Int J Clin Exp Pathol 2013;6(11):2441-2450
www.ijcep.com /ISSN:1936-2625/IJCEP1309025

Original Article

Cyclosporin A enhances the ability of trophoblasts to displace the activated human umbilical vein endothelial cell monolayers

Chuanling Tang[1], Limei Chen[1], Weirong Gu[1], Meirong Du[1], Mingqing Li[1], Qi Chen[1,2], Dajin Li[1]

[1]Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, China; [2]Department of Obstetrics and Gynaecology, Faculty of Medical and Health Sciences, The University of Auckland, Auckland 1001, New Zealand

Received September 8, 2013; Accepted October 12, 2013; Epub October 15, 2013; Published November 1, 2013

Abstract: Transformation of the spiral arteries including the displacement of vascular endothelial cells by extravillous trophoblasts is an essential prerequisite to normal placentation. However, the activated endothelial cells resist the invasion of trophoblasts, which contributes to the pathologies of some pregnant disorders. Our previous studies have demonstrated that Cyclosporin A (CsA) promotes the migration and invasion of human first-trimester trophoblasts. In the present study, we further investigated whether CsA could promote the ability of trophoblasts to displace the activated human umbilical vein endothelial cell (HUVEC) monolayers and the possible molecular mechanisms. Human choriocarcinoma Jar cells were used as a model of invasive trophoblasts. CsA pretreated JAR cells (red) were added to HUVEC monolayers (green) activated with either necrotic JAR cells or tumor necrosis factor alpha (TNFα). The ability of JAR cells to displace HUVECs from the monolayers was examined by confocal microscopy. The effects of CsA on Titin and E-cadherin expression, matrix metalloproteinases (MMPs) activity and CXCL12 secretion of JAR cells were evaluated by western blot, gelatin zymography and enzyme-linked immunosorbent assay (ELISA), respectively. We found that CsA pretreatment increased the ability of JAR cells to displace activated HUVECs from the monolayers. However, the displacement was reduced by untreated JAR cells. Moreover, CsA pretreatment up-regulated Titin expression, down-regulated E-cadherin expression, improved MMP2 and MMP9 activity, and increased the CXCL12 secretion in JAR cells. These results indicate that CsA may improve the trophoblast invasion to activated HUVEC monolayers through different downstream targets, and ultimately, improve the transformation and remodeling of spiral arteries.

Keywords: CsA, trophoblast, invasion, HUVEC, activation, remodelling

Introduction

Successful pregnancy highly depends on the adequate maternal supply of nutrients and oxygen to support the metabolic demands of the growing embryo and fetus. To achieve this requirement, the placenta has evolved to transform the uterine spiral arteries into low-resistance, high-volume channels so that a constant maternal blood can be supplied, in addition to the large area for exchange and the nutrient transporter up-regulation [1]. During this arterial remodeling, extravillous trophoblast (EVT) cells play an essential role. The EVTs colonize the decidua and migrate to the maternal uterine spiral arteries, displace and replace the endothelial cells lining the spiral arteries which become dilated conduits and incapable of maternal vasomotor control, until the end of the second trimester [2]. Abnormal spiral arteries remodeling characterized by shallow trophoblast invasion is associated to the development of major placental pathologies such as second trimester miscarriage, pre-eclampsia, pre-term birth and some cases of fetal growth restriction (FGR) [3-6]. These pathologies not only increase the risk of morbidity and mortality in pregnancy, but also have long term consequences for the health of both mother and child [7-9]. Therefore, improving the spiral arteries remodeling will facilitate normal growth and development of the foetus in utero, as well as make potential influence on health in later life.

Cyclosporin A (CsA), also called cyclosporine, is a widely used immunosuppressant to suppress graft-vs-host response in organ transplant rejection. Apart from this, it has also shown clinical importance in treatment of some autoimmune disorders [10]. Interestingly, our initial study has shown that administration with low dose CsA at the early stage of pregnancy can induce the maternal tolerance to the allogeneic fetus and improve the pregnancy outcome in murine abortion-prone CBA/J×DBA/2 matings [11]. The *in vitro* study has provided evidence that CsA at low concentrations can promote the migration and invasion of human first-trimester trophoblast cells [12, 13]. These results above suggest that CsA appears to have favorable effect on the maternal-fetal interface, and might be developed into a therapeutic intervention for the pregnancy complications related to insufficient trophoblastic invasion.

Our previous experiments have also shown that the activated endothelial cells resist the invasion of trophoblasts in vitro, which may lead to deficient spiral arteries remodeling [14]. So in the current study, we firstly used the necrotic trophoblasts or endothelial activator TNFα to induce the activation of the primary human umbilical vein endothelial cells (HUVECs), and secondly analyzed whether CsA could promote the ability of trophoblasts to displace the activated endothelial cell monolayers and furthermore investigated the possible molecular mechanisms.

Materials and methods

Tissue collection and cell culture

Umbilical cords from normal term placenta were obtained with informed consent and the approval of the Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University. The tissues were collected under sterile conditions and transported to the laboratory on ice in M199 complete medium (2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin; Gibco, NY, USA).

