Ouabain Enhances Lung Cancer Cell Detachment

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Abstract. *A human steroid hormone, ouabain, has been shown to play a role in several types of cancer cell behavior; however, its effects on cancer metastasis are largely unknown. Herein, we demonstrate that sub-toxic concentrations of ouabain facilitate cancer cell detachment from the extracellular matrix in human lung cancer cells. Ouabain at concentrations of 0-10 pM significantly enhanced cell detachment in dose- and time- dependent manners, while having minimal effect on cell viability. The detachment-inducing effect of ouabain was found to be mediated through focal-adhesion kinase and ATP-dependent tyrosine kinase pathways. Alpha-5 and beta-1 integrins were found to be down-regulated in response to ouabain treatment. Since detachment of cancer cells is a prerequisite process for metastasis to begin, these insights benefit our understanding over the molecular basis of cancer biology.*

Despite advances in chemotherapy, as well as novel strategies to overcome lung cancer, this type of cancer remains a leading cause of death (1). The majority of deaths of patients with lung cancer are due to metastasis, a process by which cancer cells spread from their origin to other parts of the patient's body (2-5). In order to elucidate molecular targets for the development of novel therapies, insights into lung cancer cell biology regarding metastasis are very important. Cancer metastasis consists of several key components (6, 7). While other steps of metastasis such as anoikis, migration, invasion, and cancer cell adhesion to the endothelial surface have been intensively studied, the molecular basis of cancer cell detachment from the original tumor is largely unknown.

This early dissemination of cancer cells from their extracellular matrix (ECM) was shown to be critical to successful metastasis and such a process occurs *via* the cascades of focal adhesion dissociation (8-11). Even though the mechanisms of cancer cell detachment are not yet defined, evidence indicates that focal adhesion kinase (FAK) and ATP-dependent tyrosine kinase (AKT) pathways play important roles in both cell detachment and cell motility (12, 13). Activation of FAK or AKT was shown to induce focal adhesion dissociation in many cell models (13, 14). Furthermore, integrins, proteins linking focal adhesion complex with components of the ECM were shown to be critical for proper cell adhesion. Among various types of integrins, alpha-5 and beta-1 integrins have garnered predominant attention in cancer research since their activity was shown to be important for adhesion in several types of cancers (15-19).

Ouabain, an endogenous substance that has been identified as a human hormone, has gained increasing attention in cancer research (20-23). Ouabain was reported found in plasma in concentrations ranging from 2-770 pM (24) and was shown to sensitize cancer cells to death induced by TNF-related apoptosis-inducing ligand (TRAIL) (25). Because cancer cells in the patient's body are likely to be exposed to ouabain and there is no report indicating effects of ouabain in regulation of cancer cell detachment, the present study aimed to elucidate the possible regulatory role of the specific substance. The knowledge gained from this study may lead to a better understanding of cancer biology and may help in the search for new molecular targets for novel anticancer strategies.

Materials and Methods

Cells and reagents. Human lung adenocarcinoma H23 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin were obtained from GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD, USA) in 37˚C with 5% CO_2-

Abbreviations: AKT, ATP-dependent tyrosine kinase; FAK, Focal-adhesion kinase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; p-AKT, Phosphorylated AKT; PBS, Phosphate-buffered saline; p-FAK, Phosphorylated-FAK; TBST, Tris-buffered saline with 0.1% Tween.

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humidified incubator. Ouabain, concanamycin A (CMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342 and propidium iodide (PI) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Matrigel was obtained from Becton Dickinson, Inc. (Mississauga, Ontario, Canada). Rabbit monoclonal antibody to integrin, p-FAK, FAK, p-AKT and AKT were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell viability assay. Cell viability was evaluated using MTT assay. Briefly, H23 cells were seeded at a density of 10^4 cells/well in 96-well plate, overnight. After that, they were treated with different concentrations of ouabain for 12 h. Cell viability was determined by incubation with MTT solution (5.0 mg/ml in phosphate-buffered saline (PBS)) and incubated at 37˚C for 4 h. The optical density was then determined by a microplate reader (Anthros, Durham, NC, USA) at 570 nM, and the percentage of viable cells was calculated relative to control cells.

Nuclear staining assay. Apoptotic and necrotic cell death were determined by Hoechst 33342 and PI co-staining. After treated with different concentrations of ouabain, cells were incubated with 10 μM of Hoechst 33342 and 5 μg/ml of PI for 30 min at 37˚C. Apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70).

Cell-ECM adhesion assay. Cell-ECM adhesion assay was performed as follows. Briefly, matrigel was thawed at 4˚C for 12 h, diluted to final concentration at 0.2 μg/ml, spread evenly on the surface of 96-well plates, and incubated at 37˚C for 12 h. Cells were treated with non-toxic concentrations of ouabain, then trypsinized to produce single cells and immediately seeded onto matrigel-coated plate. Cells were allowed to adhere to the matrigel for 30 min at 37˚C, then the cells were washed three times with PBS. Adherent cells were evaluated by MTT assay and scoring under an inverted microscope (Olympus IX51 with DP70).

Detachment assay. Suspended cells (5×10^3 cells/well) were seeded on matrigel-coated 96-well plates for 12 h. Cells were then treated with non-toxic concentrations of ouabain for 6 h. After that, cells were dissociated from matrigel by incubated with 0.1% trypsin at 37˚C for 5-10 min. The remaining cells after washing with PBS were observed under an inverted microscope (Olympus IX51 with DP70). The remaining cells were determined using MTT assay and scored under an inverted microscope (Olympus IX51 with DP70).

