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ORIGINAL ARTICLE

Calcium Mobilization and Inhibition of Akt Reduced the Binding of PEO-1 Cells to Fibronectin

Ca^{2+} Mobilizasyonu ve Akt İnhibisyonu ile PEO-1 Hücrelerinin Fibronektine Bağlanmasında Azalma

Seda Mehtap SARI KILIÇASLAN[1]*, Aysun AYRIM[2], Elif APAYDIN[3], Zerrin İNCESU[4]

[1]Anadolu University, Faculty of Education, Department of Primary Education, Eskişehir, Turkey
[2]Eskişehir Osmangazi University, Institute of Life Sciences, Eskişehir, Turkey
[3]Anadolu University, Institute of Life Sciences, Eskişehir, Turkey
[4]Anadolu University, Faculty of Pharmacy, Department of Biochemistry, Eskişehir, Turkey

ABSTRACT

Objectives: To investigate the effects of intracellular calcium (Ca^{2+}) mobilization, β-catenin and Akt signal pathways after the binding of metastatic ovarian cells to fibronectin.

Materials and Methods: The expression levels of α4β1 and αvβ6 integrin were determined using α4, β1, αv, and β6 antibodies using flow cytometry on PEO-1 cells. The effect of $[Ca^{2+}]i$ on cell adhesion capacity was investigated using RTCA after stimulating PEO-1 cells using thapsigargin and tunicamycin. The binding rate of PEO-1 cells to fibronectin was also investigated in the presence of either different concentrations of cardamonin, which inhibits the accumulation of β-catenin, or different concentrations of FPA 124, which is a specific inhibitor for the PKB/Akt signal pathway, using RTCA.

Results: RTCA analysis results showed that increasing $[Ca^{2+}]i$ through leakage of the calcium pool was strongly effective on PEO-1 cell binding to fibronectin. Extracellular calcium influx also reduced the binding of PEO-1 cells. Cell binding to fibronectin was also inhibited with a ratio of 64% in the presence of 100 μM cardamonin compared with untreated control cells. Finally, it was found that PKB/Akt inhibition with 15 μM FPA 124 decreased the binding of PEO-1 cells to fibronectin with a ratio of 88% compared with untreated control cells.

Conclusion: PEO-1 cell binding to fibronectin via integrins could be related to intracellular Ca^{2+} mobilization and Akt signaling.

Key words: Fibronectin, calcium, tunicamycin, ovarian cancer

ÖZ

Amaç: Metastatik ovaryum kanser hücrelerinin fibronektine bağlanmasından sonra, hücre içi Ca^{2+} mobilizasyonu, β-katenin ve Akt sinyal yollarının etkilerinin araştırılmasıdır.

Gereç ve Yöntemler: α4β1 ve αvβ6 integrinlerin ekspresyon düzeyleri α4, β1, αv vçe β6 antikorları kullanılarak akım sitometrisi ile PEO-1 hücrelerinde tespit edilmiştir. $[Ca^{2+}]$'un hücre adezyon kapasitesine etkisi, PEO-1 hücrelerinin tapsigargin ve tunikamisin ile uyarılmasından sonra RTCA kullanılarak araştırıldı. PEO-1 hücrelerinin fibronektine bağlanma oranı β-kateninin toplanmasını inhibe eden farklı kardamonin konsantrasyonları varlığında veya PKB/Akt sinyal yolu için spesifik inhibitör olan FPA 124'ün farklı konsantrasyonları için RTCA kullanılarak araştırıldı.

Bulgular: RTCA analiz sonuçları kalsiyum depolarından sızmasıyla artan $[Ca^{2+}]$'un PEO-1 hücrelerinin fibronektine bağlanmasını güçlü bir şekilde etkilediğini göstermiştir. Hücre dışı kalsiyum akımı PEO-1 hücrelerinin bağlanmasını azaltmıştır. 100 μM kardamonin varlığında muamele edilmemiş kontrol hücrelerine göre hücrenin fibronektine bağlanması %64 oranında inhibe olmuştur. Son olarak, 15 μM FPA 124 ile PKB/Akt inhibisyonu da PEO-1 hücrelerinin fibronektine bağlanmasını %88 oranında düşürdüğü bulunmuştur.

