

Effects of TGF beta-1 on mouse embryo implantation and expression of H2-D1 and H2-DM

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1. ABSTRACT

We investigated the mechanism that TGF-beta1 influences the immuno-environment at maternal-fetal interface and affects embryo implantation, using mouse uterine horn injection model. The expression of MHC I antigen (H2-D1) and the chaperone of MHC II antigen (H2-DM) after anti-TGF-beta1 antibody or hrTGF-beta1 treated pregnant mice were examined by real-time PCR, western blotting and immunohistochemistry. The results showed that the number of implanted embryos of anti-TGF-beta1 antibody-treated mice was decreased compared with the control. The expression of H2-D1 and H2-DM on days 6 and 7 treated uteri was increased both at mRNA and protein levels. In hrTGF-beta1 treated group, the expression of H2-D1 and H2-DM protein was decreased, and the number of implanted embryos was slightly increased. Immunohistochemical studies revealed that H2-D1 and H2-DM were mainly localized to the primary decidual zone. The anti-TGF-beta1 antibody and exogenous hrTGF-beta1 treatment altered the intensity of H2-D1 and H2-DM signal but did not change their localization. These observations suggested that injection of anti-TGF-beta1 antibody affected the number of mouse embryo implantation, and regulated the expression of H2-DM and H2-Q10.

2. INTRODUCTION

Members of TGF-beta family are pleiotropic cytokines that regulate cellular differentiation, proliferation, motility, adhesion, and apoptosis (1, 2). They are abundant in mammalian reproductive tissues and closely associated with reproduction. As a major member of TGF-beta family, TGF-beta1 has been detected in uterine deciduas, endometrium, placenta and the endoderm (3-5). TGF-beta1 contributes to the control of trophoblast invasion, decidualisation and placental development, modulation of oocyte maturation, embryo implantation, and inflammatory responses during pregnancy (6, 7). TGF-beta1 is vital because approximately 50% of TGF-beta1 gene knockout mice die *in utero* and the remainder succumbs to uncontrolled inflammation after birth (8, 9), suggesting that TGF-beta1 may function as a regulatory factor during pregnancy.

The major histocompatibility complex (MHC) is a genetic region that encodes MHC class I and class II antigens. Their main function is to present antigenic peptide to CD8+(cytotoxic) and CD4+(helper) T cells respectively, thus eliciting an immune response. DM, a class II-like heterodimer, functions as a chaperone to promote the capture of diverse peptides by MHC class II

Table 1. Primer sequences

	Forward primer 5'-3'	Reverse primer 5'-3'
H2-D1	CGCTCCGCTACTACAACCG	TCTGTGCCGCAAAGTCCG
H2-DM	TGCCTGAGTTTGCTGAGTGG	AAGTCGGTGGAAGAGGTTGC
GAPDH	TCCCACTCTTCCACCTTCG	TCTCTTGCTCAGTGTCTTG

molecules inside the endocytic compartments of APC (10). During normal pregnancy, the maternal immune system undergoes various changes that lead to tolerance of the fetus. Regulation of MHC antigen expression and tissue localization at maternal-fetus interface is critical to the implantation, survival and development of the fetus. Several lines of evidence suggested that the expression of MHC is associated with reproductive performance (11, 12).

Cytokines are small, secreted, membrane-bound proteins that act through cell-surface receptors and generally induce changes in gene expression within their target cells. They are expressed during gestation and form a complicated interdependent network of interactions taking place at different stages of reproduction. Cytokines (e.g., ILs, IFNs and TNFs) can modulate MHC expression throughout pregnancy (13). Our previous studies showed that IFN- γ and IL-1 β can modulate the expression of MHC, and IFN- γ can regulate IL-1 β and TGF- β 1 protein expression during pregnancy (14-16). Previous studies described that TGF- β 1 can prevent immune rejection of transplants by inhibiting the expression of MHC antigen, limiting T-cell activation during the immune response (17). But the effect of TGF- β 1 on MHC molecules during peri-implantation is not well understood.

In the present report, we chose to study the classical class I MHC antigen (H2-D1) and class II-like heterodimer (H2-DM) to investigate the relation between TGF- β 1 and MHC antigens. H2-D1 and H2-DM are homologous to RT1-A and RT1-DM in the rat, as we have previously reported (14, 15). Using mouse uterine horn injection model, we investigated the effect of anti-TGF- β 1 antibody or exogenous human recombinant TGF- β 1 (hrTGF- β 1) treatment on mouse embryo implantation and the expression of H2-D1 and H2-DM.

3. MATERIALS AND METHODS

3.1. Animals and tissue collection

Forty adult healthy female Kunming White mice strain (12 wk old, 25–30g body weight) were provided by the Experimental Animal Center of Institute of Genetics and Developmental Biology at the Chinese Academy of Sciences. Mice were housed in a temperature- and humidity-controlled room with a 12/12 h light and dark photoperiod, with free access to food and water. Two female were caged with a male overnight for mating. The following morning, females were checked for the presence of a vaginal sperm plug, and the day of vaginal plug was designated as day 1 of pregnancy (D1). All of the procedures were approved by the local animal care and use committee at the Institute of Zoology.

