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Anticancer effect of arsenite on cell migration, cell cycle and apoptosis in human pancreatic cancer cells

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Abstract. The standard treatment for advanced pancreatic cancer is chemotherapy, but its clinical outcome remains unsatisfactory. Therefore, the development of novel treatments for this malignancy is urgently required. In the present study, the anticancer effect of arsenite on platelet-derived growth factor (PDGF)-BB-induced migration, cell cycle and apoptosis was investigated in pancreatic cancer cells (AsPC-1 and BxPC-3), and compared with the effect on normal pancreatic epithelial (PE) cells. In the cell migration assay, arsenite clearly inhibited PDGF-BB-induced cell migration in AsPC-1 cells, but not in BxPC-3 or PE cells. Arsenite also caused cell apoptosis in AsPC-1 cells, but not in BxPC-3 or PE cells. In AsPC-1 cells, the levels of cyclin D1 and phosphorylated retinoblastoma protein decreased following treatment with arsenite, but this was not observed in BxPC-3 cells. To further examine the differences between these two cell lines, the effect of arsenite on upstream p44/p42 mitogen-activated protein kinase (MAPK) and Akt was investigated. PDGF-BB caused phosphorylation of p44/p42 MAPK and Akt in both cell lines. Pretreatment with arsenite significantly suppressed PDGF-BB-induced phosphorylation of Akt, but not of p44/p42 MAPK in AsPC-1 cells. By contrast, arsenite did not affect these molecules in BxPC-3 cells. Since the inhibition of the Akt signaling pathway markedly reduced PDGF-BB-induced migration in AsPC-1 cells, the present results strongly suggest that arsenite inhibits PDGF-BB-induced migration by suppressing the Akt signaling pathway in AsPC-1 cells. Therefore, arsenite may be a useful tool for the treatment of patients with certain types of pancreatic cancer, without causing adverse effects on normal pancreatic cells.

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Introduction

Pancreatic cancer accounts for ~5% of cancer-associated mortalities, and ranks the eighth in terms of cancer incidence worldwide (1). Due to the difficulty in early diagnosis of pancreatic cancer, the majority of patients present an advanced stage of the disease when the first symptoms appear (2). The standard treatment for advanced pancreatic cancer is chemotherapy (3). However, the median survival of patients treated with gemcitabine is not satisfactory (4). A number of studies have compared the efficacy of gemcitabine alone with gemcitabine-based combinations, including 5-fluorouracil, capecitabine, cisplatin, docetaxel, irinotecan, oxaliplatin and pemetrexed, for the treatment of pancreatic cancer, but no clear survival benefit has been demonstrated thus far (5). Therefore, current research is focused on the development of novel treatments for inoperable pancreatic cancer (6,7).

Arsenite is a natural substance with a reported medicinal use for >2,400 years (8). However, its use in recent years has been limited due to the toxicity and potential carcinogenicity of chronic arsenic administration (9). Arsenic therapy gained popularity during the 1970s, when Chinese physicians started to use arsenic trioxide as part of the treatment for acute promyelocytic leukemia (APL) (8). The results of those studies indicated that a stable solution of arsenic trioxide administered by intravenous infusion was remarkably safe and effective in patients with newly diagnosed, refractory or relapsed APL (8). The molecular mechanism of action of arsenic derivatives against APL involves induction of cell apoptosis, inhibition of cell proliferation and inhibition of angiogenesis (8), although the exact mechanistic details remain to be fully understood.

In patients with advanced pancreatic cancer, cell invasion into adjacent tissues is a major prognostic factor (10). Abnormal cell migration leads to pathological states such as invasion and metastasis of cancer (10). It has been reported that actin stress fibers generate contractile forces by pulling against focal adhesions in order to induce retraction of the rear cell membrane, which suggests that stress fibers may be important for cell migration (11). Cytoskeletal proteins such as vinculin and actinin, and several non-receptor protein tyrosine kinases, including focal adhesion kinase and members of the Src family, are involved in the organization of focal adhesion complexes (12,13). Platelet-derived growth factors (PDGFs) are known to participate in the pathogenesis, invasion and

distant metastasis of human solid tumors, and their expression levels are correlated with poor prognosis (14,15).

