the lower panel. Arrowheads, three major bands of the $PGF_{2\alpha}$ receptor transcripts; P represents ovaries treated with pregnant mares' serum gonadotrophin for 48 h; 2, 6, 11, and 13 represent ovaries obtained 2, 6, 11 and 13 days after hCG treatment, respectively.

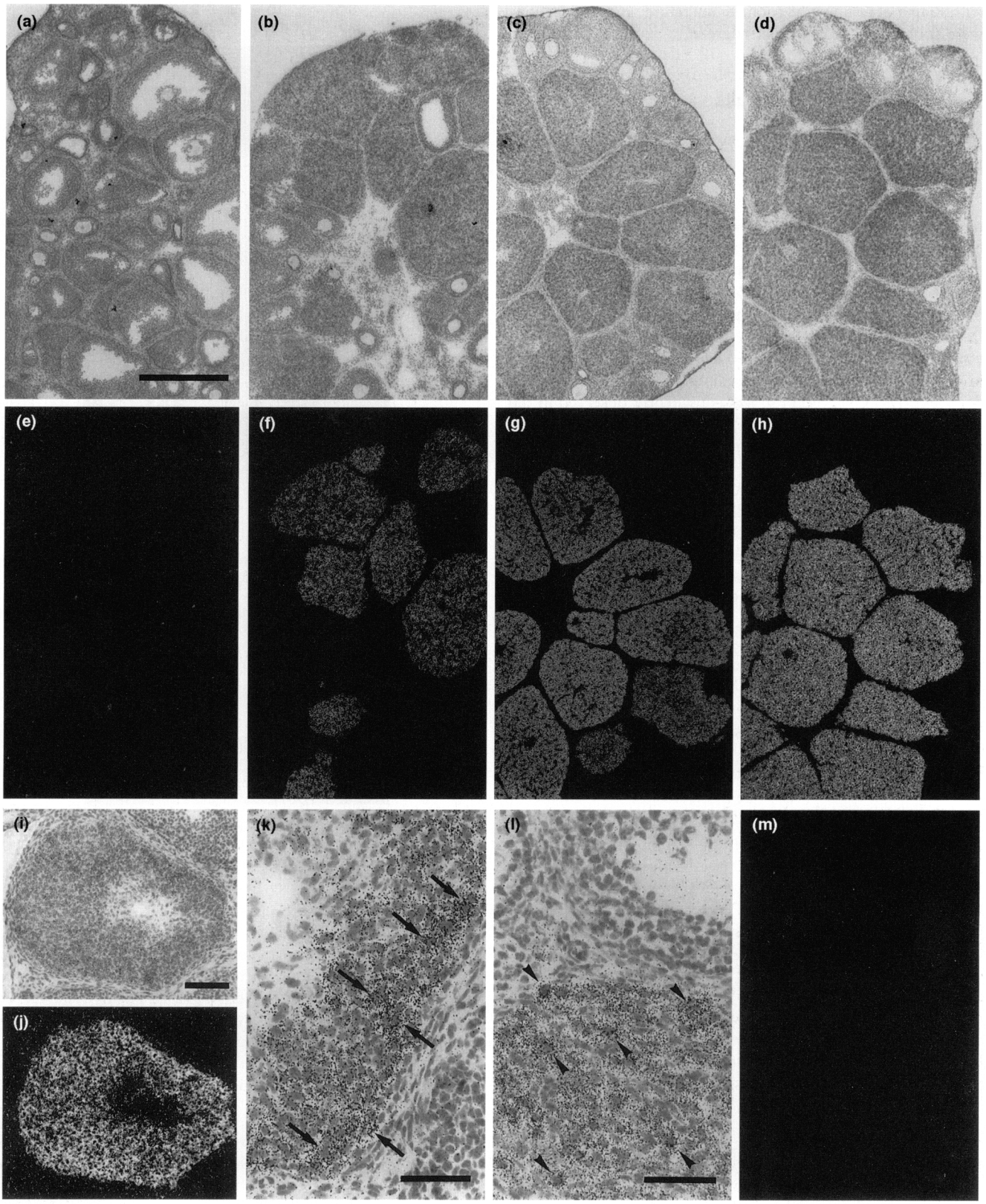
*Measurement of serum progesterone*

Blood samples were collected from each animal ($n \geq 4$) when the ovaries were removed. Measurement of progesterone was performed using a radioimmunoassay kit manufactured by Dai-ichi Radioisotope Laboratory (Tokyo). Serum concentrations of progesterone on days 8, 10, and 12 of pseudopregnancy was $295 \pm 38.1$, $31.9 \pm 4.8$, and $29.8 \pm 6.3$ (means ± SEM) nmol $l^{-1}$, respectively. The coefficients of variation of analysis of progesterone were 5.8% intra-assay and 7.8% interassay.

*Detection of oligonucleosome formation by electrophoresis and* in situ *detection of the cells undergoing DNA fragmentation (TUNEL method)*

The genomic DNA was isolated using a standard procedure and separated according to size in a 2% (w/v) agarose gel by electrophoresis. The gels were stained with ethidium bromide and viewed with a UV lightbox. The extent of DNA breakdown in fixed ovarian tissue sections was assessed using an *in situ* terminal transferase reaction to the nonisotopically labelled free 3′-ends of the DNA (Schmitz *et al.*, 1991; Thiry, 1992). *In situ* DNA labelling analysis was performed using ApopTag (Oncor, Gaithersburg). The ovary sections described above ($n \geq 4$) were fixed in 4% (w/v) neutral buffered formaldehyde, and then post-fixed in ethanol:acetic acid (2:1) at − 20°C. Endogenous peroxidase was quenched in 2.0% (w/v) hydrogen peroxide in PBS, washed with PBS, and subsequently labelled with digoxygenin-11-dUTP using a terminal transferase enzyme at 37°C for 1 h. In the negative control sections, the procedure was performed without the terminal transferase enzyme. The labelled sections were washed and subsequently incubated with anti-digoxygenin–peroxidase at 37°C for 30 min. The localization of digoxygenin–anti-digoxygenin–peroxidase complexes was detected by incubation of the slides