The uterine horn injection method was detailed previously by Ramos (18). Briefly, Gravid mice were anesthetized by injection of ethyl carbamate (20% w/v

solution, 1g/kg; Sigma) for surgery, and randomly assigned into four equal groups (n=10 per group). Two groups of pregnant mice (D2 and D3), were injected with 2 μ g/10 μ L rabbit polyclonal anti-TGF- β 1 antibody (Santa Cruz, CA) into one uterine horn, while the contra-lateral uterine horn received equal volume of 0.01M phosphate-buffered saline (PBS). Similarly, the other two groups of pregnant mice were injected with 50ng/10 μ L hrTGF- β 1 (Pep Rotech, USA). Treated animals were sacrificed on D6 and D7, respectively. The number of implanted embryos was counted in each uterine. The uteri were dissected on ice, snap-frozen in liquid nitrogen and were stored at –80°C for RNA and protein extraction, as well as cryostat sections.

3.2. Real-time PCR

Total RNA was extracted from the uteri with Trizol reagent (Invitrogen, USA) according to the manufacture's instructions. RNA pellets were gently resuspended in 30 μ L of nuclease-free water. The integrity of the purified RNA was confirmed by visualization of the 28S and 18S rRNA bands after the electrophoresis of RNA through a 1.5% (w/v) agarose-formaldehyde gel (Promega, USA). Total concentration of RNA was determined and the samples were stored at –80°C until use.

Two μ g of total RNA were reverse transcribed in the presence of 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) according to the manufacturer's instructions. Reverse transcription of total RNA was carried out in the presence of oligo-dT18 to obtain cDNA for quantitative real-time PCR.

Quantitative real-time PCR reactions were carried out using SYBR Green PCR master mix reagents (TaKaRa Biotechnology Co., Ltd.) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The primers used for target amplification are described in table 1.

Thermal cycling was conducted at 95°C for 10 s, followed by 40 cycles of amplification at 95 °C for 5 s and 60°C for 31 s. After PCR, the first derivative melting curve analysis was performed to confirm the specificity of the PCR. All samples were tested in triplicate, and each run included no-template and no-RT controls. For the mathematical analysis, it is necessary to determine the Ct value for each transcript. The Ct value represents the cycle number at which a fluorescent signal rises statistically above background. Relative quantification of gene expression was normalized to GAPDH.

3.3. Western blotting

Protein samples of the uteri (50 μ g) were separated using a 12% polyacrylamide gel for 2 h and transferred onto nitrocellulose membrane (Pall, USA). After blocking for 3 h with 5% dried fat-free milk in Tris-buffered saline-Tween (TBST) (20mM Tris-HCl, pH7.6; 137 mM NaCl; 0.1% Tween-20), the membranes were