Western blot analysis. After specific treatments, cells were washed twice with cold PBS and incubated with lysis buffer containing 20 mM TrisHCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 100 mM phenylmethyl sulfonyl fluoride, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 40 min on ice, collected whole cell lysates and centrifuged at 13,709 ×*g* at 4˚C for 15 min. The supernatants of the whole cell lysates were collected and assayed for protein content using the BCA protein assay. Loading dye was added to equal amounts of protein from each sample then, denatured by heating at 95˚C for 5 min. The proteins containing loading dye were loaded onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to separation. After separation, the proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The transferred membranes were blocked with 5% nonfat dry milk in TBST (25 mM TrisHCl pH 7.5, 125 mM sodium chloride and 0.05% Tween 20) for 1 h at room temperature. The membranes were then washed three times with TBST for 8 min and incubated with a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit antibody for 2 h. Following the incubation, the proteins were then visualized using an enhanced chemiluminescence detection kit.

Statistical analysis. Data were presented as the means±S.D. from three independent experiments. Statistical analysis was carried out using one-way ANOVA and *post-hoc* test at a significance level of $p<0.05$. SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Effect of ouabain on the viability of human lung cancer H23 cells. Firstly, we characterized the effects of ouabain on the survival of human lung cancer H23 cells. Because previous studies have reported the concentrations of ouabain and ouabain-like substances in human plasma to range from 2-770 pM, the concentration range of 0-50 pM of ouabain was selected for the present study. H23 cells were cultured in presence and absence of ouabain (0-50 pM) for 12 h, and cell viability was determined by the MTT viability assay. Figure 1A shows that when cells were treated with ouabain, at concentrations ranging from 0-10 pM, neither cytotoxicity nor proliferative effects were observed. A significant decline in cell viability was detected in cells treated with 50 pM ouabain, with approximately 70% of the cells remaining viable. Accordingly, the Hoechst33342 staining assay indicated that at 0-10 pM, ouabain did not cause apoptosis (Figure 1B and C).

Ouabain enhanced lung cancer H23 cell detachment. To elucidate the effect of ouabain on lung cancer cell detachment, we performed cell-ECM adhesion and detachment assays. For cell-ECM adhesion assay, cells were incubated with ouabain at sub-toxic concentrations (0-10 pM) for 0-6 h and were subjected to the cell-ECM adhesion assay. After trypsinization, cells were allowed to adhere to the matrigel surface for 30 min and adhered cells were determined by the MTT assay and cell counting. Figure 2 shows that treatment with ouabain significantly reduced the number of adhered cells in dose- (Figure 2 A-C) and time-dependent manners (Figure 2D-F) compared to the non-treated H23 control cells. Treatment with ouabain at 5 and 10 pM reduced the adhesion of cells to approximately 65% and 50%, respectively (Figure 2A and B).

Furthermore, we elucidated the effect of ouabain on cancer cell detachment using cell detachment assay. Cells were sparsely seeded in order to avoid cell-cell contact. After 12 h incubation, the cells were incubated with 0-10 pM ouabain for 6 h and were dissociated from matrigel surfaces by incubating with 0.1% trypsin at 37˚C for 5-10 min. The