**Fig. 2.** Expression and localization of the mRNA encoding the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor in pseudopregnant mouse ovaries primed with gonadotrophin. A series of sections 10 μm in thickness were hybridized with $^{35}$S-labelled antisense RNA probe for the $PGF_{2\alpha}$ receptor in the absence (a–l) or presence (m) of a 100-fold excess of unlabelled probe. After being exposed to the emulsion, the sections were developed and stained with haematoxylin and eosin. Figures show bright (a–d, i, k and l) and dark field (e–h, j and m) photomicrographs of the sections obtained from the ovaries treated with pregnant mares' serum gonadotrophin (PMSG) for 48 h (a and e), ovaries treated with PMSG followed by hCG for 2 days (b, f, and i–k), and ovaries obtained 6 (c, g, and l) or 11 days (d, h, and m) after hCG treatment. The first and second horizontal rows are bright and dark field micrographs of the same sections, respectively (a and e; b and f; c and g; d and h, respectively). Since the ovaries shown in a–h are on the same slide, the intensities of the signals are comparable. In micrographs b–d and i, the corpora lutea are seen in dark grey because of the dense hybridization signals. The magnifications of micrographs a–h and m are the same. Photomicrographs i–k show the hybridization signals in the corpus luteum. Arrows (k) or arrowheads (l) indicate the large luteal cells showing strong signals. Scale bars represent 500 μm (a), 100 μm (i), and 50 μm (k and l), respectively.



with diaminobenzidine at room temperature, and the sections were counterstained with methylgreen.

