RESEARCH ARTICLE

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Effects Atra on MMP-9 Activity and Integrin Expression in Choriocarcinoma Culture Cell Line Bewo (ATCC CCL-98)

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Abstract

Objective: Choriocarcinoma is characterized by aggressive growth and metastasis, and integrins and matrix metalloproteinase-9 (MMP-9) play crucial roles in its progression. ATRA (All-trans retinoic acid) has been shown to have anti-tumor effects in various cancers, poses a 15-20% malignancy risk. This study investigates the effects ATRA on MMP-9 activity and integrin expression in the choriocarcinoma cell line BeWo, and to explore the potential mechanisms underlying these effects. **Methods:** This is an experimental study using choriocarcinoma cell line BeWo. Six treatment groups were established to varying concentrations of ATR and one group served as a control without ATRA exposure. The groups were labelled with fluorescence and performed with flowcytometry to determine MMP-9 and integrin expression. Data was presented as mean ± SD and analyzed using one-way ANOVA with post-hoc tests in which p-value <0.05. **Result:** ATRA at the doses of 100, 200, 400, and 800 µg/ml significantly decreased the MMP-9 activity compared to those in the control group. Furter, ATRA at the doses of 100, 200, 400, and 800 µg/ml significantly increased the integrin expression compared to those in the control group. **Conclusion:** ATRA might decrease the MMP-9 activity as an invasive factor and increase integrin expression as an adhesive factor in the BeWo choriocarcinoma cell line that might play a role in the metastasis mechanism.

Keywords: MMP-9- integrin- choriocarcinoma- ATRA- retinoic acid

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Introduction

Choriocarcinoma had a mortality rate of almost 100% when metastases are present and approximately 60% even if hysterectomy is done for apparent nonmetastatic disease. Gestational trophoblastic neoplasms are now some of the most curable of all solid tumours, with cure rates >90% even in the presence of widespread metastatic disease [1]. In this malignant condition, the neoplastic trophoblasts continue to invade and grow without restraint, resulting in highly vascularised metastasis to organs such as the lungs, brain, and liver, as well as with poor prognosis, 3–6 especially in the pre-chemotherapy era [2].

Integrins are transmembrane heterodimeric glycoprotein receptors consisting of α and β subunits and are comprised of an extracellular domain, a transmembrane domain, and a cytoplasmic tail. Therein, integrin cytoplasmic domains may associate directly with numerous cytoskeletal proteins and intracellular signalling molecules, which are crucial for modulating fundamental cell processes and functions including cell adhesion, proliferation, migration, and survival by regulating signal transduction pathways. Based on the unique structure of integrins, including the α- and β-subunits, integrins can bind with extracellular matrix (ECM) proteins such as collagen (CO), laminin (LN), fibronectin (FN), vitronectin (VN), and some other cellular receptors [3]. Matrix metalloproteinase-9 involvement in malignant tumour progression has shifted from the original concepts of an almost exclusive role in matrix degradation, associated with tumour invasion. Currently, it has included the roles of most aspects of tumour biology, ranging from initiation and early progression to angiogenesis, dissemination, invasion and motility, the formation of the cancer stem cell niche, regulation of tumour immunological surveillance, metastatic site preparation, and the promotion of metastatic growth [4].

All-trans retinoic acid (ATRA) is one of the vitamin A natural derivatives. It can be obtained from the diet as preformed retinoids from animal sources and provitamin carotenoids from plant sources. Vitamin A plays an important part in the maintenance of normal growth, reproduction, and cell membrane integrity, mediated by nuclear receptors. The activated nuclear receptors control the expression of genes that regulate cell differentiation and growth and the induction of apoptosis. Our previous

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studies have also shown that the rates of proliferation and apoptosis, as well as the patterns of expression of oncogenes, tumour suppressor genes, cathepsin D, tissue inhibitor of metalloproteinases and telomerase, have a role in the pathogenesis of choriocarcinoma [2].