In the present study, the effect of arsenite on pancreatic cancer cell migration, proliferation and apoptosis was investigated. The results demonstrated that arsenite strongly suppressed PDGF-BB-induced cell migration by suppressing the Akt signaling pathway in AsPC-1 cells.

Materials and methods

Materials. Recombinant human PDGF-BB (catalog no. 220-BB) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, and the Akt and glycogen synthase kinase-3 beta (GSK3β) inhibitors were obtained from Merck & Co., Inc. (Kenilworth, NJ, USA). Goat polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; catalog no. sc-48166) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit polyclonal anti-proteolytically cleaved poly(adenosine diphosphate-ribose) polymerase (PARP; catalog no. 9542), rabbit monoclonal anti-cyclin D1 (catalog no. 2978), rabbit polyclonal anti-phosphorylated (phospho)-retinoblastoma protein (Rb) (catalog no. 9301), mouse monoclonal anti-phospho-p44/p42 mitogen-activated protein kinase (MAPK) (catalog no. 9106), rabbit monoclonal anti-p44/p42 MAPK (catalog no. 4695), rabbit monoclonal anti-phospho-Akt (catalog no. 5012) and rabbit polyclonal Akt (catalog no. 9272) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Amersham ECL Western Blotting Detection Reagent was used for western blotting visualization and was purchased from GE Healthcare Life Sciences (Chalfont, UK). Arsenite and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO, USA)

Cell culture. AsPC-1 and BxPC-3 pancreatic cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C with 5% CO_2. Primary normal human pancreatic epithelial (PE) cells were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan) and maintained in CSC medium (Cell Systems Corporation, Kirkland, WA, USA). Unless otherwise indicated, cells were incubated in serum-free medium for 24 h prior to be used in the corresponding experiments, as previously described (16).

Cell migration assay. Cell migration was assessed using a Boyden Transwell® chamber of 8 μm pores for AsPC-1 and BxPC-3 cells, and 3 μm pores for PE cells (Costar; Corning Incorporated, Corning, NY, USA). Cells were exposed to arsenite (10-30 μM) or the aforementioned inhibitors and incubated for 24 h. Next, cells ($5x10^4$ cells/well) were seeded onto the upper chamber in RPMI medium containing 10% FCS. Following 16-h incubation at 37˚C, cells were treated with PDGF-BB at the indicated concentrations for 36 h. Cells were then fixed and stained with 1 ml clonogenic reagent (50% ethanol and 0.25% 1, 9-dimethyl-methylene blue; Sigma-Aldrich) for 30 min. Subsequently, the cells on the upper surface of the membrane were mechanically removed, while the cells that had migrated to the lower surface of the membrane were observed under a microscope (BZ-9000 BioRevo; Keyence Corporation, Osaka, Japan). The average number of migrated cells from five randomly selected fields on the lower surface of the membrane was counted. Data were obtained from ≥3 independent experiments.

Western blot analysis. Western blot analyses were performed as described previously (17) using the Mini-PROTEAN® Electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). In brief, the cells were lysed in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 50 mM NaF, 50 mM HEPES, 1 mM Na_3VO_4 and 2 mM PMSF] and scraped from the Petri dishes. The protein lysates (5 μg) were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto an Immun-Blot® polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% skimmed dry milk in phosphate-buffered saline containing 0.1% Tween 20 for 30 min, prior to be incubated at 25˚C overnight with the indicated primary antibodies, which were diluted to 1:2,000. Donkey anti-goat (catalog no. sc-2020), goat anti-mouse (catalog no. sc-2005) and goat anti-rabbit (catalog no. sc-2004) IgG horseradish peroxidase-labeled antibodies (Santa Cruz Biotechnology, Inc.) were used as secondary antibodies and were incubated at 25˚C for 1 h at a dilution of 1:5,000. The peroxidase activity on the membrane was visualized on X-ray films (Fujifilm Co., Tokyo, Japan) by ECL detection.

Densitometry analysis. Densitometry analysis was performed using a scanner and a software package for image analysis (ImageJ version 1.32; National Institutes of Health, Bethesda, MD, USA). The respective bands were manually selected, and the intensities were measured using the 'measure' function, following automatic quantification. The background-subtracted signal intensity of each protein band was normalized to the respective controls. Data were obtained from ≥3 independent experiments.