## Results

### *Expression of mRNA encoding the $PGF_{2\alpha}$ receptor in the corpus luteum of pseudopregnant mice*

The relative abundance of mRNA encoding the $PGF_{2\alpha}$ receptor in the ovary during the oestrous cycle was investigated by performing RNA blot hybridization in the ovaries at different stages of pseudopregnancy (Fig. 1). Expression of the receptor mRNA could not be detected in untreated immature ovaries (data not shown) or ovaries obtained 48 h after PMSG treatment, while three major species of the mRNA with estimated sizes of 2.0, 2.3 and 6.0 kilobases (kb) were detected in the hCG-treated ovaries. Significant expression of mRNA encoding the $PGF_{2\alpha}$ receptor was consistently observed in the ovaries of mice on day 2; higher expression was also detected on day 6 and it reached maximum values on days 11 and 13 of pseudopregnancy.

Cellular localization of mRNA encoding the $PGF_{2\alpha}$ receptor was examined by *in situ* hybridization (Fig. 2). The hybridization signals of the receptor mRNA were specifically present in corpora lutea, but no signals were detected in the unovulated developing follicles (Fig. 2, a and e). On day 2, significant numbers of signals were detectable in all corpora lutea (Fig. 2, b and f), and large luteal cells localized near the surface of the corpus showed strong signals (Fig. 2, i–k). On day 6 of pseudopregnancy, the expression increased in most of the corpora lutea (Fig. 2, c and g), and strong signals were localized in the large cells, especially the cells in the superficial layers (Fig. 2l). On day 11 of pseudopregnancy, the highest expression was observed (Fig. 2, d and h).

### *Oligonucleosome formation in corpus luteum of pseudopregnancy*

In our pseudopregnant model, the serum concentrations of progesterone declined on day 10 (see Materials and Methods), which indicated that the onset of luteal regression (functional luteolysis) occurred on day 10. We, therefore, electrophoretically examined the ethidium bromide-stained DNA isolated from the ovaries during the mid- to late phases (Fig. 3). Slight oligonucleosome formation was seen on day 8, which reflects apoptosis involved in follicular atresia (see below). Marked oligonucleosome formation was noted on days 10 and 12. An *in situ* nick-DNA-end labelling technique was used to examine the cells that underwent apoptosis in the ovary during pseudopregnancy. Typical sections of ovaries at each stage are shown (Fig. 4). The sections shown in Fig. 4 a–c, are on the same slide. Positively-stained nuclei appeared dark brown. Slightly stained regions can be seen around positively stained nuclei but they were thought to be artefacts from the fixation. In every section during the different phases, positive staining of the atretic follicles was observed (Tilly *et al.*, 1991), and the staining intensities were constant during the different phases. In corpora lutea, no specific signals were observed in the sections on days 2 and 6 (Fig. 4a and b, respectively). In contrast,