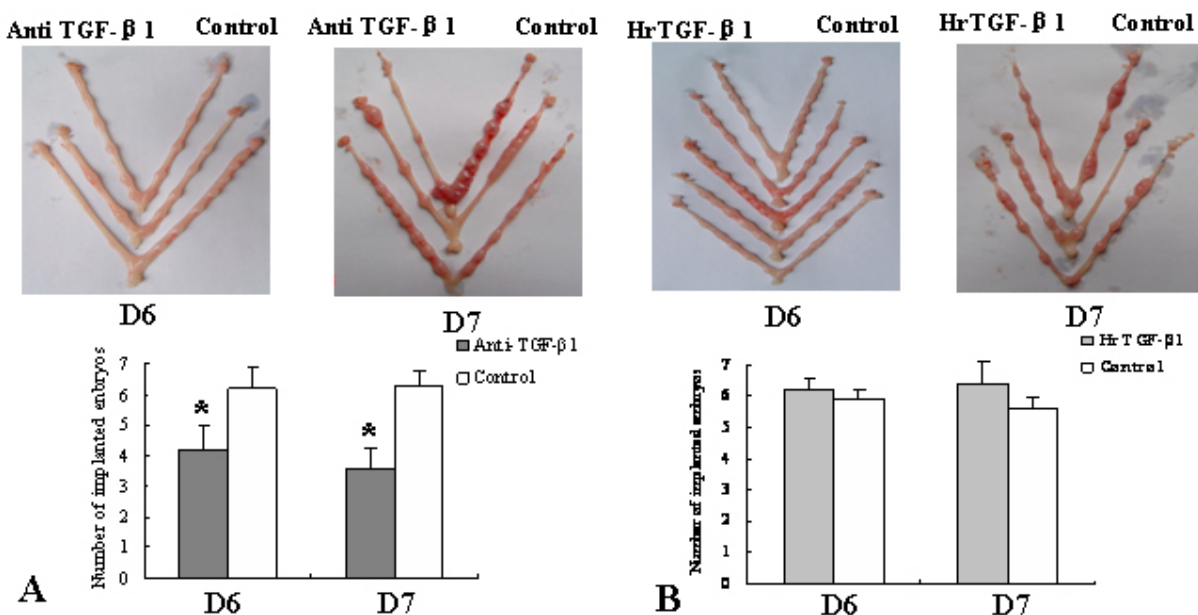


Figure 1. Effect of anti-TGF-beta1 antibody and hrTGF-beta1 on mouse embryo implantation. A: Examples of intrauterine injection of anti-TGF-beta1 antibody and their statistical analysis of implanted embryo number. Anti-TGF-beta1 antibody was injected into mouse uterine lumen on D2 and D3 of pregnancy, and their uteri were harvested on D6 and D7. Controls were treated with PBS. As shown, injection of anti-TGF-beta1 antibody severely decreased total number of implantation sites compared that of the PBS-treated controls on D6 (4.2 ± 0.34 vs. 6.2 ± 0.68 , $n = 10$, $*p < 0.05$) and D7 (3.6 ± 0.26 vs. 6.3 ± 0.36 , $n = 10$, $*p < 0.05$). B: Examples of intrauterine injection of hrTGF-beta1 and their statistical analysis of implanted embryo number. HrTGF-beta1 was injected into mouse uterine lumen on D2 and D3 of pregnancy, and their uteri were harvested on D6 and D7. Controls were treated with PBS. As shown, no obvious difference was founded in hrTGF-beta1 treatment on D6 (6.2 ± 0.45 vs. 5.9 ± 0.34 , $n = 10$, $p > 0.05$) and D7 (6.4 ± 0.26 vs. 5.6 ± 0.35 , $n = 10$, $p > 0.05$).

incubated for 1 h with the primary antibody, either a mouse anti-H2-D1 (Harlan Sera-Lab, Loughborough, UK) or a goat anti-H2-DM (Santa Cruz Biotechnology, Santa Cruz, CA). The specific protein antibody complex was detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse or rabbit anti-goat IgG (Santa Cruz) and an enhanced chemiluminescence (ECL) detection kit (Amersham, U.S.A.). Equal loading of samples was demonstrated by stripping the blots and re-probing with polyclonal goat anti-actin antibody (Santa Cruz). Band intensity was quantitated using Bio-Rad Quantity One software (Bio-Rad) and normalized to actin.

3.4. Immunohistochemistry

Sections ($10 \mu\text{m}$) from the uteri were cut on a freezing microtome and collected on 3-amino-propyl-triethoxy-silane (APES)-coated glass slides. Frozen sections were dried at room temperature, fixed with 4% paraformaldehyde in PBS for 30 min and then washed three times with PBS. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 5 min and washed in PBS. After blocking with 5% nonfat dry milk for 20 min and then normal horse serum for 20 min, sections were incubated with mouse anti-H2-D1 (Harlan) or goat anti-H2-DM (Santa Cruz) diluted to 1 to 200 in PBS at 4°C overnight. Sections were again washed in PBS and then incubated with the

secondary antibody (goat anti-mouse or rabbit anti-goat IgG conjugated with HRP) at 37°C for 1 h. The antibody stains were developed by addition of diaminobenzidine (DAB). To evaluate the specificity of the antibodies, substituting normal horse serum or goat serum for the primary antibodies served as negative controls.

3.5. Statistical analysis

Values were reported as mean \pm S.E.M. The significance of the difference was assessed by one-way ANOVA or paired t -test on SPSS 15.0 software (SPSS Software, Chicago, USA). $P < 0.05$ was considered statistically significant.

4. RESULTS

4.1. Effect of anti-TGF-beta1 antibody treatment on embryo implantation

To determine the TGF-beta1 function in pregnant mouse, anti-TGF-beta1 antibody was injected into the lumen of the normal pregnant uteri on D2 and D3 respectively, and the number of implanted embryos was counted in each uterine on D6 and D7 respectively. As shown in Figure 1A, anti-TGF-beta1 antibody treatment significantly reduced the number of implanted embryos compared with controls (d6: 4.2 ± 0.34 vs. 6.2 ± 0.68 ; d7: 3.6 ± 0.26 vs. 6.3 ± 0.36 ; $n = 10$ and $p < 0.05$)