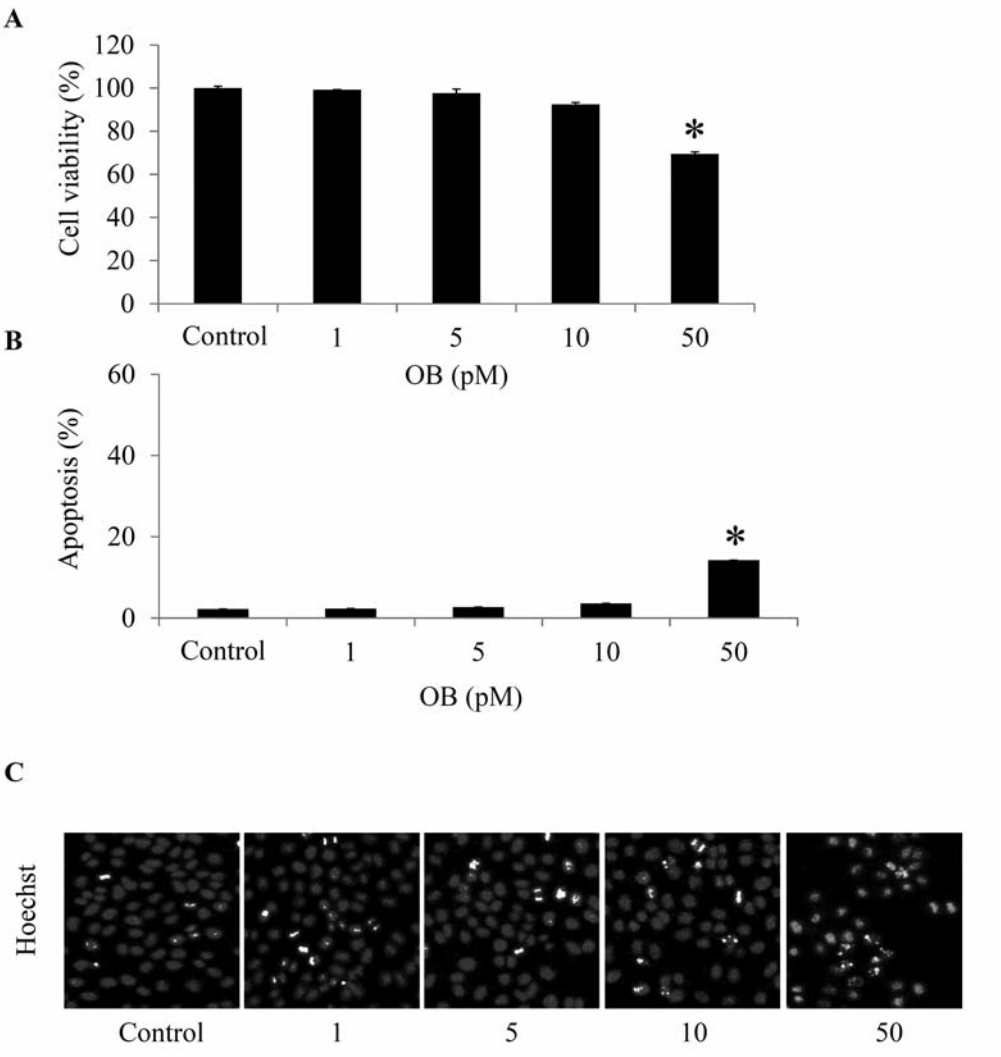

Figure 1. *Effect of ouabain (OB) on cytotoxicity in lung cancer H23 cells. A: Effect of ouabain on H23 cell viability. Cells were treated with different concentrations (0-50 pM) of ouabain for 12 h. The cell viability was analyzed using the MTT assay. B: Apoptotic cell count from Hoechst 33342 staining of ouabain-treated cells for 12 h. C: The pictures of Hoechst 33342 staining of ouabain-treated cells for 12 h. The data are the mean±S.D. (n=3). *p<0.05 versus the non-treated control.*

percentage of remaining cells to that of total cells was assessed by the MTT assay and cells scored under an inverted microscope. Figure 3 shows that treatment with ouabain significantly enhanced the detachment of cells in dose-dependent manner. Taken together, these results suggest that ouabain at physiological concentration weakened cancer cell adhesion to ECM surface and facilitate detachment.

Ouabain induces FAK and AKT activation and reduction of alpha-5 and beta-1 integrin. Having demonstrated the potentiating effect of ouabain on lung cancer cell detachment, we next examined the underlying mechanism, focusing on the expression level and activation of the proteins known to play roles in cancer cell-ECM adhesion. Cells were treated with ouabain for 6 h and expression of FAK, activated FAK (phosphorylation at Tyr 397), AKT, activated AKT (phosphorylation at Ser 473), alpha-5 integrin, and beta-1 integrin were evaluated by western blot analysis. Figure 4 indicates that treatment with ouabain significantly increased the levels of phosphorylated FAK (Tyr 397) and phosphorylated AKT (Ser 473), whereas ouabain exposure had no significant effect on the levels of total FAK and total AKT. For dose-dependent assessment, cells were treated with non-toxic concentrations of ouabain for 6 h and expression levels of these proteins were evaluated. Figure 4A and B indicate that treatment with ouabain resulted in the activation

