Autocrine regulation of TGF-β1-induced cell migration by exocytosis of ATP and activation of P2 receptors in human lung cancer cells

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Summary

TGF-β1 plays a key role in cancer progression through induction of various biological effects, including cell migration. Extracellular nucleotides, such as ATP, released from cells play a role in signaling through activation of P2 receptors. We show here that exocytosis of ATP followed by activation of P2 receptors play a key role in TGF-β1-induced actin remodeling associated with cell migration. Treatment with TGF-β1 facilitated migration of human lung cancer A549 cells, which was blocked by pretreatment with ecto-nucleotidase and P2 receptor antagonists. ATP and P2 agonists facilitated cell migration. TGF-β1-induced actin remodeling, which contributes to cell migration, was also suppressed by pretreatment with ecto-nucleotidase and P2 receptor antagonists. Knockdown of P2X7 receptor suppressed TGF-β1-induced migration and actin remodeling. These results indicate the involvement of TGF-β1-induced ATP release in cell migration, at least in part, through activation of P2X7 receptors. TGF-β1 caused release of ATP from A549 cells within 10 minutes. Both ATP-enriched vesicles and expression of a vesicular nucleotide transporter (VNUT) SLC17A9, which is responsible for exocytosis of ATP, were found in cytosol of A549 cells. TGF-β1 failed to induce release of ATP from SLC17A9-knockdown cells. TGF-β1-induced cell migration and actin remodeling were also decreased in SLC17A9-knockdown cells. These results suggest the importance of exocytosis of ATP in cell migration. We conclude that autocrine signaling through exocytosis of ATP and activation of P2 receptors is required for the amplification of TGF-β1-induced migration of lung cancer cells.

Key words: ATP, P2 receptor, Autocrine signaling, Exocytosis, TGF-β1, Cell migration

Introduction

Many cytokines and growth factors are secreted into tumor microenvironments. One of them, transforming growth factor-β1 (TGF-β1), is secreted in large amounts from many cancer cells and acts as a tumor promoter by inducing tumor angiogenesis, immune-escape and metastasis (Go et al., 1999; Mumm and Oft, 2008; Siegel et al., 2003). In several cancers, including lung cancer, high expression of TGF-β1 correlates with cancer progression and clinical prognosis (Hasegawa et al., 2001). In contrast, TGF-β1 can also act as a tumor suppressor by inhibiting proliferation and promoting apoptosis in normal epithelial cells (Rahimi and Leof, 2007). Recently, several reports have suggested that alteration of the TGF-β1 signaling pathway is implicated in the function of TGF-β1 as a tumor promoter (Miyaki et al., 1999; Gal et al., 2008; Neil et al., 2008). However, the signaling mechanisms underlying TGF-β1-induced cancer progression are not yet fully understood. In particular, it has not been established whether autocrine signaling is involved in TGF-β1-induced biological events, such as actin remodeling and migration of cancer cells.

Extracellular nucleotides, such as ATP, are released from cells in response to various stimuli through maxi-anion channels (Sabirov et al., 2001), volume-sensitive outwardly rectifying chloride channels (Hisadome et al., 2002), gap junction hemichannels (Stout et al., 2002) or exocytosis (Pangrsic et al., 2007). Recently, SLC17A9 has been identified as a vesicular nucleotide transporter (VNUT) that plays a key role in vesicular storage of ATP (Sawada et al., 2008). SLC17A9 mediates the active accumulation of nucleotides, driven by an electrochemical gradient of protons across the membrane generated by vacuolar proton-ATPase. SLC17A9 is responsible for exocytosis of ATP, in the form of ATP-containing vesicles, and is expressed in PC12 cells, type II taste cells, biliary epithelial cells and T lymphocytes (Sawada et al., 2008; Iwatsuki et al., 2009; Sathe et al., 2011; Tokunaga et al., 2010). However, Expression of SLC17A9 and vesicular exocytosis of ATP have not been reported in cancer cells.

Increased extracellular ATP is thought to activate plasma membrane purinergic P2 receptors, which are classified into two subfamilies; the ionotropic P2X1–7 receptors and the metabotropic P2Y1–14 receptors (Burnstock, 2009). Activation of P2 receptors is involved in various biological processes, such as neurotransmission, proliferation and immune response (Burnstock, 2006; Tsukimoto et al., 2007). Recently, it has

been reported that large amounts of ATP exist in tumor microenvironments (Pellegatti et al., 2008). Although the mechanism of ATP accumulation in tumor microenvironments has not been established, this extracellular ATP might activate P2 receptors on cancer cells and other bystander cells. Activation of P2 receptors has been reported to result in cancer growth, cancer cell survival and cancer-related pain (Tu et al., 2000; Gilchrist et al., 2005). Additionally, it has also been reported that activation of P2X7 receptor by exogenous ATP increases migration of C6 glioma cells and the highly aggressive breast cancer cell line MDA-MB-435s (Wei et al., 2008; Jelassi et al., 2011). Although tumor-promoting cytokines and growth factors accumulate in tumor microenvironments, it is unknown whether stimulation with growth factors or cytokines evokes ATP release from cancer cells and subsequent activation of P2 receptors.

The objective of the present study was to examine whether autocrine signaling through ATP-P2 receptors is involved in TGF-β1-induced migration of human lung cancer cells. Our results indicate that exocytosis of ATP followed by activation of P2X receptors, especially P2X7 receptor, plays an important role in TGF-β1-induced cell migration and actin remodeling, suggesting that autocrine ATP signaling is important for amplification of the cell migration.

Results

Involvement of P2 receptors in TGF-β1-induced migration of A549 cells

TGF-β1 is well known to induce migration of cancer cells, which is an essential step in tumor metastasis. TGF-β1-induced cell migration was confirmed by both a wound-healing-based assay and a Transwell assay. Initially, we examined migration by the wound-healing-based assay using the Culture-Insert system. The gap area in TGF-β1-treated samples was quickly reduced and gaps were completely closed within 24 hours, whereas the gap area in the controls was more gradually reduced and gaps were not finally closed until nearly 48 hours (Fig. 1A; supplementary material Fig. S1). The reduction of gap area was TGF-β1 dose dependent (Fig. 1B). In the wound-healing-based assay, the reduction of gap area is dependent upon a combination of migration and proliferation. To examine whether TGF-β1 influences the proliferation rate, we analyzed the cell cycle and cell division rate after treatment of A549 cells with TGF-β1. We found that TGF-β1 stimulation had no effect on the cell cycle and did not increase cell division (data not shown), suggesting that the TGF-β1-induced reduction of gap area is due to facilitation of cell migration.

In other cancer cell lines ATP has been reported to induce cell migration by activation of P2X7 receptor (Wei et al., 2008; Jelassi et al., 2011). However, it is not clear whether activation of P2 receptors is involved in TGF-β1-induced cell migration. We investigated the contribution of P2-receptor-mediated signaling to TGF-β1-induced migration of A549 cells. We analyzed the expression patterns of a number of P2 receptor subtypes in A549 cells by RT-PCR, and confirmed expression of P2X4, P2X5, P2X6 (a pseudo gene), P2X7, P2Y1, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 receptors (Fig. 1C). Next, we examined the effect of specific inhibitors of P2 receptors on TGF-β1-induced cell migration. Treatment with apyrase