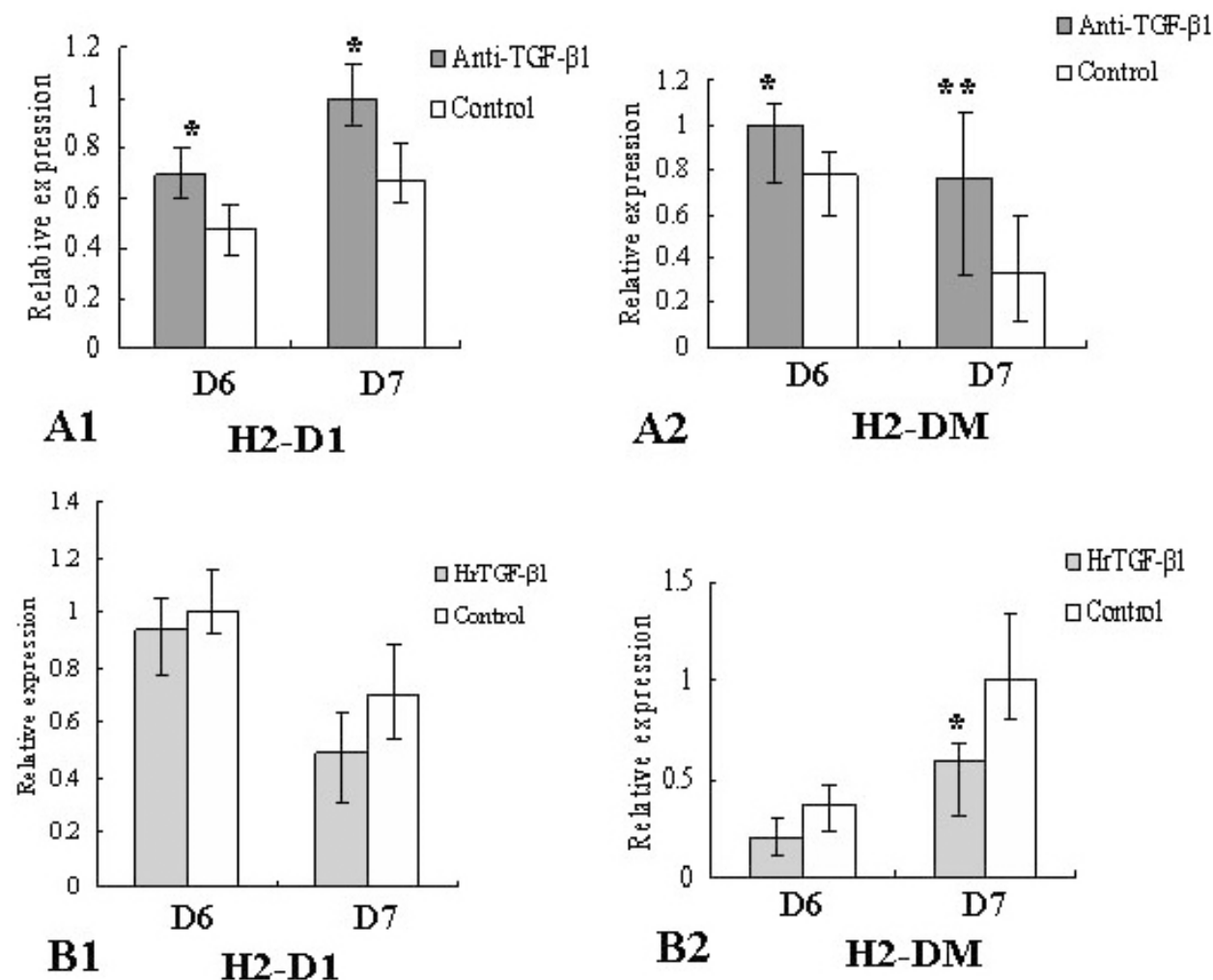


Figure 2. Effect of TGF-beta1 on mouse uteri H2-D1/H2-DM mRNA expression. Relative levels of mouse uteri H2-D1/H2-DM mRNA (presented as geometric means \times dispersion factor^{±1}) by real-time PCR. Controls were treated with PBS. A1: H2-D1 mRNA levels from intrauterine injection of anti-TGF-beta1 antibody group on D6 and D7. After the treatment, the expression of H2-D1 was increased compared with the PBS-treated controls on D6 (*P <0.05) and D7 (*P <0.05). A2: H2-DM mRNA levels from intrauterine injection of anti-TGF-beta1 antibody group on D6 and D7. After the treatment, the expression of H2-DM was increased compared with the PBS-treated controls on D6 (*P<0.05) and D7 (**P<0.01). B1: H2-D1 mRNA levels from intrauterine injection of hrTGF-beta1 group on D6 and D7. After the treatment, the expression of H2-D1 was no significant difference compared with the PBS-treated controls on D6 (P >0.05) and D7 (P >0.05). B2: H2-DM mRNA levels from intrauterine injection of hrTGF-beta1 group on D6 and D7. After the treatment, the expression of H2-DM was decreased significantly compared with the PBS-treated controls on D7 (*P<0.05), and no significant change on D6 (P >0.05).

4.2. Effect of anti-TGF-beta1 antibody treatment on H2-D1

Mouse uteri were collected on D6 and D7 after uterine horn injection of anti-TGF-beta1 antibody. Real-time PCR was employed to study mRNA expression changes of the uteri H2-D1. Figure 2A1, showed that uterine horn injection of anti-TGF-beta1 antibody increased the level of H2-D1 mRNA over that of the control in the pregnant uteri (P <0.05).

