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ORIGINAL ARTICLE

Autocrine signaling via release of ATP and activation of P2X7 receptor influences motile activity of human lung cancer cells

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Abstract Extracellular nucleotides, such as ATP, are released from cells and play roles in various physiological and pathological processes through activation of P2 receptors. Here, we show that autocrine signaling through release of ATP and activation of P2X7 receptor influences migration of human lung cancer cells. Release of ATP was induced by stimulation with TGF-β1, which is a potent inducer of cell migration, in human lung cancer H292 cells, but not in noncancerous BEAS-2B cells. Treatment of H292 cells with a specific antagonist of P2X7 receptor resulted in suppression of TGF-β1-induced migration. PC-9 human lung cancer cells released a large amount of ATP under standard cell culture conditions, and P2X7 receptor-dependent dye uptake was observed even in the absence of exogenous ligand, suggesting constitutive activation of P2X7 receptor in this cell line. PC-9 cells showed high motile activity, which was inhibited by treatment with ecto-nucleotidase and P2X7 receptor antagonists, whereas a P2X7 receptor agonist enhanced migration. PC-9 cells also harbor a constitutively active mutation in epidermal growth factor receptor (EGFR). Treatment with EGFR tyrosine kinase inhibitor AG1478 suppressed both cell migration and P2X7 receptor expression in PC-9 cells. Compared to control PC-9 cells, cells treated with P2X7 antagonist exhibited broadened lamellipodia around the cell periphery, while AG1478-treated cells lacked lamellipodia. These results indicate that P2X7-mediated signaling and EGFR signaling may regulate migration of PC-9 cells through distinct mechanisms. We propose that autocrine ATP-P2X7 signaling is involved in migration of human lung cancer cells through regulation of actin cytoskeleton rearrangement.

Keywords P2X7 receptor · Lung cancer · Cell migration · TGF-β1 · EGFR

Introduction

Cancer cells migrate within tissues during invasion and metastasis, and therefore control of cancer cell migration is an important issue in tumor treatment. However, the signaling mechanisms underlying enhanced motility of cancer cells are not yet fully understood. Various intercellular signaling molecules, including cytokines and growth factors, are secreted into the microenvironment by tumor cells and are likely to play important roles in tumor progression, including metastasis. For example, transforming growth factor-β1 (TGF-β1) is secreted by many kinds of cancer cells and has been implicated in cancer progression, including angiogenesis, immune-escape, and metastasis [1–3]. TGF-β1 promotes cell migration by inducing a phenotype change called epithelial-mesenchymal transition (EMT) [4]. During EMT, immotile epithelial cells differentiate into highly motile, fibroblast-like cells.

Extracellular nucleotides, such as ATP, are released from cells in response to various stimuli [5, 6]. It has been reported that large amounts of ATP exist in tumor microenvironments [7]. Extracellular ATP activates plasma membrane purinergic P2 receptors. P2 receptors are classified into two subfamilies, ionotropic P2X1-7 receptors and metabotropic P2Y1-14 receptors, and their activation regulates many physiological

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functions [8]. P2 receptors are also expressed in many types of cancer [9]. Among these molecules, P2X7 receptor shows increased expression in many types of cancer cells [10–14]. Furthermore, activation of P2X7 receptor has been implicated in tumor growth, as well as tumor angiogenesis and metastasis of breast cancer [6, 10–12, 15, 16]. Recently, we reported that TGF-β1 treatment induces ATP release from human lung cancer A549 cells, and we also showed that autocrine signaling through P2X7 receptor is required for the amplification of TGF-β1-induced cell migration [17]. However, the relationship between ATP-releasing ability, expression levels of P2 receptors, and motility of lung cancer cells remains unclear. In particular, it is not known whether the contribution of ATP signaling to motility of lung cancer cells depends upon the status of oncogenic gene mutation, such as constitutively active mutation of epidermal growth factor receptor (EGFR).

In the present study, we investigated whether autocrine ATP-P2X7 signaling is involved in motility of human lung cancer cells using BEAS-2B normal human bronchial epithelial cells and human lung cancer H292 and PC-9 cells. Our results indicate that ATP release and P2X7 receptor activation are involved in TGF-β1-induced migration of H292 cells and also that constitutive release of ATP and activation of P2X7 receptor contribute to the motile phenotype of PC-9 cells.

Materials and methods

Reagents and antibodies

DMEM, RPMI-1640, human recombinant TGF-β1, and SB431542 were purchased from Wako Pure Chemical (Osaka, Japan). FBS was purchased from Biowest (Nuaillé, France). Apyrase, BzATP, and ethidium bromide (EtBr) were from Sigma-Aldrich (St Louis, MO, USA). A438079 and AZ10606120 were from Tocris Bioscience (Ellisville, MO, USA). Gefitinib was purchased from LC Laboratories (Woburn, MA, USA). Rhodamine phalloidin was from Cytoskeleton, Inc. (Denver, CO, USA). Anti-P2X7 extracellular antibody was from Alomone Labs (Jerusalem, Israel). Anti-actin antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture

BEAS-2B normal human bronchial epithelial cell line and NCI-H292 human lung mucoepidermoid carcinoma cell line were purchased from DS Pharma Biomedical (Osaka, Japan). A549 human adenocarcinoma cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). PC-9 human lung adenocarcinoma cell line was from RIKEN BioResource Center (Ibaraki, Japan). BEAS-2B cells and A549 cells were grown in DMEM supplemented with 10 % fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 mg/mL). PC-9 cells and H292 cells were grown in RPMI-1640 supplemented with 10 % fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 mg/mL). Cells were grown in a humidified atmosphere of 5 % CO_2 in air at 37 °C.

