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# Interleukin-18 (IL-18) mRNA Expression and Localization of IL-18 mRNA-Expressing Cells in the Mouse Uterus

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**ABSTRACT**—Interleukin-18 (IL-18) belongs to the interleukin-1 family and was identified as an interferon-γ inducing factor. We investigated IL-18 mRNA-expressing cells in the mouse uterus. By RNase protection assay, IL-18 mRNA and α subunit of IL-18 receptor mRNA were detected in the uterus. In the uterus, IL-18 mRNA levels increased during sexual maturation. *In situ* hybridization analysis demonstrated IL-18 mRNA-expressing cells in the mouse uterus of different ages. At 21 days of age, IL-18 mRNA-expressing cells were detected in the luminal epithelial cells and stromal cells although the IL-18 mRNA signal was weak. At 42 days of age, IL-18 mRNA signal was mainly detected in the stromal cells located near the myometrium, and in some of the luminal and glandular epithelial cells. In the uterus of 63-day-old adult mice, a strong hybridization signal for IL-18 mRNA was detected at estrus, but was weak at diestrus. IL-18 mRNA was mainly detected in the glandular epithelial cells and stromal cells. The effect of estradiol-17β ($E_2$) on IL-18 mRNA-expressing cells in the uterus was examined in ovariectomized mice. In oil-treated mice IL-18 mRNA signal was localized in luminal epithelial cells and stromal cells, while in $E_2$-treated mice IL-18 mRNA signal was localized in stromal cells alone. These results suggest that the mouse uterus has an IL-18 system, and IL-18 exerts a physiological role within the uterus in a paracrine manner, and that IL-18 gene expression is regulated by estrogen.

**Key words:** interleukin-18 (IL-18), uterus, estrogen, mouse

## INTRODUCTION

Interleukin (IL) -18 was identified as an interferon (IFN) -γ-inducing factor in endotoxin-shocked mice (Okamura *et al*., 1995) and belongs to the IL-1 family. IL-18 is synthesized in the form of inactive 24 kDa precursor molecules, and cleaved by IL-1β converting enzyme (ICE; caspase 1) into biologically active 18 kDa molecules (Ghayur *et al*., 1997; Puren *et al*., 1999). IL-18 is produced in activated macrophages (Okamura *et al*., 1995), dendritic cells (Stoll *et al*., 1998), keratinocytes (Stoll *et al*., 1997; Kämpfer *et al*., 1999), intestinal epithelial cells (Takeuchi *et al*., 1997), osteoblasts (Udagawa *et al*., 1997), chondrocytes (Olee *et al*., 1999), adrenal cortex cells (Conti *et al*., 1997; Sugama *et al*., 2000), astrocytes and microglia (Conti *et al*., 1999; Prinz and Hanisch, 1999). Immune functions of IL-18 have been extensively studied. IL-18 exerted an anti-tumor effect and protective action against bacterial infection by stimulating the growth and differentiation of T-lymphocyte helper type (Th) 1 cells and by activation of natural killer (NK) cells (Ushio *et al*., 1996; Osaki *et al*., 1998). IL-18 stimulated the secretion of IL-4 and IL-13 from Th2 cells (Yoshimoto *et al*., 2000), and IL-18 together with IL-12 also stimulated the secretion of IL-2, IL-12 and INF-γ from Th1 cells.

In the uterus several cytokines and growth factors are believed to be involved in the regulation of uterine functions. Production of IL-1, IL-6 and tumor necrosis factor (TNF)-α changed throughout the estrous cycle, and synthesis of some cytokines was regulated by ovarian steroid hormones (De *et al*., 1992). In the human endometrium, IL-18 protein and mRNA were both expressed throughout the menstrual cycle (Yoshino *et al*., 2001). In human sera and amniotic fluids, IL-18 protein level increased during pregnancy, and more increased in delivery (Ida *et al*., 2000; Pacora *et al*., 2000). On the other hand, IL-18 mRNA level in human blood decreased during pregnancy (Kruse *et al*., 2000). Recently Zhang *et al*. (2003) demonstrated in pregnant mice that IL-18 was transiently expressed in decidual cells at day 5 of gestation, and in NK cells localized in the endometrium from

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day 6 to day 8 of gestation. In addition, IL-18 and IL-18 receptor (IL-18R) were expressed in endometriotic tissues (Oku *et al.*, 2004), suggesting that IL-18 is involved in pathogenesis of endometriosis in the human. However, the distribution of the IL-18-expressing cells in the uterus is not clear and there are no known changes in IL-18 expression during sexual maturation. Therefore, the aim of this study is to clarify localization of IL-18 mRNA-expressing cells in the mouse uterus and changes in IL-18 mRNA levels during sexual maturation. IL-18R functions as a heterodimer consisting of a ligand-binding chain termed IL-18Rα chain and a coreceptor termed IL-18Rβ chain (Parnet *et al.*, 1996; Torigoe *et al.*, 1997; Born *et al.*, 1998). IL-18 Rα mRNA expression in the mouse uterus was also studied in the present study.