Although previous studies have shown the effect of ATRA on choriocarcinoma, to the best of our knowledge, there has been no detailed study that examines the impact of ATRA dose on telomerase in choriocarcinoma cells [5]. Hence, this study aimed to fill the gap by investigating the effects of ATRA on choriocarcinoma BeWo culture cell line where MMP-9 activity serves as an invasion factor and integrin expression functions as an adhesion factor.

Materials and Methods

Study Design

This study was an experimental design with analysis was carried out at the end of the treatment (post control group design). The study was conducted in vitro using choriocarcinoma cell line culture BeWo (ATCC CCL-98) from American Type Culture Collection CCL-98. The study site was at the laboratory of cell culture of physiology in the faculty of medicine, Brawijaya University Malang. It lasted for three months from July to November 2020 and was designed as a double-blinded experiment with the analysis performed at the end of the treatment (post-test control group design). This study was approved by the Health Research Ethics Committee of the Faculty of Medicine of Brawijaya University, Number 400/162/K.3/302/2020.

Experimental Procedure

The study was conducted in vitro using choriocarcinoma cell line culture BeWo (ATCC CCL-98) from American Type Culture Collection CCL-98. The growth of the culture is indicated by a minimum inoculum of $5x10^5$ viable cells in 4 mL, observed under a light microscope in the culture medium. Confluent cells are characterized by cells adhering to attachment sites and establishing contact or connections with neighboring cells. The cells exhibit regular and increasingly tight spacing, with a flat surface appearance marked by visible nuclei, plasma membranes, cytoplasm, and extracellular matrix. Additionally, the cells display larger sizes as a distinguishing feature. This culture was divided into 6 treatment groups where each group was repeated 4 times. ATRA is obtained from Cayman Chemical Item No. 11017. The groups consisted of those without ATRA exposure (group I as control), the group with the ATRA exposure of 50 ug/ml (group II), 100 ug/ml (group IIII), 200 ug/ml (group IV), 400 ug/ml (group V), and 800 ug/ml (group V) that were incubated for 6 hours after ATRA exposure. Following this, the groups were labelled with fluorescence and performed with flowcytometry technic to determine MMP-9 activity and integrin expression. MMP-9 activity refers to the proteolytic enzymatic activity of Matrix Metalloproteinase-9 (MMP-9), as assessed using the monoclonal antibody MBS2025637. This activity is quantified by measuring the proportion of substrate remaining relative to the initial substrate concentration. Integrin expression refers to the expression of human integrin antibodies, specifically CD29 (Integrin beta 1), using the monoclonal antibody TS2/16 conjugated with FITC (Fluorescein Isothiocyanate) from eBioscience™, on the cell membrane of the BeWo choriocarcinoma cell line.

MMP-9 Activity Measurement

The measurement of MMP-9 activity using flow cytometry. All materials and reagents are prepared at a temperature of 18–25°C. A volume of 100 μL of the standard solution and the BeWo choriocarcinoma cell culture (ATCC-CCL-98) is added to each well. The plate is incubated for 2.5 hours at room temperature or overnight at 4°C. After incubation, the solution is discarded and the wells are washed four times with Wash Solution. The washing process is performed by filling each well with 300 μL of 1x Wash Solution. The plate is then dried using clean paper or towels.

Next, 100 μL of biotinylated MMP-9 detection antibody (1x, prepared according to Standard Preparation Step 10) is added to each well and incubated for 1 hour at room temperature. The washing process is repeated as previously described. Subsequently, 100 μL of 1x HRP-Streptavidin solution is added to each well and incubated for 45 minutes at room temperature. After incubation, 100 μL of TMB substrate reagent is added to each well, followed by incubation for 30 minutes at room temperature. To stop the reaction, 50 μL of Stop Solution is added to each well.