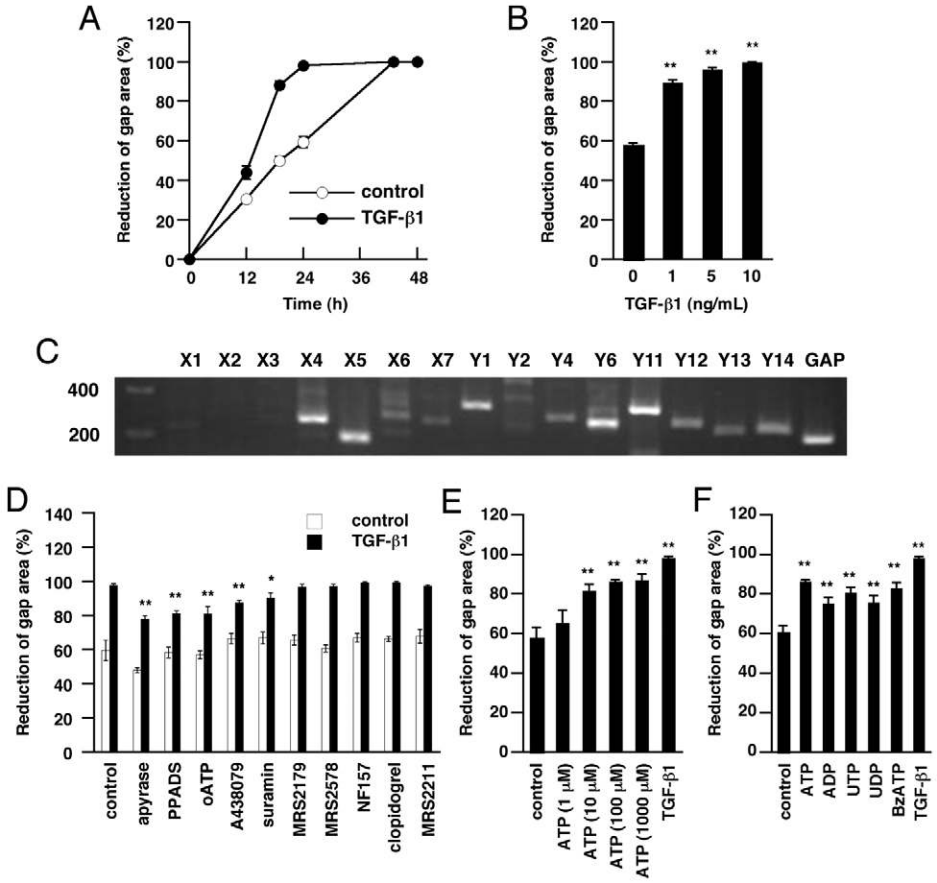

Fig. 1. Involvement of extracellular ATP in TGF-β1-induced migration of A549 cells. (**A**) Cell migration was examined by a wound-healing-based assay as described in Materials and Methods. Cells were incubated with or without TGF-β1 (5 ng/ml) for the indicated times; then photographed and analyzed. (**B**) 24 hours after TGF-β1 stimulation (1–10 ng/ml), cells were photographed and analyzed. (**C**) Total RNA was extracted from A549 cells and gene expression of each of the P2 receptors was examined by RT-PCR. (**D**) Cells were pretreated for 10 min with apyrase (20 U/ml) or for 30 min with PPADS (100 μM), oATP (100 μM), A438079 (100 μM), suramin (100 μM), MRS2179 (100 μM), MRS2578 (10 μM), NF157 (50 μM), clopidogrel (30 μM) or MRS2211 (100 μM), then incubated for 24 h with TGF-β1 (5 ng/ml). (**E**,**F**) A549 cells were incubated with ATP (1–1000 μM) or ADP (100 μM), UTP (100 μM), UDP (100 μM), BzATP (300 μM) and TGF-β1 (5 ng/ml) for 24 hours. Photographs of gap area were taken through a microscope and the percentage reduction in gap area was expressed as a bar graph. Values are means ± s.e.m. (n=5). *P<0.05 and **P<0.01: significant differences between the test groups and control group.

(ecto-nucleotidase), pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS, P2X antagonist), oxidized ATP (oATP, P2X antagonist), A438079 [P2X7 antagonist (Nelson et al., 2006)] and suramin (P2Y antagonist) significantly decreased cell migration induced by TGF-β1 (Fig. 1D). However, MRS2179 (P2Y1 antagonist), MRS2578 (P2Y6 antagonist), NF157 (P2Y11 antagonist), clopidogrel (P2Y12 antagonist) and MRS2211 (P2Y13 antagonist) did not suppress TGF-β1-induced migration (Fig. 1D). We also examined the effect of P2 ligands and agonists on migration in A549 cells without TGF-β1 stimulation. We found that cell migration was increased by ATP in a dose-dependent manner (10–1000 μM; Fig. 1E). Treatment with UTP (P2Y2, P2Y4 agonist) and 3′-O-(4-benzoyl) benzoyl adenosine 5′-triphosphate (BzATP, P2X7 agonist) also accelerated migration (Fig. 1F). The results obtained by the wound-healing-based assay suggest that activation of P2 receptors, including P2X7 receptors, plays an important role in the TGF-β1-induced migration of A549 cells.

To further investigate the involvement of P2-receptor-mediated signaling in TGF-β1-induced migration of A549 cells, we also examined the cell migration by the Transwell assay. We confirmed the increase of migration in TGF-β1-treated cells at 24 hours (Fig. 2A). The increase in migration induced by TGF-β1 was suppressed by treatment with apyrase, PPADS and A438079, but not suramin (Fig. 2B). Furthermore, treatment with ATP or BzATP induced cell migration (Fig. 2C). These data further support the idea that TGF-β1-induced migration is mediated by activation of P2X receptors, or at least P2X7 receptor, in A549 cells.

Involvement of P2 receptors in actin remodeling induced by TGF-β1

In epithelial cancer cells, TGF-β1 induces a phenotype change called epithelial mesenchymal transition (EMT), which is the process whereby immotile, polarized cells transition into highly motile, apolar fibroblastoid-like cells (Wendt et al., 2009). EMT is associated with actin remodeling, as well as changes in epithelial markers and mesenchymal markers, and has been considered as a characteristic of invasive and metastatic cells. We investigated the involvement of P2 receptor in TGF-β1-induced formation of actin stress fibers, which are also induced by TGF-β1 treatment of epithelial cells (Edlund et al., 2002). As shown in Fig. 3, TGF-β1 (5 ng/ml) increased the formation of actin stress fibers within 12 hours, whereas apyrase-treated cells exhibited only a modest polymerization of actin. Treatment with PPADS or A438079, but not suramin, also inhibited TGF-β1-induced actin polymerization, suggesting that extracellular ATP and activation of P2X receptor including P2X7 are involved in this actin polymerization. These results suggest that P2X7 would mediate the migration through actin remodeling in A549 cells. In contrast, suramin did not suppress the actin remodeling, suggesting that P2Y receptors do not contribute to TGF-β1-induced migration.

Involvement of P2X7 receptor in cell migration induced by TGF-β1

To further investigate the involvement of P2X7 receptors in TGF-β1-induced migration of A549 cells, we silenced the expression of P2X7 receptors using siRNA (a pool of three P2X7-specific siRNAs). We confirmed that protein expression of P2X7 receptor was decreased by transfection with P2X7 siRNA (Fig. 4A). In P2X7 siRNA-transfected cells, TGF-β1-induced migration was significantly (**$P<0.01$) suppressed compared with that of control siRNA-transfected cells in both the wound-healing-based assay (Fig. 4B) and the Transwell assay (Fig. 4C). Furthermore, the polymerization of actin induced by TGF-β1 was suppressed by knockdown of P2X7, and there were fewer actin stress fibers in P2X7-knockdown cells than in wild-type and control siRNA-transfected cells (Fig. 4D). Furthermore, the potent and high selective P2X7 antagonist AZ10606120 (Michel et al., 2007) also suppressed TGF-β1-induced migration

