Regulatory effect of IFN-γ on expression of TGF-β1, TβR-II, and StAR in corpus luteum of pregnant rhesus monkey[1]

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KEY WORDS transforming growth factor beta 1; transforming growth factor beta receptors; interferon type II; steroidogenic acute regulatory protein; corpus luteum

ABSTRACT

AIM: To examine expression of transforming growth factor-beta 1 (TGF-β1) and TGFβ receptor II (TβR-II) and steroidogenic acute regulatory protein (StAR) in the corpus luteum (CL) of pregnant monkeys at various stages and to study possible effect of IFN-γ on their production. **METHODS:** *In situ* hybridization and immunohistochemistry were applied to detect mRNA and protein. **RESULTS:** The expression of StAR, TGF-β1, and TβR-II in the pregnant monkey CL was progressively decreased from d 15 to d 35 of gestation. IFN-γ down-regulated the expression of TGF-β1, TβR-II, as well as StAR. **CONCLUSION:** TGF-β1 may play an important role in the CL formation and functional maintaining; IFN-γ down-regulates the expression of TGF-β1, TβR-II , and StAR.

INTRODUCTION

Corpus luteum (CL) is a transient endocrine organ formed from an ovulatory follicle. The main function of CL is to secret progesterone maintaining uterine cycling changes and pregnancy. Steroidogenic acute regulatory protein (StAR) is believed to be the key regulator of steroid hormone biosynthesis[1]. *De novo* synthesis of StAR protein is required for intramitochondrial translocation of cholesterol, the substrate of steroid biosynthesis, to the cytochrome P-450 side-chain cleavage enzyme located on the matrix side of the inner mitochrondrial membrane[2]. The appearance of StAR has been found to be precisely correlated with steroid production spatially and temporally[3]. The expression of StAR coincides well with the capacity of progesterone production in the CL and can be used as a functional marker of CL development[4].

The CL formation, development, and regression are regulated by many factors, such as hormones, cytokines, and growth factors. Transforming growth factor β (TGF-β), M_r 25 000 homodimers, are a family of growth factors including TGF-β1, 2, 3[5]. TGF-β mediate their activity by high affinity binding to the type II receptor, which has been identified as a transmembrane protein (TβR-II, M_r 70 000) with a cytoplasmic serine/threonine kinase domain[6]. TGF-β exert autocrine and paracrine functions in the tissue[5]. TGF-β was first isolated from media conditioned by transformed cells and identified as the protein responsible for the phenotypic transformation of murine fibroblast[7]. It has been demonstrated that TGF-βs mediate many cell-cell interactions, such as cellular proliferation, cellular differentiation, extracellular matrix and integrin

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modification, tissue repair, and angiogenesis[5].

TGF-βs in follicles and corpora lutea of different animals at various stages have been identified[8,9]. TGF-β1 in luteal cells may be the main functional type of TGF-β family[10] and has been demonstrated to exert an acute effect on progesterone production in bovine corpus luteum[11] and support luteal function by suppression of luteal cell apoptosis[12]. Therefore, it is suggested that TGF-β1 and TβR-II may play an important role in CL formation, functional maintaining and regression. However little information is available regarding the expression of the TGF-β1 and its receptor TβR-II in the CL of rhesus monkey.

Previous study had demonstrated that human placenta was capable of secreting IFN-γ[13] and directly exerted an inhibitory effect on placentation and implantation[14]. It is well known that progesterone secreted by CL plays an essential role in maintaining pregnancy. IFN-γ is also known to be as a luteolytic cytokine that promotes luteolysis[15,16] and reduces serum progesterone[17].

In this study, we investigated the coexpression of TGF-β1 and its receptor TβR-II in correlation with the expression of StAR in the CL at various stages and their regulation by IFN-γ.

MATERIALS AND METHODS

Reagents Polyclonal antibodies against human TGF-β1 and TβR-II were purchased from Santa Cruz (USA), the primary antibody to mouse StAR protein raised in rabbits was kindly provided by Dr Douglas M STOCCO (Texas Tech University Health Science Center, TX), DIG-RNA labelling kit, blocking reagent, alkaline phosphate conjugated anti-DIG antibody, dig-ddUTP, 4-nitro blue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim (Germany). Proteinase K was purchased from Merck (Germany). Restriction enzymes were purchased from Promega (USA). Diethyl pyrocarbonate (DEPC) and detrasulphate were purchased from Sigma.

