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IFN-GAMMA AND TNF-ALPHA INHIBIT EXPRESSION OF TGF-BETA-1, ITS RECEPTORS TBETAR-I AND TBETAR-II IN THE CORPUS LUTEUM OF PMSG/HCG TREATED RHESUS MONKEY

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1. ABSTRACT

The corpus luteum (CL) is a transient endocrine organ that secretes progesterone to support early pregnancy. Using *in situ* hybridization, immunohistochemistry and computer imaging analysis, we have investigated the expression of transforming growth factor beta 1(TGF-beta 1) , its receptors, type I (TbetaR-I) and type II (TbetaR-II) as well as steroidogenic acute regulatory protein (StAR) in the corpus luteum (CL) of the rhesus monkey at various stages of CL development. The CL was induced by injection of pregnant mare serum gonadotropin (PMSG)/human chorionic gonadotropin (hCG). The expression of TGF-beta 1, TbetaR-I and TbetaR-II as well as StAR was detected in the CL in a time-dependent manner, reaching the maximum levels on D10 (functional stage), and decreased on Day 18 (regression stage). Injection of interferon-gamma (IFN-gamma) or tumor necrosis factor-alpha (TNF-alpha) at the functional stage of CL development significantly decreased the expression of StAR, as well as TGF-beta 1, and its receptors TbetaR-I and TbetaR-II. Our results suggest that TGF-beta 1 and its receptors may play an important regulatory role in maintaining CL function, and that IFN-gamma or TNF-alpha is capable of inhibiting their expression in the CL.

2. INTRODUCTION

A CL is formed from a ruptured follicle in a dynamic process of tissue remodeling and angiogenesis. In both rodent and primate the development of CL is a rapid process with very high cellular turnover (1,2). The mature CL receives the greatest blood supply per unit tissue in the whole body and secretes the highest progesterone (3). However, if implantation is not successful, the functional phase of the CL will be terminated and luteolysis will be initiated. Therefore, tissue remodeling and angiogenesis related molecules may be involved in these processes. Just recently, we have reported the role of the plasminogen activator and its inhibitor system in the regulation of CL formation and regression in the rhesus monkey (4).

It has been suggested that the ovarian locally produced growth factors and cytokines may play an important role in regulation of CL function (3). Among a large number of locally produced ovarian growth factors, transforming growth factor betas (TGF-beta) appear to be an important candidate as an ovarian regulator, not only because it can modify the gonadotropin dependent functions (5-7), but also mediate many events of cell-cell interactions leading to cellular proliferation (8), differentiation (9), extracellular matrix and integrin modification (10), tissue repair and angiogenesis (11-15) by autocrine or paracrine functioning. TGF-betas, 25-kDa homodimers, are a family of growth factors including three closely related iso-types, TGF-beta 1,2,3 in mammal (16). TGF-betas mediate their activity by their Type I (TbetaR-I) or type II receptor (TbetaR-II)(17-20), both of which have been identified in mouse, porcine, bovine, marmoset and human corpora lutea (21-25). TGF-beta 1 has been demonstrated to play an important role in luteotropic action

of PRL by suppressing the activity of luteal 20-alpha-hydroxy-steroid dehydrogenase (20-alpha HSD) and increasing progesterone production in rat CL(7). TGF-beta 1 in luteal cells may be the main functional type of TGF-beta family (23) and exerts an acute effect on progesterone production in bovine CL (26) and supports luteal function by suppression of luteal cell apoptosis (27). Our previous studies showed that the expression of TGF-beta 1 and TbetaR-II progressively decreased at the regression stage of CL development from D15 to D35 in the pregnant monkey (28), suggesting that TGF-beta 1 and its receptors may play a role in regulating CL function in the rhesus monkey.

To assess the role of TGF-beta 1 signal pathway in the regulating CL function, StAR, a functional marker of CL development (29,30), was also detected.

IFN-gamma and TNF-alpha are potent biological response modifiers which have been known to be luteolytic cytokines released by leukocytes, macrophages in the CL (31). Several reports have demonstrated that IFN-gamma and TNF-alpha promoted luteolysis and reduced serum progesterone levels (29,32-34).