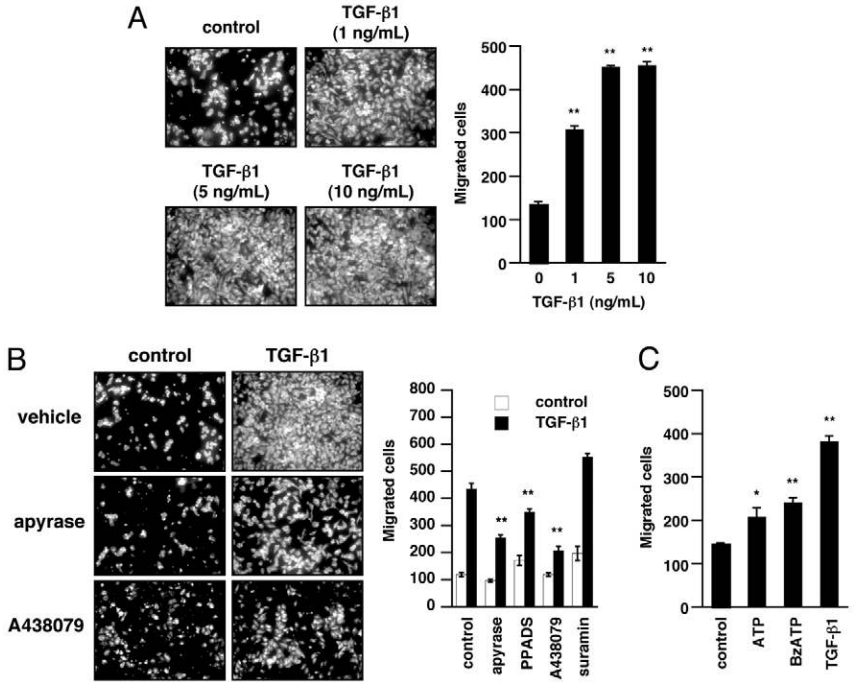

Fig. 2. TGF-β1-induced migration through activation of P2 receptor in A549 cells. (**A**) Cell migration was examined by a Transwell assay as described in Materials and Methods. A549 cells were stimulated with TGF-β1 (1–10 ng/ml) for 24 hours and the lower membrane surfaces were photographed through a microscope at 20× magnification. Migrated cells in each field were counted. (**B**) Cells were pretreated for 10 minutes with apyrase (20 U/ml) or for 30 minutes with PPADS (100 μM), A438079 (100 μM) or suramin (100 μM), then stimulated for 24 hours with TGF-β1 (5 ng/ml). (Apyrase or A438079-treated cells are shown in the left panel.) (**C**) A549 cells were treated with ATP (100 μM), BzATP (300 μM) and TGF-β1 (5 ng/ml) for 24 hours. Values are means ± s.e.m. ($n=6$–9). *$P<0.05$ and **$P<0.01$: significant differences between the test groups and control group.

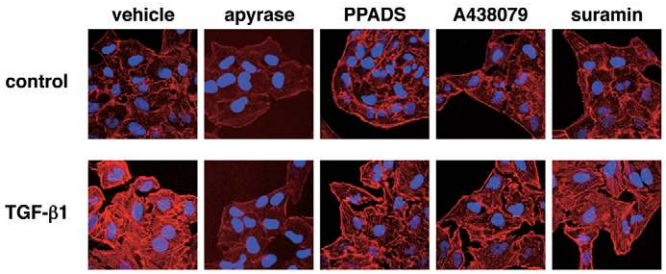

Fig. 3. Involvement of P2X receptor in TGF-β1-induced actin remodeling in A549 cells. A549 cells were pretreated for 10 minutes with apyrase (20 U/ml) or for 30 minutes with PPADS (100 μM), A438079 (100 μM) or suramin (100 μM), then incubated with vehicle (upper panel) or TGF-β1 (5 ng/ml; lower panel). After 12 hours, F-actin was stained using Rhodamine–phalloidin (red), and stained cells were analyzed using a confocal laser scanning microscope at 63× magnification. To verify the location of nuclei, cells were stained with Hoechst33258 (blue).

and actin stress fiber formation (supplementary material Fig. S2).

Thus, our results indicate that activation of P2X receptors, especially P2X7 receptors, is involved in TGF-β1-induced actin remodeling and migration. Release of ATP into the extracellular space is required for activation of P2 receptors. Because it is unknown whether cancer cells release ATP in response to TGF-β1 stimulation, we next examined ATP release from A549 cells after TGF-β1 stimulation.

TGF-β1-induced ATP release from A549 cells

To detect release of ATP from A549 cells after stimulation with TGF-β1, we measured the extracellular concentration of ATP in culture medium by using a luciferin–luciferase reaction-based assay. In this method, ATP released from cells is diluted in the culture medium and is rapidly metabolized by ecto-nucleotidases on the plasma membrane. Therefore, the detected concentration of ATP must be much lower than the real concentration at the cell surface. Before the investigation of TGF-β1-induced ATP release, we measured hypotonic stress-induced ATP release, which is well known to activate P2 receptors. Increase of extracellular ATP after hypotonic stress is involved in regulatory volume decrease through activation of P2 receptors (Wang et al., 1996). It is reported that hypotonic stress increases the concentration of ATP at the cell surface to about 100 μM (Pellegatti et al., 2005). In the previous report, cell surface concentration of ATP was measured using HEK cells expressing luciferase on the cell surface (Pellegatti et al., 2005). As shown in Fig. 5A, 10 minutes after application of hypotonic stress to A549 cells, the extracellular ATP concentration was increased about 15 nM. Therefore, if we detect an increase of extracellular ATP of about 15 nM after treatment of A549 cells with TGF-β1, using the same method, this would indicate that TGF-β1 stimulation causes release of sufficient ATP to activate P2 receptors.

We measured the extracellular ATP release from A549 cells after stimulation with 5 ng/ml TGF-β1. As shown in Fig. 5B, the extracellular concentration of ATP was increased soon after stimulation with TGF-β1 and peaked at 10 minutes. The TGF-β1-stimulated ATP release from A549 cells was dose dependent (Fig. 5C). Comparison of the increased level of extracellular ATP after hypotonic stress and TGF-β1 stimulation indicates that P2 receptors might be activated by increased ATP after TGF-β1

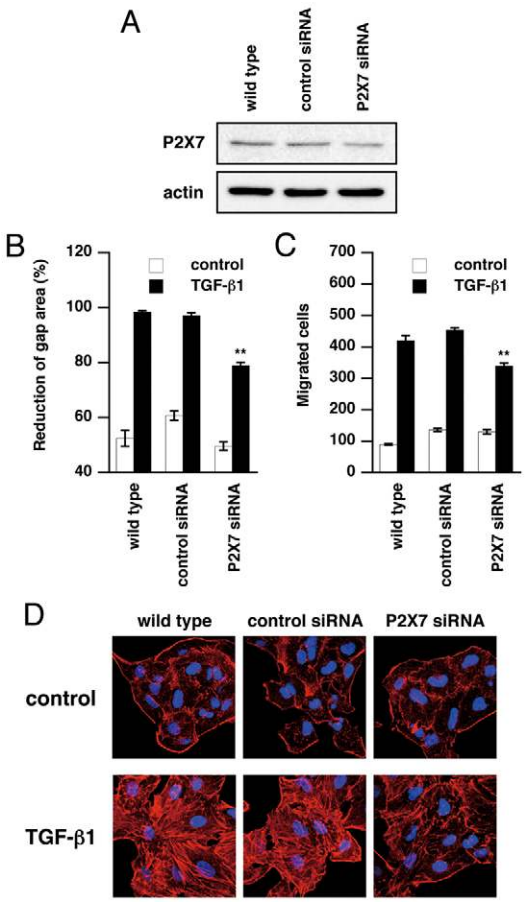

Fig. 4. Involvement of P2X7 receptor in TGF-β1-induced migration of A549 cells. (**A**) A549 cells were transfected with 10 nM P2X7 siRNA or control siRNA and incubated for 60 hours. Then the expression of P2X7 receptor was detected by immunoblotting as described in Materials and Methods. (**B**,**C**) 60 hours after transfection, TGF-β1-induced cell migration was examined using Culture-Insert (B) and Transwell (C) systems, as described in Materials and Methods. Values are means ± s.e.m. (n=6). $^{**}P<0.01$: significant differences between the test groups and control group. (**D**) 60 hours after transfection, cells were incubated with or without TGF-β1 (5 ng/ml) for 12 hours. Then F-actin was stained using Rhodamine–phalloidin (red) and stained cells were analyzed using a confocal laser-scanning microscope at 63× magnification. To verify the location of nuclei, cells were stained with Hoechst33258 (blue).

stimulation. TGF-β1-induced migration is mediated by activation of P2X receptors, or at least P2X7 receptor, in A549 cells.

