Theoretical Basis for Herbal Medicines, Tokishakuyaku-San and Sairei-To, in the Treatment of Recurrent Abortion: Enhancing the Production of Granulocyte–Macrophage Colony-Stimulating Factor in Decidual Stromal Cells

Takeshi Nagamatsu, Tomoyuki Fujii, Junko Matsumoto, Takao Kanai, Hironobu Hyodo, Takahiro Yamashita, Shiro Kozuma, Yuji Taketani

Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, Tokyo, Japan

Introduction

Elaborative interaction between maternal cells and trophoblasts is the key to placental formation, consequently leading to healthy fetal growth. Decidual stromal cells (DSCs) have close contact with extra-villous trophoblasts that invade into the uterine wall. Some kinds of cytokines, released from decidual cells, modulate the cytophysiological function of those trophoblasts. The collapse of this cell-to-cell interplay through cytokine signals at fetomaternal interface might lead to unsuccessful placental forma-
tion and then to abnormal pregnancies such as recurrent spontaneous abortion (RSA) and unexplained intrauterine growth restriction (IUGR).

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a multifunctional cytokine originally recognized as a factor implicated in the survival, proliferation and differentiation of hemopoietic cells. GM-CSF exerts its biological effect by binding to the heterodimetric receptor composed of two subunits, GM-CSF-specific alpha subunit and beta common subunit shared by interleukin (IL)-3 and IL-5. Recently, increasing evidences have suggested that GM-CSF works as a potentially important mediator for intercellular communication in reproduction. The cyclic expression of GM-CSF in endometrium controlled by ovarian steroid hormones has been reported. In human endometrium, mRNA expression of GM-CSF receptor is confirmed both in the trophoblasts and in the maternal cells in endometrium such as stromal lymphocytes and endothelial cells.

A series of works by Sarah Robertson’s group has revealed the essential role of GM-CSF in embryo development in peri-implantation period. In genetically GM-CSF-deficient mice, reduced litter size and increase of fetal death in late gestation and increase of new-born death in early post-natal life were observed, whereas implantation rates were normal. The addition of GM-CSF to culture medium of human embryo enhanced blastcyst formation, and increased the cell numbers in inner cell mass and trophectoderm preventing the apoptosis through binding to the α-subunit of GM-CSF receptor independently of β-subunit. Consequently, GM-CSF improved the incidence of placental formation and subsequent fetal growth trajectory.

Herbal medicines have established their positions as some of the treatment choices under a wide range of clinical practices in Japan, which are authorized by the Ministry of Health, Labor and Welfare of Japan. Tokishakuyaku-san (Toki) and Sairei-to (Sai) are the two herbal medicines used favorably for RSA and unexplained IUGR. Toki consists of six crude ingredients extracted from herbs, i.e. Paeoniae Radix, Atractylodis Lanceae Rhizoma, Alismatis Rhizoma, Hoelen, Cnidii Rhizoma and Angelicae Radix. Sai consists of 12, i.e. Bupleuri Radix, Alismatis Rhizoma, Pinelliae Tuber, Scutellariae Radix, Atractylodis Lanceae Rhizoma, Zizyphi Fructus, Polyporus, Ginseng Radix, Hoelen, Glycyrrhiza Radix, Cinnamomi Cortex and Zingiberis Rhizoma. Although the pharmacological mechanism of their beneficial effect has not fully been elucidated, increasing evidences have recently accumulated based on molecular-biological approach. In fact, our group previously reported that Toki and Sai, modulating the release of Th1/Th2 cytokines, have a potential to normalize cytokine balance and might give benefit to autoimmunity-related RSA cases.

In this study, we investigated the effect of Toki and Sai on GM-CSF production in decidual cells aiming at giving a new insight into the bases for the empirical usage of herbal medicine in clinical cases of RSA and IUGR.

Materials and methods

All the sample collections and the experiments in this study were conducted with the approval of the Ethical Committee of Medical Faculty, University of Tokyo.

Culture of Decidual Stromal Cells

The first trimester decidual tissues (6–10 weeks of gestation) were obtained from 12 cases of legal abortion of consenting women. The tissues were washed with phosphate-buffered saline (PBS) to remove clot and minced finely with a scalpel. The tissues were digested in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 0.05% collagenase (Wako, Osaka, Japan) for 30 min with slow stirring at 37°C. After being filtered through a 100-μm mesh, the digested solution was centrifuged at 300 × g. The collected cells were washed twice in PBS, and seeded on culture plates at a concentration of 1 · 10^6 cells/mL. DSCs were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (GibcoBRL, Grand Island, NY, USA) at 37°C. The purity of DSCs in this method was confirmed by immunocytochemical staining for vimentin (stromal cell; >98%), cytokeratin (epithelial cell; <1%) and CD45 (leukocyte common antigen; <1%). The separated DSCs were introduced to assays after refreshing the medium to remove floating cells and debris at 24 hr after seeding.