Animals Female rhesus monkeys aged 5-7 years old were used. All animals were obtained from the Monkey Colony of the Primate Research Center, Kunming Institute of Zoology, Chinese Academy of Sciences. The rhesus monkeys were permitted to use for these experiment by the Institute Ethic Committee and WHO Project Review Committee. The animals were housed under controlled environmental conditions with free access to water and food. Menstrual cycles of female rhesus monkeys were monitored for two continued menstrual cycles. Animals were permitted to mate over a period of 3 d from the day of anticipated time of ovulation after 2-cycle continued menstrual examination. Day 2 of mating was designated d1 of pregnancy. The presence of a conceptus was confirmed by ultrasound diagnosis and vectal examination. The animals were divided into 4 groups (3 monkeys in each group at least). One group was injected daily with IFN-γ (50 000 U) on d 33 and d 34. The CL were removed by abdominal surgery under anesthetization and fixed in fixative (4 % for formaidehyde in PBS, pH 7.4) on d 15, d 21, and d 35 of pregnancy, and further processed for paraffin embedding for *in situ* hybridization and immunohisto-chemistry.

Synthesis of the DIG-labeled RNA probe for TGF-b1 The DIG-labeled RNA was synthesized as previously reported[28]. In brief, the plasmids that contained the cDNA fragment of TGF-β1 was linearized with the corresponding restriction enzyme and transcripted with corresponding RNA polymerases *in vitro*. Transcription was performed using an *in vitro* transcription system, and cRNA was labeled with digoxigenin using a DIG-RNA labeling kit purchased from Boehringer Mannheim (2 μL RNA polymerase, 4 μL 5×buffer, 2 μL mix, and 1 μg linearized plasmid and Rnasin were added to an Eppendorf tube and mixed, followed by DEPC-treated water to a total volume of 20 μL, and incubated at 37 °C for 2 h). The validation of the labeled probe was evaluated with Dot blot analysis.

***In situ* hybridization** Paraffin embedded sections were deparaffinized in fresh xylene 2 times for 10 min each (2×10 min), xylene:100 % alcohol (1:1, 5 min), 100 % alcohol (5 min), 95 % alcohol (5 min), 90 % alcohol (5 min), 80 % alcohol (5 min), and 70 % alcohol. The slides were washed in DEPC-treated PBS 3 times, 5 min each time (3×5 min), and permeablized with proteinase K (10 mg/L) in PK buffer (Tris-HCl 100 mmol/L, edetic acid 50 mmol/L, pH 8.0) for 20 min. The sections were washed in PBS (5 times, 2 min each), and post-fixed with 4 % paraformaldehyde in PBS (4 °C, 5 min), the slides were washed with PBS (2×5 min) and DEPC-treated H_2O (5 min). The sections were dehydrated with serial alcohol, dried in air, and incubated in prehybridization buffer (2×SSC, 50 % deionized, formamade, RT, 2-4 h). After prehybridization, the hybridization solutions were applied onto the slides and

covered with paraffin film. Hybridization solutions were made by mixing DIG-labelled cRNA probes (20-30 ng per slide) with 100 μL hybridization buffer (2×SSC, 50 % deionized-formamide, Tris-HCl 10 mmol/L, yeast tRNA 250 mg/L , 0.5 % SDS, 1×Denhardt, DTT 10 mmol/L , 10 % dextra-sulphate). The sections were incubated at 48-50 °C for 16-20 h. At the end of hybridization ,the paraffin film were removed by incubating the slides in 4×SSC, subsequently slides were washed with 2×SSC (2×15 min, RT), 1×SSC (2×15 min, 42 °C), 0.1×SSC (2×15 min, 42 °C). Sections were washed by shaking for 10 min with buffer 1 (Tris-HCl 100 mmol/L, NaCl 150 mmol/L), and covered with buffer 2 (buffer 1 containing 1 % blocking reagent) for 1 h. Sections were incubated in humid chamber with blocking solution containing anti-DIG alkaline phosphatase antibody (Fab fragment) at a dilution of 1:200, washed by shaking in buffer 1 (3×10 min), and incubated with buffer 3 [Tris-HCl (pH 9.5) 100 mmol/L, NaCl 100 mmol/L, and $MgCl_2$ 50 mmol/L] for 5-10 min. The sections were covered with colour generating solution (1 mL buffer 3, 4.5 μL NBT solution, and 3.5 μL BCIP solution), and incubated in a humid chamber for 2-7 h in the dark. When the colour development was optimal, the reaction was stopped by incubating the slides in buffer.