Next, we investigated whether activation of TGF-β receptors and the canonical Smad pathway mediate TGF-β1-induced ATP release. When activated, the TGF-β type I receptor (TβRI) and type II receptor (TβRII) form a tetrameric receptor heterocomplex that allow direct binding of Smads and their phosphorylation by the kinase activities of the cytoplasmic domains of the TβRI. Phosphorylation of Smad3 was detected at 15 minutes after TGF-β1 stimulation and peaked at 30 minutes, indicating that TGF-β1 induces ATP release before phosphorylation of Smads (Fig. 5D). Increase of extracellular ATP at 10 minutes after TGF-β1 stimulation was blocked by the TβRI inhibitor SB431542 (Inman et al., 2002) (Fig. 5E). To investigate the involvement of ligand binding by TGF-β receptor complex, we tested the effect of a blocking antibody against

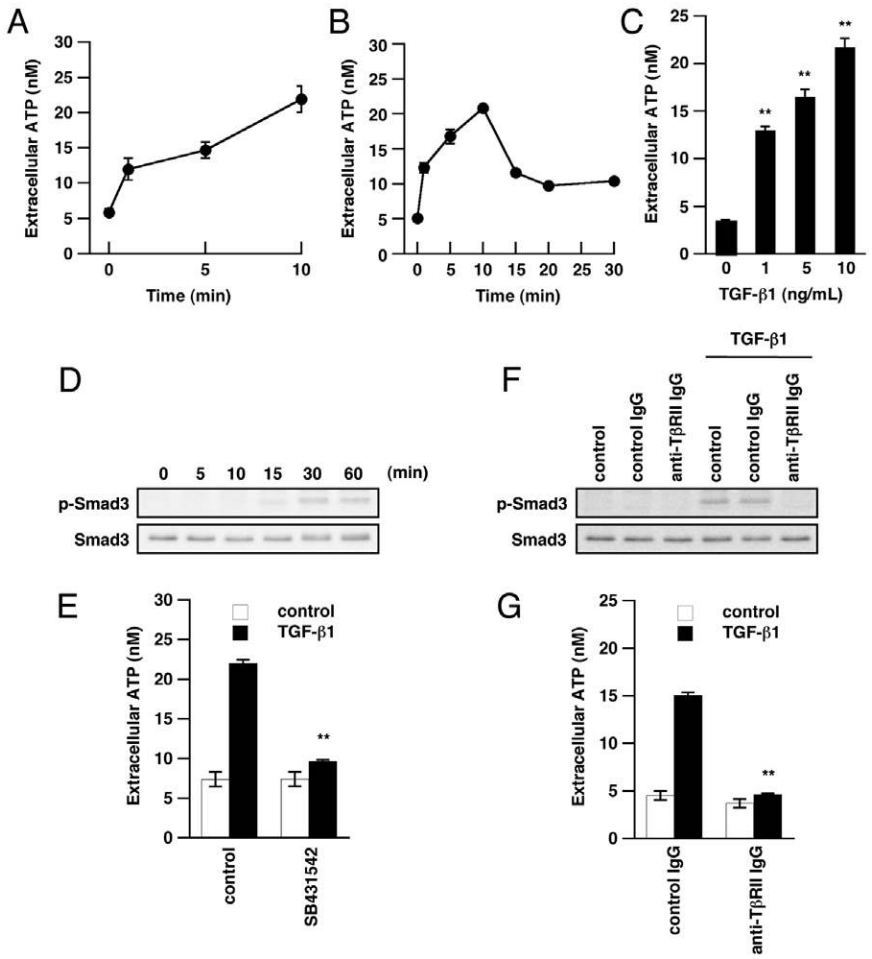

Fig. 5. TGF-β1-induced ATP release from A549 cells. (**A**) A549 cells were exposed to 50% hypotonic solution and incubated for the indicated times, then the concentration of ATP in the culture medium was measured as described in Materials and Methods. (**B**) Cells were stimulated with TGF-β1 (5 ng/ml) and incubated for the indicated times, then the concentration of ATP in the culture medium was measured. (**C**) At 10 minutes after TGF-β1 stimulation (1–10 ng/ml), the concentration of ATP in the culture medium was measured. (**D**) Cells were stimulated with TGF-β1 (5 ng/ml) and incubated for the indicated times, then the phosphorylation of Smad3 was detected by immunoblotting as described in Materials and Methods. (**E**) Cells were pretreated with SB431542 (10 μM) for 60 minutes. At 10 minutes after TGF-β1 stimulation (5 ng/ml), each supernatant was collected and the ATP concentration was measured. (**F**) Cells were pretreated for 60 minutes with control IgG (40 μg/ml) or anti-TβRII IgG (40 μg/ml), then incubated with or without TGF-β1 (5 ng/ml) for 30 minutes and phosphorylation of Smad3 was detected. (**G**) After treatment with control IgG (40 μg/ml) or anti-TβRII IgG (40 μg/ml), cells were stimulated with TGF-β1 (5 ng/ml) and the concentration of ATP in the culture medium was measured. Values are means ± s.e.m. (n=4). $^{**}P<0.01$: significant differences between the test groups and control group.

TβRII on TGF-β1-induced ATP release. TGF-β1-induced phosphorylation of Smad3 was completely blocked by pretreatment with anti-TβRII IgG, indicating that pretreatment with anti-TβRII IgG is enough to inhibit activation of TGF-β receptors (Fig. 5F). Treatment with anti-TβRII IgG inhibited increase of extracellular ATP at 10 minutes after TGF-β1 stimulation (Fig. 5G). These results suggest that TGF-β1-induced ATP release is dependent on TGF-β receptors, and occurs before phosphorylation of Smads.

Involvement of vesicular exocytosis in TGF-β1-induced ATP release from A549 cells

Next, we investigated whether vesicular exocytosis is involved in TGF-β1-induced ATP release from A549 cells. Because increase of intracellular Ca^{2+} and activation of phosphoinositide 3-kinase (PI3K) are known to regulate vesicular exocytosis (Südhof and Rothman, 2009; Lindmo and Stenmark, 2006), the involvement of intracellular Ca^{2+} elevation and PI3K activation in ATP release induced by TGF-β1 stimulation was also investigated, using 1,2-bis (2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM, an intracellular Ca^{2+} chelator) and LY294002 (an inhibitor of PI3K). The increase of extracellular ATP after TGF-β1 stimulation was significantly ($^{**}P<0.01$) suppressed by treatment with BAPTA-AM or LY294002 (Fig. 6A), indicating contributions of both intracellular Ca^{2+} and the PI3K pathway to the ATP release. This result indicates the involvement of vesicular exocytosis in the mechanism of TGF-β1-induced ATP release.