After uterine horn injection of anti-TGF-beta1 antibody on D2 and D3, western blotting results

demonstrated that the expression of H2-D1 protein in pregnant uteri of D6 and D7 was increased when compared to the control (Figure 3A 1, P <0.05 and P <0.01, respectively).

Immunohistochemical studies showed that H2-D1 staining was mainly localized in the primary decidual zone around the embryo. Anti-TGF-beta1 antibody treated group displayed a more intensely expression of H2-D1 when compared with that of control, but did not affect their localization at the maternal-fetal interface (Figure 4 A vs C).

4.3. Effect of anti-TGF-beta1 antibody treatment on H2-DM

As shown in Figure 2A2, real-time PCR results showed that uterine horn injections of anti-TGF-beta1 antibody increased the levels of H2-DM mRNA in D6 and D7 pregnant uteri ($P < 0.05$ and $P < 0.01$, respectively).

Western blotting results showed that anti-TGF-beta1 antibody treatment significantly increased the expression of H2-DM protein in D6 pregnant uteri compared with control group (Figure 3A2, $P < 0.05$), but no significant change was observed in D7 pregnant uteri ($P > 0.05$).

Protein localization of H2-DM was similar to H2-D1 at the maternal-fetal interface in uterine horn injection of anti-TGF-beta1 antibody. The intensity of the signal markedly increased in the primary decidual zone around the embryo when compared to control (Figure 4D vs F).

4.4. Effect of exogenous hrTGF-beta1 treatment on embryo implantation

In the mouse uterine horn injection model, administration of exogenous hrTGF-beta1 (50ng) was injected into the lumen of the normal pregnant uteri on D2 and D3, which was found to increase TGF-beta1 protein in normal pregnant uteri before implantation. After the treatment, the increase in the number of implanted embryos was not significant when compared to controls (Figure 1B) (D6: 6.2 ± 0.45 vs 5.9 ± 0.34 , $n = 10$, $p > 0.05$; D7: 6.4 ± 0.26 vs 5.6 ± 0.35 , $n = 10$, $p > 0.05$).

4.5. Effect of exogenous hrTGF-beta1 treatment on H2-D1

Mouse uteri were collected on D6 and D7 after uterine horn injection of hrTGF-beta1. Real-time PCR was employed to investigate the mRNA expression changes in H2-D1 expression. As shown in Figure 2B1, there was no obvious effect on expression of H2-D1 mRNA in D6 and D7 pregnant uteri after the treatment ($P > 0.05$).

After exogenous hrTGF-beta1 treatment, western blotting results demonstrated that H2-D1 protein expression was decreased in D6 pregnant uteri (Figure 3B1, $P < 0.05$) as well as in D7 pregnant uteri (Figure 3B1, $P < 0.01$) when compared to controls.

Immunohistochemical analysis showed that in uterine horn injection of exogenous hrTGF-beta1 group, the expression of H2-D1 protein, mainly localized in the decidual zone, was not affected when compared to control (Figure 4B vs C).

4.6. Effect of exogenous hrTGF-beta1 treatment on H2-DM

Real-time PCR results showed that uterine horn injection of hrTGF-beta1 decreased the levels of H2-DM mRNA in D7 pregnant uteri ($P < 0.05$), but there was no effect on the expression of H2-D1 mRNA in D6 pregnant uteri ($P > 0.05$, Figure 2B2).

Western blotting results showed that in hrTGF-beta1 treated group, the expression of H2-DM protein in D6 and D7 pregnant uteri were decreased when compared to control (Figure 3B2, $P < 0.05$ and $P < 0.01$, respectively).

Immunohistochemical studies showed that H2-DM protein, mainly localized in the primary decidual zone, was not altered following exogenous hrTGF-beta1 administration (Figure 4E vs F).

5. DISCUSSION

Embryo traffic from oviduct to the uterus for preparing implantation occurs on D3.5 or D4 in mouse. We performed uterine horn injection on D2 and D3 prior to the implantation. Because have been more interested in the effect of TGF-beta1 on early pregnancy (which is the first and most pivotal step of gestation), we did not investigate the effect of TGF-beta1 on mid pregnancy and late pregnancy. Therefore, the effect of TGF-beta1 on mouse embryo implantation was examined on D6 and D7, which are the end of peri-implantation period, using the uterine horn injection model. The surgical procedure for intra-uterine injection was performed as described previously (18, 19). However, one must ensure that the surgical process and the injection of vehicle solution (PBS) had no effect on the implanted embryos in the mouse.