The absorbance is measured at 450 nm. The average absorbance for each standard set is calculated, and the data are plotted on a standard curve using a log-log graph, with the standard concentration on the x-axis and absorbance on the y-axis. The numerical data obtained represent the remaining substrate or the residual product of the MMP-9 antibody reaction.

Integrin Expression Measurement

The measurement of integrin expression using flow cytometry. Excess microplate strips are removed from the plate frame, returned to the foil pouch containing a desiccant pack, and resealed. A volume of 10 μL of Assay Diluent RD1-78 is added to each well. 50 μL of standards, controls, or samples is added to each well, ensuring that sample addition is uninterrupted and completed within 15 minutes. The wells are sealed with adhesive strips provided and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. A plate layout is used to document the standards and samples tested.

Each well is aspirated and washed, repeating the process three times for a total of four washes. Washing is performed by filling each well with 400 μL of Wash Buffer using a spray bottle, multi-channel dispenser, or autowasher. Complete removal of liquid at each step is critical for optimal performance. After the final wash, residual buffer is removed by aspiration or decanting, and the plate is inverted and blotted dry on clean paper towels. A volume of 200 μL of human integrin conjugate is added to each well, followed by sealing with new adhesive strips.

The plate is incubated for 2 hours at room temperature. Aspirations and washes are repeated as described in the earlier step.

Subsequently, 200 µL of substrate solution is added to each well and incubated for 30 minutes at room temperature, protected from light. Following this, 50 µL of Stop Solution is added to each well. The color in the wells should change from blue to yellow. If the color appears green or uneven, gently tap the plate to ensure thorough mixing. Optical density (O.D.) readings are taken for all standards, controls, and samples, with the zero O.D. average subtracted. A standard curve is generated by plotting the average absorbance for each standard on the y-axis against the corresponding concentration on the x-axis.

Data Analysis

Data are assessed for normal distribution using the Shapiro-Wilk test. The decision criterion relies on the p-value. If the p-value is less than the significance level ($\alpha = 0.05$), the data are considered not normally distributed, and parametric tests cannot be applied.

The One-Way ANOVA (F-test) is employed to compare six sample groups regarding MMP-9 activity and integrin expression. The significant results of ANOVA were tested using the post hoc test in which p-value < 0.05. Post hoc analysis is conducted using Tukey's test if variance homogeneity is met, otherwise, Dunnett T3 is used. This analysis aims to evaluate the effect of ATRA at doses of 0 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, and 800 µg/mL on MMP-9 activity and integrin expression in BeWo choriocarcinoma cell line cultures (ATCC-CCL-98). Data presented in this study used the mean value of ± SD. The researchers used SPSS 17.0 statistical package.

Results

Figure 1 shows the the subculture of BeWo choriocarcinoma cell (ATCC CCL 98) which was observed under a microscope with 400x magnification. It was then compared based on the density level of the cultured cells and the schematic image of the shape of choriocarcinoma cell.

Table 1. MMP-9 Activity and Integrin Expression in All Group

Group	MMP-9 activity (mean ± SD)	Integrin expression (mean ± SD)
Control	0.31 ± 0.11	1.14 ± 0.18
50 µg/mL	0.45 ± 0.15	0.97 ± 0.21
100 µg/mL	2.89 ± 1.07	5.51 ± 2.04
200 µg/mL	2.13 ± 0.64	8.52 ± 0.79
400 µg/mL	5.72 ± 0.47	10.39 ± 1.84
800 µg/mL	7.19 ± 0.54	8.62 ± 0.3

Table 1 shows the histogram of mean + SD that indicates the effects of ATRA on the MMP-9 activity, in choriocarcinoma cell line BeWo based on the remaining substrate. The data were gathered in a different manner of doses (0 ug/ml, 50 ug/ml, 100 ug/ml, 200 ug/ml, 400 ug/ml, and 800 ug/ml. Based on one-way ANOVA analysis, MMP-9 activity significantly decreased at all doses compared to the control group ($p<0.05$). Following the Dunnet T3 post hoc test, it was revealed that the MMP-9 activity significantly decreased at doses 100, 200, 400 and 800 ug/ml compared to the control group. However, at the dose 50 ug/ml, there was no significant effect of giving ATRA to decrease the activity of MMP-9.