Because of the limitation of the tissue, little information is available in the literature regarding the expression and regulation of TGF-beta 1 and its receptors, TbetaR-I and TbetaR-II, in monkey CL. In this study, we have designed experiments of rhesus monkey with various time points of CL development induced by injection of PMSG/hCG and investigated the co-expression of TGF-beta 1, its receptors TbetaR-I, TbetaR-II and StAR, a functional marker of CL, in the monkey CL and further examined their possible regulation by IFN-gamma and TNF-alpha at the functional stage of CL development.

3. MATERIALS AND METHODS

3.1. Reagents

Polyclonal antibodies against human TGF-beta 1,TbetaR-I and TbetaR-II raised in rabbits 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 labeling 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. Proteinase K was purchased from Merck-Schuchardt (Hobenbrunn). Restriction enzymes, recombined human-IFN-gamma and recombined human-TNF-alpha were purchased from Promega. Diethyl pyrocarbonate (DEPC) and detrasulphate were purchased from Sigma.

3.2. Animals

Fifteen female rhesus monkeys aged 5-7 years old were divided into five groups randomly. All animals were obtained from the monkey colony of the Primate Research Center, Kunming Institute of Zoology, Chinese Academy of Sciences. The monkeys were permitted to use for these experiments by the Institute Ethic Committee and WHO Project Review Committee. The animals were housed under the controlled environmental conditions with free access to water and food. Before hormone treatment, two to three successive estrous cycles were observed, and only 26-28 day's cycling monkeys were housed for the experiment. To induce ovulation and luteinization, initial dose of 950 IU PMSG was given i.m. on the second day of menses. From day 2 to day 7, 300 IU PMSG was administered every other day, followed by treatment with 300 IU PMSG daily for an additional 5 days. On day 13, a single injection of 5000 IU hCG was administered and this day was designated as day 1 of CL development. At various stages (D5, D10, D18) of the hCG treatment, the ovaries were removed by abdominal surgery under anesthetization. The ovaries were fixed in formalin's fluid for *in situ* hybridization and immunohistochemistry. Groups of rhesus monkeys were injected with TNF-alpha (50,000 IU) or IFN-gamma(50,000IU) on day 8 and 9, and the ovaries were removed on day 10.

3.3. Synthesis of the DIG-Labeled RNA probe for TGF-beta1

The DIG-labeled RNA was synthesized as previously reported (29). In brief, the plasmids that contain the cDNA fragment of TGF-beta 1 were 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. 2 µl RNA polymerase, 4µl 5x buffer, 2µl mix, 1µg linearized plasmid and Rnasin were added to an Eppendorf tube and mixed, followed by DEPC-treated water to a total volume of 20µl, and incubated for 2h at 37C. The validation of the labeled probe was evaluated with Dot Blot analysis.

3.4. *In situ* hybridization

Paraffin embedded sections were deparaffinized in fresh xylene (2×10min), xylene:100% alcohol (1:1, 5min), 100% alcohol (5min), 95% alcohol (5min), 90% alcohol (5min), 80% alcohol (5min), 70% alcohol. The slides were washed in DEPC-treated PBS (3×5min), and permeablized with proteinase K (10µg/ml) in PK buffer (100mM Tris-HCl, 50mM EDTA, pH8.0) for 20min. The sections were washed in PBS (2×5min), and post-fixed with 4 percent paraformaldehyde in PBS (4C, 5min), the slides were washed with PBS (2×5min) and DEPC-treated H_2O (5min). The sections were dehydrated with serial alcohol, air dried and then incubated in prehybridization buffer (2×SSC, 50% deionized formamade, RT, 2-4hr).