## MATERIALS AND METHODS

### Animals

Male and female mice of the ICR strain (CLEA Japan Inc., Meguro, Tokyo, Japan) were used. They were kept in a temperature-controlled animal room (20–22°C; lights on, 07:00–21:00), and given a commercial CE-7 diet (CLEA Inc.) and tap water *ad libitum*. Estrous cycle was examined daily in the morning at least for two weeks before the start of an experiment. All animal care and experiments were performed in accordance with the Guideline for Animal Experimentation of Okayama University, Japan.

### Estradiol-17β ($E_2$) treatment

Three-week-old female mice were ovariectomized under light ether anesthesia. After one month, $E_2$ (Sigma-Aldrich Inc., St. Louis, MO, USA) at a dose of 250 ng per mouse was injected subcutaneously. $E_2$ was dissolved in sesame oil (0.1 ml). Control mice were injected sesame oil alone. After 24 hr, the uteri were collected and fixed with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.6).

### RNA extraction

RNA extraction was performed by the single-step method of Chomczynski and Sacchi (1987). Tissues collected were homogenized in guanidium thiocyanate (GTC) mixture (containing 4 M GCT, 25 mM sodium citrate; pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol). The GCT mixture was used at a volume of 1 ml per 100 mg tissue weight. Homogenates were treated with a syringe fitted with a 22-G needle in order to shear DNA. A 1/10 volume of 2 M sodium acetate (pH 4.0), an equal volume of phenol and 2/10 volume of chloroform/isoamylalcohol (49:1) were added to the homogenate and suspended. The samples were cooled on ice for 15 min and centrifuged at 4°C for 20 min. The aqueous phase was collected and an equal volume of isopropanol was added and suspended vigorously. The samples were precipitated at –80°C for 30 min and centrifuged at 4°C for 30 min. The pellet was dissolved in GTC mixture, an equal volume of isopropanol was added, and precipitated at –80°C for 30 min after centrifugation at 4°C for 30 min. Finally the pellet was washed in 75% ethanol, desiccated completely, dissolved in diethyl-pyrocarbonate (DEPC)-treated water, and stored at –80°C until use.

### RNase protection assay (RPA)

Total RNA from spleen, liver and uterus was analyzed using RNase protection Kit (Roche Diagnostics, Mannheim, Germany). RNA samples were hybridized with radiolabeled IL-18 or IL-18Rα probes at 45°C for 16 hr. Unhybridized RNAs were digested using RNase A and RNase T1 at 30°C for 1 hr. The samples were electrophoresed on a denaturing 5% acrylamide/ 8 M urea gel (30 mA

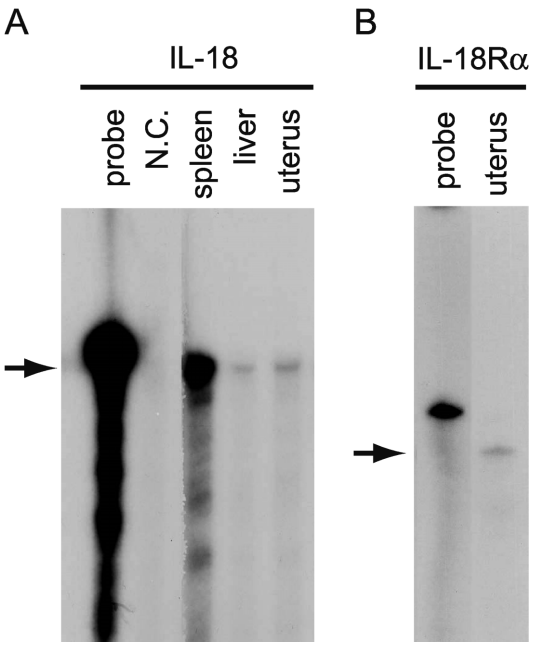


**Fig. 1.** RNase protection assay (RPA) of IL-18 and IL-18Rα mRNAs. RNA samples from mouse spleen, liver and uterus were hybridized with radiolabeled riboprobes of IL-18 (A) and IL-18Rα (B). Unhybridized RNAs were digested using RNase A and RNase T1. The samples were electrophoresed and hybridized signal was detected by autoradiography. As a negative control (N.C.) for RPA of IL-18 mRNA, yeast tRNA (Roche Diagnostics) was used instead of RNA tissue samples.

