Differential expression of peroxisome proliferator-activated receptor delta at implantation sites and in decidual cells of rat uterus

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The aim of this study was to examine the expression and regulation of peroxisome proliferator-activated receptor delta (PPAR δ) gene in rat uterus during early pregnancy by *in situ* hybridization and immunohistochemistry. PPAR δ mRNA expression in the luminal epithelium was high on day 1 of pregnancy, gradually declined from day 2 and was undetectable on day 5 of pregnancy. However, expression in the glandular epithelium began to increase from day 2 and was high on day 5 of pregnancy. There was no detectable PPAR δ immunostaining in the luminal and glandular epithelium from day 1 to day 5. On day 6 of pregnancy when embryos implanted, PPAR δ mRNA and immunostaining were intense in the subluminal stroma at implantation sites. On days 7 and 8, there was strong expression of both PPAR δ mRNA and intense immunostaining in the decidualized area near the lumen. There

was low expression of PPAR δ in the subluminal stroma and glandular epithelium under delayed implantation. After delayed implantation was terminated by oestrogen treatment and embryo implantation was initiated, both PPAR δ mRNA and immunostaining were strongly induced in the subluminal stroma. Intense PPAR δ immunostaining was observed in the decidua under artificial decidualization, while no detectable immunostaining was seen in the uninjected control horn. Retinoid X receptor (RXR α) immunostaining was seen in the subluminal stroma surrounding the implanting blastocyst on day 6 and in the decidual cells on days 7 and 8 of pregnancy. In conclusion, the high PPAR δ expression at implantation sites and in the decidual cells in rat uterus indicates that PPAR δ may play an important role during implantation and decidualization.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-dependent transcription factors. Three PPAR isoforms, PPAR α , PPAR δ (also known as PPAR β) and PPAR γ , have been cloned in mice (Guan and Breyer, 2001). PPARs must heterodimerize with retinoid X receptors (RXRs) which function as promiscuous partners for various nuclear receptors during transcriptional regulation (Kliwer *et al.*, 1992). Although PPAR α and PPAR γ have been studied extensively, much less is known about the function of PPAR δ . Expression of PPAR δ is high in brain, colon and skin, and PPAR δ may be involved in bone formation, lipid metabolism and epidermal maturation (Matsuura *et al.*, 1999; Mano *et al.*, 2000). PPAR δ deficiency in mice results in placental defects and frequent (> 90%) mid-gestation lethality, and also a marked reduction in adiposity relative to wild-type levels (Barak *et al.*, 2002). In COX-2-deficient mice, there are failures

in implantation and decidualization (Lim *et al.*, 1997). L-165,041, a PPAR δ -selective agonist, is able to restore implantation in COX-2 deficient mice. Furthermore, the co-administration of L-165,041 with 9-*cis*-retinoic acid, an RXR agonist, greatly improved implantation rate in COX2^{-/-} recipients. PPAR δ is also highly expressed in mouse uterus at the implantation site (Lim *et al.*, 1999).

The aim of the present study was to determine whether PPAR δ is essential for embryo implantation in mammalian species other than mice. *In situ* hybridization and immunohistochemistry were used to examine the expression of PPAR δ gene in rat uterus during early pregnancy and pseudopregnancy, delayed implantation and artificial decidualization, and also its regulation by steroid hormones. In addition, RXR α immunostaining during early pregnancy was performed as PPAR δ functions as a heterodimer with RXR α .

Materials and Methods

Animals and treatments

Mature rats (Sprague–Dawley strain) were caged in a controlled environment (14 h light:10 h dark). All animal

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procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. The stage of the oestrous cycle was determined by inspection of vaginal smears. Adult females were mated with fertile males of the same strain. Pregnancy was confirmed by the presence of spermatozoa in vaginal smear (day 1 = first day of vaginal sperm-positive result). Pregnancy on days 1–5 was confirmed by flushing embryos from the reproductive tracts. The implantation sites on days 6–7 were identified by i.v. injection of 0.1 ml of 1% (w/v) Chicago blue solution in 0.85% (w/v) NaCl. Pseudopregnancy was induced by keeping adult females in cages with vasectomized males and confirmed by checking for a vaginal plug (either in the vagina or on the cage floor) next morning. At least three rats were used in each stage or treatment in this study.