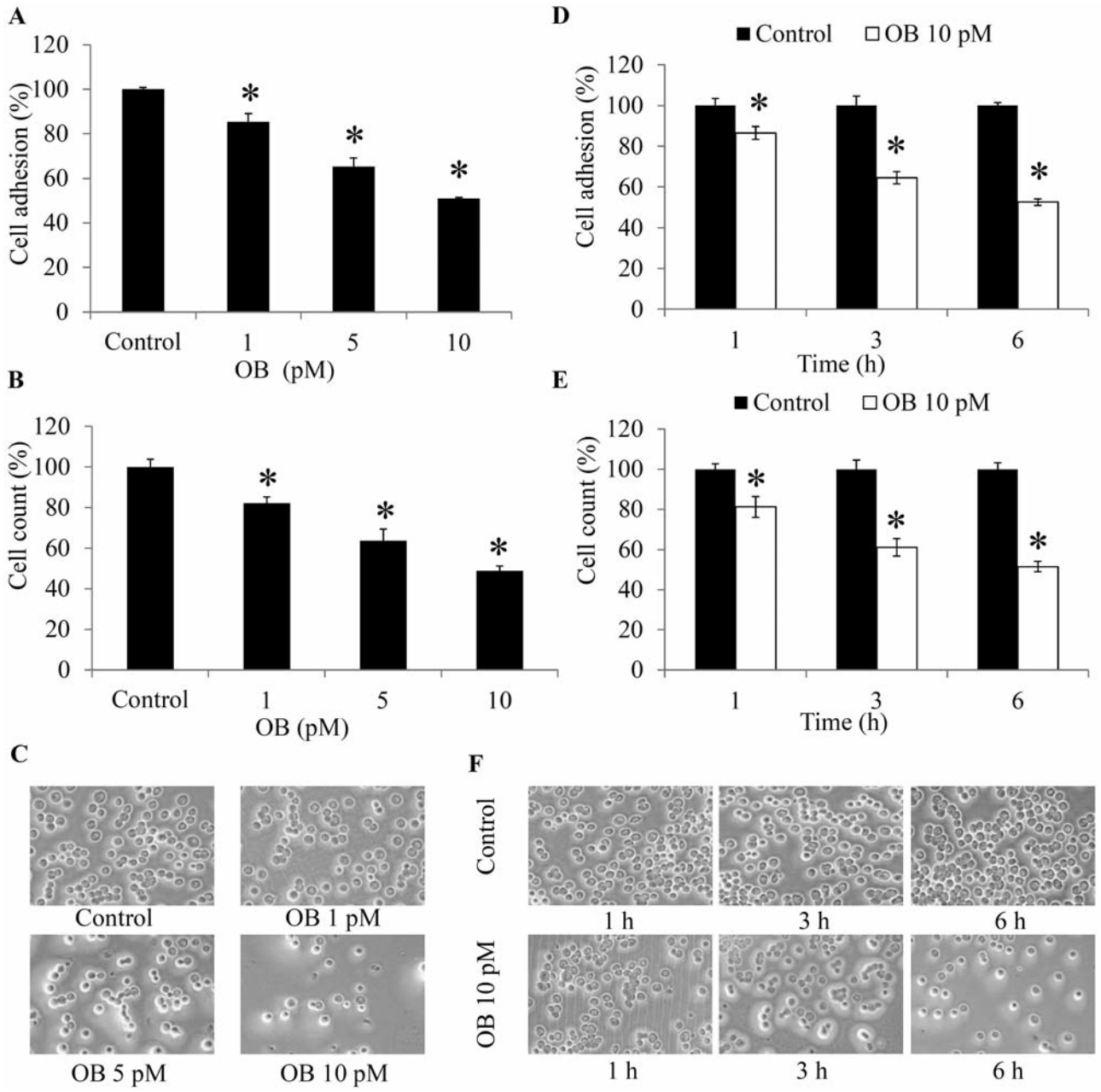

Figure 2. *Effect of ouabain (OB) on H23 cell adhesion. Cells were exposed to ouabain at different concentrations for 6 h and subjected to cell-extracellular matrix (ECM) adhesion assay. Cells were allowed to adhere to the matrigel surface for 30 min and adhered cells were determined by the MTT assay (A) and cell counting (B). C: Phase-contrast images of adhered cells. For time-dependent experiments, cells were treated with 10 pM ouabain for 1-6 h and subjected to the assay. Cell adhesion was evaluated by the MTT assay (D) and cell counting (E). F: Phase-contrast images of adhered cells. The data are the mean±S.D. (n=3). *p<0.05 versus the non-treated control.*

of FAK and AKT in a dose-dependent fashion. The time-dependent effect of ouabain on FAK and AKT activation were also evaluated. Cells were treated with 10 pM ouabain for 0-6 h, and the level of proteins was detected by western blotting. Figure 4C and D show that an increase of ouabain exposure caused an increase in activation of FAK and AKT. These results indicate the involvement of the FAK and AKT pathways in the ouabain-facilitated detachment of these cells.

As alpha-5 and beta-1 integrins have been implicated in cancer cell adhesion to the ECM surface (19), we tested whether detachment of H23 cells in the present study was associated with the decrease of these integrins. Interestingly,