Primary HUVECs were isolated from the fresh umbilical cords by a collagenase treatment according to the protocol described by Baudin [15]. Cells were seeded on gelatin-precoated culture plates and cultured in M199 complete medium containing 20% (v/v) fetal bovine serum (FBS; Gibco, NY, USA), in a 37°C-5% CO_2 humidified incubator. The culture medium was changed on the following day to remove the non-adherent cells. The cultured cell monolayer was monitored by phase-contrast microscopy. When reaching confluency, the cells were detached using a solution with 0.25% trypsin and 0.02% EDTA. In all the following experiments, HUVECs of the second or third passage were used. For each experiment, four different donor cell samples were used.

The choriocarcinoma JAR cell, one of the human trophoblast-like cell lines, was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) with the original source being the American Type Culture Collection (ATCC). Cells were cultured in DMEM/F12 complete medium supplemented with 10% FBS and maintained in 5% CO_2 at 37°C. Cells were detached by routine trypsinization every 3 to 4 days.

Immunocytochemical staining

The isolated HUVECs were grown on BD Falcon™ culture slide wells and characterized after 48 h. The cells were gently washed with serum-free culture medium, fixed with methanol and permeabilized with 0.2% Triton X-100. After being blocked with 5% BSA in PBS for 30 min at room temperature, cells were then incubated with Rabbit monoclonal von Willebrand factor (anti-vWF, Sigma Aldrich St. Louis, MO, USA), mouse monoclonal α-actin antibody (Santa Cruz, CA, USA) or mouse IgG isotypic control (Sino-America Co. Ltd, Zhejiang, China) overnight at 4 °C. Cells were then washed with PBS and further incubated with a peroxidase-conjugated secondary antibody for 60 min at 37°C. The slides were stained with DAB, and counterstained with hematoxylin. Images were observed by using an Olympus BX51 microscope (Tokyo, Japan), and recorded with a high-resolution DP70 Olympus digital camera.

Induction of cell death

Necrotic death in JAR cells were induced through a cycle of freeze/thaw as described previously [16]. Briefly, cells were rapidly frozen as cell pellets for 2 h at -70°C and then thawed at room temperature without washing, and suspended in DMEM/F12 without serum before

use. The average percent of necrosis was assessed by trypan blue positivity and Dead Cell Apoptosis Kit.

Annexin V and PI (propidium iodide) staining

The necrosis percentage of the induced JAR cells was analyzed by flow cytometry with the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Inc., UK). The freshly trypsinized JAR cells and the cells after a freeze/thaw cycle were adjusted to the same concentration and washed in cold PBS. Then, Alexa Fluor® 488 annexin V and PI working solution were added into each cell suspension for 15 min in the dark at room temperature. Control tubes of unstained cells, cells stained with PI alone, and cells stained with annexin V only were included for setting up the flow cytometric compensation. After staining, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence.

HUVECs activation assay

HUVECs were seeded into 0.1% gelatin-coated 6 well culture plates (Costar, USA) and cultured until confluent. The cells were then exposed to either necrotic JAR cells (8×10^4 per well) or human recombinant TNFα (10 ng/ml; Peprotech, USA) for 24 h [17]. To determine whether HUVECs became activated after treatment with the above factors, Western blot analysis was employed to evaluate the expression of intercellular adhesion molecule 1 (ICAM-1).

Co-culture of activated endothelial cells with trophoblasts

HUVECs were grown onto plastic microscope slide coverslips in 6-well plates until confluent and then were labeled by 1 μM CMFDA (cell tracker green, Molecular Probes, Inc., UK) for 2 h. HUVECs were then exposed to either necrotic JAR cells or TNFα for 24 h. Alive JAR cells were labeled with 1 μM CMTPX (cell tracker red, Molecular Probes, Inc., UK) and then were treated with CsA (1 μg/ml, Sigma Aldrich St. Louis, MO, USA) for 24 h. Treated and red fluorescence-labeled JAR cells (2×10^5 per well) were then added to the HUVEC monolayers after treatment with the above factors for another 24 h. Control cultures consisted of non-treated HUVEC monolayers cultured with JAR cells. All the cocultures were then washed, fixed with 4% paraformaldehyde (PFA) and the coverslips were mounted onto glass microscope slides using Citifluor fluorescent mounting medium, and then examined by a Leica model TCS SP2 confocal microscopy. The area of JAR cell "island" (red) within the HUVEC monolayers (green) was then quantified in 10 fluorescence images per groups using Image J software. The invasion index was calculated as the JAR cell ''islands'' area of the treated groups to that of the control group.

Western blot analysis

Cells were lysed on ice in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 10 mM NaF, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF and phosphatase inhibitors; Roche, USA). The lysates were centrifuged at 15 000 g for 20 min at 4 °C to get the supernatants. Equal amount of total proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking, the membrane was probed with specific primary mouse monoclonal anti-ICAM-1 (1:1000; Abcam, USA), anti-Titin (1:5000; Abcam, USA), or anti-E-cadherin (1:5000; BD Biosciences, Bedford, MA, USA), and anti-GAPDH (1:1000; Santa Cruz, CA, USA) antibodies overnight at 4 °C, then followed by incubation with HRP-conjugated secondary antibodies. After extensive washing, proteins of interest were detected by enhanced chemiluminescence system (ECL, Thermo Scientific, UK) and quantified by densitometry using Quantity One (Bio-Rad, USA).