Sonuç: PEO-1 hücrelerinin integrinler aracılığı ile fibronektine bağlanmasının hücre içi Ca^{2+} mobilizasyonu ve Akt sinyali ile ilgili olabilir.

Anahtar kelimeler: Fibronektin, kalsiyum, tunikamisin, ovaryum kanseri

*Correspondence: **E-mail:** smskilicaslan@anadolu.edu.tr, **Phone:** +90 222 335 0580 / 3433 **ORCID-ID:** orcid.org/0000-0001-7547-5519
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INTRODUCTION

The cell adhesion molecules, integrins, activate outside-in signaling pathways necessary for cell invasion by mediating attachment between cells and the extracellular matrix (ECM). The integrins participate in cell morphology, migration, proliferation, differentiation, survival[1,2], as well as apoptosis. Integrin-mediated cell attachment is necessary for cancer cell metastasis and invasion. Moreover, ECM-integrin signaling is said to have survival advantage in different types of cancer cells against many chemotherapeutic treatments.[2] Studies on this topic are usually based on integrin expressions. Signal transduction mechanisms of integrins in ovarian cells, however, have not yet been explicitly investigated.

Cell-ECM adhesiveness is generally increased in human cancers, which allows cancer cells to destroy the histologic structure. Reduced cell-ECM adhesiveness is also important for cancer invasion and metastasis.[3] Moreover, some studies showed that the adhesion capacity of highly metastatic cells could be reduced by inhibiting integrin expression or modifies the process that leads metastatic cells to anoikis, which is programmed cell death that is induced upon cell detachment from ECM.[4,5] αv integrin subunit-inhibition or αv-knockdown was shown to inhibit the colonogenic and migratory ability of human prostate cancer cells *in vitro* and *in vivo*.[4,6] Accordingly, αv integrin is said to be a promising target for cancer therapy strategies.

Ovarian cancer cells can spread in cell form or spheral form from the surface of the ovary. Therefore, metastatic ovarian cells should survive and proliferate without ECM binding. The microenvironment of cells is dynamic and contains survival factors such as cytokines, growth factors, hormones, proteases, and ECM proteins that regulate tumor cell migration, invasion, survival, and spheral forms.[7] In particular, fibronectin and vitronectin (ECM proteins) induce the formation of spheroids, adherence, and disaggregation of ovarian cancer cells. These proteins, which are disintegrated by metalloproteinase-2, increase the adhesion of ovarian cancer cells to the peritoneal region that is the nascent stage of metastasis.[8]

β-catenin is a multi-functional protein involve in the Wnt signal pathway, as well as adhesion via E-cadherin in epithelial cells.[9] In normal epithelial cells, β-catenin binds to the E-cadherin-α-catenin complex in adherent junctions. In the presence of Wnt signaling, however, β-catenin accumulates in the cytoplasm and then translocates to the nucleus due to activation of a large number of target genes including LEF/TCF genes.[10] These activated genes are attributed to the development of some diseases, especially various types of human cancers. A number of studies showed that accumulation of β-catenin was also effective in creating a suitable microenvironment for cancer progression.[11,12]

Recently, it has been shown that Akt is one of the most effective regulatory proteins in the β-catenin accumulation process. In particular, N-cadherin adhesion can lead to phosphatidylinositide 3-kinase (PI3K) mediated activation of Akt, and that might stimulate the β-catenin signaling pathway.[13] Moreover, the Akt protein also phosphorylates glycogen synthase kinase 3 beta (GSK3β) and leads to inactivating the function of GSK3β. In this case, stabilization and accumulation of β-catenin is induced.[14]

The current study aimed to investigate the role of increased Ca^{2+} via tunicamycin (TN) treatment and β-catenin-Akt signaling on the binding of metastatic ovarian cancer cells (PEO-1) to fibronectin. We investigated the expression levels of integrins that play an active role in PEO-1 binding to fibronectin using flow cytometry and immunofluorescence staining. Using real-time cellular analysis (RTCA), we showed that increasing cytoplasmic calcium in PEO-1 cells influenced cell adhesion. Inhibition of the accumulation of β-catenin and Akt signaling using specific inhibitors led to inhibition of PEO-1 adhesion to fibronectin. These results suggest a link between the adhesion of PEO-1 ovarian cells and Ca^{2+} mobilization, and the function of Akt and β-catenin.

