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RESEARCH PAPER

UDP induces intestinal epithelial migration via the $P2Y_6$ receptor

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BACKGROUND AND PURPOSE

Extracellular nucleotides are released at high concentrations from damaged cells and function through P2 receptor activation. Intestinal epithelial restitution, which is defined as cell migration independent of cell proliferation, is an important initial step in the process of wound healing. In this study, we investigated the role of extracellular nucleotides in intestinal epithelial migratory responses.

EXPERIMENTAL APPROACH

Wound-healing and trans-well migration assays were performed with a rat intestinal epithelial cell line (IEC-6). The concentrations of extracellular nucleotides released from injured IEC-6 cells were measured by HPLC. TGF-β expression was assessed by RT-PCR and ELISA.

KEY RESULTS

Scratching the monolayer of IEC-6 cells induced cell migration. Pretreatment with apyrase or MRS2578, a selective $P2Y_6$ antagonist, inhibited the wound-induced cell migration. Among the cellular nucleotides, only ATP and uridine 5′-diphosphate (UDP) were detected in the culture medium after cell wounding. Exogenously applied UDP dose-dependently enhanced the migration more effectively than ATP but did not induce proliferation. In addition, cell wounding and UDP increased the expression of TGF-β, and both the wound-induced and UDP-enhanced migration were inhibited by MRS2578 or ALK5Inhibitor (ALK5i), a TGF-β receptor blocker. Furthermore, cell wounding and UDP stimulation up-regulated the expression of $P2Y_6$ receptor mRNA, and this effect was suppressed by MRS2578 or ALK5i.

CONCLUSION AND IMPLICATIONS

Wound-induced UDP evokes intestinal epithelial restitution by activation of $P2Y_6$ receptors, which mediates *de novo* synthesis of TGF-β. In addition, the expression of $P2Y_6$ receptors is increased by cell wounding and UDP, which constitutes a positive-feedback loop for mucosal repair.

Abbreviations

UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate

Introduction

All cells contain millimolar concentrations of intracellular nucleotides, such as ATP, ADP, UTP (uridine 5′-triphosphate) and UDP (uridine 5′-diphosphate), which are released into the extracellular space in response to stimulation or injury. These extracellular nucleotides activate the P2 receptor family, which is divided into the P2X and P2Y groups. Ionotropic P2X

receptors are subdivided into $P2X_1$-X_7. G-protein-coupled P2Y receptors are subdivided into $P2Y_1$, Y_2, Y_4, Y_6 and Y_{11-14}, which have different binding affinities for each nucleotide. For instance, ATP binds to almost all P2Y receptors and has a relatively low affinity for $P2Y_6$ and $P2Y_{14}$. In contrast, UDP selectively activates the $P2Y_6$ receptor. In steady-state conditions the concentration of extracellular nucleotides is maintained at the nanomolar range, whereas after tissue or cell damage, the concentration is locally increased to submillimolar levels (Abbracchio *et al.*, 2006; Burnstock, 2006).

In the intestine, epithelial cells seal the surface of the tract and act as a barrier to prevent the invasion of deleterious compounds. Intestinal epithelial integrity is impaired by intestinal motility or digestive functions and also by alcohol consumption or drugs such as non-steroidal anti-inflammatory agents (Mammen and Matthews, 2003). Disruption of epithelial continuity increases the risk of intestinal inflammation and the subsequent development of sepsis. Therefore, intestinal epithelial wound healing is an important process for maintaining normal epithelial function and preventing serious diseases (Blikslager *et al.*, 2007). The wound-healing process is initiated by epithelial restitution, which is defined as cell migration independent of cell proliferation. To retain intestinal continuity, epithelial restitution occurs within minutes of cell injury (Feil *et al.*, 1989; Moore *et al.*, 1989). After restitution, the migrated cells subsequently proliferate and differentiate into mature cells to complete the wound-healing process (Iizuka and Konno, 2011).

As large of amounts of nucleotides are released immediately after cell damage, it has been speculated that extracellular nucleotides are one of the first signals to initiate epithelial restitution. In corneal epithelial cells, released ATP increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via P2Y receptor activation and generates the propagation of Ca^{2+} waves (Klepeis *et al.*, 2001; 2004). Klepeis *et al*. suggested that these Ca^{2+} waves are involved in promoting cell migration as the first response following injury. In addition, extracellular nucleotides, such as ATP and UDP, facilitate the production and secretion of growth factors and chemokines that enhance epithelial migration (Yin *et al.*, 2007; Grbic *et al.*, 2008; Ivison *et al.*, 2011). However, the exact role of extracellular nucleotides in the process of intestinal epithelial wound healing is still not known. Identification of the factors that induce epithelial restitution is needed to understand the mechanism of restitution that prevents epithelial disruption from developing into a serious disease. In the present study, we investigated the role of extracellular nucleotides in the process of intestinal epithelial wound healing, focusing on the initial phase of migration.