Gelatin zymography

The enzyme activity of both MMP2 and MMP9 in the culture media was measured by gelatin zymography [18]. Briefly, the conditioned medium was subjected to 10% SDS-PAGE copolymerized with 0.1% gelatin (Sigma Aldrich St. Louis, MO, USA) under non-reducing conditions. After electrophoresis, the gel was rinsed in 2.5% Triton-X 100 for 1h to remove SDS, and then incubated for 36 h at 37 °C in developing buffer (50 mM Tris–HCl, pH 7.8, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% v/v Brij 35). Thereafter, the gel was stained for 1 h with 0.5% w/v Coomassie blue R-250 until areas of gelatinolytic activity appear as clear white bands over

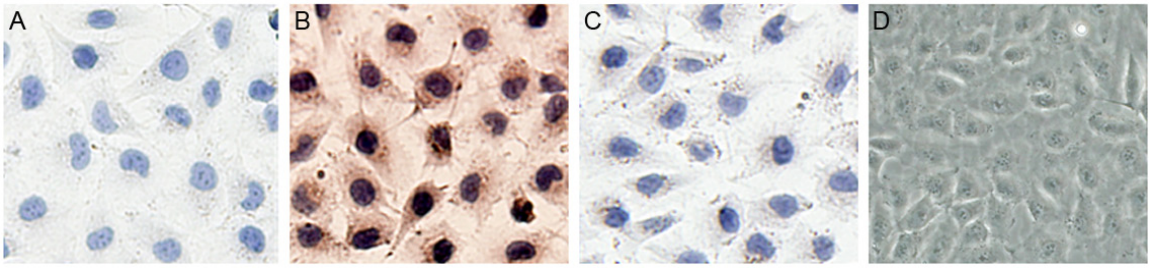

Figure 1. *Characterization of HUVECs by immunocytochemistry.* The isolated human umbilical vein endothelial cells (HUVECs) were stained moderately by anti-vWF monoclonal antibody (mAb) (B), and not by anti-α-actin mAb (A). Murine IgG served as isotypic control (C). HUVECs at confluency present "cobblestone" morphology in culture (D). Magnification: ×200.

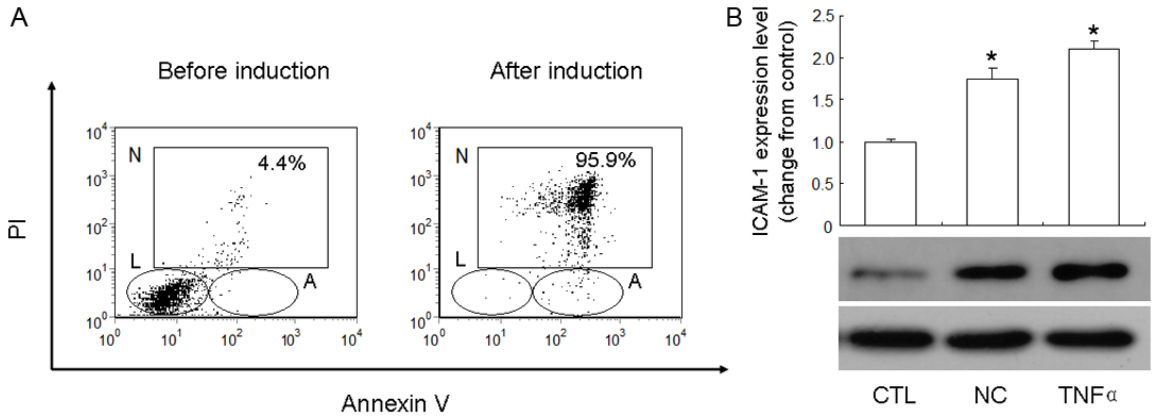

Figure 2. *HUVECs are activated by necrotic Jar cell bodies.* A: Jar cells were rapidly frozen as cell pellets for 2 h at -70°C and then thawed at room temperature. The necrosis percentage of the induced Jar cells was analyzed by flowcytometry with Annexin V Alexa Fluor® 488 and PI staining. Representative analysis of three independent experiments was presented. L, live cells; A, apoptotic cells; N, necrotic cells. B: HUVECs in 6 well culture plates were treated with TNFα (10 ng/ml) or necrotic Jar cells (8×10^4 per well) for 24 h. The expression of ICAM-1 was assessed by western blot analysis. GAPDH was used as a loading control. CTL, control; NC, necrotic Jar cells. Bottom, typical blots; top, densitometric analysis, normalized to 1 in control. $^*P<0.05$, versus control.

the uniformed blue background. The gel was photographed and analyzed by the Odyssey Infrared Imaging System (LI-COR Biosciences German version of Ltd.). The experiments were carried out in triplicate, repeated three times.

Enzyme-linked immunosorbent assay (ELISA)

JAR cells were seeded in a 24-well plate at a density of 1×10^6 cells/ml and treated with 1 μg/ml CsA for 24 h. Thereafter, the culture supernatants were harvested, centrifuged to remove cellular debris, and stored at -80°C. Human CXCL12 ELISA kit (R&D Systems, Abingdon, UK) was used to measure chemokine production in each supernatant according to the manufacturer's instructions. The ELISA assay was carried out in duplicate in three separate experiments.

Statistics

Results are given as mean ± SD. Statistical comparisons were performed either by the Student's paired t-test or by one-way analysis of variance (ANOVA) followed by a Dunnett test. Differences were accepted as statistically significant at $P<0.05$.

