Original article

All-Trans Retinoic Acid Reduces Matrix Metalloproteinase-2 and Increases E-Cadherin Levels in BeWo Choriocarcinoma Cells

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Abstract:

Background*:** Choriocarcinoma is a malignant trophoblastic tumor that can degrade the uterine basement membrane to facilitate local and distant metastasis. Matrix metalloproteinase-2 (MMP-2) is a vital proteinase produced by trophoblasts and can cleave type IV collagen in the basement membrane of the uterine epithelium. In addition, E-cadherin controls cell adhesion, which is associated with tumorprogression, and loss of E-cadherin function is associated with metastasis. Vitamin A analogues, including all-trans retinoic acid (ATRA), exhibit anticancer properties, including metastasis inhibition.This study aimed to investigate the ability of ATRA to prevent trophoblast invasion and metastasis in choriocarcinoma by assessing MMP-2 and E-cadherin activity. ***Methods: This study administered various doses of ATRA to the BeWo (American Type Culture Collection CCL-98) choriocarcinoma cell line and assessed MMP-2 and E-cadherin activity through flow cytometry. The research was performed at the Biomedical Laboratory of the Faculty of Medicine of Universitas Brawijaya Malang, East Java, Indonesia. ***Results:*** BeWo cells were exposed to 0, 50, 100, 200, 400, or 800 µg/mL ATRA. MMP-2 was downregulated after ATRA treatment. The greatest reduction in MMP-2 level was observed after 200 µg/mL ATRA treatment, but this result was not statistically significant (P = 0.550). However, ATRA treatment significantly reduced the number of BeWo cells with low E-cadherin levels (P = 0.012). ***Conclusion:*** ATRA downregulates MMP-2 and upregulates E-cadherin. Further in vitro and animal studies are required to evaluate the ability of ATRA to inhibit metastasis.

Keywords:ATRA, BeWo cell line; choriocarcinoma; MMP-2; E-cadherin

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Introduction:

Choriocarcinoma is a malignant tumor resulting from the abnormal proliferation of trophoblasts.[1] The placenta of patients with choriocarcinoma is characterized by hemorrhage, necrosis, the absence of chorionic villi, and high metastatic potential. Recently, chemotherapy has been performed for treating choriocarcinoma, but it presents risks of secondary malignancies, nausea, vomiting, alopecia, diarrhea, fever, infection, and anemia.[2] Chemotherapy induced anemia of any degree is a contributor for increasing risk of need for

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blood transfusions including higher morbidity and mortality in cancer.[3]Therefore, novel agents with high effectiveness and low toxicity are required for the successful treatment of choriocarcinoma.

Trophoblasts can degrade the basal membrane of the endometrium and invade the myometrium, leading to local and distant metastasis.[4] Moreover, trophoblasts interact with the extracellular matrix (ECM), which affects the invasion, migration, and metastasis of cancer cells.[5]Matrix metalloproteinases (MMPs) play an important role in trophoblast implantation. MMPs are endopeptidases that function for the extracellular environment degradation, telomerase action inhibition, and immunosuppressive environmental chemicals support. Trophoblast invasion begins shortly after implantation in a normal pregnancy, and various adhesion and signaling events prevent invasion beyond the myometrium. By contrast, in choriocarcinoma, trophoblasts invade the myometrium, escape the inner third of the myometrium and replace maternal vascular endothelium,leading to uncontrolled cell growth and metastasis.[6]

Several factors, such as MMPs, gonadotropin chorionic hormone, and vascular endothelial growth factor (VEGF), influence the essential endometrial modifications for implantation and placental development. Trophoblast attachment to the ECM triggers the downregulation of proteinases that play a role in destroying and invading the matrix. MMP-2 is one of the most important proteinases produced by trophoblasts. It cleaves type IV collagen at the basal membrane of the uterine epithelium. Furthermore, elevated decidual MMP-2 is associated with increased trophoblast invasion and metastasis in choriocarcinoma.[7]

Tumor growth and development are associated with cell adhesion, controlled by a member of the calcium-dependent adhesion (cadherin) superfamily: E-cadherin. The E-cadherin–catenin complex develops an adherents junction construction structure that promotes cell adhesion. Impairment of this function can promote tumor growth, invasion, and metastasis.[8]

Retinoids are vitamin A analogues that influence cell proliferation and differentiation. They inhibit cell proliferation in many cancers by attaching to intracellular retinoic acid receptors (RARs). The interaction of retinoids with specific DNA response elements, such as the retinoic acid response element (RARE), is important in activating the transcription of retinoid target genes. Other vitamin A analogues, including all-trans retinoic acid (ATRA), can reduce MMP levels and activity. ATRA was reported to be a potent chemotherapeutic agent because of its ability to regulate cell growth and differentiation.[9] ATRA can effectively regulate cell proliferation and induce apoptosis and may thereby prevent the persistent proliferation of trophoblasts in choriocarcinoma.[10]

This study investigated the ability of ATRA to prevent trophoblast invasion and metastasis in choriocarcinoma by assessing MMP-2 and E-cadherin activity in American Type Culture Collection (ATCC) BeWo CCL-98 cells. This research was performed in vitro at the Biomedical Laboratory of the Faculty of Medicine of Universitas Brawijaya Malang, East Java, Indonesia.