Immunoblotting

Cells were lysed in PBS containing 10 mM HEPES-NaOH, pH 7.4, 1 % Triton X-100, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1.04 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A, and 14 μM E-64, at 4 °C for 30 min. Lysates were centrifuged at 10,000×*g* for 15 min. After removal of cellular debris, the protein content in each sample was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein lysate were dissolved in 2×sample buffer (50 % glycerin, 2 % SDS, 125 mM Tris, 10 mM DTT) and boiled for 10 min. Aliquots of samples containing 4 μg of protein were analyzed by means of 10 % SDS-PAGE and bands were transferred onto a PVDF membrane. The blots were blocked overnight in TBST with 1 % BSA, 5 % skim milk at 4 °C and then incubated with rabbit P2X7 extracellular antibody (1:1000) for 90 min at room temperature or mouse actin antibody (1:1000) overnight at 4 °C. After having been washed with TBST, blots were incubated with goat HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Inc.) or goat anti-mouse IgG antibody-HRP (Santa Cruz Biotechnology) for 90 min at room temperature. The blots were again washed with TBST, and specific proteins were visualized with ECL Western blotting detection reagents (GE Healthcare) according to the manufacturer's instructions.

Determination of extracellular ATP concentration

Release of ATP was quantified by using the luciferin-luciferase-based Enlighten ATP assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells were grown to 100 % confluence in a 48-well plate. The culture medium was replaced with RPMI1640-based buffer containing 102 mM NaCl, 5 mM KCl, 0.4 mM $CaCl_2$, 0.4 mM $MgSO_4$, 23.8 mM $NaHCO_3$, 5.6 mM Na_2HPO_4, 11.1 mM glucose, and 10 mM HEPES-NaOH (pH 7.4). For detection of TGF-β1-induced ATP release, the cells were stimulated with 5 ng/mL of TGF-β1, and cell-conditioned buffer was obtained at 1 to 30 min after stimulation. Luciferin-luciferase reagent (100 μL) was injected into 10 μL of conditioned buffer, and the chemiluminescence was measured with a WALLAC ARVO SX multilabel counter (PerkinElmer, Inc., Waltham, MA, USA). ATP concentration

in each sample was determined by comparing the luminescence of samples with those of standards in the concentration range of 10^{-8} to 10^{-10} M.

Cell migration assay

Cell migration was analyzed by using a 24-well Transwell plate (6.5-mm diameter; 8 μm pore size polycarbonate membrane, Corning, Lowell, MA, USA). The upper compartment was seeded with 2×10^4 cells in basal culture medium and incubated for 24 h. Then, the medium was replaced with fresh medium containing TGF-β1 or other drugs. The upper chamber contained 5 % FBS instead of 10 %. After incubation for a further 24 h, cells were fixed with 4 % paraformaldehyde for 10 min at room temperature and incubated with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) and 50 μg/mL of propidium iodide (PI) for 30 min at room temperature. Nonmigrated cells on the upper surface of the membrane were removed and cells that had migrated through the membrane to the lower surface were counted by using a fluorescence microscope (BZ-9000; KEYENCE). To examine migration of PC-9 cells, migrated cells on the plate well that had become detached from the membrane were also photographed and counted.

Fluorescence imaging

For fluorescence imaging, cells were grown in glass-based 35 mm dishes (IWAKI, Tokyo, Japan). EtBr uptake assay was performed in the complete culture medium. EtBr (25 μM) was added to the culture medium, and the cells were photographed at the indicated times using a confocal laser scanning microscope (FV10i-LIV; Olympus, Tokyo, Japan) equipped with a UPLSAPO 60×1.2 NA water objective lens. Images were obtained from different fields at each time point in order to avoid cytotoxicity due to the laser beam (3–6 random fields/sample). Cells were held in an incubation chamber at 37 °C in a humidified atmosphere of 5 % CO_2 in air during the assay. For F-actin staining, cells were fixed with 4 % paraformaldehyde for 10 min at room temperature and permeabilized with 0.5 % Triton X-100 for 5 min on ice. Fixed cells were incubated with 100 nM rhodamine phalloidin for 30 min at room temperature. Stained cells were also analyzed using a confocal laser scanning microscope FV10i-LIV.

Detection of lactate dehydrogenase (LDH) release

Cells were grown in a 96-well plate. EtBr (25 μM) was added to the culture medium and incubated for 30 min at 37 °C in a humidified atmosphere of 5 % CO_2. Then supernatants were collected, and the LDH content was measured by using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany). LDH release is expressed as percentage of total content, which was determined by lysing an equal amount of cells with 1 % Triton X-100.

Statistics

Results are expressed as mean±SE. The statistical significance of differences between control and other groups was calculated by using Dunnett's test with the InStat version 3.0 statistical package (GraphPad Software, San Diego, CA, USA). The criterion of significance was set at $P<0.05$.

Results

Activation of P2X7 receptor is involved in TGF-β1-induced migration of H292 lung cancer cells, but not normal lung cells

It is well known that TGF-β1 induces migration of many kinds of cancer cells, as well as normal epithelial cells. We have previously reported that human lung cancer A549 cells release ATP in response to TGF-β1 stimulation and that activation of P2X7 receptor by extracellular ATP is involved in TGF-β1-induced migration of these cells [17].