Results

Immunocytochemical characterization for purity of HUVECs

In the present study, we firstly isolated and characterized the purity of primary HUVECs by immunocytochemistry. The isolated primary cells were almost all positive immunostaining for von Willebrand factor (vWF), one of the specific markers for endothelial cells (**Figure 1A**),

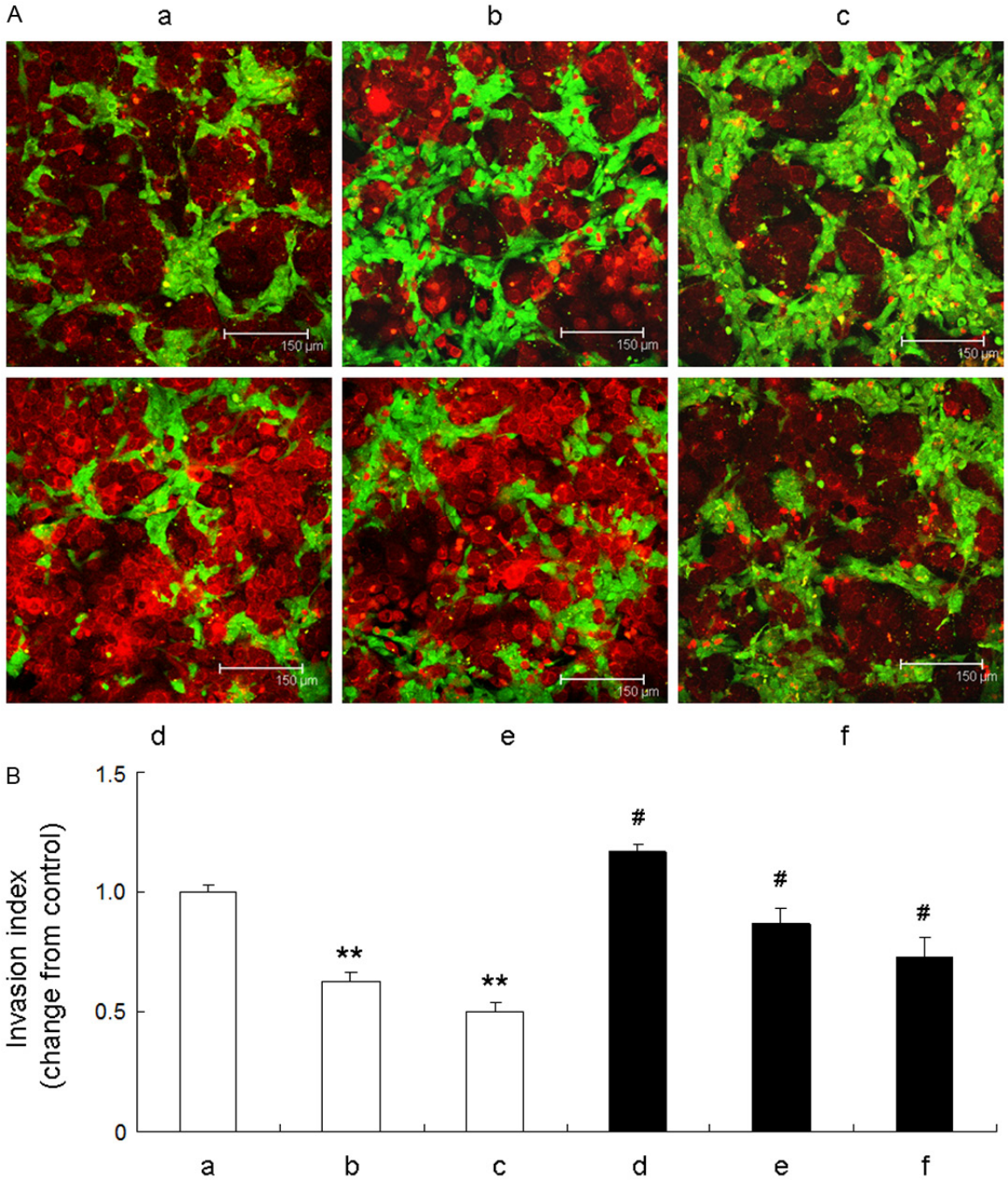

Figure 3. *CsA promotes the invasion of Jar cells to the activated HUVEC monolayers.* The HUVECs were none treated (a & d) or treated with necrotic Jar cell bodies (b & e), TNFα (c & f) for 24 h, then co-cultured with CsA-untreated (a-c) or CsA-pretreated Jar cells (d-f) for another 24 h. A: Representative confocal images showing cell ''islands'' of CMTPX (cell tracker red)-labeled Jar cells which have displaced CMFDA (cell tracker green)-labeled HUVECs from monolayers. B: The invasion index was calculated as the Jar cell ''islands'' area (red fluorescence) of treated groups to that of the control group (a). $^{**}P<0.01$, b versus a, c versus a; $^{\#}P<0.05$, d versus a, e versus b, f versus c.

and negative with antibodies against α-actin, a smooth muscle cell specific marker (**Figure 1B**). Cells were also negative for murine immunoglobulin (Ig) G which is served as isotypic control (**Figure 1C**). The purity of the isolated HUVECs was more than 98%. When reaching confluency, the monolayers of isolated endothelial cells exhibit the characteristic "cobblestone appearance" under phase-contrast microscopy (**Figure 1D**).