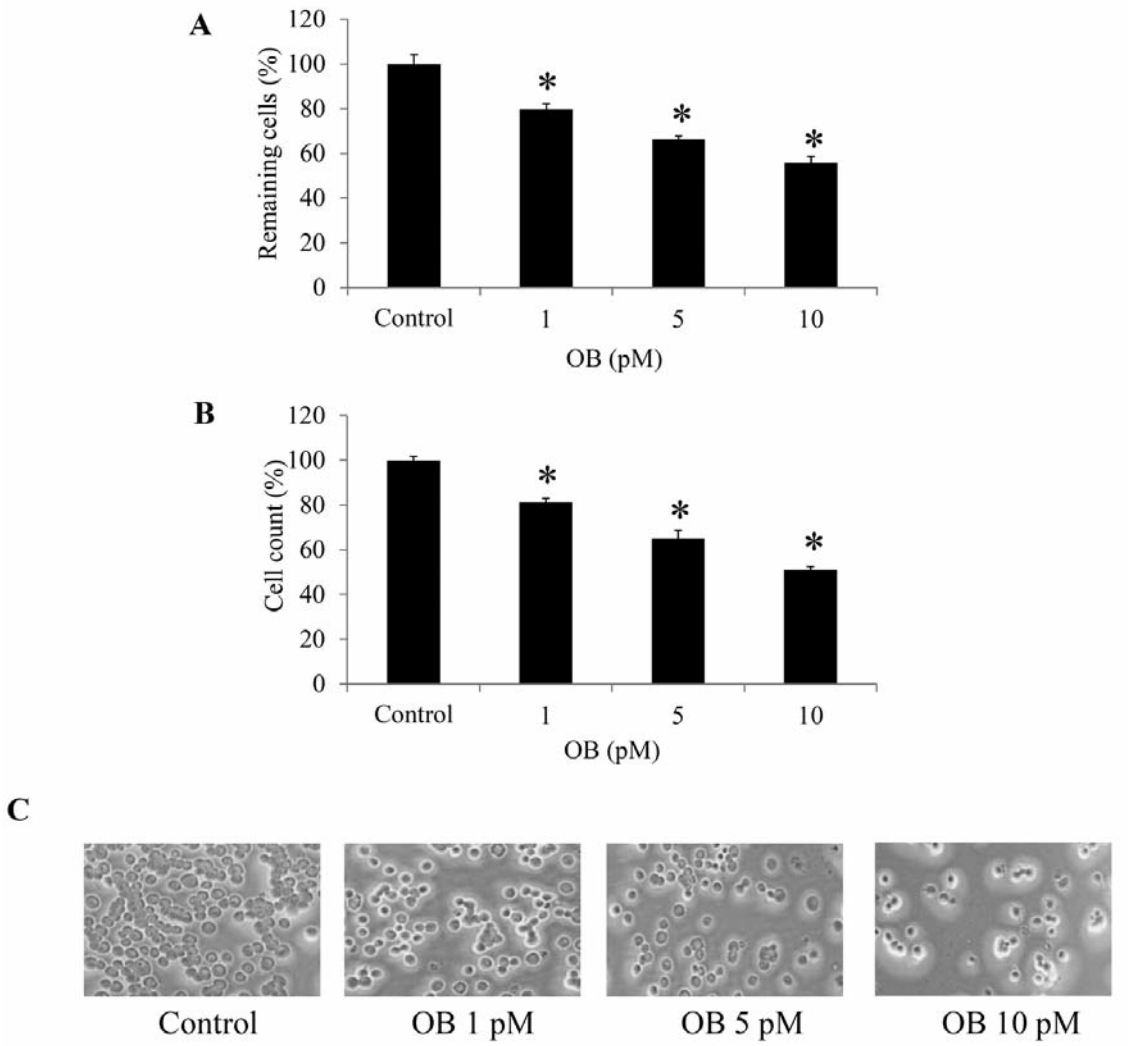

Figure 3. *For detachment assay, cells were incubated with 0-10 pM ouabain (OB) for 6 h and were dissociated from matrigel surfaces by incubated with 0.1% trypsin at 37˚C for 5-10 min. The percentage of remained cells to that of total cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (A) and cell scoring (B) under an inverted microscope. C: Phase-contrast images of remaining cells. The data are the mean±S.D. (n=3). *p<0.05 versus the non-treated control.*

ouabain treatment significantly decreased the cellular level of alpha-5 and beta-1 integrins in dose- and time-dependent manners (Figure 5). Because cellular integrins were previously shown to be tightly regulated *via* degradation through the lysosomal pathway (34, 35), we further tested whether treatment with ouabain could facilitate this degradation of integrins. Cells were treated with ouabain in the presence or absence of concanamycin A (CMA, a specific inhibitor that blocks lysosomal degradation of proteins) for 0-6 h and level of integrins was determined. Western blot analysis revealed that treatment with ouabain down-regulated alpha-5 and beta-1 integrins in a time-dependent manner and the addition of CMA significantly inhibited such effects of ouabain (Figure 5C and D). These results indicate that ouabain facilitates cancer cell detachment by mediating alpha-5 and beta-1 integrin degradation through a lysosomal mechanism. Taken together, we see that it is likely that this endogenous substance at specific biological concentrations could enhance cancer cell detachment *via* FAK and AKT activation, as well as facilitating of integrin degradation.

Discussion

Ouabain, a cardiac glycoside member isolated from plants, was found to be endogenously produced in humans and classified as a human hormone (20, 26). It has lately garnered increasing attention in cancer research. Here, we demonstrate for the first