Immunohistochemistry The paraffin-embedded sections (6 μm in thickness) were deparaffinized as described previously. In order to unmask the antigens on the tissue, sections were immersed in citric acid buffer 10 mmol/L, and boiled in microwave oven at 92-98 °C for 10 min. Endogenous peroxidase was quenched by incubating the sections with 3 % H_2O_2 in 60 % methanol for 10 min at room temperature (RT), and the slides were washed with PBS (3×5 min). then the sections were blocked with 5 % normal goat serum (20 min, RT), and further incubated with primary antibodies specific for TGF-β1 and TβR-II (1:100 diluted with PBS) at RT for 1 h. Primary antibodies were replaced by normal rabbit IgG in negative control. The sections were washed with PBS (3×5 min), and the slides were incubated with biotin-conjugated second antibodies (1:200 in dilution) for 45 min, after being washed with PBS (3×5 min), the sections were incubated with a mixture of reagent A and B prepared 30 min in advance with PBS (1:100 for each reagent) for 45 min. Sections were washed thoroughly with PBS and incubated with DAB substrate solution for 2-7 min, the nuclei of the tissue were counter-stained using haematoxylin.

Statistical analysis The data for immunohistochemistry and *in situ* hybridization were obtained from 3 individual experiments with the CL sections of 3 monkeys. One representive experiment from three similar results was shown. The relative density data of the immunohistochemistry and *in situ* hybridization represent mean±SD of 3 individual experiments from 3 monkeys in each group. Image screened from 5 different areas with the same large and at the comparative positions were transformed into grey value. All values were analyzed by *t*-test, $P<0.05$ was taken to indicate statistical significance.

RESULTS

No measurement background staining was detected in the control used for TGF-β1, TβR-II, or StAR.

Immunohistochemical localization of StAR Immunopositive StAR reactions were detected in the corpus luteum of pregnant monkeys on d 15, d 21, and d 35; the intensity of the staining among them was obviously distinguishable and a progressive decreasing expression of StAR from d 15 to d 35 was observed. Treatment with injection of IFN-γ on d 33 and 34 decreased the expression of CL StAR antigen obtained on d 35 as compared with the control group (Fig 1, 2).

Immunohistochemical localization of TGF-b1 The location of TGF-β1 antigen in CL of pregnant monkey was assessed. Intensity of the immunopositive staining from d 15 to d 35 was obviously distinguishable and steadily decreased. Treatment with injection of IFN-γ on d 33 and d 34 decreased the expression of TGF-β1 antigen obtained on d 35 as compared with the control group (Fig 3, 4).

***In situ* hybridization of TGF-b1 mRNA** The expression of TGF-β1 mRNA was detected in the corpus luteum of pregnant rhesus monkeys on d 15, d 21, and d 35. The intensity of the staining among them in the CL was obviously distinguishable and a progressive decreasing expression of TGF-β1 mRNA from d 15 to d 35 was observed. Treatment with injection of IFN-γ on d 33 and 34 decreased the expression of TGF-β1 mRNA obtained on d 35 as compared with the control group (Fig 5, 6).

Immunohistochemical localization of TbR-II Immunopositive TβR-II reactions were detected in corpus luteum of pregnant monkey on d 15, d 21, and d 35. The expression of TβR-II antigen was progres-

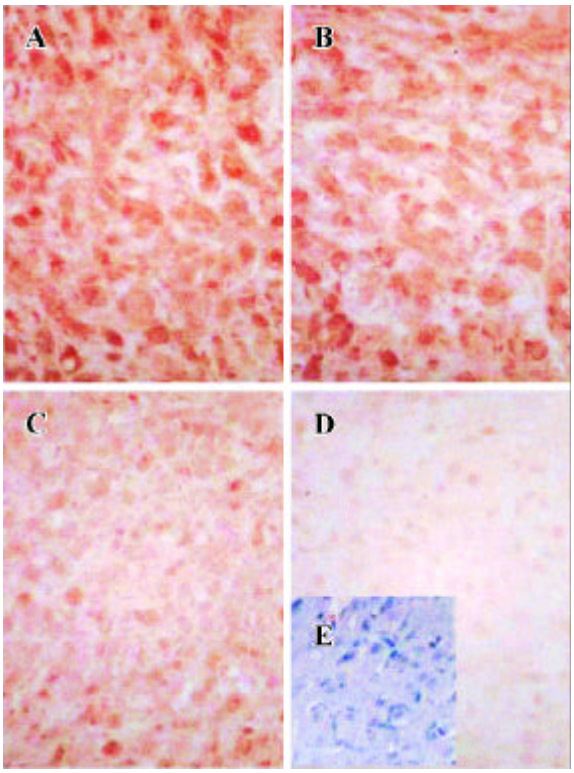

Fig 1. Immunolocalization of StAR protein in the CL of pregnant monkey. a: D 15, pregnant group; b: D 21, pregnant group; c: D 35, pregnant group; d: D 35, IFN-g -treated group; e: Negative control. (stained with haematoxylin, ×400).