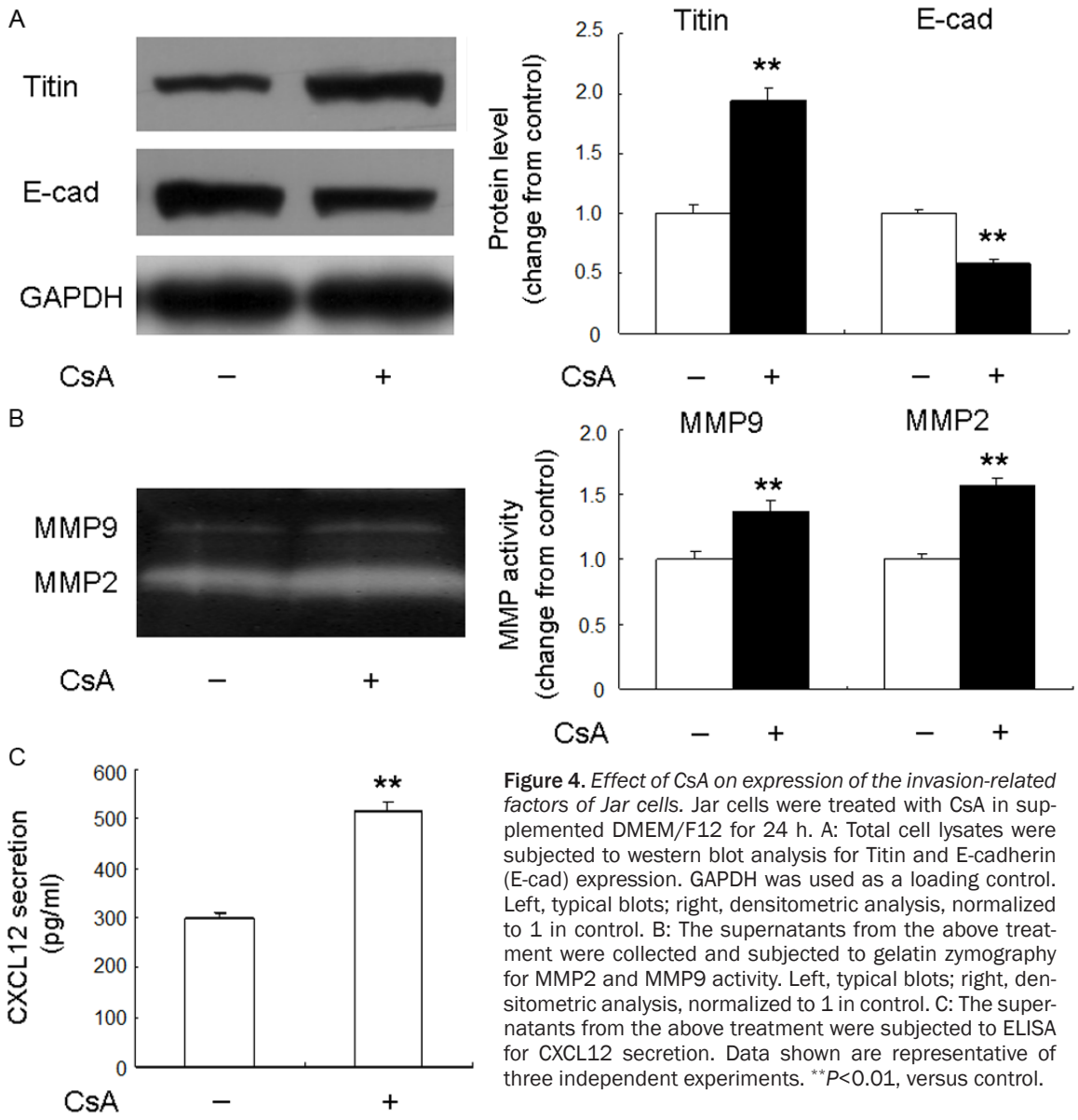

Figure 4. *Effect of CsA on expression of the invasion-related factors of Jar cells.* Jar cells were treated with CsA in supplemented DMEM/F12 for 24 h. A: Total cell lysates were subjected to western blot analysis for Titin and E-cadherin (E-cad) expression. GAPDH was used as a loading control. Left, typical blots; right, densitometric analysis, normalized to 1 in control. B: The supernatants from the above treatment were collected and subjected to gelatin zymography for MMP2 and MMP9 activity. Left, typical blots; right, densitometric analysis, normalized to 1 in control. C: The supernatants from the above treatment were subjected to ELISA for CXCL12 secretion. Data shown are representative of three independent experiments. $^{**}P<0.01$, versus control.

The role of necrotic trophoblasts on HUVECs activation

To determine whether HUVECs became activated following treatment with the necrotic trophoblasts, a cycle of freeze/thaw was used to induce the necrosis of JAR cells. The average percent necrosis assessed by trypan blue positivity was 92.9±1.7%. Staining with Alexa Fluor® 488 Annexin V and PI revealed that 95.8±1.4% of JAR cells after induction were positive for both (**Figure 2A**). These data indicate that sufficient necrotic JAR cells can be induced by this method. We then analyzed the expression of ICAM-1 to evaluate the activation of HUVECs by Western blot. TNFα which is one of the well known activators of endothelial cells was also included in our system. Our data demonstrated that the ICAM-1 expression in HUVECs increased significantly after treatment with necrotic JAR cells or TNFα for 24 h (**Figure 2B**), indicating that HUVECs can be activated by necrotic JAR cells induced in our study.