Statistical analysis. Data were analyzed by analysis of variance, followed by the Bonferroni method between the indicated pairs for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Arsenite suppressed PDGF-BB-induced migration in AsPC-1 cells, but not in BxPC-3 or PE cells. PDGF and its receptor are known to participate in pathogenesis, invasion and distant metastasis of human solid tumors, and their expression levels are correlated with poor prognosis (14,15). Therefore, the effect of arsenite on PDGF-BB-induced migration in AsPC-1, BxPC-3 and PE cells was examined in the present study. When cells were pretreated with increasing doses of arsenite for 24 h and then exposed to PDGF-BB for 36 h, the number of migrated cells was clearly reduced in

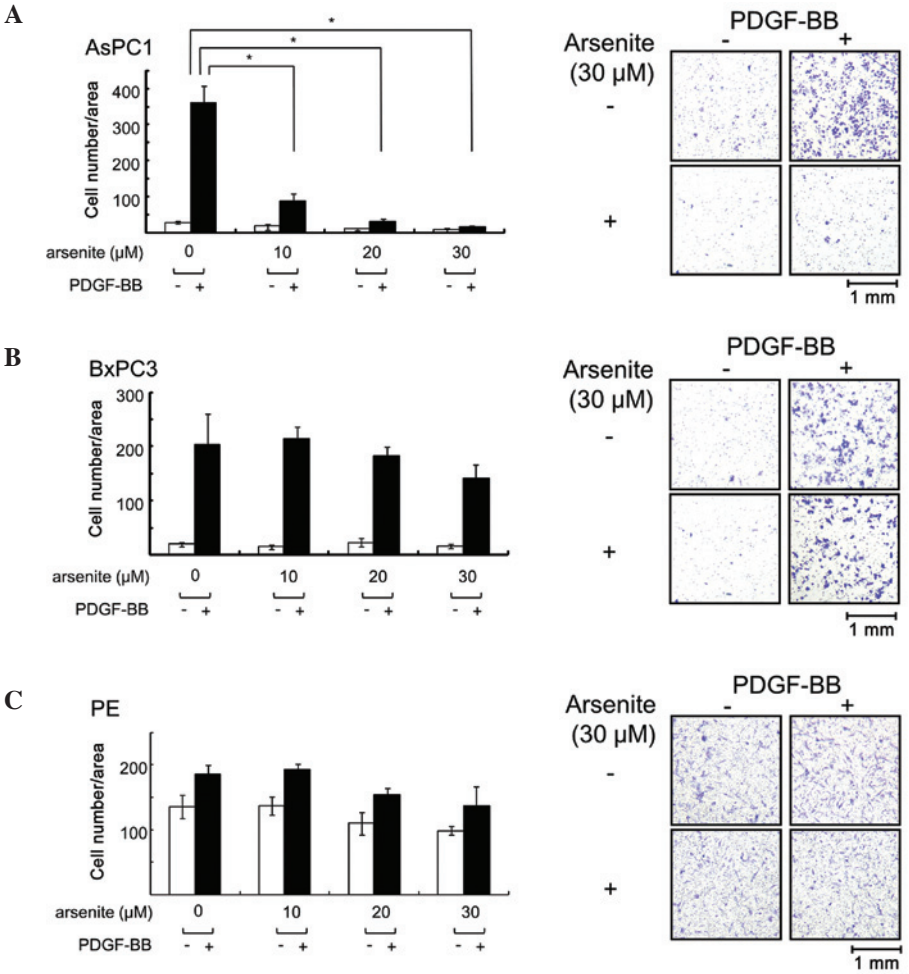

Figure 1. Effects of PDGF-BB on the migration of AsPC-1 and BxPC-3 pancreatic cancer cells, compared with normal PE cells. (A) AsPC-1, (B) BxPC-3 and (C) PE cells ($5x10^4$ cells/well) were treated with the indicated doses of arsenite for 24 h, prior to be seeded in the upper Boyden chamber. Following incubation for 16 h, cells were exposed to 30 ng/ml PDGF-BB for 36 h at 37˚C. Cells were then fixed, stained and visualized under a microscope. The average number of migrated cells from five randomly selected fields on the lower surface of the membrane was counted. Data were obtained from ≥3 independent experiments. *P<0.05 vs. controls. Right panels show representative images of the migrated cells stained with clonogenic reagent. PDGF, platelet-derived growth factor; PE, pancreatic epithelial.