Furthermore, Table 1 also provides the histogram of mean + SD that shows the effects of ATRA on the integrin expression, in choriocarcinoma cell line BeWo in a different manner of doses (0 ug/ml, 50 ug/ml, 100 ug/ml, 200 ug/ml, 400 ug/ml, and 800 ug/ml. Based on one-way ANOVA analysis, integrin expression significantly increased at all doses compared to the control group ($p<0.05$). After Dunnet T3 post hoc test was carried out, it seemed that the integrin expression did not significantly increase in ATRA treatment dose 50 ug/ml compared to the control group. In other words, there are significant differences in integrin expression due to administration of ATRA in doses 100, 200, 400 and 800 ug/ml compared to the control group and doses 50 ug/ml with $p<0,05$. Comparing the mean + SD of integrin expression between the ATRA treatment doses 100, 200, 400 and 800 ug/ml where the p-value $> 0,05$ indicated that the mean integrin expression in the ATRA group doses 100, 200, 400 and

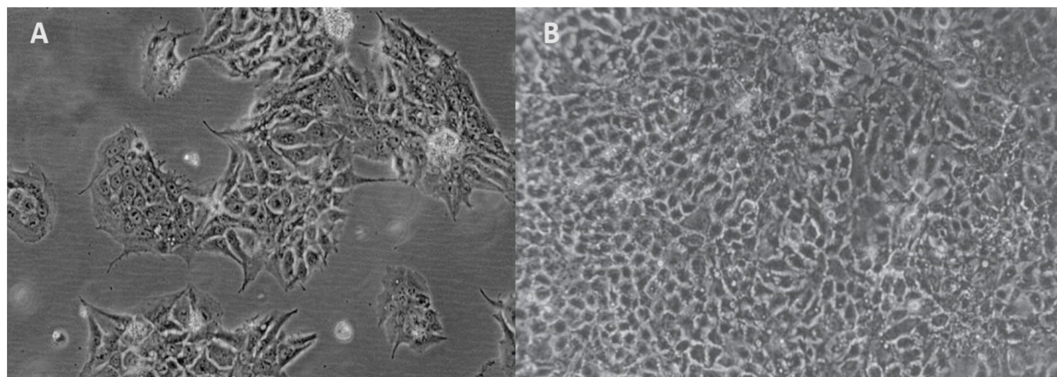

Figure 1. Microscopic Image of BeWo Choriocarcinoma Cell Culture (ATCC CCL 98) with Small Cytplasmic Amount and Large Hyperchromatic Nuclei on 400x magnification. (A). Depiction of low cell density and the schematic picture of choriocarcinoma cells. (B). Description of high cell density.