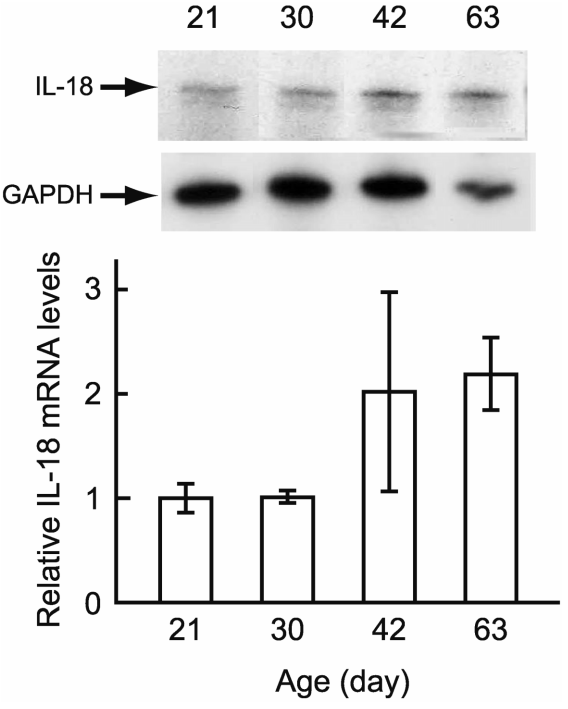


**Fig. 2.** IL-18 mRNA expression in the mouse uteri of different ages. RNA samples from mouse uterus were analyzed by RPA. Glyceraldehyde triphosphate dehydrogenase (GAPDH) mRNA levels were measured as an internal control. Data are expressed as the mean ± SEM (standard error of the means) of triplicate samples.

for 1 hr). Hybridized signals were detected by autoradiography. Glyceraldehyde triphosphate dehydrogenase (GAPDH) mRNA levels were measured as an internal control. The signal intensities were normalized relative to the intensity of GAPDH mRNA.

**Probes**

Mouse IL-18 and IL-18 Rα riboprobes were generated as follows. DNA fragments encoding parts of the mouse IL-18 and IL-18 Rα were generated from total RNA prepared from the ICR mice by RT-PCR using specific primer sets (IL-18, sense: 5'- AACAAGCTTTACTTTATACCTGAA -3', antisense: 5'-CAGAATTCGTTTGAAAGCATCATC-3'; IL-18Rα sense: 5'- CCAAAAGCTTCGTCTTGGTAGAGAA -3', antisense: 5'- AACTGAATTCAGATGATCTTAATC -3'). The cDNA fragment was subcloned into pGEM3zf(+), designated as an IL-18 and IL-18Rα riboprobe templates, sequenced, and confirmed to be cDNAs encoding parts of the mouse IL-18 and IL-18 Rα. The plasmid DNA containing IL-18 cDNA and IL-18 Rα cDNA was linearized using suitable restriction enzyme. Riboprobes were for IL-18 and IL-18 Rα synthesized with [α-$^{32}$P]rUTP (800 Ci/mmol, Amersham Biosciences, Buckinghamshire, UK) using Riboprobe System – T7/SP6 (Promega, Madison, WI, USA) for RNase Protection assay analysis, and with [α-$^{33}$P]rUTP (2500 Ci/mmol, Amersham Biosciences) for *in situ* hybridization analysis.