In this study, the number of implanted embryos of different treatment was determined. The results of statistical analysis indicated that the number of implanted embryos was significantly decreased in the anti-TGF-beta1 antibody treated horns. Accordingly, the numbers of implanted embryos were increased in the hrTGF-beta1 group compared to the control, but the effect was not observable. These results suggest that mouse embryo implantation was inhibited by anti-TGF-beta1 antibody administration. We presumed that there was a molecular regulation between TGF-beta1 and MHC antigens. In normal pregnancy, the MHC expression is low enough for an immunosuppressed environment at maternal-fetus interface. Although exogenous TGF-beta1 administration was able to decrease the expression of MHC, it is not enough to affect normal physiological activity, so an increase in the number of implanted embryos didn't occur. Exogenous TGF-beta1 administration could have markedly increased embryo implantation as it has been recently reported (20). Using abortion model, pharmaceutical bioactive TGF-beta3 injected into mouse vaginal tract reduced abortion rates. Authors explained that independent ability of TGF-beta promotes a regulatory T-cell response. Our results proved that TGF-beta1 may be involved in mouse embryo implantation, and it is propitious to pregnancy by creating a special immune microenvironment. Robertson *et al.* suggested that activation of latent TGF-beta in seminal plasma could promote maternal tolerance to paternal antigens (21). Increasing evidence suggested that fraction V BSA, a binder of TGF-beta, weakened the availability of added TGF-beta3 that reduced abortion rates (20). Also, the injection of neutralizing antibodies, specific for TGF-beta2,