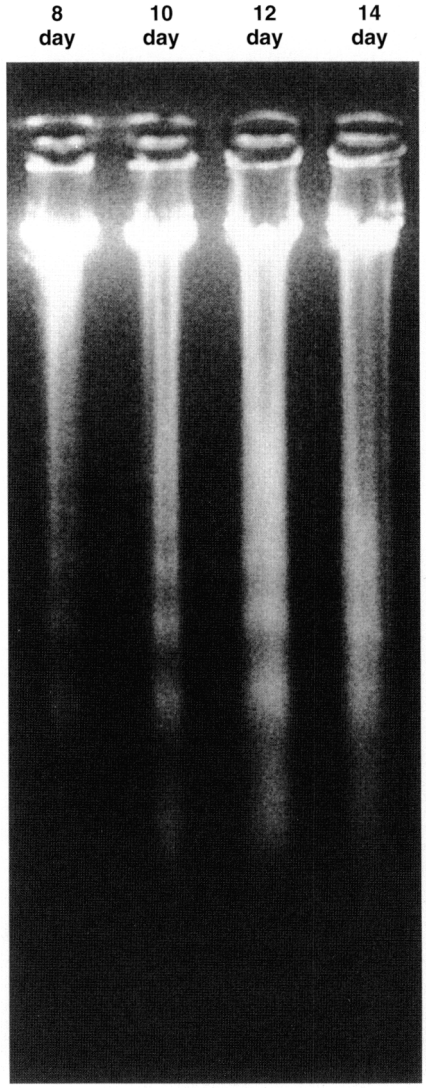
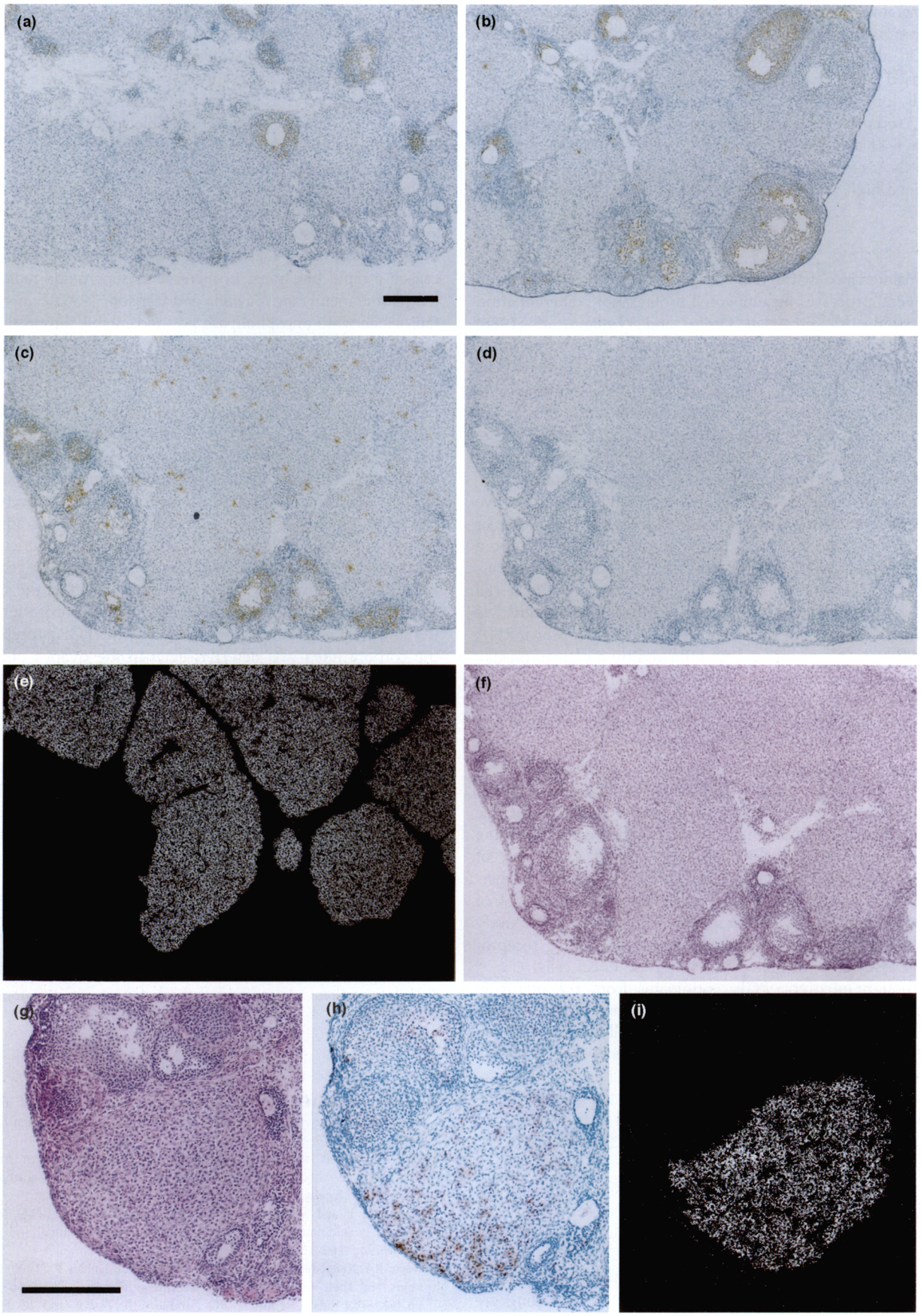