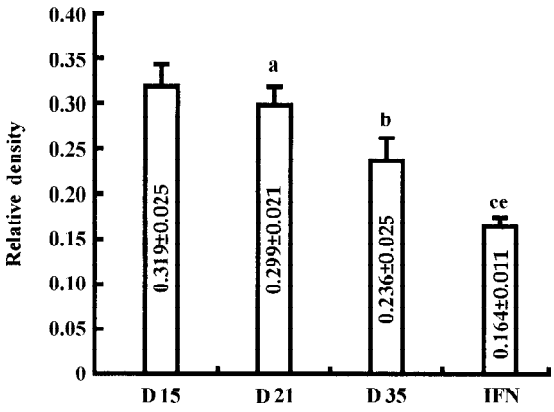

Fig 2. Relative density of StAR immunostaining in the CL of pregnant monkey. *n*=3. Mean±SD. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* D 15 group. [e]*P*<0.05 *vs* D 35 group.

sively decreased in corpus luteum of pregnant monkeys from d 15 to d 35. IFN-γ treatment on d 33 and 34 decreased the expression of TβR-II antigen as compared with the control group (Fig 7, 8).

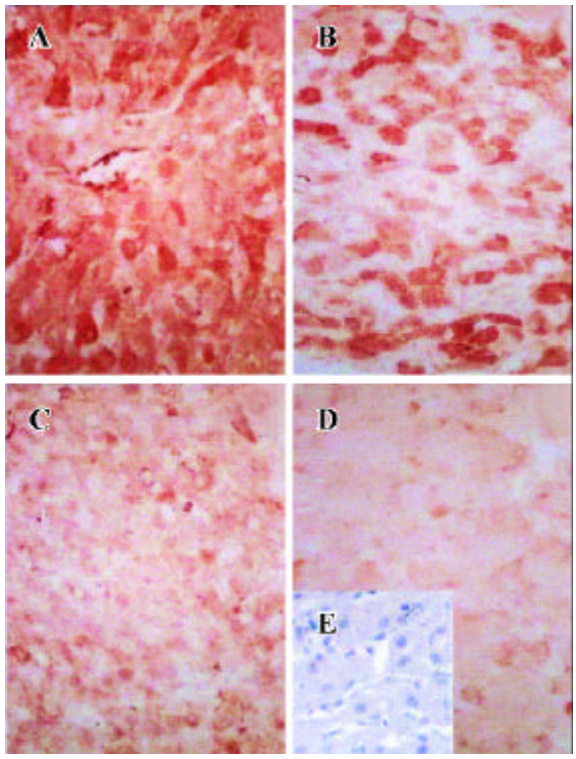

Fig 3. Immunolocalization of TGF-b1 protein in the CL of pregnant monkey. a: D 15, pregnant group; b: D 21, pregnant group; c: D 35, pregnant group; d: D 35, IFN-g-treated group; e: Negative control. (stained with haematoxylin, ×400).

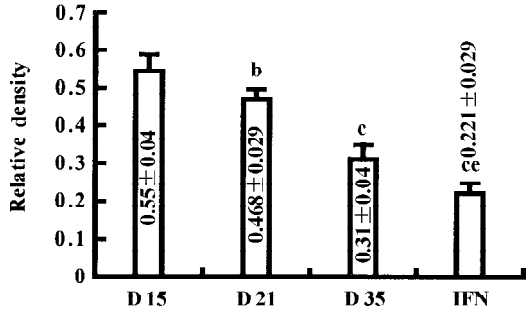

Fig 4. Relative density of TGF-b1 immunostaining in the CL of pregnant monkey. *n*=3. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* D 15 group. [e]*P*<0.05 *vs* D 35 group.