***In situ* hybridization**

Localization of IL-18 mRNA in uterine tissue sections was determined by *in situ* hybridization as described previously (Weiser *et al*., 1993). Uteri obtained from female mice of various ages were immediately fixed with 4% paraformaldehyde in 0.01 M PBS at room temperature overnight and processed for paraffin embedding. The tissues were sectioned at a thickness of 5-μm. The sections were digested with 10 μg/ml proteinase K (Nacalai tesque, Kyoto, Japan) at 37°C for 30 min and reaction of proteinase K was stopped with 0.2% (w/v) glycine in 0.01 M PBS. Sections were post-fixed with 4% paraformaldehyde in 0.01 M PBS, followed acetylation treatment with 0.1 M triethanolamine (pH 8.0) and dehydrated. The sections were placed in a moist chamber and hybridized at 50°C overnight in a solution containing 2×SSC, 1×Denhardt's solution, 10% dextran sulfate, 10 mM dithiothreitol, 0.4% yeast rRNA, 50% (v/v) deionized formamide and radiolabeled sense or antisense IL-18 riboprobes ($2\times10^5$ cpm/slide). The slides were washed in 1×SSC at room temperature for 5 min twice, and then 0.2×SSC at 50°C for 10 min twice. Unhybridized RNAs and nonspecific signals were digested using RNase A (100 mg/ml) at 37°C for 30 min. Slides were washed in 0.2×SSC at 50°C for 30 min and dehydrated. The slides were dipped in Konica NR-M2 Autoradiographic Emulsion (Konica, Tokyo, Japan), and kept at 4°C for 3–7 days in a dark box with silica gel, developed in Konicadol X (Konica), counterstained with hematoxylin.

## RESULTS

**Detection of IL-18 and IL-18Rα mRNAs in the mouse uterus**

IL-18 mRNA expression in adult mice was studied by RPA using the mouse IL-18 riboprobe (Fig. 1). Protected IL-18 mRNA signal, whose size differed from the riboprobe used, was detected in the spleen, liver and uterus (A). Protected IL-18 Rα mRNA signal was detected in the uterus (B).

**Postnatal changes of IL-18 mRNA levels in the uterus**

To determine the change of uterine IL-18 gene expression, total RNA (10 μg) obtained from uteri of various ages was analyzed by RPA (Fig. 2). IL-18 mRNA levels did not change during the prepubertal ages (21 and 30 days of age), and increased from 30 days to 42 days of age.

**IL-18 mRNA expressing cells**

Fig. 3A shows the uterus of a 21-day-old immature mouse, and Fig. 3B shows a part of the uterus of a 63-day-old adult mouse. The uterus consists of the endometrium and myometrium (m), a layer of smooth muscle cells. The endometrium consists of the luminal epithelium (le), glandular epithelium (ge) and stroma (s). Formation of uterine glands was observed from 3 weeks of age in the ICR mouse used in the present study.

Localization of IL-18 mRNA in the uterus of various ages was investigated by radioisotopic *in situ* hybridization analysis. At 21 days of age, IL-18 mRNA signal was broadly expressed in the luminal epithelium and stromal cells, although the hybridization signal was weak (Fig. 4 A, B and C). At 30 days of age, distribution and intensity of IL-18

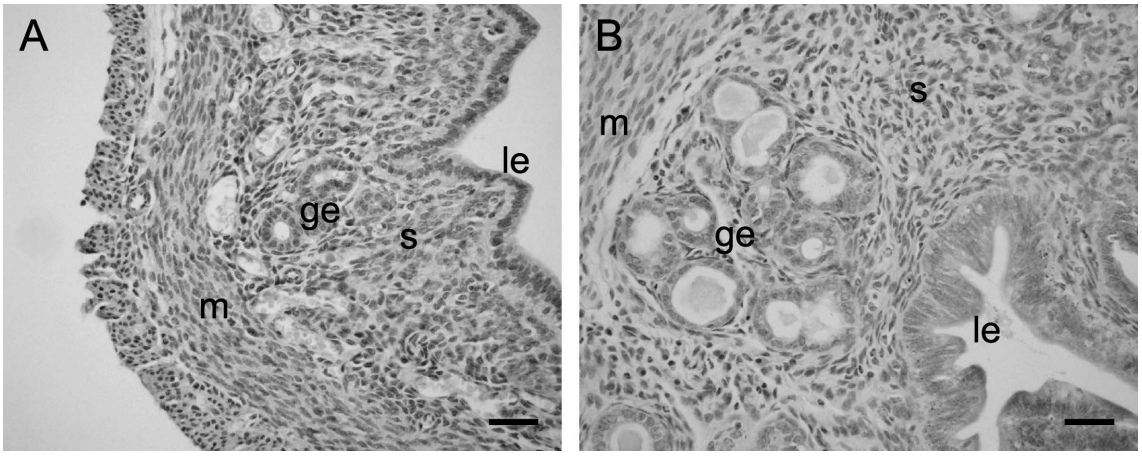


**Fig. 3.** Photomicrographs of the mouse uterus stained with hematoxylin and eosin at 21 days (A) and 63 days (B) of age (le, luminal epithelium; ge, glandular epithelium; s, stroma; m, myometrium). Bar=20 μm.