MATERIALS AND METHODS

Cell culture

The PEO-1 human ovarian cancer cell line was purchased from Public Health England (10032308) and cultured in RPMI 1640, 10% fetal bovine serum, 2 mM sodium pyruvate, and 2 mM glutamine.

Detection of integrin expression

Expression levels of αv, α4, β1, and β6 integrin were determined using specific antibodies with flow cytometry on PEO-1 cells. The cells were incubated with a 1:200 dilution of primary antibodies against integrin subunits, subsequently washed in PBS, and incubated with a 1:200 FITC-conjugated secondary antibody for 30 min at 4°C. Control cells contained either a primary antibody or an FITC secondary antibody. After washing, all samples were analyzed using a flow cytometer (Becton Dickinson, FACSAria II, Canada).

Localization of integrins on cell membrane

The localization of integrins was detected using florescence microscopy. Coverslips were coated with 50 µg/mL fibronectin. The cells were then seeded, washed with PBS, and fixed with 4% formaldehyde, washed again and then permeabilized with 0.1% Tween-20. After washing with PBS, the cells were treated with 1% bovine serum albumin (BSA). The cells were treated with specific primary integrin antibodies (1:200 dilution) overnight at +4°C, then with the FITC-conjugated secondary antibody (1:300 dilution) for 1 h at +4°C. No primary antibody was added in the control group. After washing the coverslips, they were mounted on microscope slides. The slides were examined using florescence microscopy and monitored.

Binding assays

The binding rate of PEO-1 cells to fibronectin

RTCA was used for real-time and time-dependent analysis of binding of PEO-1. RTCA measures changes in electrical

impedance as cells attach and spread in multi-well plates covered with a gold microelectrode array.[15] Electrode impedance is displayed and recorded as "cell index" values, which reflects the biologic status of the monitored cells, including the cell number, cell viability, morphology, and cell adhesion.[16]

Sixteen-well plates were coated with different concentrations (1 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL) of fibronectin, except the control wells. The wells were washed with PBS and then blocked with 1% BSA to inhibit non–specific binding. After a background impedance measurement, 5×10^4 cells/well were seeded. Impedance was monitored at 15-min intervals for 24 h. The rate of cell adhesion was calculated according to the normalized cell index (NCI) or cell index formula.

NCI = (impedance at time point n - impedance in the absence of cells) / nominal impedance value.

CI = (resistance measured at a time point - resistance measured without the cell) / 15Ω.

The role of $[Ca^{2+}]i$ on PEO-1 cell adhesion capacity

Integrins on the cell surface were activated using fibronectin (50 µg/mL). The effect of $[Ca^{2+}]i$ on cell adhesion capacity was investigated using RTCA after stimulating PEO-1 cells with IP_3-independent intracellular calcium release, thapsigargin (TG) (50 nM, 100 nM, 500 nM, 1 µM, 2 µM), and extracellular calcium channel blocker TN (0,5-1-5-10-20 µg/mL). PEO-1 cells were treated with 5, 20, 35 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], which is Ca^{2+} chelator, to create a non-calcium environment. Measurement of cell adhesion was conducted for 24 h.

The role of cardamonin and Akt on the binding ability of PEO-1 cells

Sixteen-well plates were prepared as mentioned above. After the wells were coated with 50 µg/mL fibronectin, the plates were seeded with 5×10^4 PEO-1 cells and transferred into RTCA for 2 h incubation before monitoring. After that, the cells were treated with different concentrations of cardamonin (3,13 µM, 6,25 µM, 12,5 µM, 25 µM, 50 µM, 100 µM), an inhibitor β-catenin, and FPA 124 (5 µM, 15 µM, 25 µM, 35 µM), an Akt signal pathway inhibitor. The cells were monitored at 30-min intervals for a period of 24 h. Uncoated wells were used as a control group together with the wells containing no inhibitor.