AsPC-1 cells (Fig. 1A). However, arsenite had little effect on PDGF-BB-induced cell migration in BxPC-3 cells (Fig. 1B) and PE cells (Fig. 1C). These results indicate that arsenite exerts suppressive effects on cell migration in a particular type of pancreatic cancer cells.

Arsenite induced apoptosis in AsPC-1 cells, but not in BxPC-3 or PE cells. Accumulating evidence indicates that arsenic compounds induce apoptosis in APL, multiple myeloma and human hepatoma cells (18-20). Therefore, the effect of arsenite on apoptosis in human pancreatic cells was next examined. PARP enables cells to maintain their viability, and cleavage of PARP results in apoptosis (21); thus, PARP cleavage is usually observed in cells undergoing apoptosis. As represented in Fig. 2, arsenite decreased the levels of full-length PARP in AsPC-1 cells, but not in BxPC-3 or PE cells, similarly to the results shown in Fig. 1.

Arsenite inhibited cell proliferation of AsPC-1 cells, but not of BxPC-3 cells. The effect of arsenite on cell proliferation was next investigated. The ternary complex of cyclin D1, cyclin-dependent kinase (CDK)4 and p27Kip1 requires extracellular mitogenic stimuli for the release and degradation of p27Kip1 and concomitant increase in the levels of cyclin D1, which affects the progression and phospho-Rb-dependent entry into the S phase of the cell cycle (22). Thus, increased levels of cyclin D1 and phospho-Rb promote cell cycle transition, resulting in cell proliferation. As depicted in Fig. 3, arsenite clearly decreased the levels of phospho-Rb and cyclin D1 in AsPC-1 cells (Fig. 3A). However, arsenite did not decrease, but transiently increased instead, the levels of these proteins in BxPC-3 cells (Fig. 3B). Overall, these findings strongly suggest that arsenite exhibits anticancer effects in certain types of pancreatic cancer cells. PE cells were not investigated, since the expression level of cyclin D1 and Rb were too low to be detected using western blot analysis (data not shown).

Arsenite suppressed PDGF-BB-induced Akt activation in AsPC-1 cells, but not in BxPC-3 or PE cells. The survival ability of cancer cells is largely dependent on growth factors such as epidermal growth factor (23) and PDGF-BB (14,15). Through binding to their corresponding cell surface receptor, the above growth factors activate an extensive network of