We first compared the expression of P2X7 receptor in BEAS-2B normal human bronchial epithelial cell line and three human lung cancer cell lines (A549, PC-9, H292) by means of immunoblotting. BEAS-2B cell line is derived from epithelial cells, which were isolated from normal human bronchial epithelium and infected with and adenovirus 12-SV40 virus hybrid in order to increase culture longevity [18]. We used BEAS-2B cells as noncancerous control because this cell line is nontumorigenic and retains features of human bronchial epithelial cells [18, 19]. As shown in Fig. 1a, P2X7 receptor was not expressed in noncancerous BEAS-2B cells, though it was expressed in cancer cells. It is noteworthy that P2X7 receptor was abundantly expressed in PC-9 cells compared to A549 or H292 cells. To investigate whether autocrine ATP signaling participates in cell migration, we next examined ATP release and cell migration using these cell lines. To evaluate release of ATP from cells, we measured the concentration of ATP in culture medium by using a luciferin-luciferase reaction-based assay. In BEAS-2B cells, the concentration of extracellular ATP was as low as approximately 1.5 nM and was not changed by treatment with 5 ng/mL of TGF-β1 (Fig. 1b). On the other hand, in H292 cells, the extracellular ATP concentration was increased to 10 nM after stimulation with TGF-β1 (Fig. 1c). Extracellular ATP released from cells is rapidly metabolized by ecto-nucleotidases on the plasma membrane. When degradation of extracellular ATP was inhibited by ecto-nucleotidase inhibitor ARL67156, the extracellular ATP concentration was increased after TGF-β1 stimulation in a time-dependent manner and reached to 26 nM at 30 min (Online Resource Fig. S1). These results suggest

that H292 lung cancer cells release ATP in response to TGF-β1 stimulation similarly to A549 cells, but noncancerous BEAS-2B cells do not.

Next, we examined the cell migration by means of Transwell assay. As shown in Fig. 1d, treatment with TGF-β1 (5 ng/mL) increased the number of migrated BEAS-2B cells at 24 h. The TGF-β1-induced migration of BEAS-2B cells was not affected by treatment with A438079 (P2X7 antagonist). This may reflect the lack of P2X7 receptor expression and TGF-β1-induced ATP release in BEAS-2B cells. In the case of H292 cells, however, treatment with A438079 or apyrase (ecto-nucleotidase) significantly reduced the TGF-β1-induced cell migration, suggesting that activation of P2X7 receptor by extracellular ATP, which is released in response to TGF-β1 stimulation (Fig. 1c), is involved in TGF-β1-induced migration of H292 cells (Fig. 1e). Basal migration of H292 cells was not affected by treatment with apyrase or A438079 (Fig. 1e). Based on our previous findings on A549 cells and the results here with BEAS-2B and H292 cells, it appears that release of ATP and activation of P2X7 receptor are involved in TGF-β1-induced cell migration of lung cancer cells, but not normal lung cells.

Constitutive activation of P2X7 receptor in PC-9 human lung cancer cells

We also examined ATP release and TGF-β1-induced cell migration using PC-9 human lung cancer cells. Interestingly, we detected a relatively high concentration of ATP in the culture medium of PC-9 cells (Fig. 2a). The extracellular concentration of ATP was approximately 10 nM in the absence of TGF-β1, and stimulation with 5 ng/mL of TGF-β1 did not further increase the extracellular ATP level. To examine whether PC-9 cells constitutively release ATP without TGF-β1 stimulation, we tested the effect of ecto-nucleotidase inhibitor ARL67156 on the concentration of ATP in culture medium. Treatment with ARL67156 for 2 h resulted in an increase of extracellular ATP concentration (Fig. 2b). This result indicates that PC-9 cells release a substantial amount of ATP in the absence of exogenous TGF-β1. However, treatment with TGF-β receptor type I (TβRI) inhibitor SB431542 had no effect on the ATP release, suggesting that the constitutive release of ATP is not mediated by autocrine activation of TGF-β1 receptor in PC-9 cells. PC-9 cell line is known to have a constitutively active mutation of the EGFR gene (exon 19 flame deletion) [20]. Therefore, we also tested the effect of AG1478, which is a tyrosine kinase inhibitor selective for EGFR, on ATP release from PC-9 cells. Contrary to our expectation, AG1478 increased the concentration of ATP in culture medium (Fig. 2b), indicating that activation of EGFR does not contribute to constitutive release of ATP. These data suggest that PC-9 cells constitutively release ATP through a mechanism independent of activation of TGF-β receptor or EGFR.

Considering the abundant expression of P2X7 receptor and constitutive release of ATP, P2X7 receptor could be activated in the absence of exogenous ATP in PC-9 cells. It is known that the pore associated with P2X7 receptor activation is permeable to molecules of up to 900 Da in size [21]. To investigate whether P2X7 receptor is constitutively activated in PC-9 cells, we next measured the rate of EtBr uptake in the absence of exogenous ligand. As shown in Fig. 2c–e, fluorescence was detectable in control cells by 15 min after addition of EtBr to the medium, and the fluorescence intensity increased up to 30 min. In cells pretreated with apyrase or A438079 for 30 min, uptake of EtBr was significantly reduced (Fig. 2e). Co-treatment with apyrase and A438079 also reduced EtBr uptake (Fig. 2c–e). We examined cytotoxicity by detecting lactate dehydrogenase (LDH) in the culture medium and confirmed that cell death was not induced in the experimental condition in the EtBr uptake assay (Fig. 2f). These results suggest that the P2X7 receptor signaling pathway is activated in PC-9 cells even under standard cell culture conditions due to constitutive release of ATP and abundant expression of P2X7 receptor.

Extracellular ATP and activation of P2X7 receptor influence motile activity of PC-9 cells

We next examined migration of PC-9 cells by means of Transwell assay. As shown in Fig. 3a, PC-9 cells actively migrate even in the absence of TGF-β1 stimulation, as compared to other human lung cancer cell lines. The number of migrated cells was not further increased by stimulation with 5 ng/mL of TGF-β1 (Fig. 3a). The lack of effect of TGF-β1 on cell migration is consistent with the result of ATP release assay (Fig. 2a). To investigate the contribution of P2X7 receptor-mediated signaling to the motile activity of PC-9 cells, we examined the effect of specific inhibitors and a P2X7 receptor agonist. Treatment with apyrase, A438079, and AZ10606120 (P2X7 antagonists) significantly decreased migration of PC-9 cells (Fig. 3b). We also found that cell migration was significantly accelerated by 3′–O–(4-benzoyl)benzoyladenosine 5′-triphosphate (BzATP, P2X7 agonist) (Fig. 3c). Progression of cell migration by BzATP was completely inhibited by treatment with A438079 (Fig. 3c). Furthermore, treatment with ARL67156 increased cell migration, supporting contribution of constitutive release of ATP to motile activity of PC-9 cells (Fig. 3d). These data suggest that motile activity of PC-9 cells is, at least in part, mediated by constitutive release of ATP and activation of P2X7 receptor.