Methods

Cell culture

A rat intestinal epithelial cell line, IEC-6 (Rikaken, Tokyo, Japan), was cultured in DMEM containing 100 $U \cdot mL^{-1}$ penicillin, 100 $mg \cdot mL^{-1}$ streptomycin, 4 $\mu g \cdot mL^{-1}$ insulin from bovine pancreas and 5% FBS in 95% air and 5% CO_2 at 37°C. Before all experiments, cells were deprived of serum (serum-starved) for 24 h by incubation in serum-free DMEM containing 100 $U \cdot mL^{-1}$ penicillin and 100 $mg \cdot mL^{-1}$ streptomycin.

Detection of nucleotides

Serum-starved IEC-6 cells cultured in 60 mm dishes were incubated in HEPES solution (137 $mmol \cdot L^{-1}$ NaCl, 5.4 $mmol \cdot L^{-1}$ KCl, 11.5 $mmol \cdot L^{-1}$ glucose, 1.5 $mmol \cdot L^{-1}$ $CaCl_2$, 1.2 $mmol \cdot L^{-1}$ $MgCl_2$ and 10 $mmol \cdot L^{-1}$ HEPES, pH 7.4) for 1 h. Approximately 50% of the cells were scratched with 100–1000 µL pipette tips, the medium (2 mL) was collected on ice, and the debris was immediately removed by centrifugation at x 16 000 *g* for 10 min at 4°C. The medium from unscratched cell cultures was also collected as a control. The supernatant (1 mL) of the centrifuged medium was passed through a Ministart® filter (0.2 µm pore size; Sartorius AG, Göttingen, Germany). Aliquots of 20 µL were subjected to reverse-phase HPLC (Shimadzu, Kyoto, Japan) with absorbance measured at 260 nm using a C18 column (µRPC C2/C18 ST 4.6/100; GE Healthcare, Bristol, UK). The ion-pair mobile phase consisted of 5 $mmol \cdot L^{-1}$ tetrabutylammonium hydrogen sulphate and 50 $mmol \cdot L^{-1}$ KH_2PO_4, pH 5 (solution A) or 5 $mmol \cdot L^{-1}$ tetrabutylammonium hydrogen sulphate, 50 $mmol \cdot L^{-1}$ K_2HPO_4 and 40% MeOH, pH 5 (solution B). A standard solution of nucleotides (5 $\mu mol \cdot L^{-1}$ each) and samples were injected at 1 $mL \cdot min^{-1}$; the ratio of solution B was increased to 35% from 0 to 14 min and linearly increased to 70% from 14 to 34 min. The elution times were assessed using a standard sample of nucleotides. The elution time was 1.5 min for UDP and 3.0–3.5 min for ATP.

Wound-healing migration assay

Confluent monolayers of IEC-6 cells were scratched with sterile tips of 100–1000 µL pipette to form a linear wound that was approximately 1 mm in diameter. After waiting for 10 min, the cell monolayer was washed three times with Ca^{2+}- and Mg^{2+}-free HBSS and then fresh DMEM with or without nucleotides and/or P2Y antagonists was added. Pretreatment with P2Y inhibitors or apyrase was performed 30 min before the cells were scratched/wounded. Three wounded areas were randomly photographed at 100-fold magnification with a digital camera (Nikon DS-Fi1; Nikon, Tokyo, Japan) immediately after the cells had been scratched (0 h) and after 8 h. Migration was assessed by counting the number of cells observed across the wound area by use of ImageJ software (NIH, Bethesda, MD, USA) and evaluated as a percentage of the number of cells that had migrated in cultures with untreated medium (control).

Trans-well migration assay

The trans-well migration assay was performed with a Boyden chamber. After serum starvation, 5×10^4 IEC-6 cells were seeded on the insert wells (24-well PET membrane, 8 µm pore size, BD Falcon™; BD, Franklin Lakes, NJ, USA). Nucleotides (ATP, ADP, UTP or UDP) were added to the upper and lower wells (24-well cell culture plate, BD Falcon™). After 8 h, the cells were fixed with 4% paraformaldehyde for 15 min and stained with 5% Giemsa solution for 60 min. After being washed with distilled water, the cells inside the insert well were removed with a cotton swab. Migrated cells beneath the insert wells were randomly photographed with a digital camera (Nikon 1200C) at 400-fold magnification and counted with ImageJ software. Cell migration was evaluated as the number of migrated cells per field.