Materials and Methods:

This study used the human placental choriocarcinoma cell line BeWo (ATCC CCL-98), cultured at the Physiology Laboratory of the Medical Faculty of Universitas Brawijaya Malang Central Diagnostic, Saiful Anwar Public Hospital, East Java, Indonesia.

We thawed cell cultures frozen in liquid nitrogen vapor by submerging them in a water chamber at 37°C for 2 min and then purifying them with 70% ethanol spray. We prepared the cells following a previously described method.[11]

The BeWo ATCC CCL-98 cell cultures were divided into six groups and left untreated or exposed to ATRA at 50, 100, 200, 400, or 800 µg/mL. The confluent cells were incubated for 6 h, and each condition was replicated four times. The attachment of cells and intercellular contacts were observed microscopically to determine the state of cell confluency. The confluent monolayer of cells was characterized by its spread and the flat cells covering the available surface, which had small uniform spacing and large nuclei.

The cell cultures were harvested and then fixed with absolute methanol. A cover glass was submerged in the solution in the well plate. The harvested cell cultures were retained in the solution for approximately 10 min and washed with phosphate-buffered saline (PBS) at pH 7.4 for 5 min. The washed cells were then harvested with Triton solution, washed three times with PBS, and incubated in 3% hydrogen peroxide for 20 min. The medium was supplemented with 5% fetal bovine serum and incubated for 30 min.

The cells were aliquoted for primary antibody staining and incubated overnight at 4°C. The cells were then resuspended in PBS for 5 min after incubation. After being washed three times with PBS, the cell cultures were incubated with fluorescein isothiocyanate (FITC)-labelled secondary antibody in a dark room for 60 min at 4°C. The cells were then washed three times with PBS for 5 min. The nuclei were visualized through 4′,6'-diamidino-2-phenylindole (DAPI) counterstaining. Flow cytometry analyses for MMP-2 and E-cadherin were performed using a Becton-Dickinson FACScan flow cytometer (Immunocytometry Systems, San Jose, CA, USA).

Statistical analysis

The Shapiro–Wilk test was performed as a prerequisite for parametric testing. Hypothesis testing was performed through a one-way analysis of variance. The MMP-2 and E-cadherin levels of the six groups of BeWo cells exposed to various ATRA concentrations were compared. A post hoc least significant difference test was performed to compare the effect of ATRA treatment at different doses.

Ethical Clearance:

This study was approved by the Medical Ethics Committee, RSUD Dr. Saiful Anwar, Malang, East Java (No. 400/150/K.3/302/2019). This study has no written patient consent. This study used BeWo Choriocarcinoma Cells (American Type Culture Collection CCL-98)

Results:

Flow cytometric analysis revealed that ATRA reduced MMP-2 and free E-cadherin levels [Figure 1] in the BeWo cell line in a dose-dependent manner; the untreated BeWo cells exhibited no change in MMP-2 levels. The greatest reduction was observed in the cells treated with 200 µg/mL ATRA, but this result was not even statistically significant [$P > 0.550$; Figure 2].

The untreated BeWo culture had numerous single cells with low E-cadherin [Figure 1a]. A scatter plot revealed greater numbers of cell aggregates with E-cadherin after treatment with greater ATRA concentrations. The plot indicated a trend in the BeWo cell population from low E-cadherin toward high E-cadherin levels as the dose of ATRA increased. Moreover, the proportion of single BeWo cells with low E-cadherin levels was significantly lower in the cultures treated with 50, 200, or 800 µg/mL ATRA ($P < 0.05$) than in the untreated culture [Figure 3].