Delayed implantation was induced by treating the pregnant rats on day 4 of pregnancy with progesterone (5 mg per rat, s.c.) and the rats were then ovariectomized at 08.30–09.00 h. Progesterone (5 mg per rat) was injected to maintain delayed implantation from day 5 to day 7. The progesterone-primed delayed-implantation rats were treated with oestradiol (250 ng per rat) to terminate delayed implantation. The rats were killed by stunning and cervical dislocation and uteri were collected 24 h after oestrogen treatment. The implantation sites were identified by i.v. injection of 1% Chicago blue solution into the tail.

Artificial decidualization

Mature female rats were ovariectomized and after 5 days were sensitized for the decidual cell reaction according to the procedures described by Kennedy and Ross (1997). Some of the rats were given bilateral intrauterine injection of 100 μ l of sesame oil at about 12.00 h on the equivalent of day 5 of pseudopregnancy. The animals that did not receive the intrauterine injection served as non-stimulated controls (Orlando-Mathur *et al.*, 1996). Treated rats were killed 6 days later after the intrauterine injection of oil.

Immunohistochemistry

Rat uteri were immediately fixed in Bouin's solution for 24 h, dehydrated and embedded in paraffin wax. Sections (7 μ m) were cut, deparaffinized and rehydrated. Non-specific binding was blocked in 10% (v/v) normal horse serum in PBS at 37°C for 1 h. The sections were incubated with goat anti-human PPAR δ IgG (1:150, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-human RXR α IgG (1:100, Santa Cruz Biotechnology) in 10% (v/v) horse serum at 4°C for 16 h. The sections were then incubated with biotinylated rabbit anti-goat IgG or biotinylated goat anti-rabbit IgG followed by a streptavidin–alkaline phosphatase complex and Vector

Red according to the manufacturer's protocol (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA). Endogenous alkaline phosphatase activity was inhibited by adding 1 mmol levamisole l^{-1} (Sigma Chemical Co., St Louis, MO) into Vector Red substrate solution. Moreover, goat anti-human PPAR δ was replaced with the same concentration of normal goat IgG as a negative control. The sections were counterstained with haematoxylin and mounted. The degree of staining was assessed subjectively by blind examination of the slides by two investigators.

In situ hybridization

A 430 bp rat PPAR δ cDNA fragment was reverse-transcribed and amplified with the total RNA from rat uterus using forward primer 5'-GAGGAGAAAGAGGAAGTGG-3' and reverse primer 5'-CCACCAGCTTCCTCTCTC-3' designed according to rat PPAR δ (295–724 bp, Genbank accession number U40064). The amplification of PPAR δ cDNA was done for 30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 45 s. The amplified fragment of PPAR δ was recovered from the agarose gel and cloned into pGEM-T plasmid (pGEM-T Vector System 1, Promega, Madison, WI). The orientation of PPAR δ fragment in pGEM-T plasmid was determined by a combination of the primers for T7, SP6 and PPAR δ . The cloned PPAR δ fragment was further verified by sequencing. These plasmids were linearized with appropriate enzymes for labelling. Digoxigenin-labelled antisense or sense cRNA probes were transcribed *in vitro* using a digoxigenin RNA labelling kit (T7 for sense and SP6 for antisense; Boehringer Mannheim, Mannheim).