To determine whether ATP-enriched vesicles are present in A549 cells, we stained intracellular ATP with the fluorescent ATP analogue 2′-/3′-O-(*N*′-methylanthraniloyl)-ATP (MANT-ATP) (Zhang et al., 2007). Fluorescence was observed in cytoplasmic vesicles in A549 cells. In addition, the low-pH-sensitive fluorescent probe quinacrine dihydrochloride (Lee, 1971) stained the same population of vesicles, because protons are accumulated in ATP-enriched vesicles by vacuolar proton-ATPase, forming an electrochemical gradient of protons across the membrane that serves as the driving force for ATP accumulation (Fig. 6B–D). These images indicate that ATP-containing vesicles exist in A549 cells. Next, we investigated whether A549 cells express SLC17A9, which transports nucleotides from the cytoplasm into vesicles. We detected SLC17A9 protein in cytoplasm as dot-like vesicles (Fig. 6E), suggesting that SLC17A9 is involved in the accumulation of ATP in vesicles in A549 cells. After TGF-β1 stimulation, quinacrine-positive vesicles were diminished in a time-dependent manner, indicating discharge of ATP-containing vesicles (Fig. 6F). These results suggest that ATP-enriched vesicles are present in A549 cells and TGF-β1 stimulation evokes vesicular exocytosis of ATP.

Involvement of exocytosis in ATP release, migration and actin remodeling after TGF-β1 stimulation

To further investigate whether exocytosis is involved in TGF-β1-induced ATP release and migration in A549 cells, we silenced the expression of SLC17A9 using two different shRNAs (clone ID;

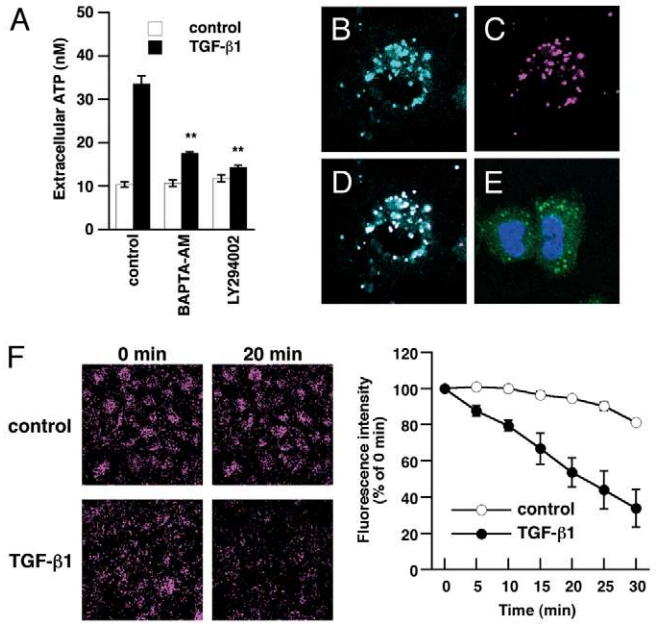

Fig. 6. Involvement of vesicular exocytosis in TGF-β1-induced ATP release from A549 cells. (**A**) Cells were pretreated with BAPTA-AM (50 μM) or LY294002 (10 μM) for 30 minutes. At 10 minutes after TGF-β1 stimulation (5 ng/ml), each supernatant was collected and the ATP concentration was measured. Values are means ± s.e.m. (n=4–8). $^{**}P<0.01$: significant differences between the test groups and control group. (**B–D**) Cells were stained with MANT-ATP (50 μM, cyan; B) and quinacrine dihydrochloride (5 μM, magenta: C) for 1 h at 37°C. Then, stained cells were analyzed by confocal laser scanning microscopy at 63× magnification. The merged image is shown in D. (**E**) Expression of SLC17A9 protein was detected by immunocytochemistry as described in Materials and Methods. To verify the location of nuclei, Hoechst33258-counterstaining (blue) is shown with SLC17A9 immunofluorescence (green). (**F**) Cells were stained with quinacrine dihydrochloride and incubated with or without TGF-β1 (5 ng/ml); then fluorescence micrographs of the same field were taken using confocal laser microscopy at the indicated times. Fluorescence intensity of each image was analyzed using the ImageJ image processing program and expressed as relative to those at 0 minutes (n=3).

red and green). In cells transfected with SLC17A9 shRNA, mRNA expression of SLC17A9 was decreased to 44% (red) and 26% (green) of that in scrambled shRNA-transfected cells (Fig. 7A). We also confirmed that protein expression of SLC17A9 was suppressed by transfection with SLC17A9 shRNA (Fig. 7B,C). The decreased SLC17A9 expression resulted in reduction of MANT-ATP accumulation in vesicles, because SLC17A9 is a nucleotide transporter on vesicles (Fig. 7D). In contrast, quinacrine-dependent fluorescence was not decreased by transfection with SLC17A9 shRNA, because protons are accumulated by proton-ATPase on vesicles.

The decreased SLC17A9 expression also resulted in suppression of ATP release in response to TGF-β1 stimulation, compared with that in scrambled shRNA-transfected cells (Fig. 8A). It seems that the consumption of vesicular ATP after TGF-β1 stimulation is completed much earlier in SLC17A9-knockdown cells, because the slightly increased extracellular concentration of ATP returned to the basal level within 10 minutes after TGF-β1 stimulation. This finding indicates a crucial role of exocytosis in ATP release after TGF-β1 stimulation.

We also investigated whether exocytosis of ATP is involved in cell migration induced by TGF-β1. When SLC17A9 was silenced with SLC17A9 shRNA (red and green), TGF-β1-induced cell migration was significantly ($^{**}P<0.01$) suppressed compared with that of scrambled shRNA-transfected cells in both the wound-healing-based assay (Fig. 8B) and the Transwell system (Fig. 8C), indicating that exocytosis of ATP contributes to the TGF-β1-induced migration.

We also examined the effect of decreased expression of SLC17A9 on formation of actin stress fibers. In scrambled shRNA-transfected cells, the actin cytoskeleton was strikingly reorganized from the periphery to stress fibers after TGF-β1 treatment. Knockdown of SLC17A9 suppressed the actin polymerization induced by TGF-β1, and actin stress fibers in knockdown cells were fewer and thinner than those in scrambled shRNA-transfected cells (Fig. 8D). Because polymerization of actin is closely related to cancer cell motility (Olson and Sahai, 2009), reduction of actin stress fibers seems to contribute to the suppression of TGF-β1-induced cell migration by knockdown of SLC17A9.

The epithelial cell–cell adhesion molecule E-cadherin and the mesenchymal cell–cell adhesion molecule N-cadherin are related to cell motility (Yilmaz and Christofori, 2010). Finally, we investigated the involvement of exocytosis of ATP in TGF-β1-induced downregulation of E-cadherin and increase of N-cadherin, which are involved in EMT. As shown in Fig. 8E, treatment with TGF-β1 reduced the expression of E-cadherin and increased the expression of N-cadherin at 48 hours (Fig. 8E). The expression of E-cadherin was also decreased in SLC17A9-knockdown cells after TGF-β1 stimulation (Fig. 8F). Similarly, TGF-β1-induced increase of N-cadherin was not affected by knockdown of SLC17A9 (Fig. 8F). These results suggest that exocytosis of ATP is involved not in the cadherin switch, but rather in actin remodeling, in the mechanism of TGF-β1-induced EMT. Moreover, this result also indicates that transfection of SLC17A9 shRNA does not abolish activation of TGF-β1 signaling itself.

Discussion

We investigated whether TGF-β1-induced cancer progression is mediated by extracellular release of ATP and activation of P2 receptors. Our results suggest that vesicular exocytosis of ATP, which causes activation of P2 receptors, plays an important role in TGF-β1-induced migration through formation of actin stress fibers.