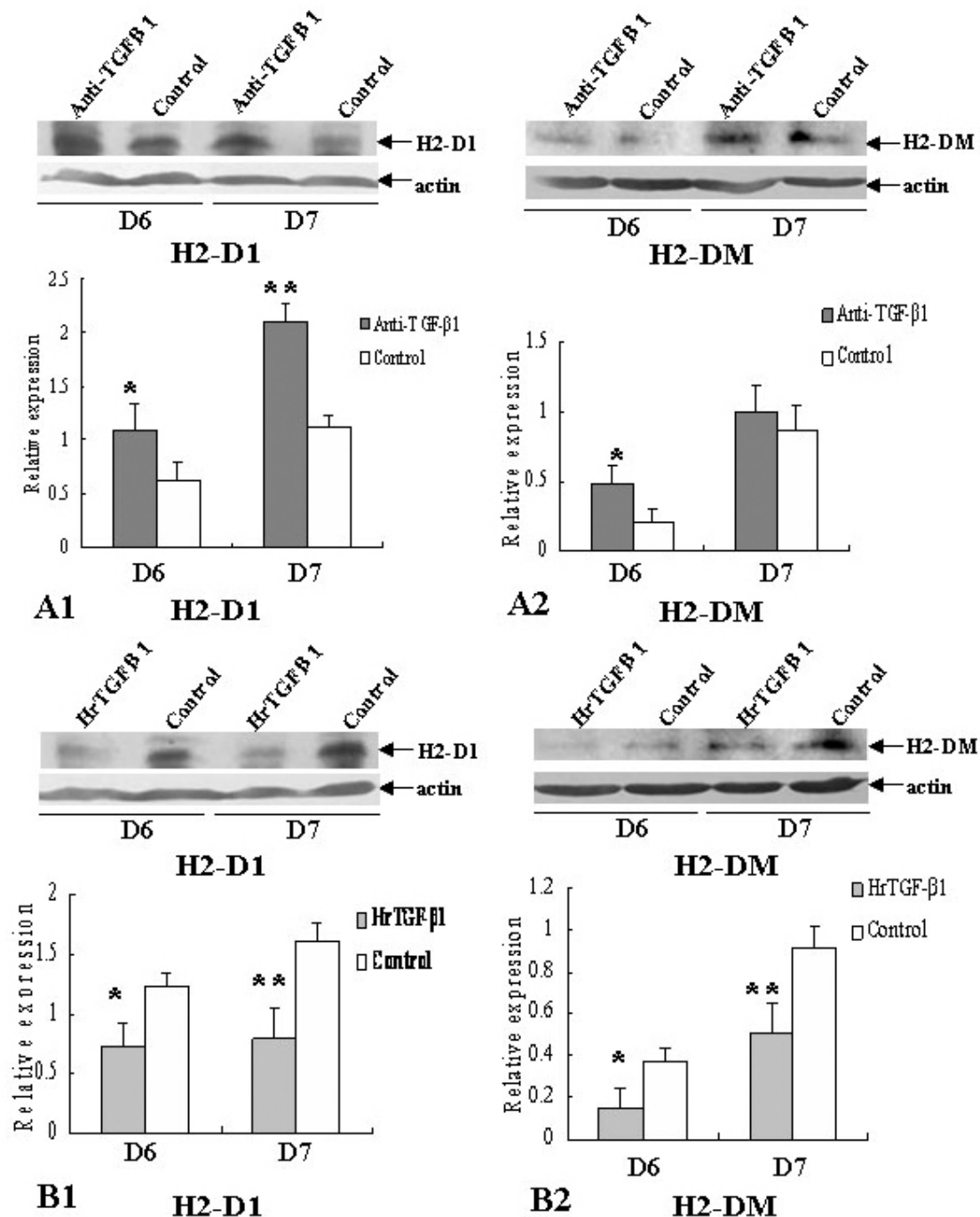


Figure 3. Effect of TGF-beta1 on mouse uteri H2-D1/H2-DM protein expression. Relative levels of mouse uteri H2-D1/H2-DM protein by western blotting. A1: H2-D1 protein levels from intrauterine injection of anti-TGF-beta1 antibody group on D6, D7. After the treatment, the expression of H2-D1 was increased compared that of the PBS-treated controls on D6 (*P < 0.05) and D7 (**P < 0.01). A2: H2-DM protein levels from intrauterine injection of anti-TGF-beta1 antibody group on D6, D7. After the treatment, the expression of H2-DM was increased on D6 compared that of the PBS-treated controls (*P < 0.05), and no significant change on D7 (P > 0.05). B1: H2-D1 protein levels from intrauterine injection of HrTGF-beta1 group on D6, D7. After the treatment, the expression of H2-D1 was decreased compared that of the PBS-treated controls on D6 (*P < 0.05) and D7 (**P < 0.01). B2: H2-DM protein levels from intrauterine injection of HrTGF-beta1 group on D6, D7. After the treatment, the expression of H2-DM was decreased significantly compared that of the PBS-treated controls on D6 (*P < 0.05) and D7 (**P < 0.01).