After prehybridization, the hybridization solution was applied onto the slides and covered with paraffin film. Hybridization solution were made by mixing DIG-labeled cRNA probes (20-30ng per slide) with 100µl hybridization buffer (2×SSC, 50% deionized-formamide, 10mM Tris-HCl, 250µg/ml yeast tRNA, 0.5%SDS, 1×Denhardt, 10mM DTT, 10% dextra-sulphate). The sections were incubated 16-20 hr at 48-50^{o}C. At the end of hybridization, the paraffin film were removed by incubating the slides in 4×SSC, subsequently slides were washed with 2×SSC (2×15min, RT), 1×SSC 2×15min, 42C,

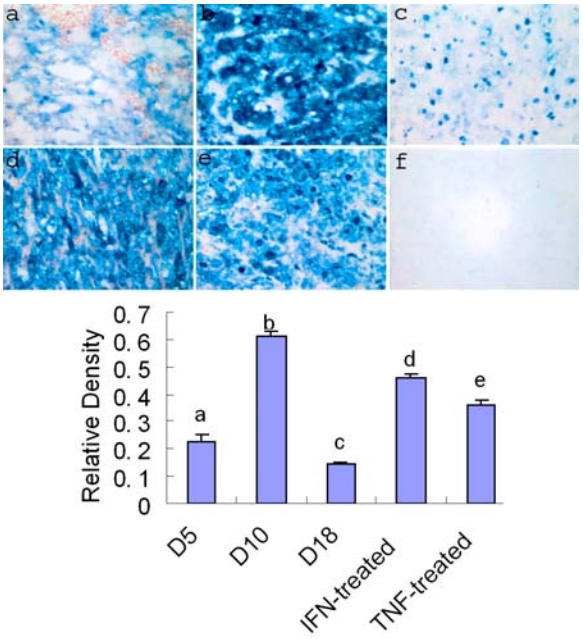

Figure 1. Top. Expression and regulation of TGF-beta 1 mRNA in the CL of the superovulated monkeys. Cryostat sections (6μm) were hybridized with a digoxigenin-labeled antisense RNA probe, a sense RNA probe was used as the negative control. Specific hybridization was localized in the steroidogenic cells. Treatment of the monkeys with IFN-gamma and TNF-alpha was carried out on Day 8 and Day 9, the CLs were removed on Day 10. The expression of TGF-beta1 mRNA was detected throughout all the stages and changed in a time-dependent manner (a,b,c). IFN-gamma (d) or TNF-alpha (e) decreased the expression of TGF-beta1 mRNA. a) D5, b) D10, c) D18, d) IFN-gamma treated group, e) TNF-alpha treated group , f) The sense probe negative control. Original magnification X 400. Bottom. Relative density of TGF beta1 mRNA in the CL of superovulated monkey. Image screened from 5 different areas with the same large and at the comparative positions were transformed into grey value by Image-Pro-Plus and analyzed by ANOVA followed by T-test. Data are the mean ± SEM of three experiments in two to three monkeys. Values with different letter subscript are significantly different ($p<0.05$)

0.1×SSC 2×15min, 42C. Sections were washed by shaking for 10 min with Buffer 1 100mM Tris-HCl, 150mM NaCl and then sections were covered with buffer 2 buffer 1 containing 1% blocking reagent for 1 hr. Sections were incubated in humid chamber with blocking solution containing anti-DIG alkaline phosphatase antibody Fab fragment at a dilution of 1:200. Sections were washed by shaking in buffer 1 3×10min , and incubated with buffer 3 [100mM Tris-HCL(pH9.5), 100mM NaCl, 50mM $MgCl_2$] for 5-10 min. Sections were covered with color generating solution [1 ml buffer 3, 4.5μl NBT solution (75mg NBT/ml 70% dimethyl formamiade), 3.5μl BCIP solution (50mg BCIP/ml 100% dimethyl formamide)], and sections were incubated in a humid chamber for 2-7hr in the dark. When the color development was optimal, the reaction was stopped by incubating the slides in buffer.

3.5.Immunohistochemistry

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

3.6. Statistical analyses

Each group in this study included three monkeys. The data for *in situ* hybridization and immunohistochemistry were obtained from at least two independent experiments with the CL sections of three monkeys. A representative photograph was shown. The relative density data of the immunohistochemistry and *in situ* hybridization represent mean ± SEM of three individual experiments from two to three monkeys in each group. Images screened from 5 different areas with the same large and at the comparative positions were transformed into grey value by Image-Pro-Plus. All data were analyzed using one-way analysis of variance (SPSS for Windows, Chicago, Illinois). If the differences were significant, a Student-Newman-Keuls test was used for post ANOVA multiple comparisons ($P < 0.05$).

