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Original Article

The role of nitric oxide in the outgrowth of trophoblast cells on human umbilical vein endothelial cells

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ABSTRACT

Objective: Embryo implantation is a complex process that requires coordinated trophoblast–endometrial interactions. Previous studies demonstrated that the identification of nitric oxide synthase (NOS) in trophoblast cells and the remodeling of the implantation process by nitric oxide (NO) support the important role of NO during implantation. However, the role of NO in trophoblast–endometrial interactions is unclear and is therefore examined in this study.

Materials and methods: We cocultured BeWo trophoblast spheroids with human umbilical vein endothelial cell (HUVEC) monolayers to mimic the trophoblast–endometrial interaction. $N^{(\omega)}$-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a competitive inhibitor of NOS, and sodium nitroprusside (SNP), an NO donor, were used to test the role of NO in the trophoblast–endometrial interaction.

Results: L-NAME diminished spheroid expansion on HUVEC monolayers in a concentration-dependent manner ($p < 0.05$). However, trophoblast spreading on HUVEC-free culture surfaces was unaffected by L-NAME treatment ($p > 0.05$). Significant suppression of spheroid expansion was found at the higher dose (1mM) of SNP ($p < 0.05$).

Conclusion: NO may be needed in the process of implantation, and an adequate but not overly NO-containing environment might be an important factor for successful implantation. This finding is worthy of further investigation.

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Introduction

Embryo implantation, a critical step in successful pregnancy, is a unique biological phenomenon [1]. The successful implantation is defined by the presence of a receptive endometrium, a functional embryo at the blastocyst developmental stage, and synchronized embryo–endometrial interactions [2,3]. Implantation begins with the apposition and attachment of the hatched blastocyst to the luminal epithelium of the uterus [4], and then trophoblast cells of the attached blastocyst invade through the luminal epithelium into the stroma to establish a relationship with the maternal vasculature [5–7]. During the implantation window, many factors are supposed to facilitate the implantation process [8], and this has contributed to the proposal of the existence of many molecular pathways addressing trophoblast penetration into the endometrial stroma [9]. However, the factors that regulate trophoblast invasion of the endometrial epithelial barrier are still uncertain, although nitric

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oxide (NO) is proposed to play an important role, because this multifunctional biomolecule is involved in human reproduction [10].

The identification of nitric oxide synthase (NOS) in trophoblast cells is the evidence that supports the important role of NO during implantation [11]. NOS includes three isoforms [12]. One is an inducible form called "iNOS," and the other two are constitutive forms—endothelial NOS (eNOS) and neuronal NOS (nNOS). iNOS, Ca^{2+} independent, is induced by cytokines and some other agents. By contrast, eNOS and nNOS, Ca^{2+}–calmodulin dependent, are involved in cellular signaling and present in almost all cells [13,14]. These three isoforms of NOS can be found in trophoblast cells of the placenta, including nNOS and eNOS in human beings [15,16] and in rhesus monkeys [17].

NO can stimulate the production of matrix metalloproteinases, which results in the remodeling of the extracellular matrix in cooperation with the process of implantation [18]. Therefore, we supposed that NO might promote the outgrowth of trophoblast cells. To elucidate the role of NO in the outgrowth of trophoblast cells, we used a coculture model in which BeWo trophoblast spheroids were cocultured with human umbilical vein endothelial cell (HUVEC) monolayers to mimic the trophoblast–endometrial interaction, and N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), a competitive inhibitor of NOS, and sodium nitroprusside (SNP), an NO donor [19–21], were used to test the role of NO in the trophoblast–endometrial interaction. Our results may have clinical significance, as they may clarify the physiological roles of NO in the implantation process.

Methods

Experimental design

To study the roles of NO in trophoblast–HUVEC interactions, we used a coculture model [22,23], in which BeWo trophoblast spheroids were cocultured with HUVEC monolayers. The effects of NO on trophoblast outgrowth on HUVEC monolayers were examined by measuring fold expansion in trophoblast spheroid areas. Then, we determined whether the spheroids themselves were targets for NO by measuring spheroid expansion on HUVEC-free culture surfaces in the presence or absence of NO.