Uteri were cut into 4–6 mm pieces and flash frozen in liquid nitrogen. Frozen sections (10 μ m) were mounted on slides coated with 3-aminopropyltriethoxy-silane (Sigma) and fixed in 4% (w/v) paraformaldehyde solution in PBS. The sections were washed in PBS twice, treated in 1% (v/v) Triton-100 for 20 min and washed again in PBS three times. After prehybridization in the solution of 50% (v/v) formamide and 5 $\times$ SSC (1 $\times$ SSC is 0.15 mol sodium chloride l^{-1} , 0.015 mol sodium citrate l^{-1}) at room temperature (20°C) for 15 min, the sections were hybridized in the hybridization buffer (5 $\times$ SSC, 50% (v/v) formamide, 0.02% (w/v) BSA, 250 μ g yeast tRNA ml^{-1} , 10% (w/v) dextran sulfate, 1 μ g ml^{-1} denatured digoxigenin-labelled antisense or sense RNA probe for rat PPAR δ) at 55°C for 16 h. After hybridization, the sections were washed in 50% (v/v) formamide/5 $\times$ SSC at 55°C for 15 min, 50% (v/v) formamide/2 $\times$ SSC at 55°C for 30 min, 50% (v/v) formamide/0.2 $\times$ SSC at 55°C twice for 30 min each, and 0.2 $\times$ SSC at room temperature for 5 min. After non-specific binding was blocked in 1% (w/v) block mix (Boehringer Mannheim) for 1 h, the sections were incubated in sheep anti-digoxigenin

antibody conjugated with alkaline phosphatase (1:5000, Boehringer Mannheim) in 1% block mix overnight at 4°C. The signal was visualized with 0.4 mmol 5-bromo-4-chloro-3-indolyl phosphate l^{-1} and 0.4 mmol nitroblue tetrazolium l^{-1} in the buffer containing 100 mmol Tris-HCl l^{-1} , pH 9.5, 100 mmol NaCl l^{-1} and 50 mmol MgCl_2 l^{-1} . Endogenous alkaline phosphatase activity was inhibited with 2 mmol levamisole l^{-1} . All of the sections were counterstained with 1% (w/v) methyl green in 0.12 mol glacial acetic acid l^{-1} and 0.08 mol sodium acetate l^{-1} for 30 min.

Results

PPAR δ mRNA in the uterus during early pregnancy

There was high expression of PPAR δ mRNA in the luminal epithelium on day 1 (Fig. 1a). After digoxigenin-labelled PPAR δ antisense cRNA probe was replaced by digoxigenin-labelled PPAR δ sense cRNA probe, there was no detectable expression in the uterus on day 1 of pregnancy (Fig. 1b). Expression of PPAR δ mRNA in the luminal epithelium gradually declined from day 2 and was undetectable on day 5 (Fig. 1c–e). However, the expression in the glandular epithelium began to increase from day 2 and was high on day 5 (Fig. 1c–e). On day 6 when embryos implanted, a high expression was seen in the subluminal stroma at implantation sites (Fig. 1f). On days 7 and 8, PPAR δ mRNA was detected in the decidualized area near the lumen (Fig. 1g). The PPAR δ mRNA was mainly seen in the mesometrial side of the decidua on day 9 (Fig. 1h). Additionally, PPAR δ mRNA was detected in the embryos from days 7 to 9 (Fig. 1h).

PPAR δ immunostaining during early pregnancy

There was no detectable PPAR δ immunostaining in the luminal and glandular epithelium from day 1 to day 5 (Fig. 2a–c). PPAR δ immunostaining was seen in the subluminal stroma at the implantation site on day 6 (Fig. 2d). On days 7 and 8, PPAR δ immunostaining was observed in the decidua near the lumen and embryos (Fig. 2f–g). On day 9, PPAR δ immunostaining was mainly in the mesometrial side of the decidua (Fig. 2h).

PPAR δ mRNA and immunostaining during pseudopregnancy

There was a low expression of PPAR δ mRNA in the luminal and glandular epithelium from day 1 to day 8 of pseudopregnancy except for a considerably high expression in the luminal epithelium on day 1 (data not shown). No detectable PPAR δ immunostaining was seen in the luminal and glandular epithelium from day 1 to day 8 of pseudopregnancy (Fig. 2e).

Expression of PPAR δ mRNA and protein in the delayed implanting uterus before and after the initiation of implantation

There was low expression of PPAR δ mRNA in the subluminal stroma and glandular epithelium under delayed implantation (Fig. 3a). After delayed implantation was terminated by oestrogen treatment and embryo implantation initiated, there was high expression of PPAR δ mRNA in the subluminal stroma and low expression in the glandular epithelium (Fig. 3b).