RESULTS

Expression and localization of integrins on the surface of PEO-1 cells

The expression and localization of α4, β1, αv, and β6 integrin subunits were investigated using flow cytometry and immunofluorescence staining. The results of the flow cytometric analysis are given in Figure 1. The expression levels of αv, α4, β6, and β1 were in a descending order with respect to expression level. The expression level of the αv integrin subunit was found to be abundant compared with the others.

PEO-1 cells were incubated with specific primary integrin antibodies and the localization of integrin subunits was demonstrated using fluorescence microscopy (Figure 2). PEO-1 cells showed low but detectable level of β6 integrin subunit localization, whereas cells exhibited considerable amounts of α4, αv, and β1 localizations over the whole cell as labelled with specific antibodies.

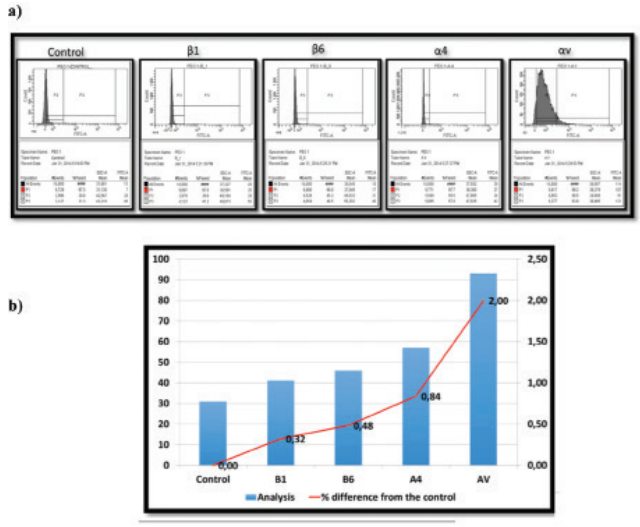

Figure 1. Flow cytometric analysis of α4, β1, αv and β6 integrin subunits expression (a), and percentage of integrin subunits expressions (b) on the surface of human ovarian cancer PEO-1 cell line. (a) Cells were first treated with α4, β1, αv and β6 primary antibodies, except the controls, and then FITC conjugated secondary antibodies. (b) The left axis is expression level, the right axis is the percentage differences from the control group. Expression levels of α4, β1, αv and β6 are higher by about 32%, 48%, 84%, and 200% than the control group, respectively

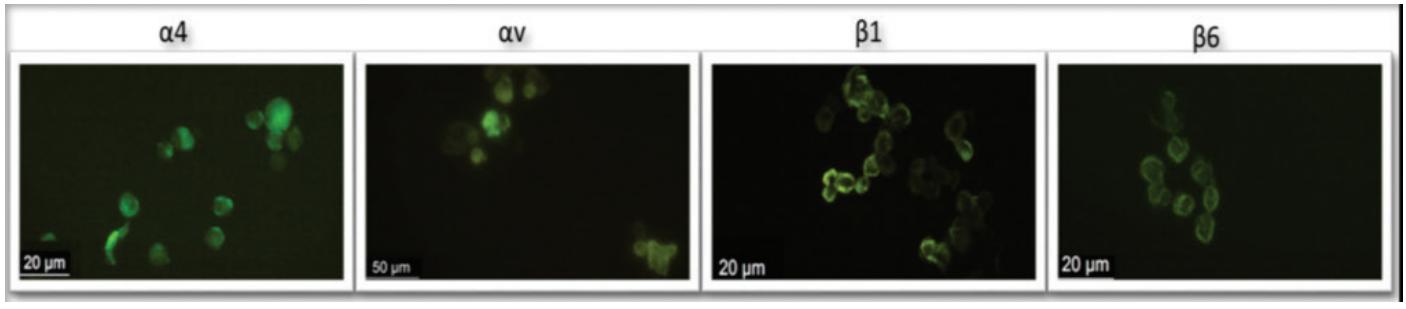

Figure 2. Immunofluorescence staining of human ovarian cancer PEO-1 cells for α4, αv, β1 and β6 integrin subunits. The cells were labelled with various primary integrin antibodies at 37°C for 1 h. After that, PEO-1 cells were incubated with FITC-conjugated secondary antibody