Proliferation assay

IEC-6 cells were seeded on 24-well plates at 1×10^5 cells per well and starved for 24 h. The medium was exchanged with fresh DMEM containing UDP (1–100 μmol·L^{-1}) or 1% FBS, and the cells were cultured for 24 h. After removal of debris, the cells were dissociated with Ca^{2+}-free Hanks buffer containing 0.1% trypsin and EDTA, and the cell number was counted with a cytometer.

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured as described previously (Nakamura *et al.*, 2011). In brief, IEC-6 cells cultured on glass coverslips were incubated with 3 μmol·L^{-1} fura-2 AM containing 0.01% Cremophor in HEPES solution in the dark at 37°C for 40 min. The cells were then placed on the stage of an inverted microscope (TE-300, Nikon) equipped with a 40-fold objective lens. The fluorescence ratio (R: F340/F380) was determined by the fluorescence signals collected every 3 s at 340 nm (F340) and 380 nm (F380) using a fluorescence imaging system (Hamamatsu Photonics, Hamamatsu, Japan). Ca^{2+}-free HEPES-buffered solution (Ca^{2+}-free solution: containing 0.5 mmol·L^{-1} EGTA instead of 1.5 mmol·L^{-1} $CaCl_2$) was used to remove extracellular Ca^{2+}. The area under the Δ ratio per time curve (AUC) was calculated to measure the increase in $[Ca^{2+}]_i$. The AUC was expressed as the mean ± SEM of 16–17 cells in each of three or four independent experiments.

Semi-quantitative RT-PCR

Total RNA from IEC-6 cells was extracted with the acid–guanidine–phenol–chloroform method using Trizol reagent. Complementary DNA was synthesized from total RNA with random primers and ReverTraAce (TOYOBO, Osaka, Japan) at 30°C for 10 min, 42°C for 60 min, 99°C for 5 min and 4°C for 10 min. PCR was performed for 28 cycles at 98°C for 10 s, 55°C for 30 s and 72°C for 40 s. The expression levels of P2Y$_6$ receptor, TGF-β, TGF-α and GAPDH were semi-quantified as the ratios to the mRNA expression of GAPDH. The following primers were used: P2Y$_6$ receptor, forward 5′-ACGCTTCC TCTTCTATGCCA-3′, reverse 5′-TAGCAGGCCAGTAAGGCT GT-3′; TGF-β, forward 5′-TAGGAAGGACCTGGGTTGGAAG-3′, reverse 5′-CGGGTTGTGTTGGTTGTAGAGG-3′; TGF-α, forward 5′-GCTAGCGCTGGGTATCCT-3′, reverse 5′-ACCACT CACAGTGCTTGCGG-3′; and GAPDH, forward 5′-TCCCTCA AGATTGTCAGCAA-3′, reverse 5′-AGATCCACAACGGATAC ATT-3′. The PCR products were subjected to electrophoresis in 2% agarose gels containing 0.2 μg·mL^{-1} ethidium bromide and visualized with an ultraviolet transilluminator. Band intensity was quantified using ImageJ. Each mRNA level was expressed as the ratio of the optical density of each product to that of GAPDH.

ELISA

A confluent monolayer of IEC-6 cells was scratched or stimulated with UDP (100 μmol·L^{-1}) in the presence or absence of MRS2578 (1 μmol·L^{-1}) for 6 h. The supernatant was collected and immediately centrifuged to remove the debris. The amount of TGF-β was measured by ELISA (eBioscience, San Diego, CA, USA).

Statistical analysis

Results are expressed as mean ± SEM. The statistical significance of differences between mean values was assessed by one-way ANOVA followed by Dunnett's test for comparison of multiple groups with the control group or by the Tukey–Kramer test for multiple comparisons.