First, we investigated whether activation of P2 receptors is involved in TGF-β1-induced migration of A549 cells. Using a wound-healing-based assay we found that pretreatment with some inhibitors of P2 receptors suppressed TGF-β1-induced migration. Considering the expression of P2X receptors in A549 cells and the inhibitory effect of P2X antagonists and P2X7 antagonist on the migration, it is suggested that P2X receptors, including P2X7 receptor, contribute to TGF-β1-induced migration. The contribution of the P2X7 receptor is supported by the facilitation of migration by BzATP, which is a specific agonist of the P2X7 receptor. However, ADP, UTP or UDP, which do not induce activation of P2X7 receptor, also partly reduced gap area. Considering that the reduction of gap area depends on not only migration but also proliferation, stimulation with these nucleotides would activate P2Y receptors and result in proliferation of A549 cells (Schafer et al., 2003). The results

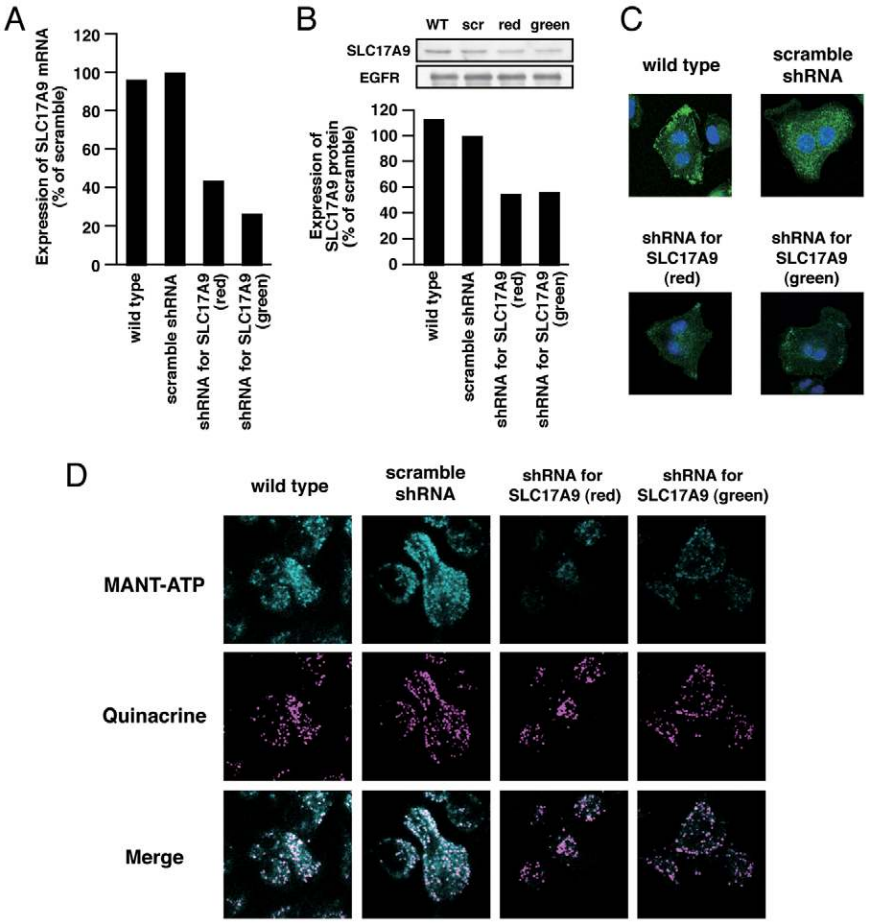

Fig. 7. Decrease in ATP-enriched vesicles in A549 cells by knockdown of SLC17A9. (**A**) A549 cells were transfected with 2 μg shRNA targeting SLC17A9 (red or green) or scrambled shRNA. Then 48 hours after transfection, total RNA was extracted and SLC17A9 gene expression was examined by measuring mRNA levels with real-time RT-PCR. (**B**) Cell membrane proteins were extracted 72 hours after transfection, and the expression of SLC17A9 was detected by immunoblotting as described in Materials and Methods. SLC17A9 expression levels were normalized to EGFR expression levels and expressed relative to those of scrambled shRNA-transfected cells. (**C**) 72 hours after transfection, expression of SLC17A9 protein was detected by immunocytochemistry. (**D**) ATP-enriched vesicles were detected using MANT-ATP (cyan, upper panels) and quinacrine dihydrochloride (magenta, middle panels) 72 hours after transfection. The merged images are shown in lower panels.

obtained by the wound-healing-based assay suggest that activation of P2 receptors, including P2X7 receptor, plays an important role in the TGF-β1-induced migration of A549 cells. We also examined cell migration using the Transwell assay. The results further support the idea that TGF-β1-induced migration is mediated by activation of P2X receptors, or at least P2X7 receptor, in A549 cells. We further confirmed the involvement of the P2X7 receptor in TGF-β1-induced migration of A549 cells by knockdown experiments. In addition, another potent and high selective P2X7 antagonist, AZ10606120, also suppressed TGF-β1-induced migration and actin remodeling, supporting the involvement of the P2X7 receptor. P2X7 receptor might mediate the migration through actin remodeling in A549 cells. It has been reported that the migration is mediated by mitogen-activated protein kinases (MAPKs), including Jun N-terminal kinase (JNK) (Huang et al., 2004) and that actin remodeling is mediated by Rho kinase (Narumiya et al., 2009). Because activation of P2X7 receptor also causes activation of JNK (Humphreys et al., 2000) and Rho kinase (Morelli et al., 2003), TGF-β1-mediated migration of A549 cells might be mediated through activation of JNK or Rho kinase as downstream events of P2X7 receptor activation.

Next, we investigated whether TGF-β1 induced ATP release from cancer cells. We found that ATP was released from A549 cells in response to TGF-β1 stimulation, which was dependent on a kinase activity of TGF-β receptor type I and ligand binding by TGF-β receptor complexes. However, TGF-β1-induced ATP release was dependent on intracellular Ca^{2+} and PI3K, suggesting the involvement of vesicular exocytosis. Furthermore, we detected ATP-enriched vesicles and expression of the vesicular nucleotide transporter SLC17A9 protein in A549 cells. Knockdown of SLC17A9 resulted in reduction of MANT-ATP accumulation in vesicles and suppression of TGF-β1-induced ATP release. These results support the idea that ATP is accumulated in vesicles through SLC17A9 in A549 cells and the TGF-β1-induced ATP release is regulated by vesicular exocytosis. This is first evidence indicating the vesicular exocytosis of ATP from cancer cells in response to cytokine stimulation.

Furthermore, we found that vesicular exocytosis of ATP is involved in migration and actin remodeling but not decrease in E-cadherin and increase in N-cadherin expression. Autocrine signaling through exocytosis of ATP and activation of P2 receptors is a novel mechanism of TGF-β1-induced cell migration. TGF-β1 is known as a potent inducer of tumor metastasis, and increase in cell motility is the first step in the process of metastasis. Therapy based on the inhibition of ATP release or P2 receptors could complement conventional treatments to prevent tumor cell metastasis. It seems worthwhile to further investigate the involvement of exocytosis of ATP followed by activation of P2 receptors in other TGF-β1-mediated events, such as production of extracellular matrix or inflammation, proliferation and angiogenesis.

This is the first report to demonstrate TGF-β1-induced-ATP signaling, which occurs before activation of Smads. It is known that TGF-β1 stimulation induces activation of a canonical Smad pathway and a non-canonical (Smad-independent) pathway.