4.RESULTS

No measurable amount staining was detected in the negative control for StAR, TGF-beta 1 or its receptors, TbetaR-I and TbetaR-II.

4.1. Expression of TGF-beta1 mRNA in the CL

As shown in Figure 1, the expression of TGF-beta1 mRNA was detected in the CL of the superovulated rhesus monkeys. The messenger RNA mainly localized in the granulosa-luteal and theca-luteal cells, fibroblasts in a time-dependent manner, reaching the maximum levels on D10, and remarkably decreased on Day 18. Injection of IFN-gamma or TNF-alpha on day 8 and 9 significantly decreased the messenger RNA production examined on day 10 as compared with the control (Figure 1)

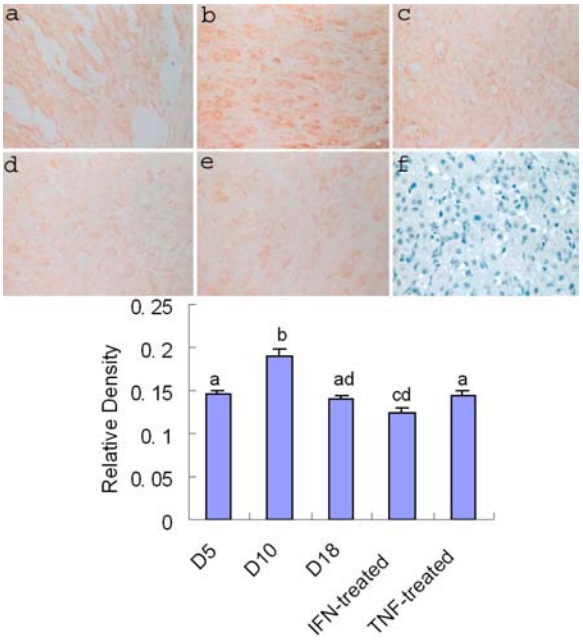

Figure 2. Top. Immunohistochemical detection of TGF-beta 1 protein in the CL of superovulated monkey. Cryostat sections (6μm) were incubated with TGF-beta 1 polyclonal primary antibody, replacement of the primary antibody with normal IgG as the negative control. Specific cytoplasmatic staining was located in the steroidogenic cells. The expression of TGF-beta 1 was detected throughout all the stages, and changed in a time-dependent manner. Injection of IFN-gamma or TNF-alpha on D8 and D9 decreased the expression of TGF-beta 1 examined on D10. a) D5, b) D10, c) D18, d) IFN-gamma treated group, e) TNF-alpha treated group, f) negative control (stained with haematoxylin). Original magnification x 400. Bottom. Relative density of TGF-beta 1 immunostaining in the CL of superovulated monkey at various stages. The CLs were obtained as indicated in the Figure1 top. Image screened from 5 different areas with the same large and at the comparative positions were transformed into grey value by Image-Pro-Plus and analysed by ANOVA followed by T-test. Data are the mean ± SEM of three experiments in three monkeys. Values with different letter subscript are significantly different ($p<0.05$).

4.2. Immunohistochemical localization of TGF-beta 1 in the CL

TGF-beta 1 antigen in the CL of the superovulated monkey was assessed. Intensity of the immunopositive staining from D5 to D18 was obviously distinguishable. Expression of TGF-beta 1 protein peaked on D10 (mid-luteal phase) and decreased on D18 (late phase). Treatment with IFN-gamma and TNF-alpha on Day 8 and 9 significantly decreased the expression of TGF-beta1 antigen in the CL obtained on D10 as compared with the control group (Figure 2).