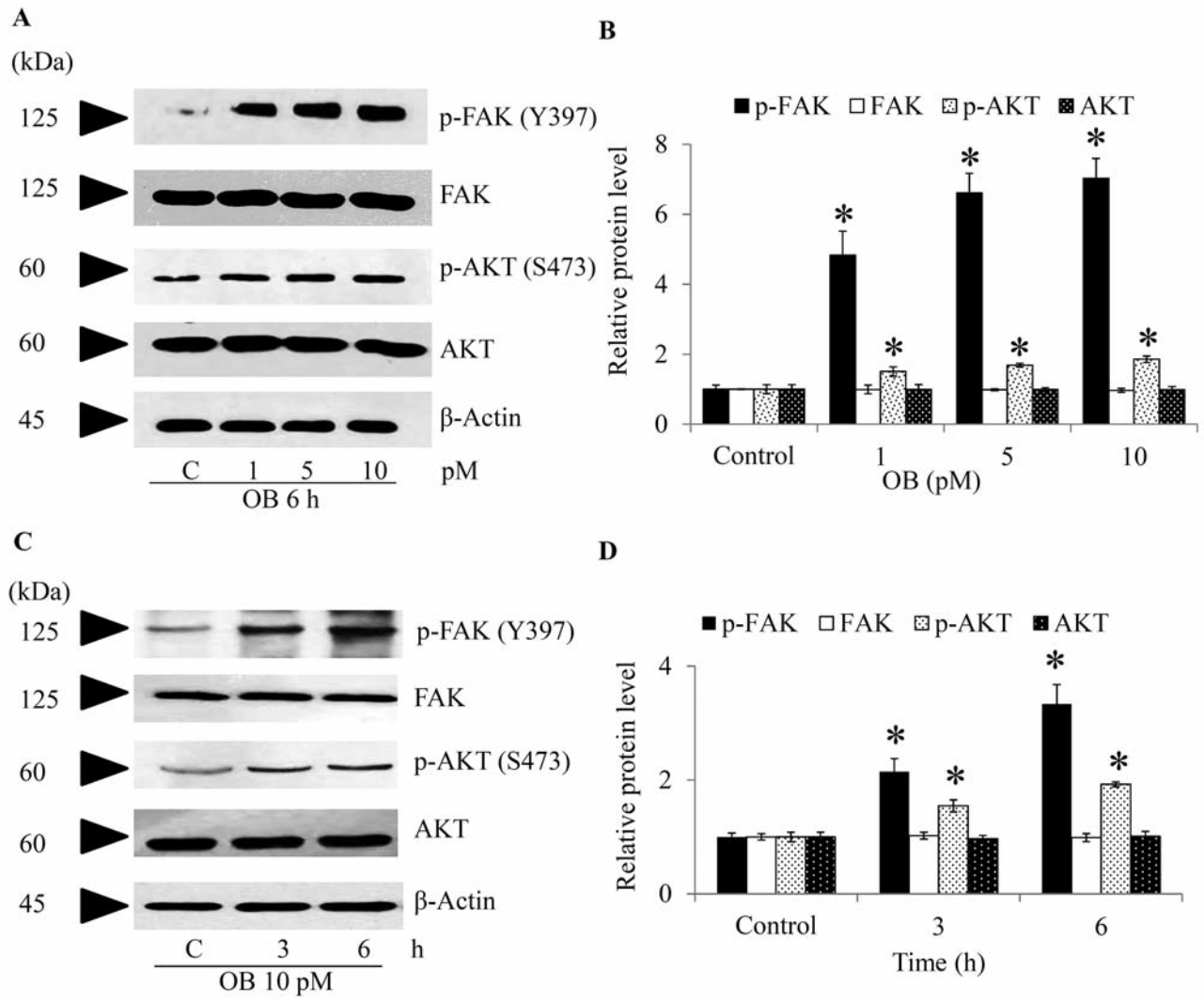

Figure 4. *Ouabain (OB) activates the focal-adhesion kinase (FAK)-ATP-dependent tyrosine kinase (AKT) pathways. (A) Cells were treated with 0-10 pM ouabain for 6 h, and the expression of phosphorylated FAK, total FAK, phosphorylated AKT, and total AKT were determined by western blotting. To confirm equal loading of the samples, the blots were reprobed with β-actin antibody. C: Cells were treated with 10 pM ouabain for 0-6 h and proteins were evaluated. B and D: The immunoblot signals were quantified by densitometry. The data are the mean±S.D. (n=3). *p<0.05 versus the non-treated control.*

time that ouabain at physiological concentrations (0-10 pM) facilitates lung cancer cell detachment. Even though detachment of cancer cells from their original tumor is accepted as an important initial process of cancer metastasis, there are only few studies to elucidate the cellular mechanisms of cell detachment.

Interestingly, ouabain and its related compounds are found in human plasma at different concentrations depending on the status of individuals as well as methods of assessment. In most cases, plasma ouabain concentrations are reported to be between 2-770 pM (24). The present study aimed to investigate the effect of this endogenous substance at its physiological levels, we therefore selected concentrations of 0-50 pM; however, ouabain at concentrations of more than 50 pM caused significant cytotoxicity towards human lung cancer cells. This result is consistent with the previous experiments indicating that ouabain possesses activity against many types of cancers (21, 22, 25, 27). Besides its direct cytotoxicity, we previously found that ouabain at non-toxic concentrations sensitized TRAIL-mediated death by down-regulating anti-apoptotic myeloid cell leukemia sequence-1 (MCL-1) protein in H292 and H460 cells (25).

Cell-ECM adhesion plays an important part for survival of the cancer cells by activating the survival signal through integrin-AKT-dependent mechanisms (28). Although the