Cell Proliferation Assay

Decidual stromal cells were incubated with Toki or Sai at concentrations ranging from 50 to 800 μg/mL for
72 hr and the number of viable cells was evaluated with WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting kit 8) (Dojin-do, Tokyo, Japan). WST-8 is converted to reduced yellow formazan by dehydrogenase activities in viable cells. According to manufacturer’s instruction, WST-8 solution was added at 1:100 in culture medium. After 2-hr incubation, the light absorbance (test wavelength OD 450 nm, reference wavelength 655 nm) was measured by a microplate reader.

Enzyme-Linked Immunosorbbent Assay

The concentrations of GM-CSF in culture supernatants were measured using ELISA kit (Quantikine ELISA kit) (R&D systems, Minneapolis, MN, USA). All the procedures were performed according to manufacture’s instructions. The minimal detectable dose of the kit was 0.26 pg/mL.

Reverse Transcriptase Reaction and Real-Time PCR

Total RNA of the cells was isolated using RNeasy Kit (Qiagen, Hilden, Germany) according to manufacturer’s instruction. The amount of isolated RNA was assessed spectrophotometrically. The total RNA was reverse transcribed into cDNA using Rever Tra Ace kit (Toyobo, Osaka, Japan) in the volume of 40 μL including 8 μL 5× RT buffer, 4 μL dNTP, 2 μL RNA inhibitor, 2 μL Rever Tra transcriptase, 2 μL random primer and 22 μL sample RNA in a thermal cycler (30°C for 10 min; 42°C for 20 min; 99°C for 5 min). Real-time polymerase chain reaction (PCR) was carried out to semi-quantify mRNA amounts of GM-CSF and beta-actin using Light Cycler system (Roche Diagnostics, Lewes, UK). PCR was performed in a total volume of 20 μL mixture including 5 ng cDNA, 2 μL Light-cycler FastStart Reaction Mix SYBR Green1 (Roche Diagnostics), 0.5 μm each primer and 3 mm MgCl2. After 10 min denaturing at 95°C, 40 cycles of amplification were carried out (95°C denaturation for 15 s, 60°C annealing for 10 s, and 72°C extension). The time for extension depended on the PCR product size (a second/each 25 bp). The primer sequences used in PCR were as follows: GM-CSF; sense 5’-ATGTTGAATGCTCATCCAGGAGGC-3, antisense 5’-CCCATTTCTCTGGCATGCGAGT-3 (GenBank accession no. M10663), and beta-actin; sense 5’-CGA CAA CGG CTC CGG CAT GTG C-3, and antisense 5’-CGT CAC CGG AGT CCA TCA CGA TGC-3 (GenBank accession no. X00351). Beta-actin mRNA was quantified in each sample as an internal control to normalize the level of mRNA among samples. After amplification program, PCR products were analyzed by melting curve to confirm the amplification specificity.

Statistical Analysis

All data obtained in this study were analyzed by one-way repeated-measures analysis of variance using In-Stat software (Graf Pad Software, San Diego, CA, USA). All the values were presented as mean ± S.E., and P = 0.05 was considered to be significant. In each experiment, ‘n’ means the number of decidual tissues from different women.

Fig. 1 The effect of Tokishakuyaku-san (Toki) and Sairei-to (Sai) on cell proliferation. Decidual stromal cells (DSCs) were cultured for 72 hr with Toki or Sai at different concentrations ranging from 50 to 800 μg/mL. The viable cell numbers were assessed by WST-8 as described in Materials and methods. The shown data represent the mean ± S.E. of seven separate cultures of DSCs derived from different decidual tissues. DSC proliferation was inhibited under the presence of Sai dose-dependently, while Toki did not affect the cell proliferation.
Results

The Effect of Sai and Toki on the Proliferation of Cultured DSCs

WST-8 assay was conducted to see whether Sai and Toki affect the cell proliferation of cultured DSCs (Fig. 1). The addition of Sai at concentrations of more than 100 showed dose-dependent reduction in cell number, while no significant difference was seen between 50 µg/mL of Sai addition and control. Toki addition up to 800 µg/mL did not affect the cell proliferation, showing the marked difference from the growth inhibition of Sai.

The Effect of Sai and Toki on GM-CSF Production in Cultured DSCs

Granulocyte–macrophage colony-stimulating factor was not detected in the fresh culture medium regardless of the presence of Sai or Toki. The GM-CSF concentrations in DSC culture supernatant were examined at 72 hr after the addition of Sai or Toki using enzyme-linked immunosorbent assay. The assessed concentration was set in the range 0.1–400 µg/mL, considering the results of WST-8 assay which showed the marked interference of cell viability at the concentration above 800 µg/mL. GM-CSF concentration increased significantly in the presence of Sai or Toki in a dose-dependent manner (Fig. 2).