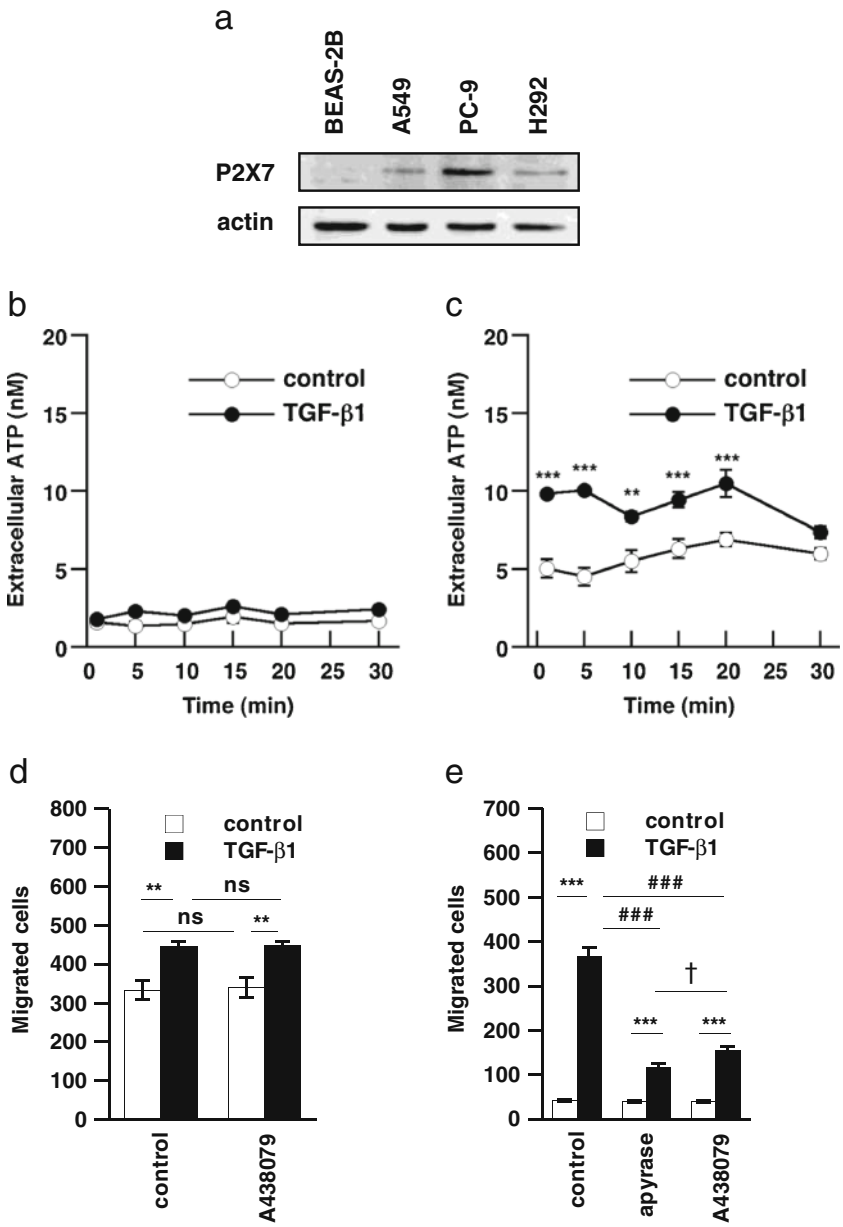
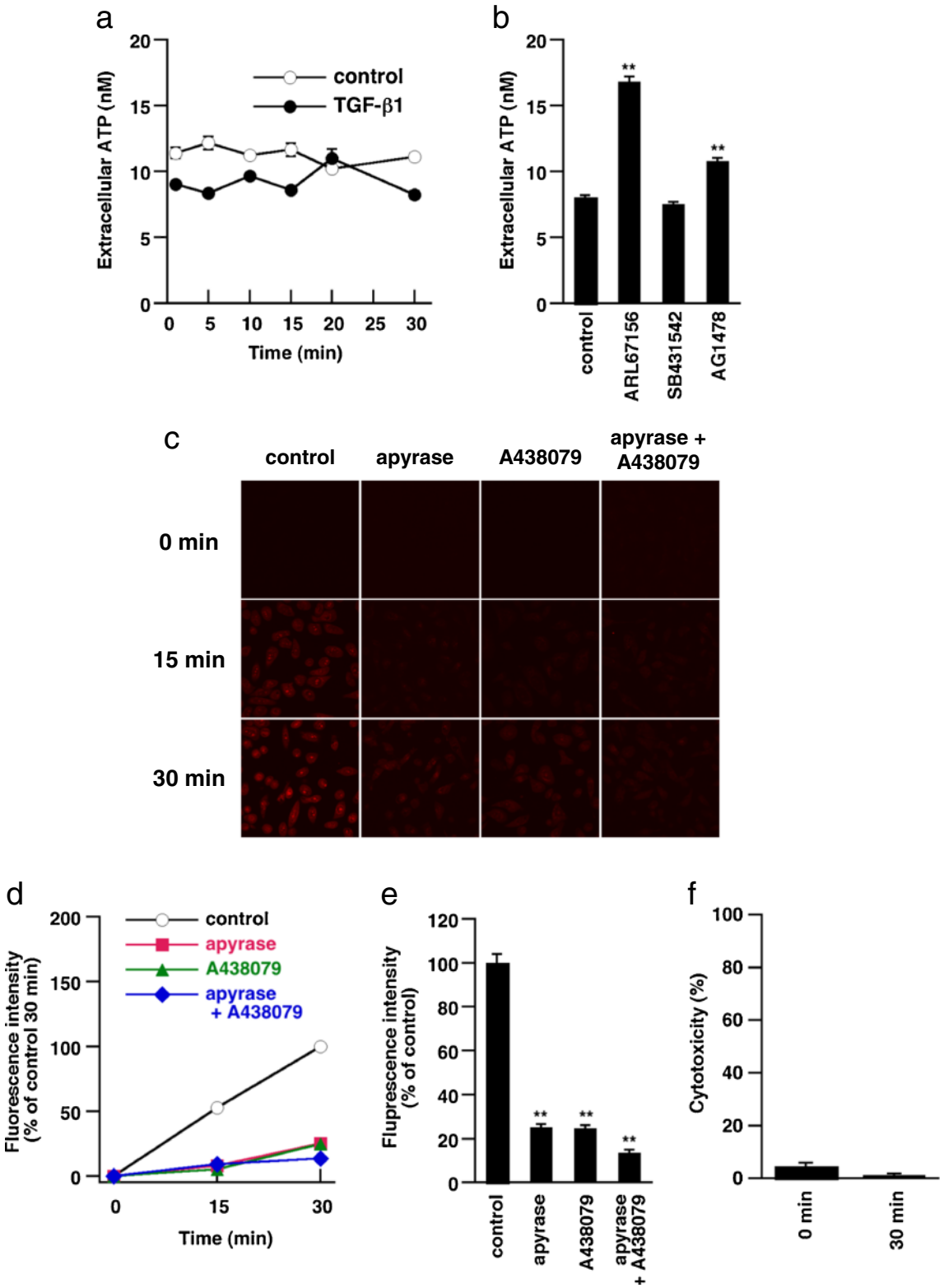