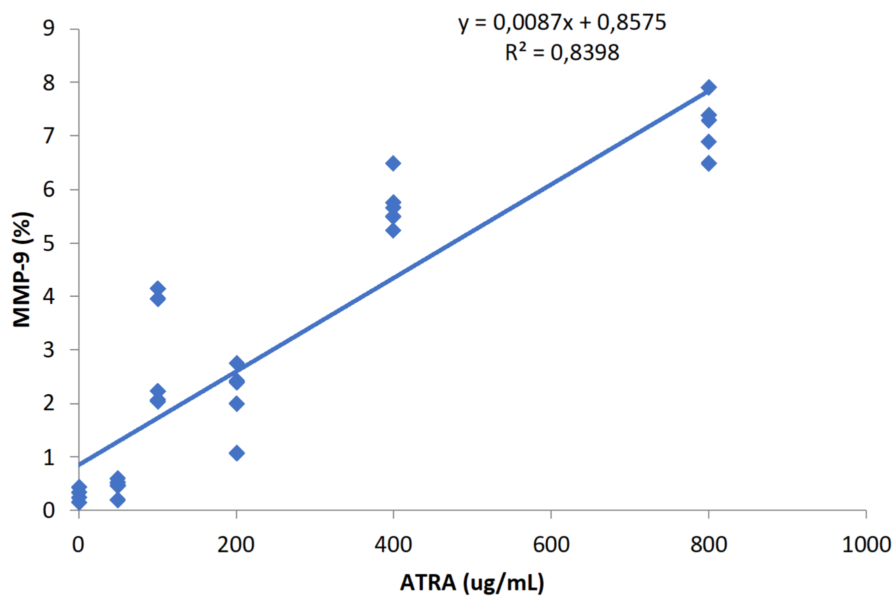

Figure 2. Scatter Plot of the Effect of ATRA on MMP-9 Activity Seen from the Remaining Substrate

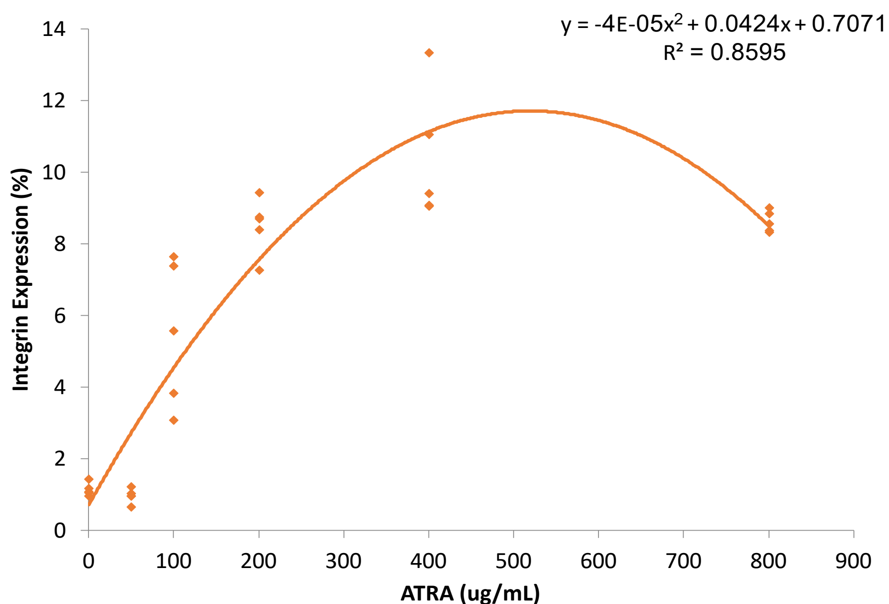

Figure 3. Scatter Plot of the Effect of ATRA on Integrin Expression with Polynomial Regression

800 ug/ml were not statistically significant in increasing integrin expression.

Table 1 also illustrated that starting from the control group, the mean integrin expression increased in the ATRA group administered at a dose of 100 ug/ml to a dose of 800 ug/ml. The administration of ATRA with a higher dose, from 100 ug/ml to 800 ug/ml, indicated an increase in integrin expression. Statistically, it was shown that the increase in integrin expression was significantly found in the ATRA group with a dose of 100, 200, 400 and 800 ug/ml. The mean integrin expression of ATRA administration with higher doses, namely the 200, 400 and 800 ug/ml doses, was not significant from the 100 ug/ml ATRA administration. From the statistical analysis, it was proven that the effect of giving ATRA at a higher dose was not different from that of giving ATRA at a dose of 100 ug/ml.