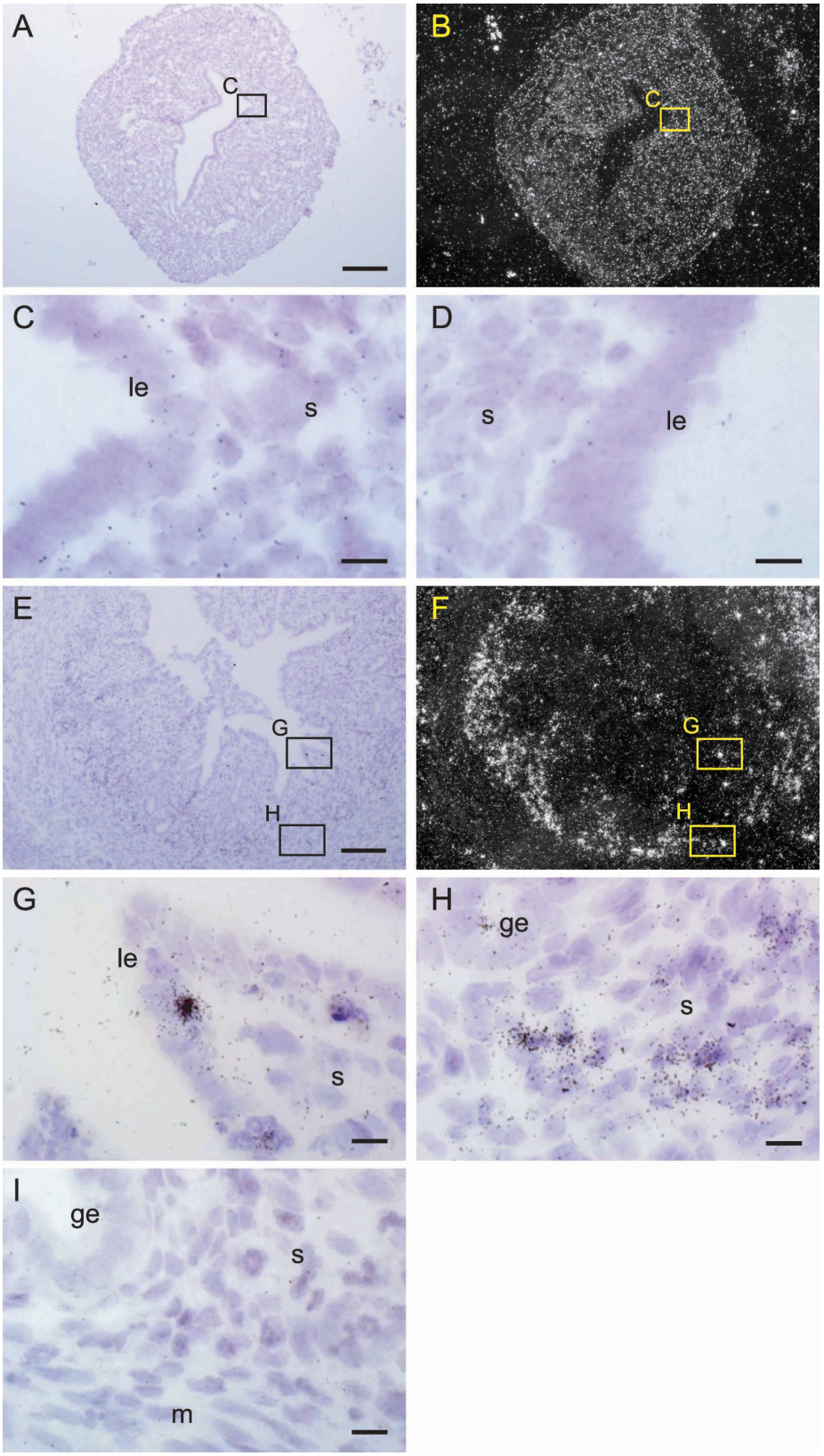


**Fig. 4.** *In situ* hybridization of IL-18 mRNA in the mouse uterus. Bright-field (A and E) and dark-field (B and F) micrographs of the same sections of uterus at 21 days of age (A–D) and 42 days of age (E–I). No signals were detected when the sense probe was used for hybridization (D, I). le, luminal epithelium; s, stroma. Rectangles in A, B, E and F indicates the fields of photographs of C, G and H. At 21days of age IL-18 mRNA hybridization signal was seen in epithelial cells and stromal cells although their intensity was weak (C). At 42 days of age IL-18 mRNA signal was seen in luminal and glandular epithelial cells (G and H), and stromal cells (H). Bar=100 μm (A and E) and 20 μm (C, G, H and I).