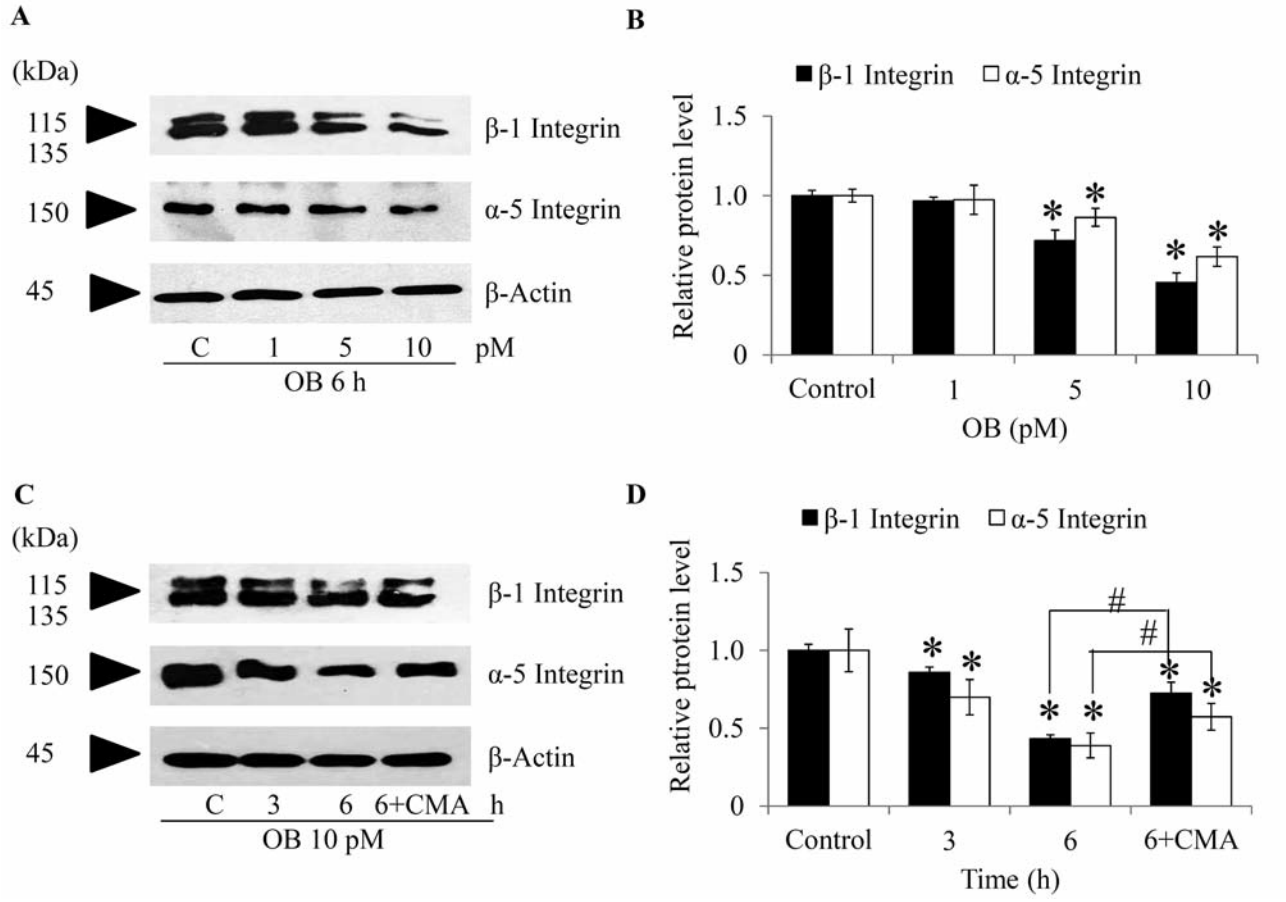

Figure 5. *Ouabain (OB) down-regulates beta-1 and alpha-5 integrins via lysosomal pathway. A: Cells were treated with 0-10 pM ouabain for 6 h, and the expression of beta-1 and alpha-5 integrins were determined by western blotting. To confirm equal loading of the samples, the blots were re-probed with β-actin antibody. C: Cells were treated with 10 pM ouabain for 0-6 h in the presence or absence of lysosomal inhibitor concanamycin A (CMA) and proteins were evaluated. B and D: The immunoblot signals were quantified by densitometry. The data are the mean±S.D. (n=3). *p<0.05 versus the non-treated control.*

detachment of cells will disrupt such survival and trigger detachment-mediated apoptosis (anoikis), certain cancer cells have the ability to resist anoikis and further spread (29, 30). For cell detachment, integrin binding to extracellular compartments must be disrupted and previous studies showed that such dissociation of integrin resulted from the activation of FAK and AKT pathways (10, 11, 31). In certain studies, FAK action was shown to involve downstream AKT (32). In addition, activation of AKT was found to regulate internalization and recycling of integrin (33). The internalization of integrins, under certain conditions, leads to degradation of cellular integrins *via* the lysosomal pathway (34, 35). Consistent with this finding, our results indicate that ouabain-mediated cell detachment involves FAK and AKT activation, and down-regulation of cellular integrins. Furthermore, using specific inhibitors of lysosomal degradation pathway, we found that ouabain reduced cellular integrins through the lysosomal pathway.

In summary, the present study demonstrated the possible role of ouabain at its physiological levels on the detachment of lung cancer cells. Ouabain exposure activated the FAK-AKT signaling pathway and reduced the level of integrins through lysosomal degradation. The knowledge gained from the present study may benefit our understanding on cancer biology and cancer metastasis.

Conflicts of Interest

The Authors declare that there are no conflicts of interest regarding this research.

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References

1 Jemal A, Siegel R, Xu J and Ward E: Cancer statistics. CA Cancer J Clin *60*: 277-300, 2010.

2 Yokoi K, Kamiya N, Matsuguma H, Machida S, Hirose T, Mori K and Tominaga K: Detection of brain metastasis in potentially operable non-small cell lung cancer: A comparison of CT and MRI. Chest *115*: 714-719, 1999.

3 Park HY, Kim YH, Kim H, Koh WJ, Suh GY, Chung MP and Kwon OJ: Routine screening by brain magnetic resonance imaging decreased the brain metastasis rate following surgery for lung adenocarcinoma. Lung cancer *58*: 68-72, 2007.

4 Coleman RE: Metastatic bone disease: Clinical features, pathophysiology and treatment strategies. Cancer Treat Rev *27*: 165-176, 2001.

5 Iordanidou L, Trivizaki E, Saranti S, Georgakopoulos A, Bolanos N, Baltagiannis N and Koutsiouba P: Is there a role of whole body bone scan in early stages of non-small cell lung cancer patients. J BUON *11*: 491-497, 2006.

6 Fidler IJ: The pathogenesis of cancer metastasis: The "seed and soil" hypothesis revisited. Nat Rev Cancer *3*: 453-458, 2003.

7 Coghlin C and Murray GI: Current and emerging concepts in tumour metastasis. J Pathol *222*: 1-15, 2010.

8 Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell *44*: 646-674, 2011.