The binding rate of PEO-1 cells to fibronectin

PEO-1 cells were examined using RTCA to determine the binding of cells in various concentrations (1-100 µg/mL) of fibronectin. The results, shown in Figure 3, indicate that PEO-1 cells bound to fibronectin at all concentrations compared with the control cells. The cells showed a higher percentage of binding to 50 µg/mL fibronectin (cell index 5-6 at 24 h), whereas low-concentration fibronectin (1 µg/mL) supported little cell adhesion. The result suggests that 50 µg/mL fibronectin would be a suitable concentration for use in further studies.

Effects of calcium on PEO-1 cell binding

Figure 4 depicts that the involvement of Ca^{2+} increases in the mechanism of cell adhesion to fibronectin. PEO-1 cells were plated onto 16-well plates that were previously coated with 50 µg/mL fibronectin in the presence or absence of various concentrations of TN, TG or EGTA. The results demonstrated that integrin-mediated PEO-1 adhesion to fibronectin was reduced by Ca^{2+} mobilization from both the extracellular and intracellular Ca^{2+} pool. The addition of all Ca^{2+} mobilization compounds resulted in a decrease in CI down to zero at the 24 h time point because of the cells becoming rounded and detaching from the well bottom.

Figure 4a demonstrates that the change in cell index occurred within the first few hours of exposing PEO-1 cells to all concentrations of TN. The rate of adhesion of PEO-1 cells to fibronectin decreased in a dose-dependent manner. TN concentrations of 0.5 and 1 µg/mL indicated no effects on PEO-1adhesion ability as compared with the control cells. The proportion of adherent cells reduced to a minimal level by the presence of 20 µg/mL TN. The results suggest that extracellular calcium mobilization reduced the adhesion of highly metastatic PEO-1 cells to fibronectin, and might induce anoikis of PEO-1 cells; however, the latter remains unclear.

The addition of all concentrations of TG reduced cell adhesion to fibronectin in a time-dependent manner as compared with the control cells (CI: 0.4) (Figure 4b). After 24-h incubation, even the addition of the lowest concentration (50 nM) of TG decreased the attachment of PEO-1 cells to fibronectin to zero. The results suggest that the adhesion of PEO-1 cells to fibronectin was decayed by the inhibition of the endoplasmic reticulum pump via TG treatment.

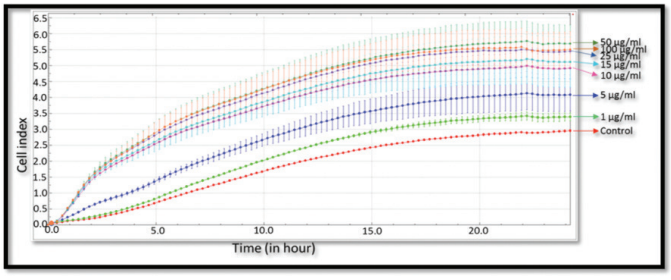

Figure 3. The binding ability of PEO-1 cells to fibronectin by RTCA. PEO-1 cells were allowed to incubate for 24 h on 16-well plates coated with 1-50 µg/mL fibronectin concentrations. The control wells were not coated with fibronectin. As mentioned in the Materials and Methods section, impedance was monitored at 15-min intervals for 24 h

Treatment of PEO-1 cells with various concentrations of EGTA also reduced the rate of adhesion within 5 h and then remain unchanged (Figure 4c).

The role of the PKB/Akt signal pathway on PEO-1 cell binding to fibronectin

FPA-124 was used to inhibit the protein kinase B (PKB)/Akt signal pathway. The impact of inhibiting PKB/Akt on cell binding was tested with different concentrations (5 µM, 15 µM, 25 µM, 35 µM) of FPA 124 (Figure 5). The percentage of the impact of different FPA-124 concentrations on cell binding at 24 h in PEO-1 is given in Figure 5b.

