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Recombinant human soluble thrombomodulin as an anticoagulation therapy improves recurrent miscarriage and fetal growth restriction due to placental insufficiency – The leading cause of preeclampsia

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ABSTRACT

Introduction: Placental insufficiency is one of the major risk factors for growth restriction and preeclampsia. The aim of this study is to investigate whether recombinant human Thrombomodulin(r-TM) improves fetal conditions and physiological outcomes.

Methods: We used CBA/J × BALB/C mice as a control and CBA/J × DBA/2 mice – a well-studied model of recurrent spontaneous miscarriage. Pregnant mice received daily subcutaneous injections of r-TM or saline from day 0–15. The fetal resorption rate, fetal weight, and litter size were calculated at day 15. Additionally, we analyzed the mRNA expression of angiogenic factors and the concentration of soluble Flt-1 (sFlt-1) using the ELISA kit.

Results: The rate of fetal resorption in CBA/J × DBA/2 mice treated with r-TM was significantly lower compared with mice without r-TM treatment. Additionally, fetal weight and litter size were also significantly higher in the r-TM treated mice. Fibrinogen deposition in the labyrinth area of the CBA/J × DBA/2 mice treated with r-TM was significantly lower compared with deposits in the mice untreated with r-TM. As well, r-TM significantly increased the gene expression level of VEGF and Flt-1 mRNA in the placentas of the CBA/J × DBA/2 mice. r-TM treatment also significantly decreased the production of sFlt-1 protein in the placentas of preeclampsia-like diseased mice.

Conclusion: r-TM as an anticoagulation therapy has the potential for the medical treatment of recurrent miscarriage and fetal growth restriction due to improved angiogenic factors. Additionally, r-TM treatment has the potential for the recovery of preeclampsia.

1. Introduction

Recurrent pregnancy loss (RPL), defined as 3 or more consecutive pregnancy losses, affects about 1% of couples. Women who have experienced repeated miscarriages may undergo numerous expensive tests to try to identify an individual cause. While patients may discover a uterine malformation, hormonal imbalance, thrombophilia or chromosomal aberration as the cause of their RPL, about 40–50% remain with the diagnosis of idiopathic RPL [1]. Additionally, when these patients do have a successful pregnancy, they are at a higher risk for adverse perinatal complications such as growth restriction, preeclampsia, and preterm delivery. Placental insufficiency is one of the major risk factors for growth restriction and preeclampsia [2]. Placental insufficiency can result from deep placentation disorders, but also from other risk factors such as thrombophilia [3]. Angiogenesis is the development of new blood vessels from preexisting vasculature and is a crucial event for fetal growth and placental vascular development [4]. A balance of angiogenic and antiangiogenic factors is essential for a successful pregnancy [5,6]. The placental production of soluble Flt-1,which comprises the extracellular domains of the VEGF receptor-1, has a potent antiangiogenic molecule associated with decreased circulating levels of free VEGF and PLGF in preeclampsia [7].

Abbreviations: r-TM, recombinant human Thrombomodulin; s Flt-1, soluble Flt-1; VEGF, vascular endothelial growth factor; PLGF, placental growth factor; RPL, Recurrent pregnancy loss; CBA/JxDBA/2, DBA/2-mated female CBA/J; LPSs, lipopolysaccharides; APC, Activated protein C; DIC, disseminated intravascular coagulation; DAB, 3, 3–diaminobenzidine.

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DBA/2-mated female CBA/J (CBA/J × DBA/2) mice are a well-studied model of immunologically mediated pregnancy loss and share similar features with recurrent human miscarriage [8] [9]. Embryos derived from CBA/J × DBA/2 mice showed an increased resorption rate compared with control matings (CBA/J × BALB/c), and surviving fetuses show significant growth restriction [10]. Previous reports have shown that the high frequency of abortion in the CBA/J × DBA/2 model may be explained by exposure to bacterial lipopolysaccharides (LPSs) at the time of mating [9] [11]. Moreover, CBA/J × DBA/2 mice recapitulate key preeclamptic symptoms and pathological changes, which are hypersensitivity to angiotensin II, proteinuria, and renal glomeruloendotheliosis [12].

Thrombomodulin (TM, CD141 or BDCA-3) is an integral transmembrane glycoprotein expressed on the surface of vascular endothelial cells. TM serves as a co-factor for thrombin, the enzyme which regulates protein C activation, and inhibits thrombin activity. Activated protein C (APC) cleaves and inhibits coagulation cofactors FVIIIa and FVa, resulting in the downregulation of the activity of the coagulation system [13]. The absence of TM causes the fatal arrest of placental morphogenesis in mice, thus leading to fetal loss which is likely due to the tissue factor-dependent blood coagulation cascade at the fetus-maternal interface [14]. Recent reports have shown that the expression of TM is lower in preeclampsia, and that decreased TM expression impairs placental cell dysfunction [15].