Materials

The chemicals and reagents used were as follows: ATP and UTP sodium salts, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS), DMEM, suramin sodium salt, potassium phosphate dibasic solution, AG1478, apyrase, insulin from bovine pancreas and potassium phosphate monobasic solution (Sigma, St. Louis, MO, USA), ADP and UDP sodium salts (Yamasa, Chiba, Japan), MRS2578 (Tocris Bioscience, Ellisville,MO, USA), TRIzol reagent and 10 000 U penicillin + 10 000 mg·mL^{-1} streptomycin (Invitrogen, Carlsbad, CA, USA), ReverTraAce, 5 × RT Buffer (TOYOBO), TaKaRa Ex Taq™, 10 × ExTaq Buffer, and dNTP mixture (Takara Bio Inc., Shiga, Japan) tetrabutylammonium hydrogen sulphate and ALK5i (Wako, Osaka, Japan).

Results

UDP contributes to cell migration induced by wound stimulation

To investigate the effect of nucleotides on the modulation of intestinal epithelial wound healing, we used a rat intestinal epithelial cell line (IEC-6). Because several of its characteristics are consistent with *in vivo* conditions, this cell line has been reported to be well suited for studying the process of mucosal healing (McCormack *et al.*, 1992).

First, we investigated the possible involvement of endogenous nucleotides in intestinal epithelial migration after wound formation by performing a wound healing assay. As shown in Figure 1A, scratching the epithelial cell monolayer with a pipette tip stimulated cell migration measured at 8 h. The accelerated migration was significantly ($P < 0.01$) inhibited by apyrase (30 U·mL^{-1}), an enzyme that catabolizes nucleoside triphosphates and diphosphates to monophosphates. Next, we examined the effect of P2 receptor antagonists to determine the nucleotide receptor subtype responsible for cell migration (Figure 1B). Suramin (100 μmol·L^{-1}), an antagonist of a wide spectrum of P2Y receptors but with very low affinity for P2Y$_6$, and PPADS (100 μmol·L^{-1}), an antagonist of P2X receptors, did not inhibit the wound-induced cell migration (Figure 1B). In contrast, MRS2578 (1 μmol·L^{-1}), a selective antagonist of P2Y$_6$ receptors, significantly inhibited the migration (Figure 1B). These results suggest that the cell migration stimulated by wound formation is mainly due to the activation of P2Y$_6$ receptors, which may be mediated by UDP, a selective P2Y$_6$ agonist, released from the injured epithelial cells.

UDP is released from wounded intestinal epithelial cells

We performed HPLC to analyse the amount of extracellular nucleotides in the culture medium after wound formation. The concentration of nucleotides released into the medium at resting state (without wound stimulation) was almost negligible with our HPLC system (Figure 2B). ATP and UDP, but not ADP or UTP, were detected in the culture medium