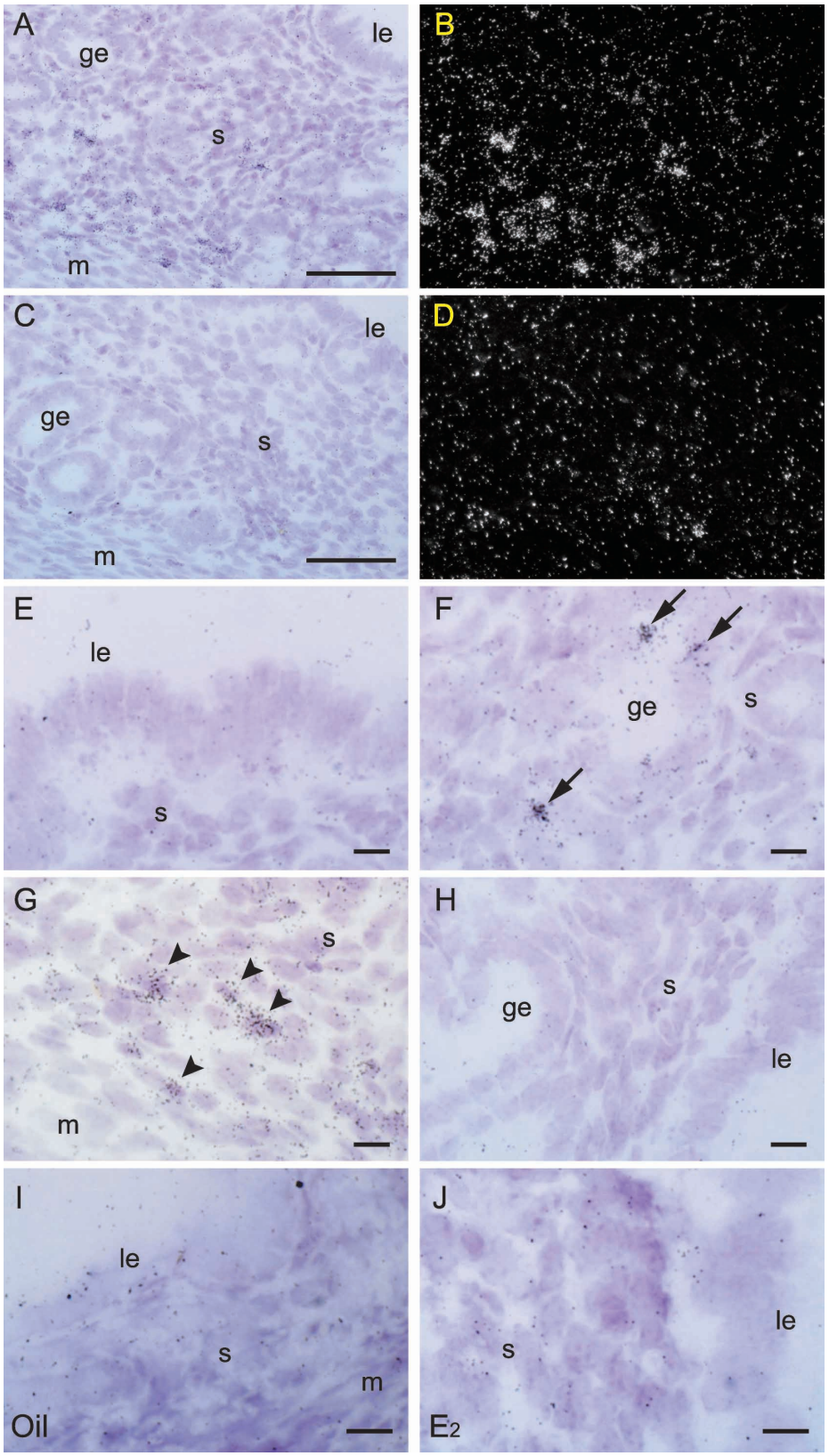


**Fig. 5.** *In situ* hybridization of IL-18 mRNA in the mouse uterus. Bright-field (A and C) and dark-field (B and D) micrographs of the same sections of uterus at 63 days of age (estrus: A and B) and (diestrus: C and D). No signal was detected when the sense probe was used for hybridization (H). IL-18 mRNA hybridization signal was seen in glandular epithelial cells and stromal cells (A, B and F). In estradiol-17β (E2) -treated ovariectomized mice IL-18 mRNA signal was seen only in stromal cells (J), and in oil-treated ovariectomized mice IL-18 mRNA signal was seen in luminal epithelial cells and stromal cells (I). le, luminal epithelium; ge, glandular epithelium; s, stroma. Bar=50 μm (A and C), and 20 μm (E–J).

mRNA signal were similar to those of 21-day-old mice (data not shown). At 42 days of age, IL-18 mRNA signal was mainly detected in the stromal cells located near the myometrium, and in some of the luminal and glandular epithelial cells (Fig. 4E–H). In the uterus of 63-day-old adult mice, a strong hybridization signal for IL-18 mRNA was detected at estrus (Fig. 5A and B), but IL-18 mRNA signal appeared weak at diestrus (Fig. 5C and D). IL-18 mRNA signal was mainly expressed in the glandular epithelial cells and stromal cells (Fig. 5 F and G), and in the luminal epithelial cells IL-18 mRNA signal was not detected (Fig. 5E). IL-18 sense probe was used as a negative control for *in situ* hybridization, and no hybridization signal was detected (Fig. 4D, I, and Fig. 5H).

**Effect of $E_2$ treatment on uterine IL-18 mRNA expression**

To investigate the effect of estrogen on uterine IL-18 mRNA expression, ovariectomized mice were given $E_2$ (250 ng/mouse) subcutaneously. The IL-18 mRNA signal was localized in the luminal epithelial cells and stromal cells in the oil-treated mice (Fig. 5 I), while in the $E_2$-treated mice IL-18 mRNA signal was localized in the stromal cells alone (Fig. 5 J). In the stromal cells the IL-18 mRNA signal of $E_2$-treated mice appeared not to differ from that of control mice.