The novel biological agent recombinant human soluble thrombomodulin (r-TM ART-123, Asahi Kasei Pharma Co., Tokyo, Japan) was recently approved and has been used clinically in Japan for disseminated intravascular coagulation (DIC) treatment [16]. This agent regulates the imbalanced coagulation system by reducing the excessive activation of thrombin. The histologic features of the placenta in fetal loss often reveal hemosiderin and increased fibrin [17], and r-TM may treat these hypercoagulable states. Recently, the administration of r-TM has been shown to rescue fetal tissue oxygenation and growth in preeclampsia rat models [18].

The aim of this study is to investigate whether pregnant CBA/J × DBA/2 mice treated with r-TM as an anticoagulation therapy improves maternal and fetal conditions, as well as physiological outcomes including the increased expression of angiogenic factors.

2. Materials and methods

2.1. Tissue samples

2.1.1. Mice

Inbred CBA/J, DBA/2, and BALB/c mice from The Charles River Laboratory (Kanagawa,Japan) were used. 8–10-wk-old virgin female CBA/J mice were mated with 8–14-wk-old, BALB/c or DBA/2 males. Females were inspected daily for vaginal plugs, and the presence of a vaginal plug was designated as day 0 of pregnancy. Pregnant females were killed at day 15. The frequency of fetal resorption was calculated on day 15 (number of resorptions/total number of formed fetuses and resorptions). Fetal weight and fetal size (measured from head to rump using Vernier calipers) were also determined. All experimental procedures were performed in compliance with the guidelines of the Osaka Medical College Animal Care and Use Committee (No. 28090). Pregnant mice received daily subcutaneous injections of human recombinant thrombomodulin (r-TM) or saline from day 0–15.

2.1.2. Immunohistochemistry

Mouse placenta samples were fixed in formaldehyde and embedded in paraffin prior to sectioning for immunohistochemical studies. The placentas were stained for fibrinogen with a polyclonal rabbit antihuman fibrinogen/fibrin antibody that cross-reacts with mouse fibrinogen ab34269 (Abcam Inc., Cambridge, MA, USA).

An HRP-labeled secondary antibody and 3,3′-diaminobenzidine (DAB) as a substrate were used to develop the reaction. Antigen retrieval using a boiling citrate solution (pH 6) for 20 min was performed prior to fibrinogen immunostaining and analyzed by light microscopy (Nikon Eclipse 50i,Nikon, Tokyo, Japan) at 40× magnification. In the areas with strong fibrinogen staining, immunohistochemical expression was quantified in accordance with the Allred scoring system as follows: 〔1〕 a proportion score was assigned, which represented the estimated proportion of positive-staining fibrinogen (0, none; 1, 1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, >2/3), and 〔2〕 an intensity score was assigned, which represented the average intensity of positive areas (0, none; 1, weak; 2, intermediate; and 3, strong).

The proportion and intensity scores were then added to obtain a total score which ranged from 0 to 8. The numerical scoring was confirmed by a second independent examination where the investigator was blinded to the initial score. The scores obtained from three different specimens and three different areas were calculated as the average ± standard deviation.

2.1.3. RNA extraction and quantitative RT-PCR

Total RNA was purified from the placental tissues using the RNeasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The purity of the RNA was evaluated with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific Inc. Wilmington, USA) by measuring the absorbance at 260 and 280 nm. OD260/280 ratios greater than 1.90 were considered to indicate that the samples were acceptable for further processing. All RNA samples met this purity requirement. Reverse transcription was then performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructionsand in a 38 μl reaction volume with T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with settings as follows: 25 °C for 10 min, 42 °C for 50 min, and 70 °C for 15 min. VEGF, FLT1, PLGF and beta actin gene expression was evaluated by quantitative PCR using predesigned mouse VEGF (Mm00437306_m1), FLT1 (Mm00438980_m1), PLGF (Mm00435613_m1), beta actin (Mm00607939_s1), TaqMan gene expression assays, and the TaqMan Gene Expression Master Mix in the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Samples were assayed in triplicates in a 20-μl reaction volume with the following thermal cycler settings: 1 cycle of 50 °C for 2 min and 95 °C for 20 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Beta actin was evaluated as an internal control to normalize VEGF, FLT1, PLGF expression. A negative control without cDNA was included.

2.1.4. ELISA

50 μl of blood on day 15 of pregnancy was collected to measure the concentration of the secreted sFlt-1. Serum levels of sFlt1 were measured by commercially available ELISA kits following the manufacturer's instructions (R&D systems, Minneapolis, MN, USA). The optical density was measured at 450 nm using a microplate ELISA reader (SPECTRA MAX 190; Molecular Devices, Tokyo, Japan). The average of the duplicate readings for each standard, control, and individual samples was used for the analysis.

2.2. Statistical analyses

Data are expressed as mean plus or minus standard deviation. Statistical analyses were conducted using the Student *t*-test to compare differences in means. Associations were considered to be statistically significant if the value of P was less than 0.05. Data were processed using Excel Statistics for Windows.