To determine the effect of ATRA on MMP-9 activity and integrin expression in BeWo choriocarcinoma cell culture (ATCC CCL 98), specific testing using regression analysis was performed. Based on the analysis, the regression coefficient was 0.0087 with a p-value of 0.000. The coefficient of determination (R-square) of 83.98% indicated that the diversity of data that were explained by the effect of ATRA on MMP-9 activity was only 83.98%. A high R-square value indicated that the linear model could accurately explain the effect of ATRA on MMP-9 activity. The addition of ATRA could affect the decrease in MMP-9 activity from the remaining 83.98% of the substrate. The regression coefficient of 0.0087 implied that if the dose of ATRA was increased, each increase in the ATRA dose of 100 ug / mL was predicted to decrease MMP-9 activity by 0.87% as seen in Figure 2.

The results of the polynomial regression analysis are set out in Figure 3. As shown in the Figure, the

R-square value was 0.8595 or 85.95%. It means that the administration of ATRA was capable of increasing the integrin expression by 85.95%.

Discussion

Matrix metalloproteinases (MMPs) are grouped in a family of zinc-dependent and calcium-dependent endopeptidase. The enzymes play an important role in maintaining the balance between matrix degradation and the reconstruction of tissue homeostasis. The gelatinases such as Gelatinase-A and B (also known as MMP-2 and MMP-9 respectively) digest gelatin and collagen via repeating motives such as Fibronectin type II [6]. ATRA induces cell growth and differentiation/division of cells and increases cell migration in various cell types through binding to the nuclear retinoic acid receptor (RAR) which is included in the steroid/thyroid/retinoid hormone receptor family [7].

Matrix Metalloproteinase hydrolyzes the basement membrane and other components of the extracellular matrix. The MMP interacts with cell surface receptors by regulating their biological activities including migration, angiogenesis, and apoptosis [6]. This study aims to determine the effect of ATRA on MMP-9 activity in BeWo choriocarcinoma culture (ATCC CCL 98). The results showed that ATRA had a significant effect on MMP-9 activity. In other words, there was a significant difference in MMP-9 activity that was caused by the administration of ATRA at different doses. Using the post hoc test with the Dunnet T3 test at various doses, it was found that there was no significant difference in the average activity of MMP-9. It can be inferred from the remaining substrate between the control group and the ATRA group with a dose of 50 ug / mL with a p-value of 0.763 ($p > 0.05$). While the comparison of the control group with the administration of ATRA at a dose of 100 ug / mL, 200 ug / mL, 400 ug / mL, and 800 ug / mL, the p-value was less than 0.05 ($p < 0.05$). From this test, it was proven that there was a significant difference in the mean activity of MMP-9, seen from the rest of the substrate, in the ATRA group with a dose of 100 ug / mL, 200 ug / mL, 400 ug / mL, and 800 ug / mL compared to the control group or group without ATRA administration and the dose group 50 ug / mL.

The results of this study are in line with the study conducted by Axel, et al. They argue that ATRA can inhibit the expression and activity of MMP-9 and reduce human arterial smooth muscle cell migration by regulating MES degradation [8]. The results are also consistent with that of Wang et al, who reported that the administration of IFN-β and retinoids acid therapy could inhibit MMP-9 expression and migration of breast cancer culture cells (MCF-7). Yet, the specific mechanism of this pathway is still under further investigation [9]. In a study conducted by Papi et al., the expression of MMP-9 and MMP-2 decreased by 39% in glioblastoma culture cells having given ATRA exposure through Western blot assay analysis and confirmed by a real-time PCR test. It was found that retinoids induce MMP inhibition at the mRNA level and in harmony with a 40% increase in TIMP expression which functions as a regulator of MMP catalysis [10].

In the process of invading choriocarcinoma cells, the degradation of the extracellular matrix and metastases is strongly associated with the presence of invadopodia, which are protrusive and adhesive structures in cancer cells. These invadopodia have been reported to secrete Matrix Metalloproteinase 9 (MMP-9) as a proteolytic that can degrade collagen in the extracellular matrix of the basement membrane. As mentioned earlier, the MMP-9 is a gelatinase subgroup of MMP that is capable of degrading denatured interstitial collagen (gelatin), lamin, elastin, fibronectin, and collagen in the basement membrane. Most of the MMPs are secreted as an inactive proprotein. It is usually activated when there is an activation signal from the cell nucleus. An active MMP-9 will degrade collagen type IV, which is the main structural component of the basement membrane.