DISCUSSION

In rheus monkey, implantation window opens on d 8-d 13 after ovulation. The luteal-placental shift occurs near the end of the third week (d 21) of gestation and the placenta forms completely around d 35 of pregnancy[18]. Placenta secrets progesterone from its forming and gradually replays the corpus luteal function,

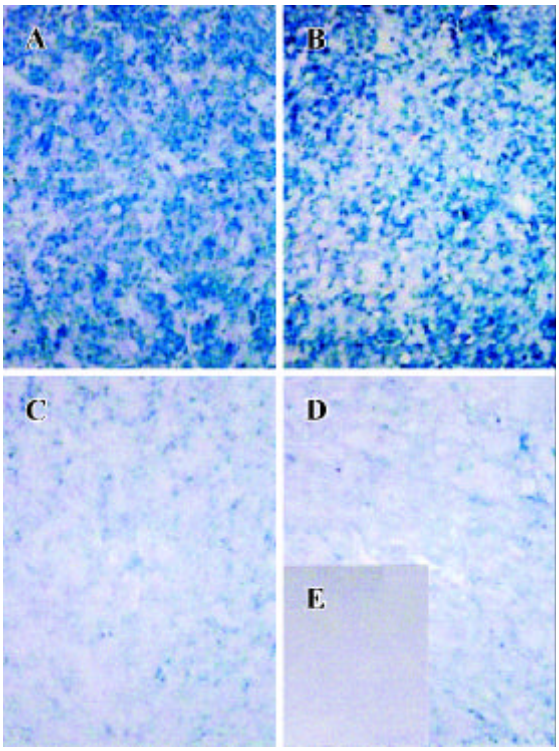

Fig 5. Localization of TGF-b1 mRNA in the CL of pregnant monkey. a: D 15, pregnant group; b:D 21, pregnant group; c: D 35, pregnant group; d: D 35, IFN-g-treated group; e: Negative control, replacement of antisense probe with sense probe. Original magnification×200.

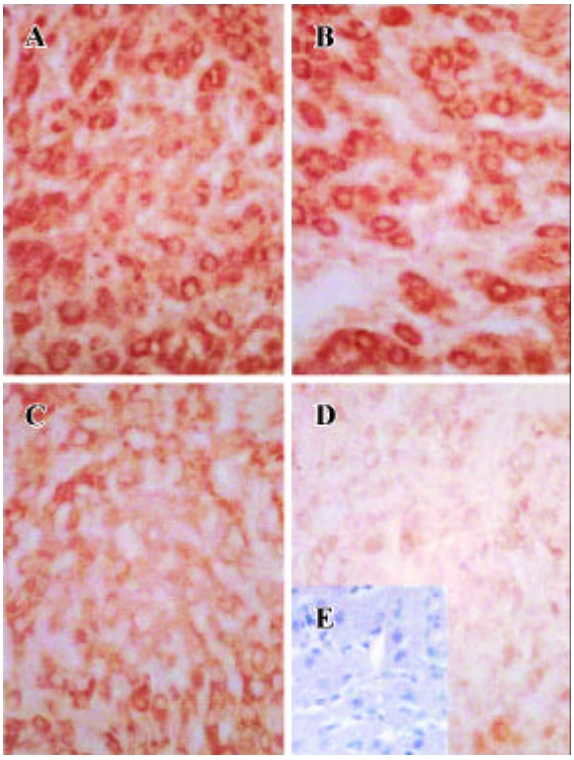

Fig 7. Immunolocalization of TGF-bRII protein in the CL of pregnant monkey. a: D 15, pregnant group; b: D 21, pregnant group; c: D 35, pregnant group; d: D 35, IFN-g-treated group; e: Negative control (stained with haematoxylin). Original magnification×400.

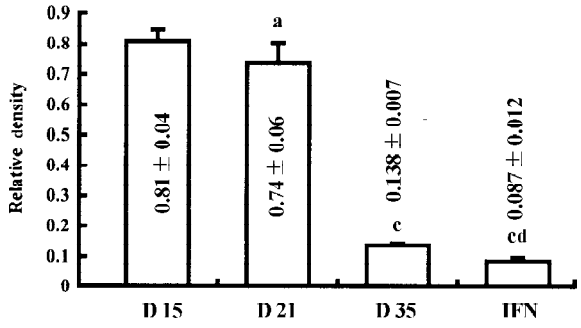

Fig 6. Relative density of TGF-b1 mRNA in the CL of pregnant monkey. *n*=3. Mean±SD. [a]*P*>0.05, [c]*P*<0.01 *vs* D 15 group. [d]*P*>0.05 *vs* D 35 group.