There was no detectable PPAR δ immunostaining in the uterus under delayed implantation (Fig. 3c). After delayed implantation was terminated by oestrogen treatment and embryo implantation initiated, intense PPAR δ immunostaining was seen in the subluminal stroma and implanting blastocyst (Fig. 3d).

PPAR δ immunostaining under artificial decidualization

There was no detectable immunostaining in the uninjected control horn (Fig. 3e), whereas intense PPAR δ immunostaining was observed in the decidua under artificial decidualization (Fig. 3f).

RXR α immunostaining during early pregnancy

There was basal RXR α immunostaining in the uteri on days 1–5 of pregnancy (Fig. 4a–d). On day 6 when embryos implanted, RXR α immunostaining was strong in the subluminal stroma immediately surrounding the implanting blastocyst (Fig. 4e). RXR α immunostaining was strongly detected in the decidua near the lumen on day 7 (Fig. 4f) and in a larger area of the decidua on day 8 (Fig. 4g), but no corresponding immunostaining was seen when rabbit anti-human RXR α was replaced by normal rabbit IgG (Fig. 4h).

Discussion

In the present study, both PPAR δ mRNA and immunostaining were strongly detected in the subluminal stroma at the implantation site on day 6 of pregnancy in rats. However, these results were not seen on day 5 of pregnancy. High expression of PPAR δ was also reported in the subluminal stroma at implantation sites in mouse uterus (Lim *et al.*, 1999). Furthermore, although no PPAR δ signals were observed in rat uterus under delayed implantation, both PPAR δ mRNA and immunostaining were also highly induced in the subluminal stroma at the implantation site once delayed implantation was terminated by oestrogen treatment and the embryo had implanted. Interestingly, RXR α immunostaining was detected in the subluminal stroma at the implantation site in the rat uterus. These data showed that PPAR δ expression was specifically stimulated by the presence of active blastocysts since no corresponding signals were seen in the rat uterus on day 6 of pseudopregnancy in this study. PPAR δ may be important too for implantation

Fig. 1. *In situ* hybridization of peroxisome proliferator-activated receptor delta (PPAR δ) mRNA in rat uterus on days (a) 1, (c) 2, (d) 4, (e) 5, (f) 6, (g) 8 and (h) 9 of pregnancy. After digoxigenin-labelled PPAR δ sense cRNA was used for probe, no signal was seen in rat uterus on day 1 of pregnancy (b). Arrow: implanting blastocyst; $\blacktriangle$, $\blacktriangledown$: luminal epithelium; $\triangle$: glandular epithelium; st: stroma; myo: myometrium; *: mesometrial side. Scale bars represent 30 μ m.

Fig. 2. Peroxisome proliferator-activated receptor delta (PPAR δ) immunostaining in rat uterus on days (a) 1, (b) 4, (c) 5, (d) 6, (f) 7, (g) 8 and (h) 9 of pregnancy. There was no detectable PPAR δ immunostaining in rat uterus on day 6 of pseudopregnancy (e). Arrow: implanting blastocyst; ▲: luminal epithelium; △: glandular epithelium; st: stroma; myo: myometrium; *: mesometrial side. Scale bars represent 30 μ m.

Fig. 3. No detectable peroxisome proliferator-activated receptor delta (PPAR δ) (a) mRNA or (c) immunostaining was observed in rat uterus during delayed implantation. After delayed implantation was terminated by oestrogen treatment and the embryo was implanted, there was strong PPAR δ mRNA expression in the subluminal stroma (b) and strong PPAR δ immunostaining in the luminal epithelium (d). There was no detectable PPAR δ immunostaining in the control uterus (e), whereas a strong PPAR δ immunostaining was observed in the decidual cells under artificial decidualization (f). Arrow: implanting blastocyst. Scale bars represent 30 μ m.

in rats through heterodimerizing with RXR α as in mice. In the mouse uterus, COX-2 expression was co-localized with PPAR δ at implantation sites (Chakraborty *et al.*, 1996). As PGI $_2$ was the most dominant prostaglandin and no PGI $_2$ receptor (IP) expression was seen at the implantation site of mouse uterus, COX-2-derived PGI $_2$ might mediate embryo implantation via PPAR δ (Lim *et al.*, 1999). PGI $_2$ -mediated activation of PPAR δ was also reported in colorectal cancer (Gupta *et al.*, 2000). Additionally, intracellular PGI $_2$ formed by expressing

prostacyclin synthase in human embryonic kidney 293 cells could promote apoptosis by activating endogenous PPAR δ (Hatae *et al.*, 2001).