The fundamental functions of MMP-9 are the further cleavage of the collagenase degradation products into smaller peptides. Then, this peptide is cleaved by another protease. One of the main implications of MMP-9 in the development of choriocarcinoma cancer is their role in the degradation of the extracellular matrix thereby allowing cancer cells to migrate out of the primary tumour for invasion and metastasis via blood vessels and lymph vessels to distant organs. ATRA is believed to have an effect of inhibiting cell migration in cases of human par adenocarcinoma (A549) by reducing light chain expression of myosin-kinase and myosin light chain phosphorylation [7].

All-Trans Retinoic Acid (ATRA) is an active metabolite of vitamin A in the retinoic group. It affects chromosome transcription and plays a role in the processes of proliferation, apoptosis, and cell differentiation. The ATRA will regulate and inhibit the activity of genes, including genes related to MMP-9 activity [11]. As an effective signalling molecule, ATRA regulates gene activity by binding to specific intracellular receptors, such as retinoic acid receptors (RAR-α, -β, -γ) and retinoid X receptors (RXR-α, -β, -γ) in the cell nucleus and Cytoplasmic Retinoic Acid Binding Protein 1 and 2 (CRABP 1 and 2) in the cytoplasm. After that, cells undergo gene changes, one of which is those related to MMP such as stromelysin-1, collagenase and gelatinase. This MMP-related gene is suppressed by the action of retinoids which are antagonistic in nature so that MMP-9 activity is reduced. Thus, ATRA can suppress MMP-9 activity through intracellular gene regulation. In this study, MMP-9 activity was measured by measuring the levels of the remaining active substrate that had been labelled and checked through a flowcytometric technique. This study then indicated that the administration of ATRA at various doses to the choriocarcinoma cell culture had a significant effect on the reduction of MMP-9 activity. Therefore, the hypothesis that ATRA can reduce MMP-9 activity is proven.

Integrins are involved and play an important role in signal transduction in the modulation of cell adhesion. Based on the unique structure of the integrins, including α and β subunits, integrins can be bound to extracellular matrix (MES) proteins such as collagen,

laminin, fibronectin, vitronectin, and other cellular receptors. These related proteins provide the basis for modulating fundamental cell processes and various biological outcomes including proliferation, migration, cell differentiation, and apoptosis by regulating signal transduction pathways [3]. The integrin-mediated adhesion of cells to components of the extracellular matrix is essential for the organization, maintenance and repair of various tissues. The cell adhesion process is complex and has a series of steps including the binding to the extracellular matrix, receptor grouping, and the recruitment of cytoskeletal elements [12].

Retinoids, vitamin A and their analogues play an important role in modulating the proper balance between cell adhesion and proliferation necessary for the maintenance of proper cell homeostasis. The biologically active metabolites of vitamin A, all-trans-retinoic acid and 9-cis-retinoic acid are reported to mediate a number of cellular functions including differentiation, proliferation, adhesion, transmigration, and expression of newer integrins. All-trans-retinoic acid (t-RA) and 9-cis-retinoic acid (9-cis-RA) activate retinoic acid receptors (RAR α, β, and γ) and retinoid X receptors (RXR α, β, and γ). Retinoids have been documented to have a role in regulating integrin-dependent cellular adhesion by modifying the expression of various adhesion or proliferation signalling proteins through complex processes [13].