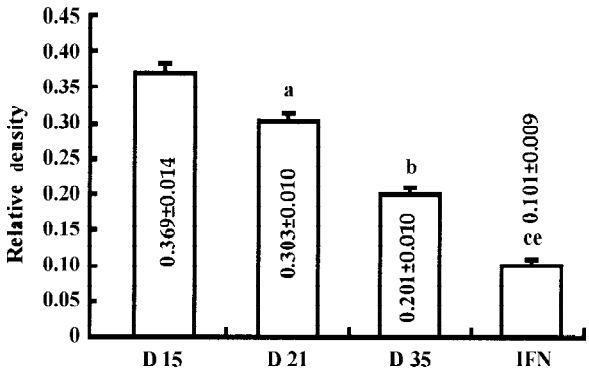

Fig 8. Relative density of TbR-II immunostaining in the CL of pregnant monkey. *n*=3. Mean±SD. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* D 15 group. [e]*P*<0.05 *vs* D 35 group.

therefore plasma progesterone level could not represent the truthful CL function. Because of the limitation of monkey CL materials available for progesterone measurement, the changes of StAR expression are consistent with the profile of progesterone in monkeys[19], therefore StAR could be used as a functional marker of CL development[4]. Our results showed that StAR was immunopositive in luteal cells and the intensity of staining was decreased in a time-dependent manner from d 15 to d 35, suggesting that the CL function was reducing in the same manner.

TGF-β1 and its receptor II localized on the granulose-luteal cells, theca-luteal cells, and fibroblasts in the CL of early pregnant monkeys. This observation is consistent with the previous reports[20,21]. Human granulose-luteal cells[20,22], marmoset corpus luteum

accessoria[9], and mouse[8] and rat corpus luteum[23] were capable of secreting TGF-β1 . Wehrenberg *et al*[21] have recently reported that TβR-II localized in the marmoset ovary. The present results were the first to show the presence of TGF-β1 and TβR-II in the corpus luteum of pregnant rhesus monkey. The expression of TGF-β1 mRNA and antigen in the CL from d 15 to d 35 was decreased in a time-dependent manner, as the same profile as StAR expression. It is therefore suggested that TGF-β1 may play an important role in corpus luteum formation and functional maintaining. Previous studies have showed that TGF-β induced significant acute effect on the release of progesterone in a dose-dependent manner, while it inhibited relaxin release in large luteal cells[24]. The 80 %-90 % of TGF-β activity in luteal cell conditioned medium of moderate-term monolayer culture measured was TGF-β1[10]. Although the precise function of TGF-β1 in corpus luteum is not clear, it may be possible to play a role in mediating CL angiogenesis, tissue remodeling by stimulating production of various ECM proteins, like fibronectin, various collegens, proteoglycans, and their integrin receptor subunits in the cells[25-27]. The changes of TGF-β1 expression in the CL were correlated with the changes of progesterone production. The increase in progersterone release by TGF-β might be partly explained by its inhibitory action on 20α-dihydro-progesterone as demonstrated in rat luteal cells in culture[28]. TGF-β may be also capable of activating the intracellular pathways related to protein kinase C activity[5].

TGF-β mediate their activity by high affinity binding to the type II receptor[6], this is consistent with our result that a progressive decrease in TGF-β parallel with its receptor TβR-II from d 15 to d 35 in the pregnant CL was observed.

Two-day IFN-γ treatment decreased the TGF-β1 antigen significantly, while the change of TGF-β1 mRNA was not significantly different between the two groups (P=0.056). It is possible that the level of TGF-β1 mRNA was so low on d 35 that the effect of IFN-γ was not significantly observed, or IFN-γ could inhibit TGF-β1 production in post-transcribe level.

Treatment of the monkeys with IFN-γ decreased both TGF-β1 and its receptor II, which was well correlated with the changes of StAR expression in the CL and also with our previous data in rat CL[29], suggesting that IFN-γ may induce CL regression mediated by down-regulation of TGF-β1 and Tβ-II. IFN-γ could abolish TGF-β1-stimulated adhesion of human mononuclear phagocytes to fibronectin and laminin[30], while TGF-β1 inhibited IFN-γ secretion[31]. TGF-β1 and IFN-γ provide opposing signals each other in many tissues[32].

In conclusion, TGF-β1 and TβR-II may play an essential role in CL functional maintaining; IFN-γ may exert a direct inhibitory effect on placentation as demonstrated previously on one hand, and affect the CL function, partly by down-regulation of TGF-β1 and TβR-II as well as StAR, on the other.

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