Figure 5a demonstrates the attachment of the control cells (without FPA-124; CI: 1.9). The cell index of the group treated with 5 µM FPA-124 inhibitor is close to that of control group (CI: 1.4 after 24-h incubation). Increasing the concentration of inhibitor by about three times showed the different effects on PEO-1 cell attachment to fibronectin. For the first few hours, cell attachment to fibronectin increased after adding 15 µM FPA inhibitor; however, longer incubation times reduced the binding of PEO-1 cells to the background level (CI: 0.3). The results suggest that FPA inhibitors might cause rapid focal contacts between cell-fibronectin in a dose dependent manner. Higher

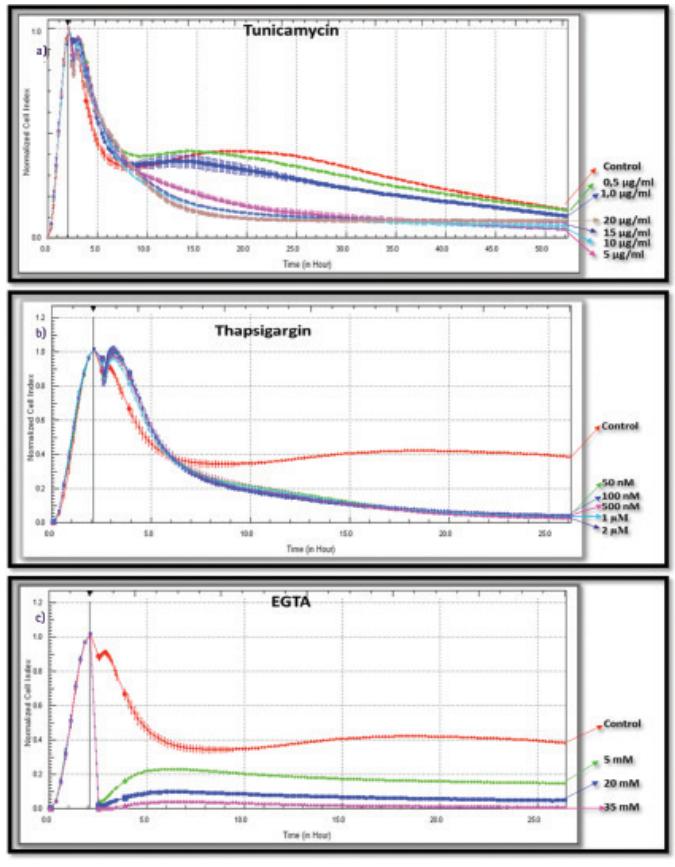

Figure 4. Effects of TN, TG and EGTA on PEO-1 binding to 50 µg/mL fibronectin after 24 h. (a) PEO-1 cells were treated with 0.5-1-5-10-20 µg/mL of TN; (b) with 50-100-500 nM-1-2 µM TG and (c) with 5-20-35 mM of EGTA for 24 h after being plated onto 50 µg/mL fibronectin. The rate of cell adhesion was calculated according to the NCI formula

concentrations of FPA-124 (25 and 35 μM) inhibited the binding of cells to fibronectin immediately after being added to the culture medium.

The role of cardamonin on PEO-1 cell binding to fibronectin

The effects of cardamonin on PEO-1 cell binding to fibronectin were investigated using RTCA and adding various concentrations (3.13, 6.25, 12.5, 25, 50 and 100 μM) of cardamonin. The results are shown in Figure 6a and b. The addition of 100 μM cardamonin to the cells inhibited cell binding to fibronectin by about 64%. With the exception of 3.13-μM cardamonin treatment, all other concentrations of cardamonin inhibited cell binding to fibronectin as compared with the control cells plated on uncoated wells. Treatment of cells with the lowest concentration (3.13 μM) of cardamonin stimulated the rate of cell binding to fibronectin at around 13% as compared with the control cells. The results showed that the degradation of β-catenin in the cytoplasm by cardamonin had an important role in PEO-1 cell binding to fibronectin.