4.3. Immunohistochemical localization of TbetaR-II in the CL

Immunopositive TbetaR-II reaction was detected in the CL on Day 5, Day10 and Day18 of the superovulated monkeys. The expression of the TbetaR-II peaked in the middle luteal phase (D10) and significantly reduced during the late phase (D18). Two day's treatment of the CL with IFN-gamma or TNF-alpha decreased the TbetaR-II production as compared with that in the control group (Figure 3)

4.4. Immunohistochemical localization of TbetaR-I in the CL

Immunohistochemical observation indicated that immunoreactive TbetaR-I was present in the CL, with the highest intensity in the functional stage (D10). Treatment with IFN-gamma or TNF-alpha on Day 8 and 9 significantly decreased the expression of TbetaR-I protein obtained on D10 as compared with that of the control group (Figure 4).

4.5. Immunohistochemical localization of StAR in the CL

The StAR has been demonstrated to be a reliable functional marker of CL development, the expression of StAR antigen in the CL was also examined. It peaked on D10, and significantly reduced during the late luteal phase (D18). Two day's IFN-gamma or TNF-alpha treatment of the CL decreased the StAR production, as compared with that in the control group (Figure 5).

5. DISCUSSION

In our previous study we have examined the expression of TGF-beta1 and its receptor TbetaR-II as well as StAR at various stages of CL development and looked at the action of IFN-gamma on their expression at the regression phase of CL development in the pregnant monkey (28). To further explore the possible action of the cytokines, the effect of both IFN-gamma and TNF-alpha on TGF-beta 1 and its two receptors, TbetaR-I and TbetaR-II, as well as StAR in the functional stage of CL development was further examined to confirm the possible demise action on CL development. In the present study, we have designed experiments using a well-defined PMSG/hCG-induced luteinized rhesus monkey model and demonstrated that the expression of TGF-beta1 was observed with a strong staining at early and middle luteal phase and reduced in the late phase. This observation is partly consistent with the initial report in human (25). The changes of TGF-beta 1 was the same profile as that of the StAR expression**,** the CL functional marker molecule (29,30), in a time- and space-dependent manner in the CL, suggesting that TGF-beta 1 may play an important role in the CL formation and functional maintaining. Previous studies have shown that TGF-beta exerted a significant effect on progesterone production in a dose-dependent manner in the large luteal cells (26). Evidence was also showed that TGF-beta 1 could suppress the activity of luteal 20 alpha-hydroxy-steroid dehydrogenase (20-alpha HSD) in the rat luteal cells (7), which catabolizes progesterone into biologically inactive 20-dihydroprogesterone. In addition, TGF-beta 1 supported luteal function by reducing the cell apoptosis in the human luteinized granulosa cells (27). Although the precise function of TGF-beta 1 in the CL is not clear, it may be possible to mediate CL angiogenesis, tissue

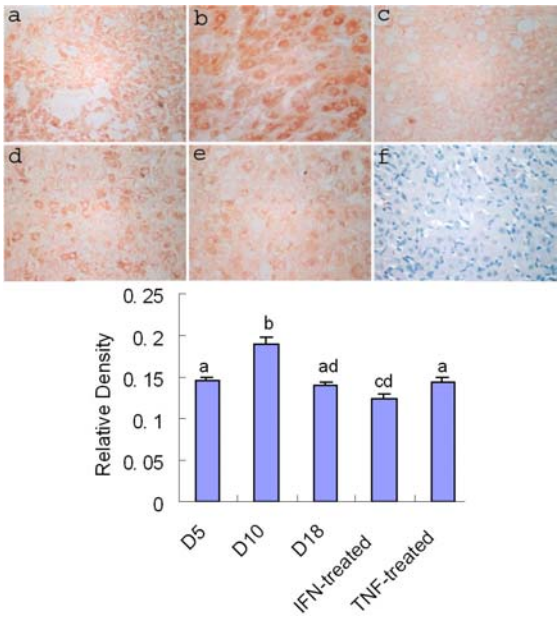

Figure 3. Top. Immunolocalization of TbetaR-II protein in the CL of superovulated monkey at various stages (a,b,c), IFN-gamma treated group (d) and TNF-alpha treated group (e). Positive immunoreactivity was observed in the steroidogenic cells. The expression of TbetaR-II was detected throughout all the stages and changed in a time-dependent manner, IFN-gamma or TNF-alpha decreased the expression of TbetaR-II. a) D5, b) D10, c) D18, d) IFN-gamma treated group, e) TNF-α treated group, f) negative control (stained with haematoxylin), replacement of the primary antibody with normal IgG. Original magnification x 400. Bottom. Relative density of TbetaR-II immunostaining in the CL of superovulated monkey at various stages. Image screened from 5 different areas with the same large and at the comparative positions were transformed into grey value by Image-Pro-Plus and analyzed by ANOVA followed by T-test. Data are the mean ± SEM of three experiments in two to three monkeys. Values with different letter subscript are significantly different ($p<0.05$).