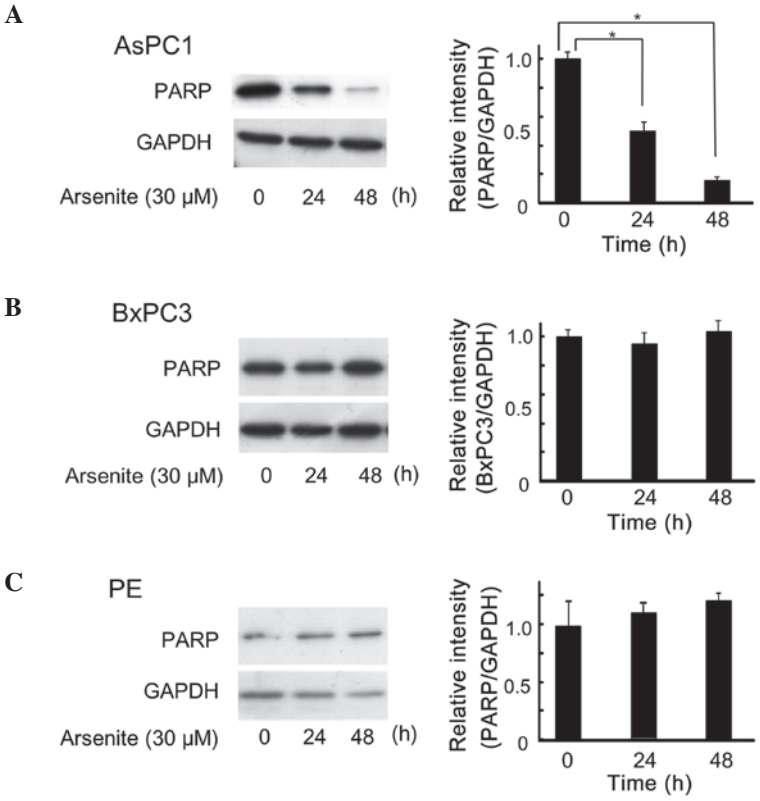

Figure 2. Effects of arsenite on PARP cleavage in AsPC-1, BxPC-3 and PE cells. (A) AsPC-1, (B) BxPC-3 and (C) PE cells were exposed to arsenite (30 μM) for the indicated time periods. Protein extracts were then harvested and examined by western blotting using anti-PARP and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies. Background-subtracted signal intensity of each protein band was normalized to GAPDH. Data are presented as the mean ± standard deviation of triplicate assay. *P<0.05. PARP, poly(adenosine diphosphate-ribose) polymerase; PE, pancreatic epithelial; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

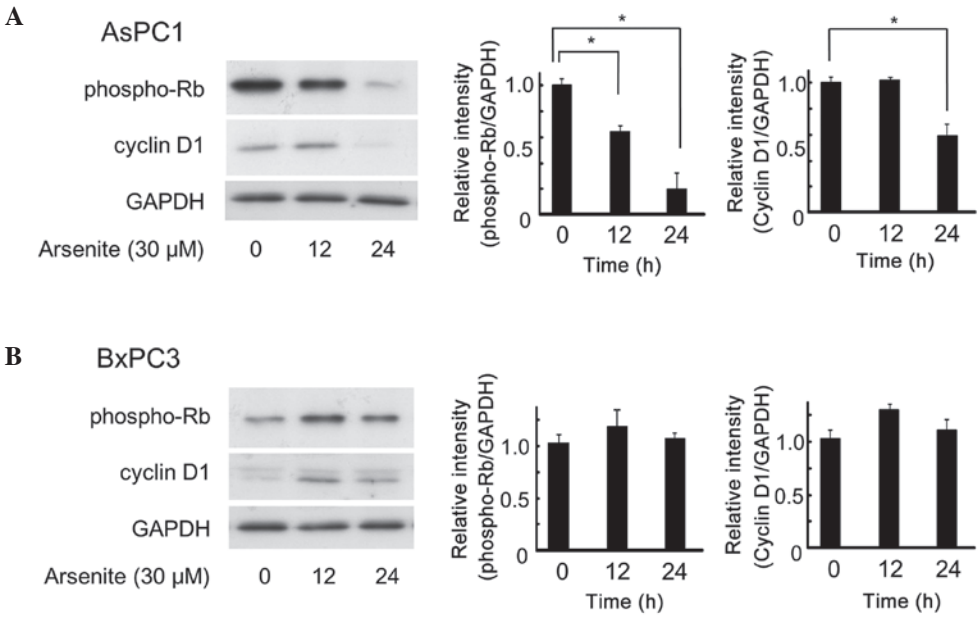

Figure 3. Effects of arsenite on the expression levels of phospho-Rb and cyclin D1 in AsPC-1 and BxPC-3 human pancreatic cancer cells. (A) AsPC-1 and (B) BxPC-3 cells were exposed to arsenite (30 μM) and incubated for the indicated time periods. Protein extracts were then harvested and examined by western blotting using antibodies against phospho-Rb, cyclin D1 and glyceraldehyde-3-phosphate dehydrogenase, which served as control. Background-subtracted signal intensity of each protein band was normalized to GAPDH. Data are presented as the mean ± standard deviation of triplicate assay. *P<0.05. Phospho, phosphorylated; Rb, retinoblastoma protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

signal transduction pathways that include activation of the Ras/p44/p42 MAPK and PI3K/Akt pathways (23). In order to elucidate the mechanism underlying the suppressive effect of arsenite on the migration of AsPC-1 cells, but not on that of BxPC-3 or PE cells, western blotting was performed to examine the effect of arsenite on PDGF-BB-induced