Fig. 1 TGF-β1-induced migration is mediated by release of ATP and activation of P2X7 receptor in H292 lung cancer cells, but not BEAS-2B normal lung cells. **a** The expression of P2X7 receptor in BEAS-2B, A549, PC-9, and H292 cells was detected by immunoblotting as described in "Materials and methods". **b**, **c** BEAS-2B cells (**b**) or H292 cells (**c**) were stimulated with TGF-β1 (5 ng/mL) and incubated for the indicated times, then the concentration of ATP in the culture medium was measured as described in "Materials and methods". **d**, **e** Cell migration was examined by means of Transwell assay as described in "Materials and methods". BEAS-2B cells (**d**) or H292 cells (**e**) were pretreated for 30 min with apyrase (20 U/mL) or A438079 (100 μM) and then stimulated for 24 h with TGF-β1 (5 ng/mL). The lower membrane surfaces were photographed through a microscope at ×20 magnification, and migrated cells in each field were counted. Values are means± SE (n=4–10). A significant difference between the indicated group and control group is indicated by *** or ### (P<0.001), ** (P<0.01), †(P<0.05)

Motile phenotype of PC-9 cells is dependent on constitutive activation of EGFR signaling pathway and also on a P2X7-mediated pathway

As mentioned above, it has been reported that PC-9 cells harbor mutation in EGFR, and their EGFR tyrosine kinase is active even in the absence of exogenously added ligand. We found that migration of PC-9 cells was strongly suppressed by treatment with EGFR tyrosine kinase inhibitor AG1478 or gefitinib (Fig. 4a). This result may indicate that constitutive activation of EGFR is also involved in motile activity of PC-9 cells. Furthermore, expression of P2X7 receptor was reduced by AG1478 or gefitinib, suggesting that the expression of P2X7 receptor is, in part, mediated by activation of EGFR pathway (Fig. 4b). As shown in Fig. 4c, the suppressed migration of AG1478-treated cells was only slightly enhanced by stimulation with BzATP, supporting the idea that the motile phenotype of PC-9 cells might be predominantly mediated by the EGFR signaling pathway.

The actin cytoskeleton plays a crucial role in cellular movement and is strictly regulated during migration. To investigate the mechanisms underlying the suppressive effects of blockade of EGFR and P2X7 receptor on migration of PC-9 cells, we also investigated the effect of blockade of EGFR and P2X7 receptor on actin skeleton arrangement. As shown in Fig. 4d, control PC-9 cells had actin-rich lamellipodial protrusions at both ends of their long axis (left panels, arrowhead). In contrast, AG1478-treated cells completely lacked



lamellipodia around the cell periphery (Fig. 4d, middle panels). Instead, these cells extended filopodia along their edges (arrow) and showed increased cell-cell adhesion. Cells treated with A438079 showed broad lamellipodia that were often spread around most of the cell periphery (Fig. 4d, right panels, arrowhead). These observations suggest that the EGFR signaling pathway is required for lamellipodia formation, whereas a P2X7-dependent pathway might be involved

◀ **Fig. 2** Constitutive activation of P2X7 receptor in PC-9 cells. **a** PC-9 cells were incubated with or without TGF-β1 (5 ng/mL) for the indicated times, and the concentration of ATP in the culture medium was measured. **b** PC-9 cells were treated with ARL67156 (50 μM), SB431542 (10 μM), or AG1478 (10 μM) for 2 h. Then, each supernatant was collected and the ATP concentration was measured. Each value represents the mean±SE (n=4). Significant differences between the indicated groups and control group are indicated by ** (P<0.01). **c** EtBr uptake assay was performed as described in "Materials and methods". PC-9 cells were pretreated for 30 min with apyrase (20 U/mL) or A438079 (100 μM) and incubated with EtBr for the indicated times. Fluorescence of EtBr (*red*) was detected using a confocal laser scanning microscope at ×60 magnification. **d**, **e** Fluorescence intensity of each cell was analyzed using the ImageJ image processing program and expressed as relative to those of control cells at 30 min. Each value represents the mean±SE (n=25–38). **e** Significant differences between the indicated groups and control group at 30 min are indicated by **(P<0.01). **f** Thirty minutes after incubated with EtBr, the content of LDH in the culture medium was measured as described in "Materials and methods". Values are means±SE (n=4)

in restricting lamellipodial broadening and polarization of protrusive activity in cell migration.