9 Weiss L and Ward MP: Cell detachment and metastasis. Cancer Metastasis Rev *2*: 111-127, 1983.

10 Nagano M, Hoshino D, Koshikawa N, Akizawa T and Seiki M: Turnover of focal adhesions and cancer cell migration. Int J Cell Biol, 2012: 310616. doi: 10.1155/2012/310616, 2012.

11 Broussard JA, Webb DJ and Kaverina I: Asymmetric focal adhesion disassembly in motile cells. Curr Opin Cell Biol *20*: 85-90, 2008.

12 Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M and Yamamoto T: Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*: 539-543, 1995.

13 Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, Van RF, Lee KW, Donowitz M, Tsichlis PN and Larue L: The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res *63*: 2172-2178, 2003.

14 Hamadi A, Bouali M, Dontenwill M, Stoeckel H, Takeda K and Ronde P: Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. J cell Sci *118*: 4415-4425, 2005.

15 Truong H and Danen EH: Integrin switching modulates adhesion dynamics and cell migration. Cell Adh Migr *3*: 179-181, 2009.

16 Hynes RO: Integrins: Bidirectional, allosteric signaling machines. Cell *110*: 673-687, 2002.

17 Schaffner F, Ray AM and Dontenwill M: Integrin α5β1, the fibronectin receptor, as a pertinent therapeutic target in solid tumors. Cancers *5*: 27-47, 2013.

18 Hirasawa M, Shijubo N, Uede T and Abe S: Integrin expression and ability to adhere to extracellular matrix proteins and endothelial cells in human lung cancer lines. Br J Cancer *70*: 466-473, 1994.

19 Roca-Cusachs P, Gauthier NC, Del Rio A and Sheetz MP: Clustering of alpha(5)beta(1) integrins determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. Proc Natl Acad Sci USA *106*: 16245-16250, 2009.

20 Blaustein MP: Physiological effects of endogenous ouabain: Control of intracellular Ca^{2+} stores and cell responsiveness. Am J Physiol Cell Physiol *264*: 1367-1387, 1993.

21 Huang YT, Chueh SC, Teng CM and Guh JH: Investigation of ouabain-induced anticancer effect in human androgen-independent prostate cancer PC-3 cells. Biochem Pharmacol *67*: 727-733, 2004.

22 Ozdemir T, Nar R, Kilinc V, Alacam H, Salis O, Duzgun A, Gulten S and Bedir A: Ouabain targets the unfolded protein response for selective killing of HepG2 cells during glucose deprivation. Cancer Biother Radiopharm *27*: 457-463, 2012.

23 Gasper R, Vandenbussche G and Goormaghtigh E: Ouabain-induced modifications of prostate cancer cell lipidome investigated with mass spectrometry and FTIR spectroscopy. Biochim Biophys Acta *1808*: 597-605, 2011

24 Gottlieb SS, Rogowski AC, Weinberg M, Krichten CM, Hamilton BP and Hamlyn JM: Elevated concentrations of endogenous ouabain in patients with congestive heart failure. Circulation *86*: 420-425, 1992.

25 Chanvorachote P and Pongrakhananon V: Ouabain downregulates Mcl-1 and sensitizes lung cancer cells to TRAIL-induced apoptosis. Am J Physiol Cell Physiol *304*: C263-C272, 2013.

26 Ogawa H, Shinoda T, Cornelius F and Toyoshima C: Crystal structure of the sodium-potassium pump (Na^+,K^+-ATPase) with bound potassium and ouabain. Proc Natl Acad Sci USA *106*: 13742-13747, 2009.

27 Winnicka K, Bielawski K, Bielawska A and Miltyk W: Apoptosis-mediated cytotoxicity of ouabain, digoxin and proscillaridin A in the estrogen independent MDA-MB-231 breast cancer cells. Arch Pharm Res *30*: 1216-1224, 2007.

28 Bolos V, Gasent JM, Tarruella SL and Grande E: The dual kinase complex FAK-Src as a promising therapeutic target in cancer. Onco Targets Ther *3*: 83-97, 2010.

29 Frisch SM and Screaton RA: Anoikis mechanisms. Curr Opin Cell Biol *13*: 555-562, 2001.

30 Rennebeck G, Martelli M and Kyprianou N: Anoikis and survival connections in the tumor microenvironment: Is there a role in prostate cancer metastasis?. Cancer Res *65*: 11230-11235, 2005.

31 Ivaska J, Vuoriluoto K, Huovinen T, Izawa I, Inagaki M and Parker PJ: PKCepsilon-mediated phosphorylation of vimentin controls integrin recycling and motility. EMBO J *24*: 3834-3845, 2005.

32 Wang S and Basson MD: Protein kinase B/AKT and focal adhesion kinase: two close signaling partners in cancer. Anticancer Agents Med Chem 11: 993-1002, 2011.

33 Li J, Ballif BA, Powelka AM, Dai J, Gygi SP and Hsu VW: Phosphorylation of ACAP1 by Akt regulates the stimulation-dependent recycling of Integrin β1 to control cell migration. Dev Cell *9*: 663-673, 2005.

34 Johannes L and Popoff V: Tracing the retrograde route in protein trafficking. Cell *135*: 1175-1187, 2008.

35 Grant BD and Donaldson JG: Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol *10*: 597-608, 2009.

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