## DISCUSSION

This study for the first time demonstrates IL-18 mRNA expression and localization of IL-18 mRNA-expressing cells in the mouse uterus by RPA and *in situ* hybridization analysis. IL-18Rα mRNA was also detected in the mouse uterus. These findings suggest that IL-18 and its receptor are expressed in the mouse endometrium, although we did not analyze the expression of IL-18 and IL-18 Rα protein. These results suggest the presence of the IL-18 system in the mouse uterus. From the data obtained from the present study it is difficult to determine the physiological role of IL-18 in the mouse uterus. However, it is highly likely that IL-18 is involved in the regulation of immune response and uterine functions.

We found temporal and spatial changes in IL-18 mRNA expression in the mouse uterus. Uterine IL-18 mRNA levels were low during the prepubertal ages, and increased at peripubertal and adult ages. *In situ* hybridization analyses demonstrated that localization of IL-18 mRNA-expressing cells changed during the sexual maturation in the mouse uterus. At 21 and 30 days of age, IL-18 mRNA-expressing cells were detected in the uterine luminal epithelial cells and stromal cells. At 42 and 63 days of age, IL-18 mRNA-expressing cells were detected in the uterine stromal cells and glandular epithelial cells. In the adult mouse uterus, IL-18 mRNA levels in the uterine glandular epithelial cells and stromal cells changed with the estrous cycle. IL-18 mRNA levels in both cell types appeared to be higher at estrus than at diestrus. Thus, the presence of changes in IL-18 mRNA levels during the pre- and peripubertal ages and with the estrous cycle suggests that IL-18 expression is closely associated with ovarian function.

Estrogen treatment reduced IL-18 mRNA expression in the uterine epithelial cells of ovariectomized mice. These findings show that estrogen is involved in the regulation of IL-18 mRNA expression, which accounts for the changes in IL-18 mRNA expression with the estrous cycle. On the other hand, estrogen effect on IL-18 mRNA expression in the uterine stromal cells was less clear. Uterine luminal epithelial cells and stromal cells may have different regulatory mechanism of IL-18 production. This is supported by the findings that IL-18 gene has multiple transcription start sites (Tone *et al.*, 1997; Sugama *et al.*, 2000). It is also possible that other sex hormones, such as progesterone, regulate IL-18 gene expression in the uterine endometrial cells.