Discussion

In this study, we investigated the involvement of ATP signaling in motility of human lung cancer cells, focusing on the role of the P2X7 receptor. Our results indicate that autocrine ATP-P2X7 signaling plays a significant role in TGF-β1-dependent and TGF-β1-independent cell migrations.

First, we found that P2X7 receptor was expressed in A549, PC-9, and H292 lung cancer cell lines, whereas it was not expressed in BEAS-2B normal human bronchial epithelial cells. Functional expression of P2X7 has already been reported in various cancer cells. Considering that BEAS-2B cells did not express P2X7 receptor, it is possible that expression of P2X7 receptor is upregulated during malignant transformation of lung epithelial cells. Whereas it has also been reported that expression of P2X7 receptor is decreased in several epithelial cancer tissues, including cervical cancer, uterine cancer, and bladder cancer [22], the expression pattern of P2X7 might differ with organs and types of cancer.

We found that H292 cells released ATP in response to TGF-β1 stimulation, whereas BEAS-2B did not. Furthermore, we also found that TGF-β1-induced migration of H292 cells was suppressed by blockade of P2X7 receptor. Considering that blockade of P2X7 did not affect migration of H292 cells in the absence of TGF-β1, basal level of extracellular ATP might not contribute to basal migration of H292 cells. These results suggest that autocrine ATP-P2X7 signaling is involved in TGF-β1-induced migration of H292 cells, but not BEAS-2B cells. However, TGF-β1-induced cell migration was not completely inhibited by apyrase, suggesting that ATP-independent mechanisms might also contribute to TGF-β1-induced migration in H292 cells. In addition, considering that apyrase exerted slightly stronger inhibitory effect on TGF-β1-induced migration than A438079, it is possible that other P2 receptor, other than P2X7, could be partly involved in TGF-β1-induced ATP-dependent migration of H292 cells. For example, it has been reported that activation of P2Y2 receptor also increases cell motility in breast cancer cells [23]. Taken together with our previous report of involvement of ATP-P2X7 signaling in TGF-β1-induced cell migration of A549 cells [17], it is possible that involvement of autocrine signaling mediated by release of ATP and activation of P2X7 receptor is a distinctive feature of cancer cell migration. Induction of migration of epithelial cells by TGF-β1 is essential for physiological processes such as wound healing [4]. We speculate that blockade of P2X7 receptor might suppress cancer cell migration without affecting physiological migration of normal epithelial cells, although further work will be needed to confirm this idea.

Next, we assayed extracellular release of ATP by PC-9 human lung cancer cells, which abundantly expressed P2X7 receptor and found that these cells constitutively release ATP in the absence of TGF-β1 stimulation. We confirmed P2X7-mediated pore formation by means of EtBr uptake assay, confirming that P2X7 receptor is activated in PC-9 cells even in the absence of exogenous ligand. This EtBr uptake was not accompanied by cell death. In addition, we also found that treatment with P2X7 antagonists for 48 h resulted in suppression of proliferation of PC-9 cells (Online Resource Fig. S2). These results suggest that constitutive activation of P2X7 receptor does not induce cell death, but rather contribute to cell proliferation despite permeabilization. Although the detected levels of extracellular ATP in our experimental method were lower than the concentration generally needed to activate P2X7, ATP released from cells is diluted in the culture medium and is rapidly metabolized by ecto-nucleotidases. Therefore, the concentration of ATP on cell surface might high enough to activate P2X7 receptor.

Furthermore, we found that PC-9 cells actively migrate even in the absence of exogenous TGF-β1. In contrast to A549 or H292 cells, stimulation of PC-9 cells with TGF-β1 did not increase their motility. Although PC-9 cells were unresponsive to TGF-β1 in terms of both ATP release and cell migration, we confirmed that TGF-β1 induces expression of fibronectin or collagen I in PC-9 cells (Online Resource Fig. S3). These observations may indicate that the lack of effect of TGF-β1 on ATP release and cell migration is not due to a defect of the TGF-β receptor itself. It is known that activation of TGF-β receptors leads to activation of downstream signaling pathways, including the canonical Smad-dependent pathway and noncanonical Smad-independent pathways, such as MAPK-dependent pathway. It has been reported that TGF-β1-induced expression of fibronectin is mediated by a c-Jun N-terminal kinase-dependent, Smad4-independent pathway [24]. Since expression of Smad2/3 in