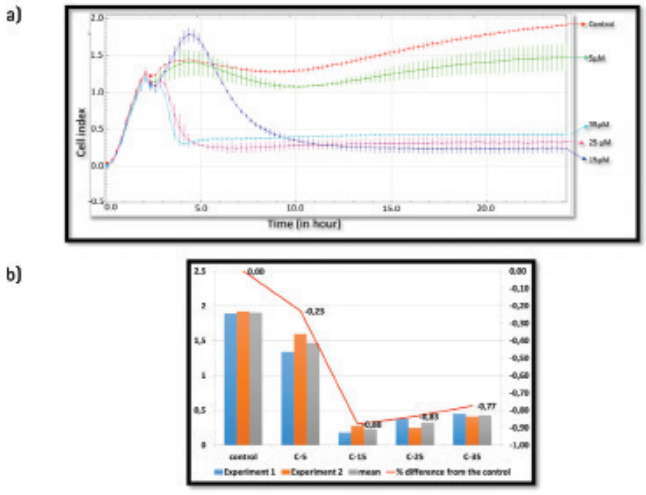

Figure 5. The role of PKB/Akt molecules on PEO-1 cell binding to fibronectin (a) and the impact of different FPA-124 concentrations on cell binding at 24 h (b). 16-wells plates were prepared as described in Materials and Methods. Various concentrations (5-35 μM) of FPA-124 inhibitor were added to each well. No inhibitor was added to the control groups. (b) The left axis is the cell index at 24 h, the right axis is the percentage differences from the control group

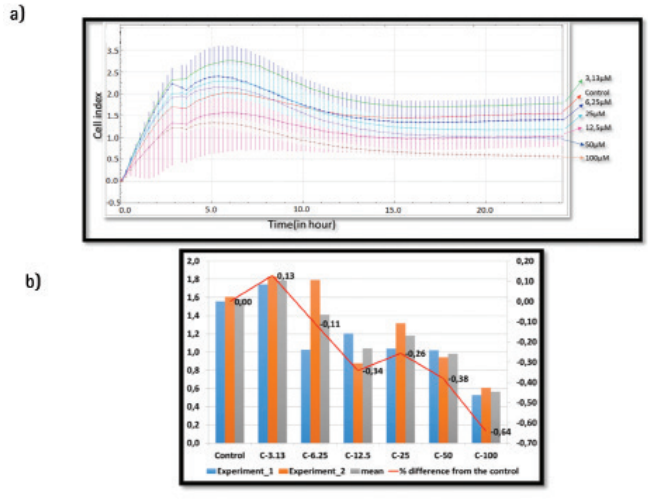

Figure 6. The effect of cardamonin on binding of PEO-1 cells to fibronectin (a) and the percentage of the impact of different cardamonin concentrations on PEO-1 cell binding to fibronectin at 24 h (b). Wells were coated with 50 μg/mL fibronectin at +4°C overnight. The cells were transferred into each well and then incubated with cardamonin (concentration range between 3.13 μM and 100 μM) for 24 h. The results represented are the means of the two wells. (b) The left axis is the cell index at 24 h, the right axis is the percentage differences from the control group

DISCUSSION

Adhesion and integrin activation are important to metastasize ovarian cancer cells onto the peritoneal mesothelial surface of the abdominal cavity[17], and in the resistance to anoikis of epithelial ovarian cancer spheroids. It has been reported that some binding proteins might be involved in the implantation of ovarian cancer metastasis.[18] CD44 in particular, which is a receptor for the ECM protein hyaluronic acid, plays a crucial role in mediating the mesothelial binding of ovarian cancer cells and β1 integrins such as α5β1 integrin (a receptor for fibronectin), α3β1 (a receptor for fibronectin, collagen and laminin) and αv (a receptor for fibronectin and vitronectin) expressed by ovarian cancer cells; and ovarian cancer cell binding to peritoneal mesothelium.[18] In our experiment, we found that the expression level of αv was two-fold higher than in the control group in PEO-1 cells. The other integrin expression levels of α4, β1, and β6 subunits were higher at around 32%, 48%, and 84%, respectively, than in the control group. The αvβ1 fibronectin receptor most likely contributes to integrin-mediated binding of PEO-1 cells. These results are consistent with previous findings. Carduner et al.[19] reported that αv had an important role in ovarian cancer progression. Lessan et al.[20] informed that β1 integrin may responsible for adhesion of ovarian cancer cells.