**Fig. 3.** Ethidium bromide-stained genomic DNA isolated from mouse ovaries during the mid- to late phase of pseudopregnancy (20 μg per lane). The day of pseudopregnancy is given at the top of the figure.

**Fig. 4.** Appearance of apoptotic cells and the expression of mRNA encoding the $PGF_{2\alpha}$ receptor in the corpora lutea during the late phases of pseudopregnancy in mice. A series of sections 10 μm in thickness was used for *in situ* analysis of DNA fragmentation of the apoptotic cells (a–d and h). Positively-stained nuclei appear dark brown. Slightly stained regions are seen around the positively stained nuclei, but they were thought to be artefacts from the fixation. After staining for the DNA strand breaks, the sections were counterstained by methyl green. The adjacent sections were hybridized with a $^{35}S$-labelled antisense RNA probe for $PGF_{2\alpha}$ receptor (e and i, darkfield). The sections obtained 2 (a), 6 (b), 11 (c–f), or 13 days (g–i) after hCG treatment are shown. Photomicrographs of c–f or g–i are the continuous adjacent sections of the same ovary, respectively. Micrographs (f) and (g) show the sections counterstained by haematoxylin and eosin. The micrograph of d shows the section of a negative control for DNA end staining. The magnifications of micrographs are the same. Scale bars represent 250 μm.



degenerating cells with DNA breakdown signals were seen in the corpora lutea of the ovaries on days 11 and 13 (Fig. 4c and h). On day 13, the number of degenerating luteal cells increased compared with day 11 (Fig. 4h). Furthermore, both signals for DNA strand breaks and for mRNA encoding the $PGF_{2\alpha}$ receptor were co-expressed in the same corpus luteum on days 11 and 13 (Fig. 4, c and e; h and i). Thus, expression of mRNA encoding the $PGF_{2\alpha}$ receptor in the luteal cells remained high until the cells exhibited DNA fragmentation.

## Discussion

The maintenance of the female reproductive cycle is achieved by several processes including cell proliferation, differentiation and cell death. Kerr *et al.* (1972) and Wyllie *et al.* (1980) indicated on the basis of histological observations that luteal regression is elicited by a highly specific programmed cell death called apoptosis. Sawyer *et al.* (1990) confirmed the histological appearance of luteal regression as apoptosis, and Zeleznik *et al.* (1989) demonstrated the activity of a $Ca^{2+}$–$Mg^{2+}$-dependent endonuclease, one of the DNases known to associate with programmed cell death, in rat luteal cells. Considering the known role of $PGF_{2\alpha}$ in luteal regression, we have suggested that $PGF_{2\alpha}$ receptor-mediated intracellular signalling directly influences the apoptosis of rodent corpora lutea. However, the temporal relationship between the expression of mRNA encoding the $PGF_{2\alpha}$ receptor and luteal cell apoptosis has not been established. In the present study, we demonstrated that the high expression of the receptor mRNA in the luteal cells on day 6 was followed by a plateau on day 11. In our pseudopregnant model, functional luteolysis, which is a decline of steroidogenesis, occurred on day 10. Similarly, electrophoretic or *in situ* analyses revealed that slight DNA fragmentation occurred in the luteal cells on days 10 and 11, and marked oligonucleosome formation was observed on days 12 and 13. Since nuclease activation is thought to be a relatively late event in apoptosis (Gavrieli *et al.*, 1992), the luteal cells may receive 'death signals' at the beginning of the late phase before day 10 when the highest expression of mRNA encoding the $PGF_{2\alpha}$ receptor was observed. Furthermore, the increased expression was maintained until most of the luteal cells underwent apoptosis. Thus, the present study has demonstrated that the expression of the $PGF_{2\alpha}$ receptor closely associates with apoptosis in mouse luteal cells.