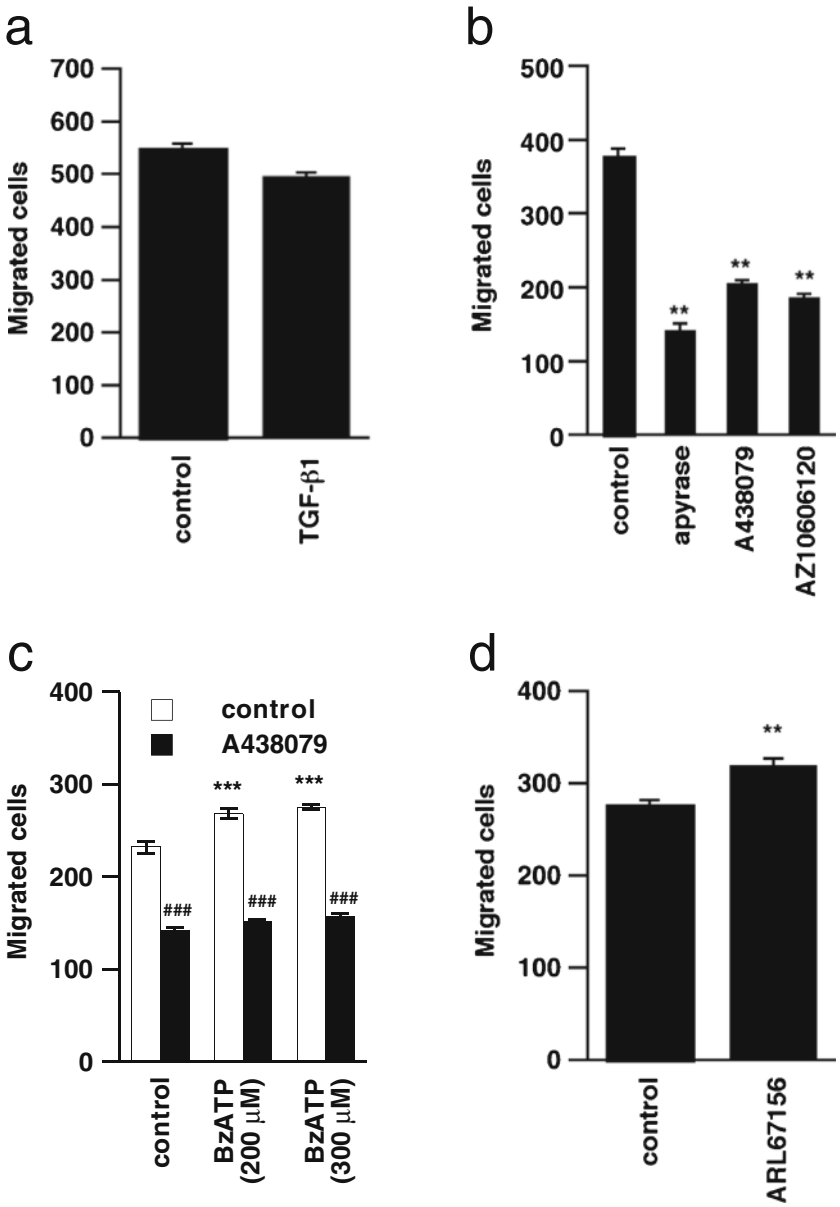


Fig. 3 Involvement of P2X7 receptor in motile activity of PC-9 cells. **a** Cell migration was examined using Transwell systems as described in "Materials and methods". PC-9 cells were incubated with or without TGF-β1 (5 ng/mL) for 24 h. **b** Cells were treated with apyrase (20 U/mL), A438079 (100 μM) or AZ10606120 (10 μM) for 24 h. Then, cell migration was examined. **c** Cells were pretreated for 30 min with A438079 (100 μM), and then stimulated for 24 h with BzATP (200–300 μM). **d** Cells were incubated with ARL67156 (50 μM) for 24 h and cell migration was examined. Each value represents the mean±SE (n=8–18). Significant differences between the indicated groups and control group are indicated by *** or ### (P<0.001), ** (P<0.01)

PC-9 cells is low [25], the Smad-dependent canonical TGF-β1 signaling pathway might be aberrant in PC-9 cells. We also found that the motile activity of PC-9 cells was suppressed by treatment with ecto-nucleotidase or P2X7 antagonists, suggesting that constitutive activation of P2X7 receptor is involved in the motility of these cells. Our results indicate that constitutive release of substantial amounts of ATP and autocrine activation of P2X7 receptor contribute to basal migration of PC-9 cells, though P2X7 receptor is not involved in basal migration in H292 cells, which had low basal level of extracellular ATP. Involvement of P2X7 receptor is supported by acceleration of migration by BzATP, which is completely abolished in the presence of A438079. PC-9 cells are known to harbor active mutation in EGFR. Thus, our results suggest that constitutive activation of EGFR also contributes to basal migration in PC-9 cells. It has also been reported that EGFR activation by EGF resulted in morphological change and increase of cell migration in A549 and HK2 lung cancer cell lines [26]. Our results also indicate that the expression of P2X7 receptor in PC-9 cells is mediated by activation of the EGFR pathway. Thus, activation of EGFR signaling could be implicated in motility of lung cancer cells.

The results of Transwell assay and microscopic analysis suggest that activation of EGFR and P2X7 receptor contribute to migration of PC-9 cells in distinct ways. Cell migration is a dynamic process, including changes in the actin cytoskeleton and cell adhesion. The Rho GTPases play a pivotal role in regulating the actin cytoskeleton. Among the Rho GTPase family protein, RhoA, Rac1, and Cdc42 have been most widely studied in connection with cell migration and have

been implicated in formation of stress fibers, lamellipodia, and filopodia, respectively [27, 28]. In addition, it has been shown that Rac1 and RhoA exhibit mutual antagonism in migrating cells [29, 30]. In this study, PC-9 cells showed actin-rich lamellipodia at both ends of their long axis. This suggests enhanced activation of Rac1, probably because of activation of the EGFR-Ras axis by mutation of EGFR [31]. In fact, inhibition of EGFR resulted in disappearance of lamellipodia and decrease of motile activity, while formation of filopodia was observed. This might be because Cdc42-induced filopodia are more apparent after Rac inhibition [27]. On the contrary, PC-9 cells treated with P2X7 antagonist exhibited broad lamellipodia extending in multiple directions. This suggests that P2X7-dependent signaling contributes to restricting lamellipodia broadening. It is known that activation of P2X7 receptor causes activation of Rho kinase [32, 33]. Therefore, blockade of P2X7 receptor would suppress activation of the Rho pathway and result in aberrant activation of Rac1.