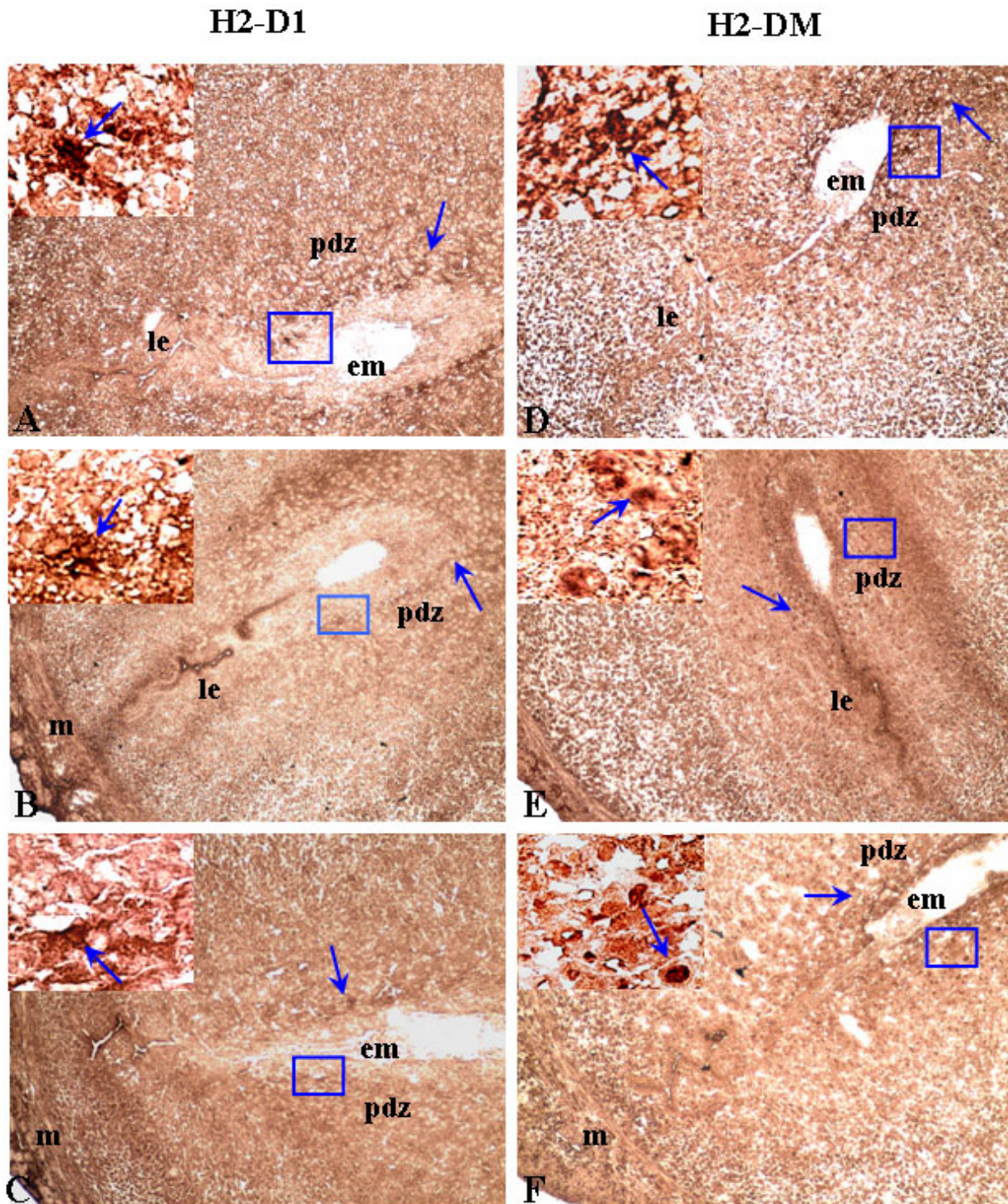


Figure 4. Immunohistochemical localization of H2-D1/H2-DM in mouse uteri. H2-D1/H2-DM was immunostained with anti-H2-D1 antibody/ anti-H2-DM antibody on D6 and the stain was developed with 3, 3' diaminobenzi-dine and H_2O_2 . Mouse uterine injection anti-TGF-beta1 antibody displayed a strongly expression of H2-D1 (A) and H2-DM (D) in the primary decidual zone, compared with the hrTGF-beta1 treatment (B, E) and the PBS treatment (C, F) respectively. Their localization did not changed in the pregnant uterine after the treatment. The photographs are shown at $\times 100$ original magnification. Rectangular selection indicate magnified original figure. Blue arrows represent a positive immunoreaction. em = embryo; m = myometrium; pdz = primary decidual zone.

into the blastocoel of mouse embryos markedly reduced the rate of implantation (22).

Apoptosis and immune privilege at maternal-fetus interface are the uppermost aspects restricting implantation, and our study focused on the latter aspect. More attention has been focused on the role of the MHC antigens in implantation because they are considered as crucial immune regulation factors at maternal-fetus interface. The MHC is connected with the survival of the early embryo as a 'semi-allograft transplant' during pregnancy (23). Thus, we hypothesized that TGF-beta1 affects mouse embryo implantation *via* regulation of MHC expression at maternal-fetus interface, this prompted us to examine the expression of H2-D1 and H2-DM in the pregnant mouse uteri injected with anti-TGF-beta1 antibody or hrTGF-beta1. Our results showed that intra-uterine injections of anti-TGF-beta1 antibody increased the expression of H2-D1 and H2-DM, and in exogenous hrTGF-beta1 treated group, the expression of H2-D1 and H2-DM was decreased in the mouse pregnant uteri. These results suggested that TGF-beta1 might be involved in the regulation H2-D1 and H2-DM gene expression during mouse pregnancy.

TGF-beta1 has been shown to inhibit the expression of MHC class II in a number of cell types (24). The TGF-beta1 deficient mouse showed an autoimmune inflammatory phenotype associated with enhanced expression of both class I and II MHC molecules (17, 25). Also, enhanced expression of MHC class II proteins was found in salivary glands of TGF-beta1 knockout mice (26). As expected, the ability of systemically administered TGF-beta1 to ameliorate or inhibit autoimmune disease coincides with a reduction in the local expression of MHC class II molecules (27). The synergism between TGF-beta1 and tacrolimus leads to the generation of Langerhans cells, reduced expression of MHC II molecules (28). All these data put together suggest that TGF-beta1 could modulate the expression of MHC, and our findings provided further proof to this scenario.

Many researchers focus on tissue distribution of MHC at maternal-fetus interface during pregnancy. Trophoblast cells fail to express MHC class II antigens (29). Classic MHC class I disappear from the cells (30) or have a very low expression (31). Whilst at the same time, extravillous cytotrophoblast cells express the non-classical MHC class I antigen, HLA-G, which may protect cytotrophoblast from maternal MHC non-restricted natural killer (NK) cell attack (32). Juretic suggested that the decidual dendritic cells of the first trimester of pregnancy also express MHC class II (33). Our studies showed that H2-D1 and H2-DM distributed in the primary decidual zone of mice uterus. The location did not change after injection of anti-TGF-beta1 antibody or hrTGF-beta1. However, a strong immunostaining can be found in anti-TGF-beta1 antibody treatment, which supported the results of PCR and western blotting.

In addition, the present study provides a new clue to explain the crosstalking and interaction between cytokines taking part in the immune regulation at the maternal-fetal interface. Our previous work reported that IFN-gamma can decrease TGF-beta1 protein expression during pregnancy, and IFN-gamma can enhance MHC

expression (17, 18). This study revealed that TGF-beta1 decreased the expression of MHC during pregnancy, suggesting a balance between IFN-gamma and TGF-beta1 during implantation, which might be inter-repressed and affect implantation *via* regulation of MHC expression.

In summary, anti-TGF-beta1 antibody affected mouse embryo implantation, and increased the expression of H2-D1 and H2-DM. Super-physiological doses of TGF-beta1 decreased their expression and did not affect mouse embryo implantation significantly.

6. ACKNOWLEDGMENTS

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Abbreviations: TGF: transforming growth factor; MHC: major histocompatibility complex; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor

Key Words: TGF-beta1, H2-D1, H2-DM, Embryo Implantation, Uterine Horn Injection

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