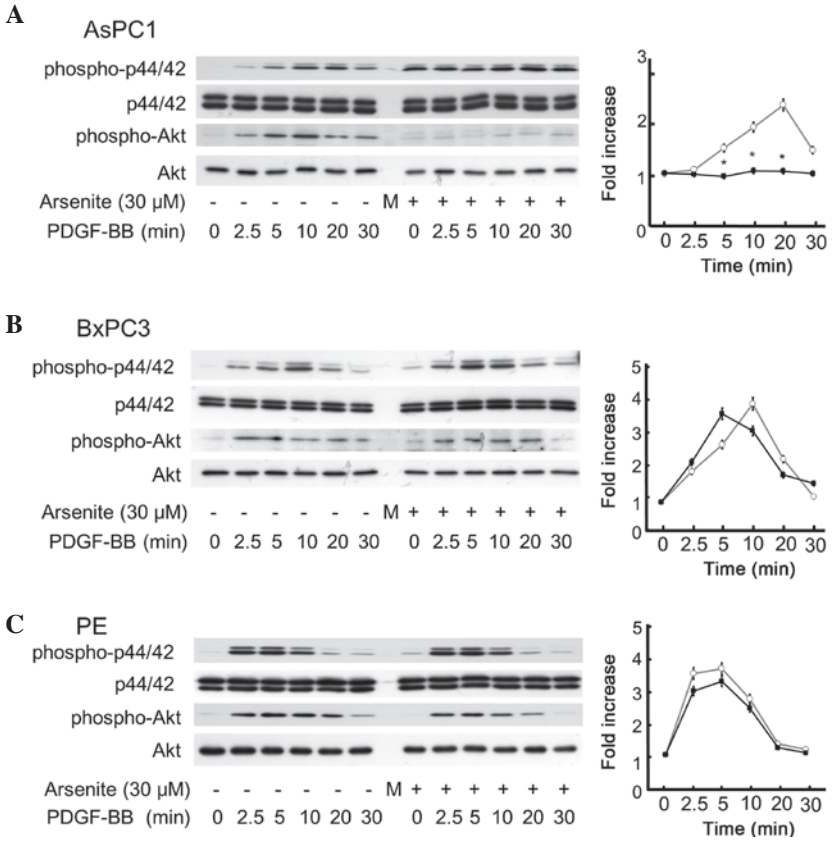

Figure 4. Effects of arsenite on the phosphorylation of p44/p42 MAPK and Akt in AsPC-1, BxPC-3 and PE cells. (A) AsPC-1, (B) BxPC-3 and (C) PE cells were exposed to arsenite (30 μM) and incubated with 30 ng/ml platelet-derived growth factor-BB for the indicated time periods. Protein extracts were then harvested and examined by western blotting using specific antibodies against phospho-p44/p42 MAPK, p44/p42 MAPK, phospho-Akt and Akt. Background-subtracted signal intensity of each protein band was normalized to Akt. Data are presented as the mean ± standard deviation of triplicate assay. *P<0.05 vs. cells without arsenite exposure. PE, pancreatic epithelial; PDGF, platelet-derived growth factor; phospho, phosphorylated; MAPK, mitogen-activated protein kinase; M, marker.

phosphorylation of p44/p42 MAPK and Akt. Arsenite had negligible effect on p44/p42 MAPK in AsPC-1, BxPC-3 and PE cells (Fig. 4). By contrast, arsenite significantly inhibited PDGF-BB-induced Akt phosphorylation in AsPC-1 cells (Fig. 4A), but did not influence PDGF-BB-induced Akt phosphorylation in BxPC-3 or PE cells (Fig. 4B and C, respectively). These results indicate a similar trend to that observed for cell migration, apoptosis and proliferation (Figs. 1-3).