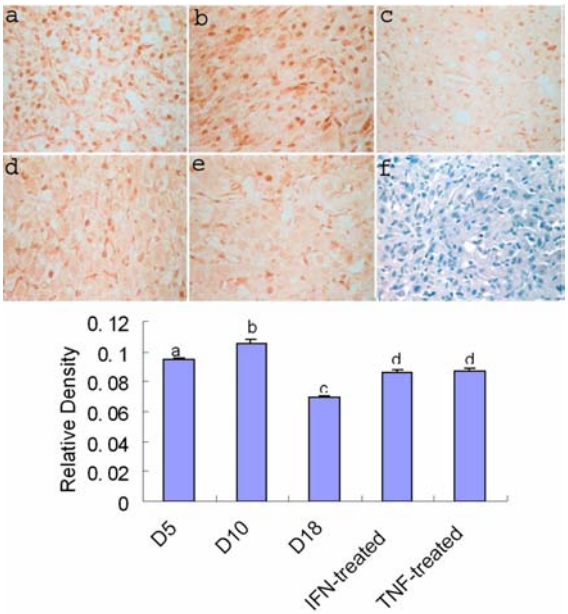

Figure 4. Top. Immunolocalization of TbetaR-I protein in the CL of superovulated monkey at various stages. Cryostat sections (6μm) were incubated with TbetaR-I polyclonal primary antibody, replacement of the primary antibody with normal IgG as the negative control. Specific cytoplasmatic staining was located in the steroidogenic cells. The expression of TbetaR-I was detected throughout all the stages and changed in a time-dependent manner, IFN-gamma or TNF-alpha decreased the expression of TGF-beta1. a) D5, b) D10, c) D18, d) IFN-gamma treated group, e) TNF-alphatreated group, f) negative control (stained with haematoxylin). Original magnification x 400. Bottom. Relative density of TbetaR-I immunostaining in the CL of superovulated monkey at various stages. The CLs were obtained as indicated in the Figure 4 top. Image screened from 5 different areas with the same large and at the comparative positions were transformed into grey value by Image-Pro-Plus and analyzed by ANOVA followed by T-test.. Data are the mean ± SEM of three experiments in three monkeys. Values with different letter subscript are significantly different ($p<0.05$).