Chemicals and reagents

L-NAME, an NOS inhibitor, and SNP were purchased from Sigma Chemical Co. (St Louis, MO, USA). These reagents were dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co.). Final concentrations of DMSO in all cell cultures were < 0.5%, which had no significant effect on the viability of HUVEC and BeWo cells. 5-Chloromethylfluorescein diacetate (CellTracker Green CMFDA) was purchased from Molecular Probes (Eugene, OR, USA).

HUVEC cell culture

HUVECs were purchased from the National Health Research Institute Cell Bank (Hsinchu, Taiwan) and cultured following the standard protocol [24].

Trophoblast spheroid formation

Human BeWo choriocarcinoma cells (CCL-98; American Type Culture Collection) were used to produce trophoblast spheroids. Cells were maintained in a Ham F-12K nutrient mixture (Life Technologies, 3175 Staley Road Grand Island, NY 14072, USA) supplemented with 15% fetal calf serum at 5% CO_2 and 37°C, and subcultured every 3 days with trypsinization. For the generation of spheroids, 2×10^6 BeWo cells in 10 mL of full-growth medium were plated onto an uncoated 100-mm plastic Petri dish (Alpha Plus, Taoyuan, Taiwan) [25,26]. Abundant spheroidal cell masses formed after a 24-hour culture, as a result of spontaneous cell aggregation. Spheroids with a diameter of 50–100 μm (sizes close to that of an implanting blastocyst) were selected under a dissecting microscope (Nikon SMZ645; Nikon Corp., Tokyo, Japan) for use in all the experiments that followed.

Measurement of spheroid outgrowth on endometrial epithelial cell monolayers

A trophoblast spheroid–HUVEC coculture model was used, as described previously [26]. The trophoblast spheroids became firmly attached to HUVEC monolayers after 60 minutes of coculture, and then began to flatten and expand. After 24 hours of coculture, the areas of trophoblast outgrowth became several times the original spheroid sizes. A quantitative method was used to examine the effects of various drugs on spheroid expansion on HUVEC monolayers. The HUVEC monolayers were prepared by seeding cells at 3×10^5/100 μL/well in 96-well plates that had been marked before with quadrants on the exterior surface under each well to allow the localization of the same sets of spheroids at different coculture time points. After 3 hours of incubation, confluent HUVEC monolayers formed and the culture medium was refreshed. Then, the HUVEC monolayers were treated with various drugs or the corresponding vehicles for 60 minutes or overnight, followed by the addition of trophoblast spheroids to the HUVEC monolayers at about two to five spheroids/well. The spreading of spheroids was observed at different coculture intervals (1 hour and 24 hours) under an inverted microscope (Nikon Diaphot; Nikon Corp.) and photographed using a cooled charge-coupled device camera system (Photometrics CoolSNAP fx; Roper Scientific Inc., Tucson, AZ, USA). To delineate the margins of trophoblast outgrowth, the spheroid–HUVEC coculture was labeled with 10μM CellTracker Green CMFDA, a nontoxic fluorescent probe, for 30 minutes before photographing at 24 hours of coculture. Because the individual BeWo trophoblast cells spread far more extensively on the culture plate than the HUVEC cells, CellTracker staining appeared dimmer for the BeWo cells and brighter for the HUVEC cells. These staining features were used to demarcate the boundary of trophoblast outgrowth on HUVEC monolayers. The areas of spreading spheroids were quantified using the Scion Image Software system (based on the NIH Image, Scion Corporation, Frederick, MD, USA). The spheroid areas at 1 hour of coculture were regarded as their original sizes, and fold expansion in spheroid areas was calculated at 24 hours of coculture.

Statistical analysis

Data were expressed as the mean ± standard error of the mean. Statistical significance between groups was determined by one-way analysis of variance using the general linear model, followed by Fisher *post hoc* least significant difference test; $p < 0.05$ was considered statistically significant. The concentration-dependent effect was analyzed by simple linear regression of all response data against concentration levels of the treatment. All analyses were performed using the SAS program (SAS Institute Inc., Cary, NC, USA) on a Pentium IV-based personal computer.

Results

To examine whether NO can regulate trophoblast outgrowth on HUVEC monolayers, we introduced L-NAME, an NOS inhibitor, to HUVEC monolayers 60 minutes before the delivery of trophoblast spheroids. The concentrations of L-NAME were adjusted to between

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