PI3K, Akt and GSK3β inhibitors suppressed PDGF-BB-induced migration in AsPC-1 cells. Akt regulates multiple biological processes, including cell survival, proliferation and growth (24). In addition, GSK3β is a critical downstream element of the PI3K/Akt signaling pathway, and its activity can be inhibited by Akt-mediated phosphorylation of GSK3β (25,26). Since arsenite significantly suppressed PDGF-BB-induced Akt phosphorylation in AsPC-1 cells (Fig. 4A), several inhibitors of the Akt pathway were used to confirm that arsenite suppressed the migration of AsPC-1 cells by inhibiting the components of this pathway. When cells were pretreated with LY294002 (a PI3K inhibitor) or with Akt or GSK3β inhibitors, and then exposed to PDGF-BB, PDGF-BB-induced migration of AsPC-1 cells was significantly inhibited (Fig. 5), thus suggesting that arsenite suppressed PDGF-BB-induced migration of AsPC-1 cells by inhibiting the Akt signaling pathway.

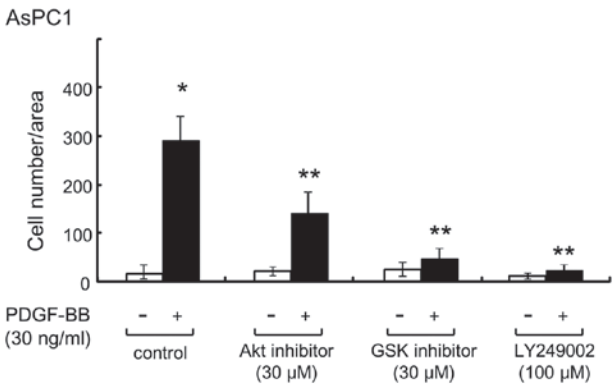

Figure 5. Effect of various inhibitors on PDGF-BB-induced migration in human pancreatic cancer AsPC-1 cells. AsPC-1 cells were pretreated with LY294002 (an inhibitor of phosphatidylinositol 3-kinase), Akt inhibitor or glycogen synthase kinase-3 beta inhibitor for 16 h, and then exposed to 30 ng/ml PDGF-BB for 36 h. Cells were then fixed, stained with clonogenic reagent and visualized under a microscope. The average number of migrated cells from five randomly selected fields on the lower surface of the membrane was counted. Data were obtained from ≥3 independent experiments. *P<0.05 increase vs. control; **P<0.05 decrease vs. control. PDGF, platelet-derived growth factor; GSK3β, glycogen synthase kinase-3 beta.

Discussion

The present study demonstrated that PDGF-BB, which is important in invasion and metastasis of various types of

human cancer (14,15), induced cell migration in AsPC-1 and BxPC-3 pancreatic cancer cells as well as normal PE cells. Notably, pretreatment with arsenite significantly inhibited PDGF-BB-induced migration of AsPC-1 cells but not of BxPC-3 or PE cells. In AsPC-1 cells, exposure to arsenite induced PARP cleavage and decreased the levels of cyclin D1 and phosphorylated Rb, indicating that arsenite induces apoptosis and cell cycle arrest in AsPC-1 cells. Since PDGF-BB induced phosphorylation of p44/p42 MAPK and Akt in these cells, the involvement of these kinases in PDGF-BB-induced cell migration was examined. The results indicated that arsenite suppressed PDGF-BB-induced phosphorylation of Akt, but not of p44/p42 MAPK in AsPC-1 cells. In addition, it was confirmed that the inhibition of the Akt signaling pathway suppressed the migration induced by PDGF-BB in these cells. Taken together, the present results strongly suggest that arsenite inhibits PDGF-BB-induced cell migration by suppressing the Akt signaling pathway in certain types of pancreatic cancer cells.

The present authors previously reported that Rho kinase, which is a downstream kinase of Rho, negatively regulates the migration of colon cancer cells (27). In that study, a Rho kinase inhibitor induced colon cancer cell migration by disrupting focal adhesion formation via the Akt pathway. In the present study, it was demonstrated that the PDGF-BB-induced cell migration mediated by Akt activation was inhibited by arsenite. Therefore, the effect of arsenite on focal adhesion-associated molecules should be investigated in future studies. Although further studies are required on the detailed mechanism of arsenite-induced suppression of the Akt signaling pathway, arsenite may be considered an attractive tool for the treatment of patients with certain types of pancreatic cancer without exerting adverse effects on normal pancreatic cells.

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