The effect of CsA on the invasion of activated HUVEC monolayers by JAR cells

To test the effect of CsA on human trophoblast cell invasion to activated HUVEC monolayers, we co-cultured CMTPX (cell tracker red)-labeled

JAR cells with CMFDA (cell tracker green)-labeled HUVEC monolayers for 24 h. The confocal fluorescence images demonstrated that CsA pretreatment increased the displacement of JAR cells to resting HUVECs. However, less HUVECs were displaced from the monolayers by JAR cells when HUVECs were activated by TNFα or necrotic JAR cells than the inactivated control monolayers. After pretreatment of JAR cells with CsA for 24 h, the co-culture results showed that more of the activated endothelial cells were displaced from the monolayers by CsA-pretreated JAR cells in comparison to untreated cells (**Figure 3A**).

Quantification analysis of the JAR cell ''islands'' area (red fluorescence) that have displaced green fluorescence-labeled HUVECs from monolayers indicated that activated HUVECs restricted the invasion by JAR cells, and CsA pretreatment could significant enhance the invasion of JAR cells to the normal or activated HUVEC monolayers. When HUVECs were activated by TNFα or necrotic JAR cells, the invasion index of untreated JAR cells was (0.504±0.036) and (0.629±0.040) as compared to the control. After CsA pretreatment, the invasion index to the activated HUVEC monolayers increased to (0.732±0.075) and (0.865±0.067), respectively (**Figure 3B**).

The effect of CsA on expression of the invasion-related factors of JAR cells

To better understand the role of CsA enhancement of trophoblast invasion to the HUVECs, we analyzed the effect of CsA on expression of the invasion-related factors of JAR cells. The western blot results showed that CsA could significantly up-regulate the expression of Titin, a giant protein responsible for the elasticity and extensibility of the sarcomere, as well as down-regulate the expression of E-cadherin, one of the invasion suppressors (**Figure 4A**). The gelatin zymography analysis displayed that the enzyme activity of MMP2 and MMP9 was improved after CsA treatment (**Figure 4B**). Both MMP2 and MMP9 exert their proteolytic activity through secretion into the extracellular matrix. The ELISA results demonstrated that CsA induced nearly a 2-fold increase in the CXCL12 level in the supernatant of JAR cells (**Figure 4C**).

Discussion

CsA is a most common and powerful immunosuppressive drug that has a major impact on improving patient outcome following organ transplantation. It is known to act by binding to a cytoplasmic protein, cyclophilin in lymphocytes, articularly in T-lymphocytes, that ultimately inhibits IL-2 production, leading to an impairment in effector T-cell functioning [19]. In addition to its immunosuppressive effects on T-cells, CsA could modulate the biological behaviors of different kinds of carcinoma cells. It has been reported that CsA treatment of adenocarcinoma cells results in striking morphological alterations, including membrane ruffling and numerous pseudopodial protrusions, increased cell motility, and anchorage-independent growth [20]. Similarly, Walsh SB *et al* have demonstrated that CsA directly alters the phenotype of cutaneous squamous cell carcinomas to an invasive and aggressive tumor-type by enhancing expression of proteins regulating epithelial-mesenchymal transition (EMT) [21].

Trophoblast invasion and displacement of vascular endothelial cells were very important for remodeling of spiral arteries. Inadequate trophoblast invasion is believed to result in the pathogenesis of various major pregnancy-associate disorders. Our previous studies have demonstrated that CsA promotes the migration and invasion of human first-trimester trophoblast cells, and improves the outcome of murine pregnancy [11-13]. These findings suggest that CsA may help to improve the JAR invasion to vascular endothelial cells and contribute to transform the spiral arteries and establish the sufficient artery remodeling.

Our previous study also observed that the activated endothelial cells resist the invasion of trophoblasts *in vitro*, which may lead to deficient spiral arteries remodeling [14]. Therefore, we dedicated to investigate whether CsA could promote the ability of trophoblasts to displace the activated endothelial cell monolayers. In the present study we have demonstrated that the necrotic trophoblasts can activate the primary HUVECs similar with TNFα, one of the cytokines increased at the maternal-fetal interface in preeclampsia [22], and that the HUVECs activated in this manner resists the displacement by trophoblast. These results raise the possibility that increased necrotic trophoblast

cell cellular debris in the spiral arteries could limit trophoblast invasion. Our data have also shown that more of the normal or activated endothelial cells are displaced from the monolayers by CsA-pretreated trophoblasts in comparison to CsA-untreated cells, suggesting that CsA pretreatment could significantly enhance the invasion of trophoblast cells to the normal or activated HUVEC monolayers.