Although COX-2-deficient mice showed multiple failures in implantation and decidualization (Lim *et al.*, 1997), the implantation in the PPAR δ -deficient mice was normal (Barak *et al.*, 2002). This finding indicates that other molecules may compensate for the loss of PPAR δ . Microsomal prostaglandin E synthase was strongly expressed at the implantation site of mouse

Fig. 4. Retinoid X receptors (RXR α) immunostaining in rat uteri on days (a) 1, (b) 2, (c) 3, (d) 5, (e) 6, (f) 7 and (g) 8 of pregnancy. No immunostaining was seen in rat uterus on day 8 of pregnancy when rabbit anti-human RXR α antibody was replaced with normal rabbit IgG (h). Scale bars represent 30 μ m.

uterus (Ni *et al.*, 2002). EP2, a subtype of PGE₂ receptor, was also highly expressed at implantation sites (Lim and Dey, 1997). It is possible that PGE₂ may compensate for the loss of PPAR δ and mediate embryo implantation. However, PGE₂ was able partially to restore implantation in COX-2-deficient mice, whereas COX-2-derived prostacyclin (PGI₂) or PPAR δ agonists greatly improved the implantation in these deficient mice (Lim *et al.*, 1999).

PPAR δ expression was also detected in the decidua on days 7–9 of pregnancy in rat uterus. As intense PPAR δ immunostaining was also observed in the decidua under artificial decidualization, PPAR δ expression in decidua may be independent of the presence of the implanting embryos. The data in the present study are consistent with the pattern of PPAR δ expression in mouse uterus (Lim *et al.*, 1999). PPAR δ was highly expressed in the decidualized cells under normal pregnancy and artificial decidualization in mouse uterus (Lim *et al.*, 1999). COX-2 expression in mouse uterus also showed a similar expression pattern (Chakraborty *et al.*, 1996). Although there was a failure in decidualization in COX-2-deficient mice (Lim *et al.*, 1997), cPGI₂ (a PGI₂ agonist) was able to restore decidualization in these mice (Lim *et al.*, 1999). Additionally, targeted knockout of PPAR δ by homologous recombination resulted in placental defects and frequent (> 90%) mid-gestation lethality (Barak *et al.*, 2002). Furthermore, RXR α is highly expressed in endometrial cells on days 1–8 of pregnancy (Lim *et al.*, 1999). Abundant expression of RXR α was also reported in the mouse decidua (Mangelsdorf *et al.*, 1992). In the present study, RXR α immunostaining was strongly detected in the decidua on days 7 and 8 of pregnancy. These data indicate that PPAR δ may also play an important role during rat decidualization through forming a heterodimer with RXR α .

There was a difference in the expression pattern between PPAR δ immunostaining and mRNA in the present study. Although PPAR δ mRNA expression was high in the luminal epithelium on day 1 of pregnancy, PPAR δ immunostaining was not detected. This difference between PPAR δ mRNA and protein content may result from a post-transcriptional regulation since the PPAR δ mRNA expression was greater than the amount of PPAR δ protein. This difference was also noticed in mouse uterus between microsomal prostaglandin mRNA and protein (Ni *et al.*, 2002). However, PPAR δ immunostaining was detected in the myometrial cells on day 6 of pregnancy, whereas no PPAR δ mRNA expression was seen in these cells. Additionally, the PPAR δ immunostaining in the decidua on day 9 was also stronger than the PPAR δ mRNA expression. This result may reflect the fact that either PPAR δ mRNA in these locations has a long half-life or PPAR δ protein accumulates from other locations.

In conclusion, expression of PPAR δ was high at implantation sites and in the decidual cells in rat uterus, and PPAR δ may play an important role during implantation and decidualization as it does in mice.

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