This study aims to determine the effect of ATRA administration on MMP-9 activity in BeWo choriocarcinoma cell culture (ATCC-CCL 98). The results showed that ATRA had a significant effect on MMP-9 activity at different doses. Based on the results of the Dunnet T3 test, it was proven that ATRA had a significant effect on MMP-9 activity starting from the dose group of 100 ug / mL, 200 ug / mL, 400 ug / mL, and 800 ug / mL. The results were also reported by Mellisa et al, 2017 where there was an increase in cell adhesion by 40% after given exposure to retinoids (RARγ). It can be seen from the Integrin binding for all subunits in erythroleukemia K562 cell culture, and an increase of 70% in integrins, α5β1 subunit. This study also revealed that retinoids modulated integrin expression on the cell surface via the α5β1 subunit. For that reason, this study proves that retinoids through RARγ can increase the expression of cell surface integrins [13].

Strikingly, results of the earlier studies also suggest that retinoids improve immune cell localization by regulating the integrin receptor repertoire. For instance, a study conducted by Wang et al, showed that retinoids significantly induce the expression of integrin subunits especially β1 in cutaneous T lymphocyte lymphoma. Retinoids currently have an intervening mechanism of action on the induction of apoptosis including cessation of growth of G1 and G2 / M cells. The present study claims that if cutaneous T-cell lymphoma cells have an initial and natural response to retinoids by increasing the expression and function of integrin molecules in the intestine, it can be inferred that this response can influence malignant cells to stop growing and increasing apoptosis. Retinoids themselves activate the nuclear receptor families RAR and RXR to obtain cellular changes. Unfortunately, the detailed molecular mechanisms that inform the types of nuclear receptors that are involved and required in the action of retinoid therapy in cutaneous T cell lymphoma are still unknown [14].

The latest paradigm regarding retinoid-based therapy is that retinoids play a role in inducing apoptosis and stopping cell growth. Retinoids also play a role in increasing the expression of α4β7 integrins by increasing their receptors in the intestinal mucosa circulation in cutaneous T cell lymphoma [14]. Integrins are isolated from serum, platelet release processes, and extracellular matrix (ECM). They are also on interactions with cell receptors (integrins). The biological function of vitronectin is mainly in the maintenance of vascular hemostasis (thrombosis and fibrinolysis), cell adhesion, and tissue regeneration. Research on breast cancer cells found that breast cancer cells express αvβ3 integrin. Integrins in breast cancer cells use the integrin protein in the extracellular matrix.

This activation will increase the expression of mTOR and mRNA translation which will facilitate tumour cell invasion. Increased expression of mTOR initiates the initiation of eIF4E from the 4E-BP1 repressor protein. The eIF4E protein binds to vitronectin for tumour cell invasion. Research on cancer cells found that retinoids are antagonistic to mTOR [15]. In this study, the integrin expression was measured using flowcytometry techniques. The administration of various doses of ATRA to the choriocarcinoma cell culture increased integrin expression. Therefore, in this study, the administration of ATRA could significantly increase integrin expression. Based on the results and discussion of this study, it can be concluded that the administration of All Trans Retinoic Acid (ATRA) in various doses to the BeWo choriocarcinoma cell culture (ATCC CCL 98) can significantly reduce MMP-9 activity and administration of All Trans Retinoic Acid (ATRA) in various doses to BeWo choriocarcinoma cell culture (ATCC CCL 98) can significantly increase the integrin expression.

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Author Contribution Statement

: Conceptualization, Nurseta, T.; data collection, Wardani, K.R.P.; data validation Wardani, K.R.P.; data analysis, Wardani, K.R.P.; writing original draft preparation, Indrawan, I.W.A.; writing review and editing.; Nurseta,T. and Gunawan, D.A.; reading and approving the final manuscript, all authors.

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Ethical Declaration

This study was approved by Health Research Ethics Commission General Hospital Dr. Saiful Anwar, number:

400/162/K.3/302/2020.

Conflict of Interest

The authors declare that there is no conflict of interest in this study. The principal investigator can provide the data of this study if requested

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