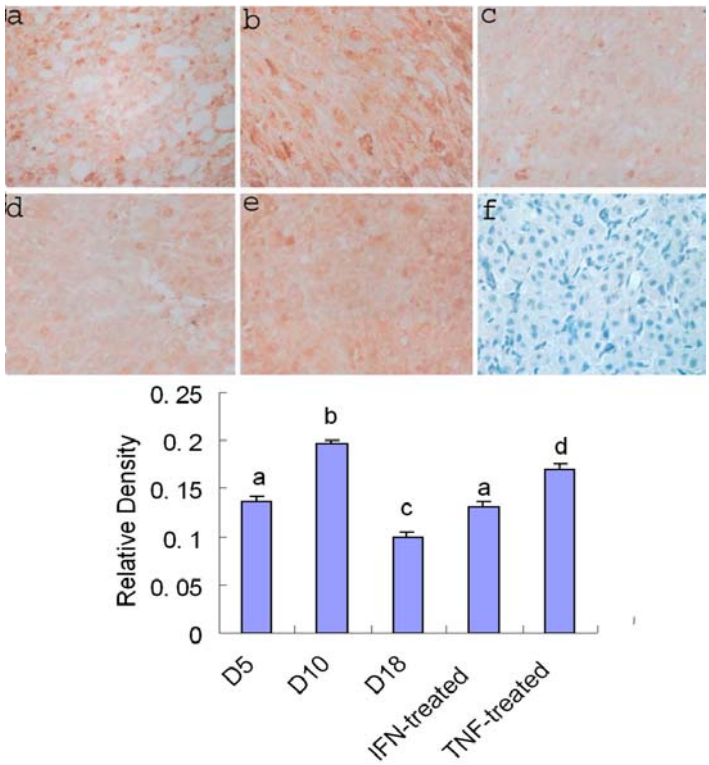

Figure 5. Top. Immunolocalization of StAR protein in the CL of superovulated monkey at various stages. Cryostat sections (6μm) were incubated with StAR polyclonal primary antibody, replacement of the primary antibody with normal IgG as the negative control. Specific cytoplasmatic staining was located in the steroidogenic cells. The expression of StAR was detected throughout all the stages and changed in a time-dependent manner, IFN-gamma or TNF-alpha decreased the expression of StAR. a) D5, b) D10, c) D18, d) IFN-gamma treated group, e) TNF-alphatreated group, f) negative control (stained with haematoxylin). Original magnification x 400. Bottom. Relative density of StAR immunostaining in the CL of superovulated monkey at various stages. The CLs were obtained as indicated in the Figure 5 top . Image screened from 5 different areas with the same large and at the comparative positions were transformed into grey value by Image-Pro-Plus analysed by ANOVA followed by T-test. Data are the mean ± SEM of three experiments in three monkeys. Values with different letter subscript are significantly different ($p<0.05$).

remodeling by stimulating the production and deposition of extracellular matrix which is known to be important factors in cellular proliferation, differentiation, angiogensis and steroid synthesis (7-15). We have also demonstrated in this study that TbetaR-I and TbetaR-II co-localized in the luteal cells with TGF-beta 1, the pattern and cellular distribution of the TGF-beta 1 two receptors, TbetaR-I and TbetaR-II, were similar to that of the TGF-beta 1. The biological activities of TGF-beta 1 are known to be mediated through binding to its receptor TbetaR-II and then TbetaR-I, both of which are transmembrane proteins with a cytoplasmic serine/threonine kinase domain (20). TbetaR-I has been suggested to be the functional TGF-beta receptor, whereas type II receptor is essential for the binding of TGF-betas**.** TbetaR-II directly binds TGF-betas, enabling TGF-beta to recognize TbetaR-I and to form the receptor complex, and then to generate the intracellular signal through SAMD pathway (17-20).

Treatment of the monkeys at the functional phase of CL development with IFN-gamma or TNF-alpha decreased the expression of TGF-beta 1 and its receptors, TbetaR-I and TbetaR-II, as well as the CL functional marker molecule StAR. We have demonstrated previously that StAR is a reliable functional marker of CL function in both rat and monkey (4,29,32), and injection of IFN-gamma or TNF-alpha in rat was capable of inhibiting CL progesterone and StAR production in the PMSG/hCG induced pseudopregnant rat (29). In the present study we have further demonstrated that the changes of TGF-beta 1 and its receptors TbetaR-I and TbetaR-II in the primate CL were well correlated with the changes of StAR expression and down-regulated by IFN-gamma or TNF-alpha. Because IFN-gamma could neutralize TGF-beta 1-stimulated adhesion of human mononuclear phagocytes to fibronectin and laminin (33), while TGF-beta1 inhibited IFN-gamma secretion (34), therefore it is possible that IFN-gamma or TNF-alpha down-regulates progesterone production by decreasing progesterone biosynthesis through reducing StAR expression on one hand, and increases progesterone digestion by inhibiting the expression of TGF-beta 1 on the other, which might promote progesterone production by inhibiting 20-HSD catabolizing progesterone into biologically inactive 20-dihydroprogesterone and block CL cell apoptosis (7). Because the limited number of animals, and only the descriptive methods were used in this study, coincidential changes of the observed molecules may be present. To further clear the molecular mechanism, the

cellular and molecular details should be further examined using an in vitro system by blocking or enhancing the action of TGF-beta 1 and its receptors to look at their actions on the CL development.

6. ACKNOWLEDGMENTS

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