Factors that are involved in the human trophoblast invasion at the maternal-fetal interface include growth factors, cell adhesion molecules, cytokines, hormones, endocrine factors, oxygen concentrations and hemodynamics [23-25]. This complex interplay acts both temporally and spatially to get appropriate invasion of trophoblasts. Titin, is a giant protein that functions as a molecular spring which is responsible for the elasticity and extensibility of the sarcomere. Recent study has demonstrated that Titin mutation defines roles for angiogenic remodelling and vascular morphogenesis by affecting endothelial morphogenesis [26]. Our results have shown that CsA significantly upregulates Titin expression in JAR cells. Therefore, the CsA-induced Titin expression may contribute to trophoblast migration and invasion through altering elasticity and extensibility, and remodeling the maternal vascular system. E-cadherin is required for the maintenance of stable adherent junctions and acts as an invasion suppressor [27]. Evidence is emerging that E-cadherin may act as a key regulator of trophoblast migration and invasion [28, 29]. Our findings have indicated that CsA also downregulates E-cadherin expression in JAR cells, suggesting that CsA may help to lose cell adhesive properties mediated by E-cadherin and enhance the migration and invasion of trophoblasts. Cell invasion is facilitated by degradation of the extracellular matrix of the microenvironment by various proteinases, including the MMPs. Among members of MMP family, MMP2 and MMP9 are high expressed in trophoblasts and critical for trophoblast invasion [30]. Our gelatin zymography analysis has displayed that CsA increases the enzyme activity of MMP2 and MMP9 in JAR cells, which may be favorable to the degradation of ECM and improvement of trophoblast invasion. CXCL12 is a unique chemokine playing various biological functions together with its receptor CXCR4. Our previous studies have shown that first-trimester human trophoblasts secrete CXCL12 that can promote cell proliferation and invasion in an autocrine manner [31]. It has also been reported that CXCL12 induces gene expression of early growth response-1 (Egr-1) and vascular endothelial growth factor (VEGF), and enhances VEGF-induced cell proliferation in human arterial endothelial cells [32]. Our ELISA results have demonstrated that CsA induced a significant increase in CXCL12 secretion, which may play a role in promotion of trophoblast invasion and angiogenesis during embryo implantation.

In summary, we have confirmed that the primary HUVECs activated by the necrotic trophoblasts restrict the displacement by trophoblast and CsA pretreatment enhances the invasion of trophoblasts to resting or activated HUVEC monolayers. Our experiments further demonstrated that CsA may improve the trophoblast invasion and spiral arteries remodeling through regulating the expression of Titin, E-cadherin, MMP2, MMP9 and CXCL12. Although the effect of CsA on HUVECs is not included here, the results above suggest that CsA appears to have favorable effect on sufficient trophoblast invasion and on adequate remodeling of the spiral arteries during placental development. Our study provides some useful insights to the treatment of miscarriage, preeclampsia, and other pregnancy-related diseases resulting from deficient trophoblast invasion.

Acknowledgements

This study was supported by Major International Joint Research Project of National Natural Science Foundation of China (NSFC) 30910103909 to D-J Li, National and Shanghai Leading Academic Discipline Project 211XK22 to D-J Li, NSFC 81070537 to M-R Du, Shanghai PuJiang Talent Program 10PJ1401600 to M-R Du, Youth Foundation of Shanghai Municipal Health Bureau 2012Y032 to C-L Tang, and NSFC 81300505 to C-L Tang.

Declaration of conflict of interest

None.

Address correspondence to: Dr. Dajin Li, Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics & Gynecology, Shanghai Medical School, Fudan University, 419 Zhao Zhou Road, Shanghai, 200011, China. Tel: +86-21-63457331; Fax: +86-21-63457331; E-mail: djli@shmu.edu.cn; Dr. Qi Chen, Department of Obstetrics and

Gynecology, Faculty of Medical and Health Sciences, The University of Auckland, 85 Park Road, Grafton, Auckland 1001, New Zealand. Tel: +64-9-3737599x89519; Fax: +64-9-3035969. E-mail: q.chen@auckland.ac.nz

References

[1] Harris LK. Trophoblast-vascular cell interactions in early pregnancy: how to remodel a vessel. Placenta 2010; 31: S93-S98.

[2] Huppertz B, Peeters LL. Vascular biology in implantation and placentation. Angiogenesis 2005; 8: 157-167.

[3] Ball E, Bulmer JN, Ayis S, Lyall F, Robson SC. Late sporadic miscarriage is associated with abnormalities in spiral artery transformation and trophoblast invasion. J Pathol 2006; 208: 535-542.

[4] Kadyrov M, Schmitz C, Black S, Kaufmann P, Huppertz B. Pre-eclampsia and maternal anaemia display reduced apoptosis and opposite invasive phenotypes of extravillous trophoblast. Placenta 2003; 24: 540-548.

[5] Kim YM, Bujold E, Chaiworapongsa T, Gomez R, Yoon BH, Thaler HT, Rotmensch S, Romero R. Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. Am J Obstet Gynecol 2003; 189: 1063-1069.

[6] Khong TY, De Wolf F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. Br J Obstet Gynaecol 1986; 93: 1049-1059.

[7] Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 2008; 359: 61-73.

[8] Moster D, Lie RT, Markestad T. Long-term medical and social consequences of preterm birth. N Engl J Med 2008; 359: 262-273.

[9] Barker DJ, Martyn CN, Osmond C, Hales CN, Fall CH. Growth in utero and serum cholesterol concentrations in adult life. BMJ 1993; 307: 1524-1527.

[10] Bach JF. The contribution of cyclosporine A to the understanding and treatment of autoimmune diseases. Transplant Proc 1999; 31: 16S-18S.