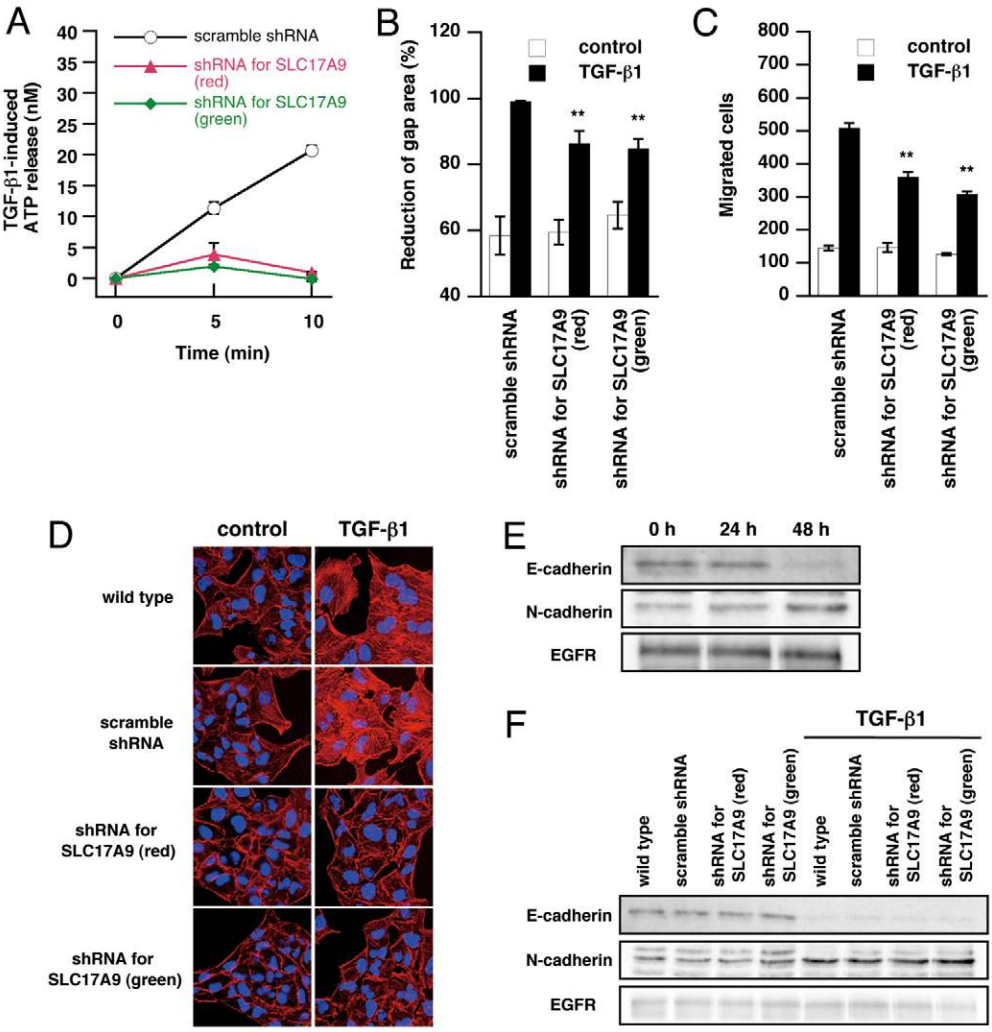

Fig. 8. Involvement of SLC17A9-dependent ATP release in TGF-β1-induced migration and actin remodeling. (**A**) Cells were transfected with shRNA targeting SLC17A9 (red or green) or scrambled shRNA and incubated for 72 hours. The transfected cells were stimulated with TGF-β1 (5 ng/ml) and further incubated for the indicated times; then the concentration of ATP in the culture medium was measured as described in Materials and Methods. (**B**,**C**) 72 hours after transfection, TGF-β1-induced cell migration was examined using Culture-Insert (B) and Transwell (C) systems, as described in Materials and Methods. The transfected cells were incubated with vehicle or TGF-β1 (5 ng/ml) for another 24 hours. Values are means ± s.e.m. ($n=8$). $^{**}P<0.01$: significant differences between the test groups and control group. (**D**) 72 hours after transfection, cells were incubated with or without TGF-β1 (5 ng/ml) for 12 h. Then F-actin was stained using Rhodamine–phalloidin (red) and stained cells were analyzed using a confocal laser-scanning microscope at 63× magnification. To verify the location of nuclei, cells were stained with Hoechst33258 (blue). (**E**) Non-transfected A549 cells were incubated with TGF-β1 (5 ng/ml) for the indicated times. Then, cell membrane proteins were extracted and expression of E-cadherin and N-cadherin was detected by immunoblotting as described in Materials and Methods. (**F**) 72 hours after transfection, cells were incubated with or without TGF-β1 for another 48 hours and expression of E-cadherin and N-cadherin was detected. EGFR was detected as a loading control.

Therefore, it is possible that ATP release and activation of P2 receptors are involved in a non-canonical TGF-β1 signaling pathway, such as the MAPK pathway. If cancer progression coincides with alteration of ATP release via exocytosis or expression of P2 receptors, this might be an explanation for the switching of TGF-β1 function from tumor suppression to tumor progression. These results appear to warrant further investigation of the exocytotic ATP-releasing ability and expression of P2 receptors of a range of malignant and normal cells.

Because released ATP would activate P2 receptors on bystander cells too, it seems that bystander cells might be affected by stimulation with TGF-β1, suggesting enhancement of TGF-β1 signaling in the tumor microenvironment. Furthermore, ATP release also causes accumulation of adenosine (a metabolite of ATP) in the extracellular space around tumors, which activates adenosine-P1 receptors (A1, A2A, A2B, A3). Adenosine-induced activation of A2A receptors on T cells inhibits activation of effector T cells (Stagg and Smyth, 2010), so TGF-β1-induced ATP exocytosis might play a role in tumor immune evasion. Adenosine also has a pro-angiogenic effect (Stagg and Smyth, 2010). It is known that activation of P2X and P2Y receptors plays an important role in cancer proliferation and cancer malignancy. Therefore, TGF-β1-induced exocytosis of ATP might be expected to regulate both P2-receptor-mediated cancer progression, including cell migration, and P1-receptor-mediated immune suppression and angiogenesis. In addition to TGF-β1,

other cytokines, such as epidermal growth factor, might also induce ATP release and activation of P2 receptors that participate in cancer growth and progression. Our results thus open up a range of new possibilities for research on the mechanisms of cancer progression.

In conclusion, we have shown here that TGF-β1 stimulation evokes ATP release from A549 cells and that vesicular exocytosis contributes substantially to TGF-β1-induced ATP release. In addition, the released-ATP-mediated activation of P2 receptors, at least P2X7, appears to be involved in the acceleration of cell migration and the actin remodeling induced by TGF-β1. We suggest that exocytosis of ATP and autocrine, positive feedback through P2 receptors would be required for effective induction of cell migration by TGF-β1.

Materials and Methods

Reagents and antibodies

DMEM, human recombinant TGF-β1 and SB431542 were purchased from Wako Pure Chemical (Osaka, Japan). FBS was purchased from Biowest (Nuaillé, France). LY294002, apyrase, PPADS, oATP, suramin, BzATP and anti-N-cadherin antibody were purchased from Sigma-Aldrich (St Louis, MO). A438079, MRS2179, MRS2578, NF157, clopidogrel, MRS2211 and AZ10606120 were purchased from Tocris Bioscience (Ellisville, MO). BAPTA-AM was from Dojin (Tokyo, Japan). Rhodamine–phalloidin was purchased from Cytoskeleton, Inc. (Denver, CO). Anti-human TGF-βRII antibody and normal goat IgG control were purchased from R&D Systems, Inc. (Minneapolis, MN). Rabbit monoclonal anti-phospho-Smad3 (Ser423/425) antibody, rabbit monoclonal anti-Smad3 antibody and rabbit monoclonal anti-epidermal growth factor receptor (EGFR) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-P2X7 extracellular antibody was purchased from Alomone Labs (Jerusalem, Israel). Anti-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-E-cadherin antibody (Clone 36B5) was purchased from Thermo Fisher Scientific (Waltham, MA).