Discussion:

Retinoids, which include β-carotene, retinol, retinal, isotretinoin, ATRA, 9-cis retinoic acid, and 13-cis retinoic acid, are products of vitamin A or analogues metabolism. The human body absorbs retinoids from animal and plant sources, and they play essential roles in cell proliferation, differentiation, and death or apoptosis. Furthermore, retinoids are involved in immunity, nervous function, and embryonic development. ATRA is a carboxylic acid form of vitamin A with two nuclear retinoid receptors (RARs and retinoid X receptors [RXRs]) that act as ligand-dependent transcription factors. ATRA is a natural ligand that binds with high affinity to RARs but does not bind to RXRs. Many researchers have performed in vitro or in vivo experiments to evaluate the chemoprotective effects of ATRA.[12]ATRA can inhibit the progression of various types of cancers, and its effects include antiproliferative, proapoptotic, and anti-metastatic effects.[13]

The anticancer effects of ATRA on choriocarcinoma have been extensively studied. Research using choriocarcinoma cell lines such as JAR and JEG-3 has demonstrated that the anticancer effects of ATRA are related to its antioxidant profile.[11] ATRA have ability to inhibit oxidative stress leads to decrease of reactive oxygen species and oxidative stress.[14] ATRA in combination with methotrexate and/or without actinomycin D may exert a strong proapoptotic effect on choriocarcinoma cells and prevent resistance to chemotherapy agents.[15] In addition, ATRA treatment of BeWo cells reduced telomerase activity, which is important in the apoptotic process and inhibition of tumor growth.[11]

Choriocarcinoma is a malignant tumor originating from trophoblasts. Trophoblasts can penetrate the uterine epithelium and the basal membrane and invade the stroma of the decidua, both of which are crucial to appropriate embryo implantation in normal pregnancies. Trophoblasts have an invasive phenotype and produce proteinases required for uterine matrix degradation and migration through the degraded matrix. MMP-2 is a vital proteinase produced by trophoblasts and is primarily present in the distal column of cytotrophoblasts and invasive extra villous cytotrophoblasts that influence the degradation of ECM of the uterine epithelium.[7]

Trophoblasts can produce MMPs, including MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92

kDa), that degrade the uterine basement membrane.[16] Abnormal MMP-2 levels in placental tissues lead to choriocarcinoma progression by promoting tumor invasion and metastasis. Previous studies have demonstrated the ability of ATRA to inhibit MMP-2 in various tumor types.[13]

In this study, ATRA reduced MMP-2 levels, but this result was nonsignificant. MMP-2 was upregulated after 50 µg/mL ATRA treatment but gradually decreased after that. ATRA treatment can upregulate or downregulate MMP-2, depending on the RAR signaling pathway and level of intracellular calcium ions, in a dose- and time-dependent manner. The upregulation of MMP-2 by ATRA is a primary cause of the retinoic acid syndrome. Additional in vitro and animal studies of ATRA effects are necessary to evaluate this hypothesis.[13] ATRA, a water-soluble molecule, can effectively control gene expression signals by scattering rapidly to bind with particular RARs and RXRs. Genes related to MMPs such as stromelysin-1, collagenases, and gelatinases are the regulatory targets of retinoids. Moreover, ATRA may reduce both MMP-2 production and MMP-2 mRNA.[17]

E-cadherin is a calcium membrane glycoprotein that facilitates cell-cell connection with Ca^{2+} ions. Loss of E-cadherin function is associated with malignancy and tumor progression and can lead to abnormal differentiation, deep invasion, and metastasis. ATRA upregulates E-cadherin, which is important for preventing cancer metastasis.[18] In this study, ATRA treatment significantly reduced the number of BeWo cells with low E-cadherin levels ($P = 0.012$).

The ability of ATRA to prevent cell adhesion alteration is associated with its ability to downregulate β1 integrin-linked protein kinase (ILK). Excessive ILK can damage intercellular connections, and a lack of cell anchorage to the ECM promotes migration and metastasis. Elevated ILK is contrary to E-cadherin function. The inhibition of ILK by ATRA may reverse such effects and thus explain ATRA-induced E-cadherin upregulation.[9]

Conclusion:

In this study, ATRA downregulated MMP-2 and upregulated E-cadherin. Both MMP-2 and E-cadherin are important in choriocarcinoma metastasis. Further in vitro studies and animal studies are required to evaluate the ability of ATRA to inhibit metastasis.

Conflicting interest

The authors declare no conflict of interest

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Figure Legends

Figure 1: Effects of ATRA on MMP-2 and E-cadherin in BeWo cell line: (a) negative control; (b–f) 50, 100, 200, 400, and 800 µg/mL ATRA, respectively. Q1: Low MMP-2 and high E-cadherin, Q2: High MMP-2 and high E-cadherin, Q3: Low MMP-2 and low E-cadherin, Q4: High MMP-2 and low E-cadherin

Figure 2: Effects of ATRA on MMP-2 in BeWo cell line

Figure 3: Effects of ATRA on free E-cadherin in BeWo cell line