Although luteolytic $PGF_{2\alpha}$ originates from the uterus in sheep and cattle, recent reports have suggested that a significant amount of $PGF_{2\alpha}$ is synthesized locally in the corpora lutea in rats (Boone *et al.*, 1993; Olofsson and Leung, 1994). In fact, using tissue samples of pseudopregnant rats, Olofsson and Selstam reported that the $PGF_{2\alpha}$ content in the corpus luteum was increased on day 11, and then increased further on day 13 of pseudopregnancy (Olofsson and Selstam, 1988). If the $PGF_{2\alpha}$ content in the corpora lutea is markedly increased at the beginning of the late phase, then it must become higher at the end of the late phase. Furthermore, it has also been suggested that intraluteal PG production is essential for structural luteolysis induced by prolactin in rats (Sánchez-Criado *et al.*, 1987). The present study together with these previous reports strongly suggests that $PGF_{2\alpha}$ and its receptor signalling are involved in luteal cell apoptosis.

A number of experiments showed that programmed cell death associates with endogenous endonuclease activity (Wyllie *et al.*, 1980; McConkey *et al.*, 1990). $Mg^{2+}$–$Ca^{2+}$-dependent endonuclease is known to be one of these DNases, and its activation was reported in regressing rat luteal cells (Zeleznik *et al.*, 1989). It is possible in this respect that $PGF_{2\alpha}$ and its receptor stimulate this enzyme, since the $PGF_{2\alpha}$ receptor increases the intracellular $Ca^{2+}$ concentration via Gq-phospholipase C (Leung *et al.*, 1986; Ito *et al.*, 1994; Sugimoto *et al.*, 1994). However, this activity does not fully account for the actions of $PGF_{2\alpha}$ (Boone *et al.*, 1993). Recently, it was demonstrated that treatment of luteal tissue with $PGF_{2\alpha}$ caused a rapid but transient increase in superoxide radicals in the plasma membrane (Sawada and Carlson, 1991). Superoxide radicals activate phospholipase $A_2$, and inhibit the production of cAMP and progesterone (Gatzuli *et al.*, 1991; Ikebuchi *et al.*, 1991). Thus, at least two pathways may be involved in $PGF_{2\alpha}$ and its receptor signalling.

$PGF_{2\alpha}$ may be a physiological mediator in the induction of follicular rupture (Carvalho *et al.*, 1989). $PGF_{2\alpha}$ concentrations increase in the preovulatory fluid of several species (Priddy *et al.*, 1989). If $PGF_{2\alpha}$ induces follicular rupture, expression of mRNA encoding the $PGF_{2\alpha}$ receptor can be detected in preovulatory follicles. In the present study, by the administration of PMSG, follicles developed and became enriched in the ovaries, but no significant expression of the $PGF_{2\alpha}$ receptor was detected. However, 2 days after hCG injection, the signals were detected in the large luteal cells in the corpora lutea. In the study reported here the expression of the $PGF_{2\alpha}$ receptor in the corpora lutea was detected even in the ovaries obtained 24 h after hCG injection (data not shown). Since ovulation usually occurred about 12 h after hCG treatment, the mRNA encoding the $PGF_{2\alpha}$ receptor should have been induced soon after ovulation. Therefore, it is possible that the transcription of mRNA encoding the $PGF_{2\alpha}$ receptor starts when granulosa cells differentiate into luteal cells. However, a more detailed analysis is required to elucidate the transcriptional regulation of the $PGF_{2\alpha}$ receptor during follicular maturation, ovulation and luteinization of ruptured follicles.

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