The Effect of Sai and Toki on mRNA Expression of GM-CSF in DSCs

Granulocyte–macrophage colony-stimulating factor expression at mRNA level was semi-quantitatively assessed by real-time PCR. The amount of mRNA was examined at 24 hr in culture with the addition of Sai or Toki. Stepwise increment was observed in the presence of either Sai or Toki among three conditions: control, 10 µg/mL and 100 µg/mL, indicating that DSCs up-regulate GM-CSF expression at mRNA level under the presence of Sai and Toki (Fig. 3).
Discussion

Decidual stromal cells contribute to the successful formation of placenta by releasing a wide variety of cytokines. Among them, the pivotal role of GM-CSF has been demonstrated not only in physiology of feto-placental unit but also in the etiology of some pathological situations. A significant increase of GM-CSF being confirmed in the peripheral blood of normal pregnant women was not observed in pregnant RSA patients. A high abortion rate in DBA/2-mated CBA/J female mouse was ameliorated by administration of GM-CSF. The percentage of cyclophosphamide-treated embryos exhibiting limb malformations was shown to decrease significantly following GM-CSF administration, suggesting a possible role of GM-CSF in modulating teratogen-induced effects.

In this study, we revealed that both Toki and Sai enhanced the release of GM-CSF protein from DSCs. Furthermore, we also confirmed the increase of GM-CSF mRNA amount in those cells by adding Toki or Sai. Our findings suggest that clinical efficacy of Toki and Sai might be derived from increased production of a pregnancy-protective cytokine, GM-CSF. More precisely, administration of Toki and Sai might remedy the shortage of GM-CSF in RSA and IUGR, and consequently contribute to the healthy fetal growth. The following should be noticed concerning this study. Clinical dosages of Toki and Sai are 7.5 g/day, and their serum level is estimated to be 200–300 µg/mL after 1 week of administration. Therefore, the level ranges of Toki and Sai examined in this study would be practical.

However, the addition of Sai inhibited cell proliferation in DSC culture in this study, while the proliferation was not affected in the presence of Toki. Similar phenomenon in different types of cells has been reported in previous studies. Bupleuri Radix, one of major ingredients of Sai, is involved in the inhibition of nifedipine-induced proliferation and collagen synthesis in gingival fibroblasts. Sai inhibits serum-induced DNA synthesis of rat mesangial cells by suppressing Raf-1/extracellular signal-related kinase cascade. Considering those cells in previous studies share the nature of stromal origin with DSC, the common paradigm might exist behind our finding and theirs. At present, however, it is unclear whether the growth inhibition of DSCs induced by Sai is favorable for the pregnancy outcome or not. Therefore, further research elucidating this point is necessary to establish the optimized protocol of Sai.

According to the previous work, the decidual change of endometrium is accompanied with the increase of GM-CSF expression in decidual stroma, suggesting the involvement of GM-CSF in the decidual function. In this study, Toki enhanced GM-CSF production without giving impact on cell proliferation in DSCs. It can be presumed that the GM-CSF promotion induced by Toki modifies the decidual function. Interestingly, Toki has been reported to stimulate progesterone production in rat luteal cells by enhancing luteal steroid genesis, leading to the transformation of endometrium into decidua. In this context, Toki might exert its efficacy through two independent modes of action, affecting ovarian luteal cells and the stromal cells in uterine endometrium.
Our study demonstrated that both Sai and Toki enhanced GM-CSF release from cultured DSCs, and the reaction followed the increase of GM-CSF mRNA in the cells. A previous study of other group indicated that Sho-saiko-to, a herbal medicine having efficacy for viral liver cirrhosis, enhanced GM-CSF production in the peripheral mononuclear cells. Considering most of galenical components in Sho-saiko-to are shared with Sai, the same mechanism might be commonly behind our finding and theirs. The molecular pathway, however, in which those herbal medicines affect GM-CSF expression, is not elucidated. According to a previous report, an anti-oxidant, pyrrolidine dithiocarbamate, preventing the degradation of mRNA in cultured pulmonary epithelial cells, increased GM-CSF expression. It has been demonstrated that the pharmacological effects of Toki and Sai are partly based on their anti-oxidant action. Therefore, the promotion of GM-CSF expression observed in the present study might be related to anti-oxidant pathway.

It remains to be investigated whether our in vitro findings are actually identical to in vivo phenomenon. The measurement of GM-CSF concentration in serum samples from patients treated with Toki and Sai is an interesting approach. However, the peripheral concentration might not reflect the increase of GM-CSF locally induced in decidua because of the interference of GM-CSF production derived from peripheral lymphocytes and other organs.

In conclusion, we revealed both Toki and Sai promoted the production of GM-CSF in DSCs. Considering the role of GM-CSF in embryonic development, clinical benefit of these herbal medicines in the treatment of RSA and IUGR might be based on their pharmacological reaction related to GM-CSF. Our observations in the present study may provide the rationale to the empirical usage of these medicines in the treatment of RSA and IUGR.

Acknowledgment

We thank Tsumura & Co. (Tokyo, Japan) for providing the Toki and Sai.

References


13 Takei H, Nakai Y, Hattori N, Yamamoto M, Kurauchi K, Sasaki H, Aburada M: The herbal medicine Toki-


