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Effects of different arginine concentrations on angiogenic protein production induced by HeLa cells

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ABSTRACT

Objectives: This in vitro study investigated the effects of different arginine (Arg) concentrations on angiogenic protein expressions of HeLa cells and endothelial cells (ECs) after stimulation. In addition, an inducible nitric oxide (iNO) synthase inhibitor (1400 W) was used to investigate the possible role of iNO in angiogenesis.
Methods: Endothelial cells and HeLa cells were treated with different concentrations of Arg and 1400 W: Arg 0, 50, 100, and 1000 μmol/L; Arg 100 μmol/L + 1400 W 10 μmol/L; and Arg 1000 μmol/L + 1400 W 10 μmol/L for 24 h. Then, ECs and HeLa cells were cocultured for 2 h, and the supernatant in the transwell was collected for analysis of angiogenic protein secreted. The expression of CD51/CD61 by ECs was also analyzed.
Results: The productions of vascular endothelial growth factor, basic fibroblast growth factor, prostaglandin E_2, and matrix metalloproteinase-2 were higher with Arg 100 and 1000 μmol/L than with Arg 0 and 50 μmol/L Arg, and this was consistent with the expression of CD51/CD61 by ECs. Inhibition of iNO production resulted in lower angiogenic protein expressions comparable with groups with low Arg administration.
Conclusion: The findings of this study suggest that Arg administration at levels similar to or higher than physiologic concentrations enhance the production of angiogenic protein and iNO may partly play a role in promoting angiogenesis in the presence of HeLa cells.

Introduction

Arginine (Arg) is a non-essential amino acid for healthy adults with plasma concentrations of ~100 μmol/L [1]. Arg has been shown to possess numerous useful physiologic properties. Previous reports have shown that supplemental dietary Arg accelerates wound healing, enhances immune function, and improves survival in injured conditions [2–4]. Also, Arg is fundamental for the function of T lymphocytes [5,6]. Studies in tumor-bearing mice and patients with cancer have indicated that increased metabolism of L-Arg inhibits the T-lymphocyte response [7,8]. Several animal tumor models and many clinical trials have demonstrated that immunotherapy in subjects with advanced tumors failed to achieve a therapeutic response as a result of loss of the T-cell response [9]. Arg supplementation is important in restoring T-cell antitumor function. Arg is often used in immunonutrition regimens. A previous study has shown that immunoenhancing diets containing Arg decrease the infection rate in the postoperative period for patients with head, neck, and esophageal cancers [10]. However, a report by Park et al. [11] described an increase in tumor proliferation markers in patients with breast cancer treated with dietary Arg supplements. Previous studies also have shown that Arg deprivation arrests the growth of cells and causes the death of malignant cells [12,13].

Arginine is the substrate of nitric oxide synthase (NOS) and a precursor of nitric oxide (NO). NO can act as a signal transducer and cellular messenger in homeostasis and host defense.

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Previous studies have shown that NO plays a critical role in antipathogenic and tumoricidal responses of the immune system [14,15]. However, NO has also been implicated as a deleterious agent in various pathophysiologic conditions including cancer [16,17]. Some studies have shown that tumors expressing NOS are more aggressive than their counterparts without NOS expression [16,17]. The Arg–NO-mediated modulatory effect on various cancers remains controversial.

Cancer was the leading cause of death in Taiwan in 2008. Cervical cancer was ranked eighth in mortality among all patients with cancer [18]. Because plasma Arg concentrations are decreased and protein–energy malnutrition is often present in patients with cancer [19], an Arg-supplemented formula may be recommended for patients with cancer. However, human gynecologic cancers are malignant tumors with high levels of NOS activity [20]. A study by Wheatley et al. [21] also found that Arg deprivation reduced DNA synthesis and protracted the cell cycle in HeLa cells. We speculated that Arg supplementation might promote the progression of cervical cancer. Therefore, we designed this in vitro study using a transwell apparatus to investigate the interactions between endothelial cells (ECs) and a cervical cancer cell line (HeLa). ECs and HeLa cells were cocultured at different Arg concentrations (0, 50, 100, and 1000 μmol/L). Several angiogenic factors including CD51/CD61 (αvβ3), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and matrix metalloproteinase (MMP) produced by cancer cells and ECs were analyzed. N(-[3-(aminomethyl)benzyl]acetamidine) (1400 W) is a highly selective inhibitor for inducible NOS (iNOS) [22]. In this study, we used 1400 W to investigate the possible role of inducible NO (iNO) in angiogenic protein production.