[11] Du MR, Dong L, Zhou WH, Yan FT, Li DJ. Cyclosporin a improves pregnancy outcome by promoting functions of trophoblasts and inducing maternal tolerance to the allogeneic fetus in abortion-prone matings in the mouse. Bilo Reprone 2007; 76: 906-914.

[12] Du MR, Zhou WH, Dong L, Zhu XY, He YY, Yang JY, Li DJ. Cyclosporin A promotes growth and invasiveness in vitro of human first-trimester trophoblast cells via MAPK3/MAPK1-mediated AP1 and Ca^{2+}/calcineurin/NFAT signaling pathways. Biol Reprod 2008; 78: 1102-1110.

[13] Tang CL, Zhao HB, Li MQ, Du MR, Meng YH, Li DJ. Focal adhesion kinase signaling is necessary for the cyclosporin A-enhanced migration and invasion of human trophoblast cells. Placenta 2012; 33:704-711.

[14] Chen Q, Stone PR, McCowan LM, Chamley LW. Activated endothelial cells resist displacement by trophoblast in vitro. Placenta 2007; 28:743-747.

[15] Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. Nat Protoc 2007; 2: 481-485.

[16] Fadok VA, Bratton DL, Guthrie L, Henson PM. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. J Immunol 2001; 166: 6847-6854.

[17] Hung TH, Charnock-Jones DS, Skepper JN, Burton GJ. Secretion of tumor necrosis factor-alpha from human placental tissues induced by hypoxia-reoxygenation causes endothelial cell activation in vitro: a potential mediator of the inflammatory response in preeclampsia. Am J Pathol 2004; 164: 1049-1061.

[18] Toth M, Fridman R. Assessment of Gelatinases (MMP-2 and MMP-9) by Gelatin Zymography. Methods Mol Med 2001; 57: 163-174.

[19] Kockx M, Jessup W, Kritharides L. Cyclosporin A and atherosclerosis--cellular pathways in atherogenesis. Pharmacol Ther 2010; 128: 106-118.

[20] Hojo M, Morimoto T, Maluccio M, Asano T, Morimoto K, Lagman M, Shimbo T, Suthanthiran M. Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature 1999; 397: 530-534.

[21] Walsh SB, Xu J, Xu H, Kurundkar AR, Maheswari A, Grizzle WE, Timares L, Huang CC, Kopelovich L, Elmets CA, Athar M. Cyclosporine A mediates pathogenesis of aggressive cutaneous squamous cell carcinoma by augmenting epithelial-mesenchymal transition: Role of TGFβ signaling pathway. Mol Carcinog 2011; 50: 516-527.

[22] Zhou P, Luo X, Qi HB, Zong WJ, Zhang H, Liu DD, Li QS. The expression of pentraxin 3 and tumor necrosis factor-alpha is increased in preeclamptic placental tissue and maternal serum. Inflamm Res 2012; 61: 1005-1012.

[23] Chen JZ, Sheehan PM, Brennecke SP, Keogh RJ. Vessel remodelling, pregnancy hormones and extravillous trophoblast function. Mol Cell Endocrinol 2012; 349: 138-144.

[24] Knöfler M. Critical growth factors and signalling pathways controlling human trophoblast invasion. Int J Dev Biol 2010; 54: 269-280.
[25] Rosario GX, Konno T, Soares MJ. Maternal hypoxia activates endovascular trophoblast cell invasion. Dev Biol 2008; 314: 362-375.
[26] May SR, Stewart NJ, Chang W, Peterson AS. A Titin mutation defines roles for circulation in endothelial morphogenesis. Dev Biol 2004; 270: 31-46.
[27] Perez-Moreno M, Jamora C, Fuchs E. Sticky business: orchestrating cellular signals at adherens junctions. Cell 2003; 112: 535-548.
[28] Shih IeM, Hsu MY, Oldt RJ 3rd, Herlyn M, Gearhart JD, Kurman RJ. The role of E-cadherin in the motility and invasion of implantation site intermediate trophoblast. Placenta 2002; 23: 706-15.
[29] Arimoto-Ishida E, Sakata M, Sawada K, Nakayama M, Nishimoto F, Mabuchi S, Takeda T, Yamamoto T, Isobe A, Okamoto Y, Lengyel E, Suehara N, Morishige K, Kimura T. Up-regulation of alpha5-integrin by E-cadherin loss in hypoxia and its key role in the migration of extravillous trophoblast cells during early implantation. Endocrinology 2009; 150: 4306-4315.
[30] Staun-Ram E, Goldman S, Gabarin D, Shalev E. Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. Reprod Biol Endocrinol 2004; 2: 59.
[31] Neuhaus T, Stier S, Totzke G, Gruenewald E, Fronhoff S, Sachinidis A, Vetter H, Ko YD. Stromal cell-derived factor 1alpha (SDF-1alpha) induces gene-expression of early growth response-1 (Egr-1) and VEGF in human arterial endothelial cells and enhances VEGF induced cell proliferation. Cell Prolif 2003; 36:75-86.
[32] Wu X, Li DJ, Yuan MM, Zhu Y, Wang MY. The expression of CXCR4/CXCL12 in first-trimester human trophoblast cells. Biol Reprod 2004; 70: 1877-1885.