In the uterine gland, IL-18 mRNA-expressing cells were detected from 30 days of age. The uterine gland secretes mucous secretions into the uterine cavity. IL-18 secreted in the uterine cavity may have a protective role for the uterine cavity as suggested by Yoshino *et al.* (2003). Interestingly, Takeuchi *et al.* (1997, 1999) found IL-18 expression in the intestinal epithelial cells, and its expression was elevated by hyperosmotic stress. Thus, uterine IL-18, secreted from the glandular epithelial cells and luminal epithelial cells, may be involved in the protective actions against bacteria and osmotic stress.

IL-18 regulates cytokine expression. Th1- and Th2-releasing cytokines were found to be regulated by IL-18 (Robinson *et al.*, 1997; Yoshimoto *et al.*, 2000; Pollock *et al.*, 2003). IL-1β-inducing fever responses were reduced by IL-18 (Gatti *et al.*, 2002). IL-18 regulated secretion of other cytokine such as TNF-α, IL-6 and IL-8 in the human vascular endothelial cells, smooth muscle cells and macrophages (Gerdes *et al.*, 2002). It is necessary to investigate the relation of IL-18 to other cytokines and steroid hormones by *in vitro* analysis. IL-8, in particular, is considered an autocrine growth factor for the proliferation of endometrial stromal cells (Arici *et al.*, 1998). Hence, analysis of the interaction of IL-8 and IL-18 in the regulation of endometrial functions is needed.

Several recent papers suggest the involvement of IL-18 in the regulation of pregnancy and onset of labor (Ida *et al.*, 2000; Kruse *et al.*, 2000; Pacora *et al.*, 2000; Yoshino *et al.*, 2001; Zhang *et al.*, 2003). In human endometrial stromal cells, cyclooxygenase-2 (Cox-2) mRNA and prostaglandin E(2) (PGE2) levels were elevated by IL-1β (Tamura *et al.*, 2002). Therefore, IL-18 produced in the mouse uterus may stimulate PGE2 release through the production of Cox-2 levels. On the other hand, IFN-γ inhibited IL-1β-induced PGE2 production in human myometrial cells (Hertelendy *et al.*, 2002). It is possible that IL-18 produced in the uterine stromal cells stimulates the production of IFN-γ in immune cells localized in the endometrium, and then IFN-γ inhibits the production of PGs in the myometrium.

Endometriosis is one of the common diseases of the woman. The etiology of endometriosis is not clear, but

endometriotic tissue has characteristics of endometrial epithelial and stromal tissues. IL-18 treatment stimulated Cox-2 induction in endometriotic tissues, leading to the production of prostaglandins (PGs) as shown in the normal uterine tissues, and IL-18 concentrations in the peritoneal fluid of endometriosis patients were higher than those in non-endometriosis samples (Oku *et al*., 2004). These findings suggest that IL-18 is associated with the pathogenesis of endometrium.

IL-18 induced apoptosis in various immune and non-immune cells through Fas-Fas ligand system of the TNF system (Ohtsuki *et al*., 1997; Okano and Yamada, 2000; Mariño and Cardier, 2003; Chandrasekar *et al*., 2004; Rodriguez-Galan *et al*., 2005). Interestingly, Kimura-Shimmyo *et al*. (2002) showed that combined treatment of IL-12 and IL-18 induced the apoptosis of the epithelial cells of the lacrimal gland and salivary gland in mice, which was dependent upon the production of INF-$\gamma$ and nitric oxide (NO), and not upon Fas-Fas ligand system and perforin-dependent cytotoxic T cells. These results suggest the involvement of IL-18 in remodeling of epithelial tissue in exocrine glands like lacrimal glands and salivary glands. We found expression of IL-18 mRNA in the epithelial cells of uterine glands. This finding raises the possibility that IL-18 secreted from the glandular epithelial cells induces apoptosis of neighboring tissue or of its own cells. Since the uterine tissues change dynamically during the estrous cycle and pregnancy, it is reasonable to assume that the uterus has a well-controlled and organized tissue remodeling system. The IL-18 system in the uterus is a candidate of the remodeling system in the uterus.

In conclusion, the present study demonstrated that IL-18 and IL-18R$\alpha$ mRNA expressed in the mouse uterus. Estrogen appears to be involved in the regulation of uterine IL-18 mRNA expression. IL-18 is considered to participate in the regulation of uterine function, anti-bacterial protection, immune response, and tissue remodeling.

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