Materials and methods

Cell culture

Human umbilical vein endothelial cells were isolated from an umbilical cord vein according to the method of Jaffe et al. [23]. The umbilical vein was cannulated, washed with phosphate buffered saline (PBS; Sigma Chemical Co., St. Louis, MO, USA) and perfused with PBS containing 0.1% collagenase for 10 min at 37 °C in 5% CO_2. ECs were collected and established as a primary culture in medium-199 (Gibco Life Technologies, Rockville, MD, USA) containing 20% fetal bovine serum (FBS; Gibco Life Technologies), 20 mM $NaHCO_3$, 25 mM HEPES, antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL), heparin sodium 10 IU/mL, and endothelial cell growth supplement 15 mg/L (Gibco Life Technologies) at 37 °C in an environment with 5% CO_2. Cells were serially passaged two to three times for the experimental assay. ECs (1×10^5 cells/well) from second subcultures were grown on fibronectin-coated inserts (3-μm pore size, 6.4 mm; Becton Dickson, Franklin Lakes, NJ, USA) until the monolayer was confluent. HeLa cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan), and cultured in L-15 medium supplemented with 10% FBS and a penicillin/streptomycin mixture. Adherent monolayer cultures were maintained at 37 °C in 5% CO_2. Cells were routinely trypsinized (0.05% trypsin/ethylenediaminetetra-acetic acid) and subcultured in flasks. ECs and HeLa cells were incubated in medium-199 (with 20% heat-inactivated FBS) with different concentrations of Arg and 1400 W: Arg 0 μmol/L, Arg 50 μmol/L, Arg 100 μmol/L, Arg 1000 μmol/L, Arg 100 μmol/L + 1400 W 10 μmol/L, and Arg 1000 μmol/L + 1400 W 10 μmol/L for 24 h. The viability of human umbilical vein endothelial cells and HeLa cells after incubation was greater than 95% as confirmed by trypan blue staining.

Measurements of MMP-2, MMP-9, VEGF, bFGF, and PGE_2 in the supernatant

The ECs were placed in the upper chamber and HeLa cells in the lower chamber of a transwell apparatus unit (Becton Dickson). After the EC–HeLa cell interaction had proceeded for 2 h, solutions in the upper chambers of the transwells were collected and centrifuged at $125 \times g$ for 10 min for further analysis. Concentrations of MMP-2, MMP-9, VEGF, and bFGF were measured using commercially available sandwich enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA). PGE_2 concentrations were measured by competitive enzyme-linked immunosorbent assay. Acetylcholinesterase covalently coupled to PGE_2 was used as the enzymatic tracer (R&D Systems). Procedures followed the manufacturer's instructions.

Measurements of CD51/CD61 expression by ECs

The EC surface expressions of CD51/CD61 were measured after the EC–HeLa cell interaction had proceeded for 4 h in the transwell coculture system. After removing the supernatant, ECs were washed twice with PBS, and the pellets were incubated with iced medium containing 2 mM ethylenediaminetetra-acetic acid to detach adherent ECs. Then the pellets were incubated with 100 μL of medium-199 (FBS free, containing 2 mM ethylenediaminetetra-acetic acid) for a further 30 min at 4 °C with the addition of fluorescein isothiocyanate–conjugated mouse anti-human CD51 and phycoerythrin-conjugated mouse anti-human CD61 (Serotec, Oxford, UK). The suspension was collected into a tube and resuspended in 500 μL of PBS (containing 0.3 mL of 350 mM formaldehyde). The percentages of CD51/CD61 expressed on ECs were analyzed by flow cytometry (Coulter, Miami, FL, USA). Fluorescence data were collected and the results are presented as a percentage of CD51/CD61-presenting cells in 5×10^3 ECs.

Tube formation assay

The tube formation assay was performed as previously described [24]. Twenty-four–well plates were coated with 100 μL of Matrigel per well and incubated at 37 °C for 30 min to promote jelling and then seeded with ECs (1×10^4 cells). After being cultured with various concentrations of Arg and 1400 W as described earlier and stimulated with supernatant of HeLa cells for 18 h at 37 °C, the formation of capillaries by ECs was observed using a microscope (400×). Three random measurements of each of four wells at each culture condition were made. Each experiment was repeated at least three times.

Statistical analysis

Data are expressed as the mean ± standard deviation. Results are representative of three independent experiments. Differences among groups were analyzed by analysis of variance using Duncan's test. $P < 0.05$ was considered statistically significant.

Results

MMP-2, MMP-9, VEGF, bFGF, and PGE_2 concentrations in the supernatant

Concentrations of VEGF, and bFGF were higher with Arg 100 and 1000 μmol/L than with Arg 0 and 50 μmol/L. PGE_2 levels were higher with Arg 1000 μmol/L than in the other groups. MMP-2 concentrations were higher in the Arg 100 and 1000 μmol/L groups than in the other groups. There were no differences in MMP-9 concentrations among all groups. Increments in MMP-2, VEGF, bFGF, and PGE_2 levels were abolished when 1400 W was added (Table 1).

CD51/CD61 expression by ECs

The CD51/CD61 expression by ECs was higher with Arg 100 and 1000 μmol/L when stimulated with HeLa cells than with Arg 0 and 50 μmol/L. There were no differences in percentages of CD51/CD61-expressing cells among the Arg 0 and 50 μmol/L and the groups with 1400 W administration (Fig. 1).

Tube formation of ECs

Cells incubated with Arg 0 and 50 μmol/L had large numbers of unorganized cells (Fig. 2A,B), whereas the Arg 100 and 1000 μmol/L groups formed more organized tubes than did the Arg 0 and 50 μmol/L groups (Fig. 2C,D). The enhanced EC differentiation in the Arg 100 and 1000 μmol/L groups was abrogated when 1400 W was administered (Fig. 2E,F).