Cell culture

A549 human adenocarcinoma cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 mg/ml) in a humidified atmosphere of 5% CO_2 in air at 37℃.

Cell migration assay

Cell migration was tested in wound-healing-based assays using Culture-Inserts (Ibidi, Martinsried, Germany). After cell adherence, the Culture-Inserts were removed and the cells were stimulated with TGF-β1 or ATP. 12–48 hours after stimulation, the remaining gaps were photographed at five randomly chosen areas through a microscope (BZ-9000; KEYENCE, Osaka, Japan). Reduction of gap areas was calculated using Photoshop (Adobe Systems Incorporated, San Jose, CA) and the ImageJ image processing program (NIH). TGF-β1-induced cell migration was also analyzed by using 24-well Transwell plates (6.5 mm diameter; 8 μm pore size polycarbonate membrane, Corning, Lowell, MA). The upper compartment was seeded with A549 cells (2×10^4 cells) in basal culture medium. After 24 hours, the medium was replaced with fresh medium containing TGF-β1 or ATP. The upper chamber contained 5% FBS instead of 10%. After incubation for a further 24 hours, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, and incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and 50 μg/ml propidium iodide (PI) for 30 minutes at room temperature. Non-migrated cells on the upper surface of the membrane were removed and cells that had migrated through the membrane to the lower surface were counted using a fluorescence microscope (BZ-9000; KEYENCE).

RT-PCR

Total RNA was isolated from A549 cells using a Fast Pure RNA kit (Takara Bio, Shiga, Japan) and the first-strand cDNA was synthesized from total RNA with PrimeScript Reverse Transcriptase (Takata Bio). Specific primers were designed with PrimerQuest[SM] (Integrated DNA Technologies, Inc., Coraville, IA) and synthesized by Sigma Genosys (Sigma-Aldrich). The sequences of specific primers for human P2 receptors are shown in supplementary material Table S1. GAPDH mRNA was determined as a positive control. PCR was carried out by incubating each cDNA sample with the primers (0.5 μM each), PrimeSTAR® HS DNA Polymerase (0.625 U: Takara Bio) and deoxynucleotide mix (0.2 mM each: Takara Bio). Amplification was carried out for 35 cycles (denaturation at 94℃ for 30 seconds, annealing at 60℃ for 30 seconds, extension at 72℃ for 1 minute). The products were then subjected to 2% agarose gel electrophoresis. Bands were stained with ethidium bromide (Sigma-Aldrich) and photographed.

Small interfering RNA (siRNA) transfection

A549 cells were transfected with 10 nM P2X7 siRNA (a pool of three target-specific 19–25 nt siRNAs) or control siRNA-A (Santa Cruz) using HiPerFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturers' instructions. Decreased protein expression of P2X7 was confirmed at 60 hours after transfection.

Fluorescence imaging

For F-actin staining and immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, and permeabilized with 0.5% Triton X-100 for 5 minutes on ice. For staining of F-actin, fixed cells were incubated with 100 nM Rhodamine–phalloidin for 30 minutes at room temperature. For detection of SLC17A9, after blocking with 10% FBS-containing PBS for 6 hours, fixed cells were incubated overnight with rabbit polyclonal anti-human SLC17A9 antibody (Sawada et al., 2008) (1:1000) at 4℃, and then further incubated with FITC-conjugated anti-rabbit IgG antibody (1:200) for 1 hour. Counterstaining with Hoechst33258 (10 μg/ml) was used to verify the location and integrity of the nuclei. To visualize intracellular ATP localization in vesicles, A549 cells were incubated for 1 hour with 50 μM MANT-ATP, 5 μM quinacrine dihydrochloride in RPMI1640-based buffer at 37℃. Stained cells were analyzed using a confocal laser scanning microscope (TCS SP2; Leica, Mannheim, Germany) equipped with a HCX PLApo 63× 1.32 NA oil objective lens. Leica confocal software (TCS SP2, version 2.6.1) was used for image acquisition and processing.

Determination of extracellular ATP concentration

The release of ATP was quantified by using the luciferin–luciferase-based Enlighten ATP assay system (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, A549 cells were grown in a 48-well plate to 100% confluence. 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), then the cells were stimulated with TGF-β1 in the concentration range of 1–10 ng/ml or 50% hypotonic stress. Between 1 and 30 minutes after stimulation, cell-conditioned buffer was obtained. Luciferin–luciferase reagent (100 μl) was added to 10 μl of conditioned buffer and the chemiluminescence was measured with a WALLAC ARVO SX multilabel counter (PerkinElmer, Inc., Waltham, MA). ATP concentration in each sample was determined by comparing the luminescence of samples with those of standards in the concentration range 10^{-8} to 10^{-10} M.

Short hairpin RNA plasmid (shRNA) transient transfection

Transient transfection with shRNA was performed using the SureSilencingTM shRNA Plasmid Kit for human SLC17A9 (SA Biosciences, Frederick, MD). Two different shRNA plasmids targeting SLC17A9 or the scrambled shRNA plasmid (negative control) were transfected by electroporation with the Amaxa system (Nucleofector solution T and Nucleofector program X-01) (Lonza, Walkersville, MD). Forty-eight hours after transfection, a decrease in SLC17A9 mRNA was confirmed. Decreased protein expression of SLC17A9 was also confirmed at 72 hours after transfection.

Real-time RT-PCR

Total RNA was isolated from A549 cells using a Fast Pure RNA kit (Takara Bio). The first-strand cDNA was synthesized from total RNA with PrimeScript Reverse Transcriptase (Takara Bio). The cDNA was used as a template for real-time PCR analysis: reactions were performed in a Stratagene Mx3000P® QPCR system (Agilent Technologies, La Jolla, CA). The sequences of specific primers for human SLC17A9 were 5′-AGTCTGTGGTCTTTGCATCAGCCT-3′ (sense), 5′-TGT-TGGCCACACCAAACAGAAAGC-3′ (antisense). GAPDH mRNA was determined as a positive control. Each sample was assayed in a 20 μl amplification reaction mixture, containing cDNA, primers mixture (5 μM each of sense and antisense primers) and 2× KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems, Woburn, MA). The amplification program included 40 cycles of two steps, comprising heating to 95℃ for 3 seconds and to 60℃ for 30 seconds. Fluorescent products were detected at the last step of each cycle. The obtained values were within the linear range of the standard curve and were normalized to GAPDH mRNA expression.

Immunoblotting

Protein expression on whole-cell or cell-membrane fractions were measured by immunoblotting. Cell membrane fractionation was carried out using the Plasma Membrane Protein Extraction Kit according to the manufacturer's instructions (BioVision, Mountain View, CA). Briefly, A549 cells were homogenized using a digital homogenizer (Iuchi, Osaka, Japan) in 1 ml ice-cold homogenize buffer. Homogenates were centrifuged at 700 ***g*** for 10 minutes at 4℃, and the supernatants were centrifuged at 10,000 ***g*** for 30 minutes at 4℃. The pellet consisted of total cell membranes. Whole-cell or total-cell membrane protein was 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. The protein content in each sample was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). 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 6 μg (whole cell) or 1 μg (total cell membrane) 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 5% skimmed milk or 1% BSA at 4℃. They were then incubated with rabbit P2X7 antibody (1:200) for 90 minutes at room temperature, or overnight with mouse actin antibody (1:1000), rabbit phospho-Smad3 antibody, rabbit Smad3 antibody (1:1000), rabbit polyclonal anti-SLC17A9 antibody (1:5000), rabbit EGFR antibody (1:1000), mouse E-cadherin antibody (1:1000) or mouse N-cadherin antibody (1:1000) at 4℃. 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 minutes at room temperature. The blots were again washed with TBST, and specific proteins were visualized using ECL western blotting detection reagents (GE Healthcare) according to the manufacturer's instructions.

Statistics

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

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