Fibronectin has been detected in ovarian cancer cell metastases and is also present in ascites.[21,22] Fibronectin contributes to the formation, adhesion, and disaggregation of ovarian cancer cell spheroids.[23] Therefore, the investigation of the binding capacity of ovarian cancer cells to fibronectin should be useful for targeted therapies. The binding studies herein suggest that PEO-1 cells could be able to adhere to fibronectin in a dose dependent-manner and the maximum binding rate was reached after adhesion of cells to 50 μg/mL fibronectin. The binding capacity of PEO-1 cells was also measured in the presence or absence of calcium because integrin-mediated cell adhesion is regulated by extracellular Ca^{2+} ions[24], and an increased calcium concentration in the cytoplasm is essential for various signal transduction pathways such as proliferation and apoptosis and thus is also crucial in cancer.[25] TN induces endoplasmic stress by inhibiting glycosylation and leads to the accumulation of unfolded proteins. Endoplasmic stress has a critical role in regulating cell death.[26] Our results clearly indicated that increased calcium levels in the cytoplasm of PEO-1 cells by either extracellular or intracellular stores was essential for the binding of these cells to fibronectin. Increasing intracellular calcium in PEO-1 cells reduced cell adhesiveness and also

might have caused programmed cell death induced after cell detachment from the ECM.

PKB/Akt is a serine/threonine kinase that has an important role in cell survival, cell proliferation, invasion, and adhesion to the ECM in various types of cells such as human umbilical vein endothelial cells[27], breast cancer cell[28], and lung adenocarcinoma.[29] The overexpression of PI3K/Akt-related genes has been observed in ovarian cancer tissues.[30] Moreover, fibronectin adhesion promotes metastatic behavior on these types of cancer cells through the focal adhesion kinase–PI3K/Akt pathway.[31] Xing et al.[32] suggested that Akt activation promoted by fibronectin might have a critical role in cell survival. In the present study, inhibition of PKB/Akt with the FPA inhibitor decreased the rate of adhesion of PEO-1 cells to fibronectin in a dose dependent manner, suggesting that PKB/Akt participates in the signaling of adhesion in these cells.

In the present study, we found that the inhibition of β-catenin by cardamonin reduced PEO-1 cell binding to fibronectin. β-catenin is an activated downstream signal molecule from Akt, and Akt-mediated phosphorylation of β-catenin results in its accumulation in the cytosol and translocation into the nucleus, thus upregulating genes related to cell proliferation such as c-Myc, cyclin D1 and D2.[33] The function of β-catenin may be necessary for the adhesive and signaling responses required for cancer. The inhibition of β-catenin activity causes suppression of several cancer hallmarks, which might be useful as a putative drug target.[34] Pramanik et al.[35] found that inhibition of β-catenin signaling blocked pancreatic tumor growth. Verma et al.[36] showed that small interfering RNAs directed against β-catenin reduced β-catenin–dependent gene expression and growth of colon cancer cells. In this study, we found that the binding ability of PEO-1 ovarian cells to fibronectin decreased with treatment of 100 μM cardamonin, suggesting that β-catenin might be involved in the adhesion process of PEO-1 cells to fibronectin as well as Akt. This process could be mediated by integrin, especially the αv subunit, but further experiments are required to investigate the role of the αv subunit under these circumstances.

CONCLUSION

In conclusion, PEO-1 cell binding to fibronectin via integrins could be related to intracellular Ca^{2+} mobilization and Akt signaling. However, the sensitivity of these cells against anoikis still remains to be investigated.

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Conflict of Interest: No conflict of interest was declared by the authors.

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