Since release of ATP and activation of P2X7 receptor are involved in motile activity of human lung cancer cells, inhibition of P2X7 receptor might be an effective strategy to prevent metastasis. The mutation status of EGFR is an important determinant of treatment choice for non-small cell lung cancer. In our study, inhibition of P2X7 receptor suppressed both TGF-β1-induced migration of H292 cells (wild-type

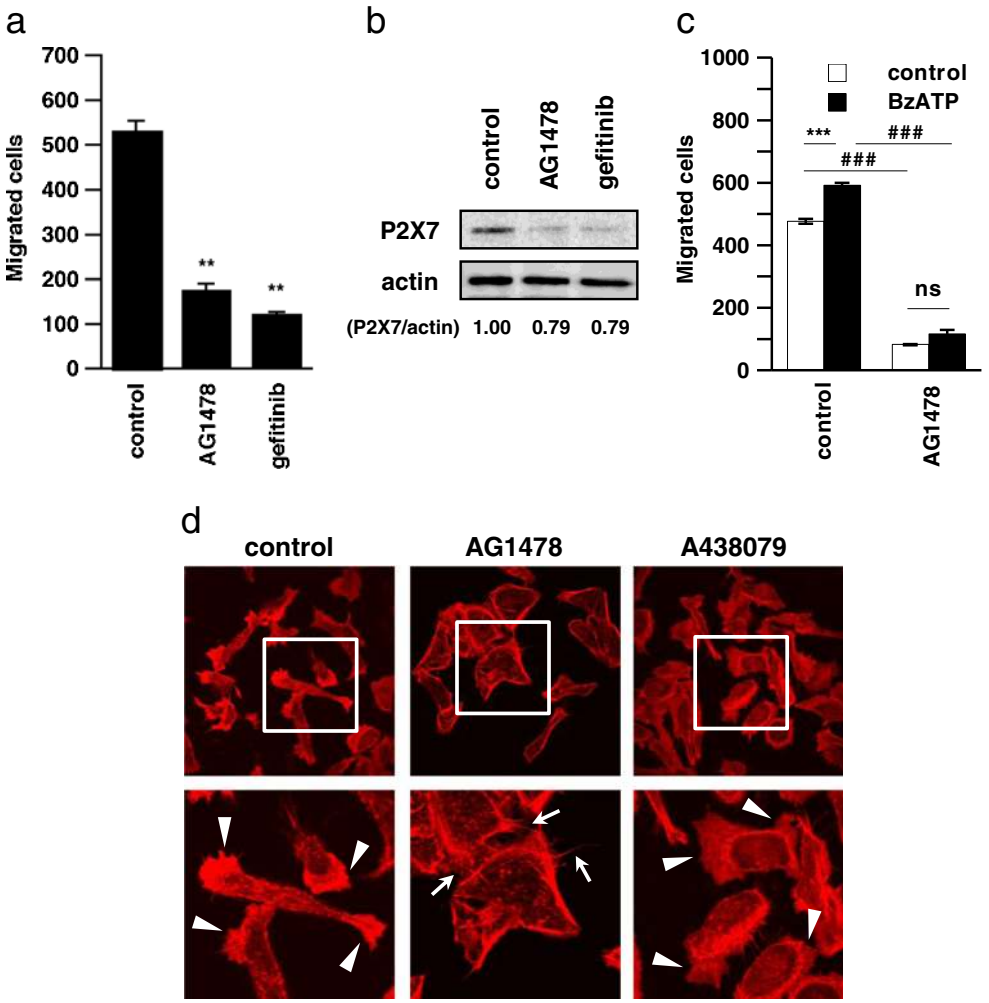


Fig. 4 EGFR signaling and P2X7 receptor signaling are both involved in migration of PC-9 cells, regulating different aspects. **a** Cell migration was examined using Transwell systems. PC-9 cells were pretreated with AG1478 (10 μM) or gefitinib (0.1 μM) for 24 h and allowed to migrate for another 24 h. **b** PC-9 cells were treated with AG1478 (10 μM) or gefitinib (0.1 μM) for 24 h and the expression of P2X7 receptor was detected by immunoblotting. The band intensities were quantified by densitometry and expressed as relative to those of each control. **c** Cells were incubated with or without AG1478 (10 μM) for 24 h and then stimulated with BzATP (300 μM) for another 24 h in a serum concentration gradient. Each value represents the mean±SE (n=8–10). Significant differences between the indicated groups and control group are indicated by *** or ### (P<0.001). **d** PC-9 cells were incubated with AG1478 (10 μM) or A438079 (100 μM) for 24 h. Then, F-actin was stained using rhodamine phalloidin (*red*), and stained cells were analyzed using a confocal laser scanning microscope at ×60 magnification. Boxed regions are shown at higher magnification below. *Arrowheads* indicate lamellipodia. *Arrows* indicate filopodia

EGFR) and migration of highly motile PC-9 cells (EGFR mutant). Therefore, therapy based on inhibition of P2X7 receptor could be applicable to a broad range of lung cancer patients, regardless of the mutation status of EGFR. Furthermore, because activation of P2X7 receptor has been implicated in tumor malignancy, such as rapid growth and angiogenesis, P2X7 receptor-targeting therapy might be expected to have a potent antitumor effect.

ATP is released from cells through various mechanisms, including exocytosis [34], maxi-anion channels [35], volume-sensitive outwardly rectifying chloride channels [36], and gap-junction hemichannels [37]. Although the mechanism of release of ATP from cancer cells has not been clearly established, it is possible that the ability to release ATP is correlated with malignancy or motility of cancer cells. We think it would be worthwhile to further investigate ATP-releasing ability and its mechanism in a wide range of cancer cells and normal cells.

In conclusion, we have shown here that both inducible and constitutive release of ATP and activation of P2X7 receptor are implicated in migration of H292 and PC-9 human lung cancer cells. In EGFR-mutant PC-9 cells, which have a highly motile phenotype, EGFR-mediated expression of P2X7 receptor and autocrine activation of P2X7 receptor by constitutive release of ATP appear to be involved in motile activity through a distinct mechanism from that of EGFR signaling. Our findings suggest that P2X7 receptor antagonists may be candidates for lung cancer therapy to inhibit metastasis.

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