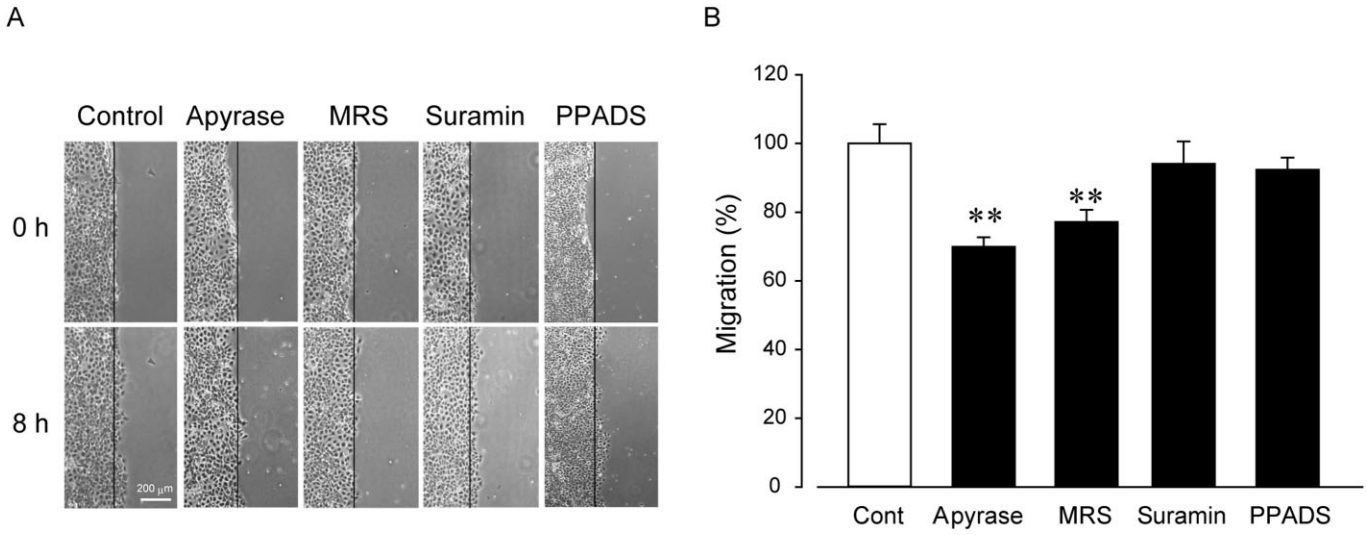

Figure 1

Wound stimulation enhances intestinal epithelial migration through $P2Y_6$ receptors. (A) The confluent monolayer of IEC-6 cells was pretreated with apyrase (30 U·mL^{-1}), MRS2578 (1 μmol·L^{-1}), suramin (100 μmol·L^{-1}) or PPADS (100 μmol·L^{-1}) for 30 min before being scratched with a pipette tip. The monolayer was photographed immediately (0 h) and 8 h after the scratch formation. (B) After 8 h, the number of cells that crossed the wounded area was counted with the ImageJ software at three different locations in each experiment. The values are expressed as a percentage compared with the control. Data are means ± SEM from four independent experiments. $^{**}P < 0.01$ as compared with the control.

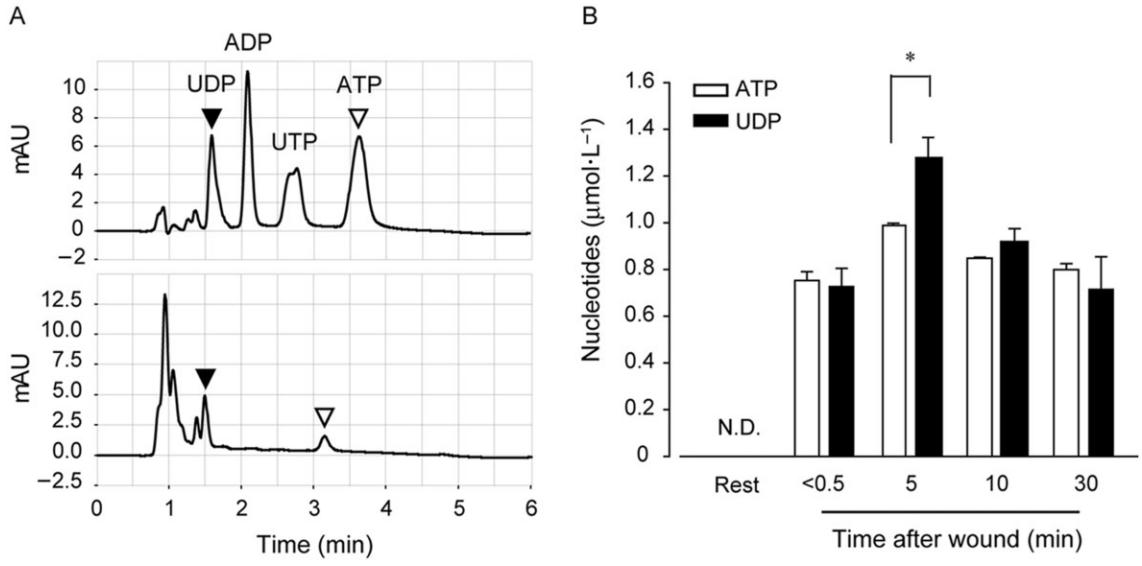

Figure 2

UDP is released into culture medium after wounding. (A) Typical reverse-phase HPLC graph showing the presence of standard nucleotides (ATP, ADP, UTP and UDP: 5 μmol·L^{-1}) (upper panel). Lower panel shows the HPLC pattern of the medium of injured cells. The signal peaks of UDP and ATP were detected at 1.5 and 3.0–3.5 min after injection of the nucleotides respectively. (B) The concentration of the nucleotides in the medium after cell scratch formation at the indicated time. The data are expressed as means ± SEM obtained from four independent experiments. $^{*}P < 0.05$ as compared with ATP.

collected just after implementation of the scratch (<0.5 min; Figure 2A). The concentrations of both ATP and UDP peaked 5 min after the wound formation (Figure 2B) and then time-dependently decreased. The peak concentration of UDP (1.44 ± 0.01 μmol·L^{-1}) was significantly higher than that of ATP (1.10 ± 0.9 μmol·L^{-1}).

UDP enhances the intestinal epithelial cell migration via $P2Y_6$ receptor

In the wound-healing assay, among the nucleotides exogenously applied to the cells (ATP, ADP, UTP and UDP), only UDP enhanced cell migration due to cell wounding at 8 h

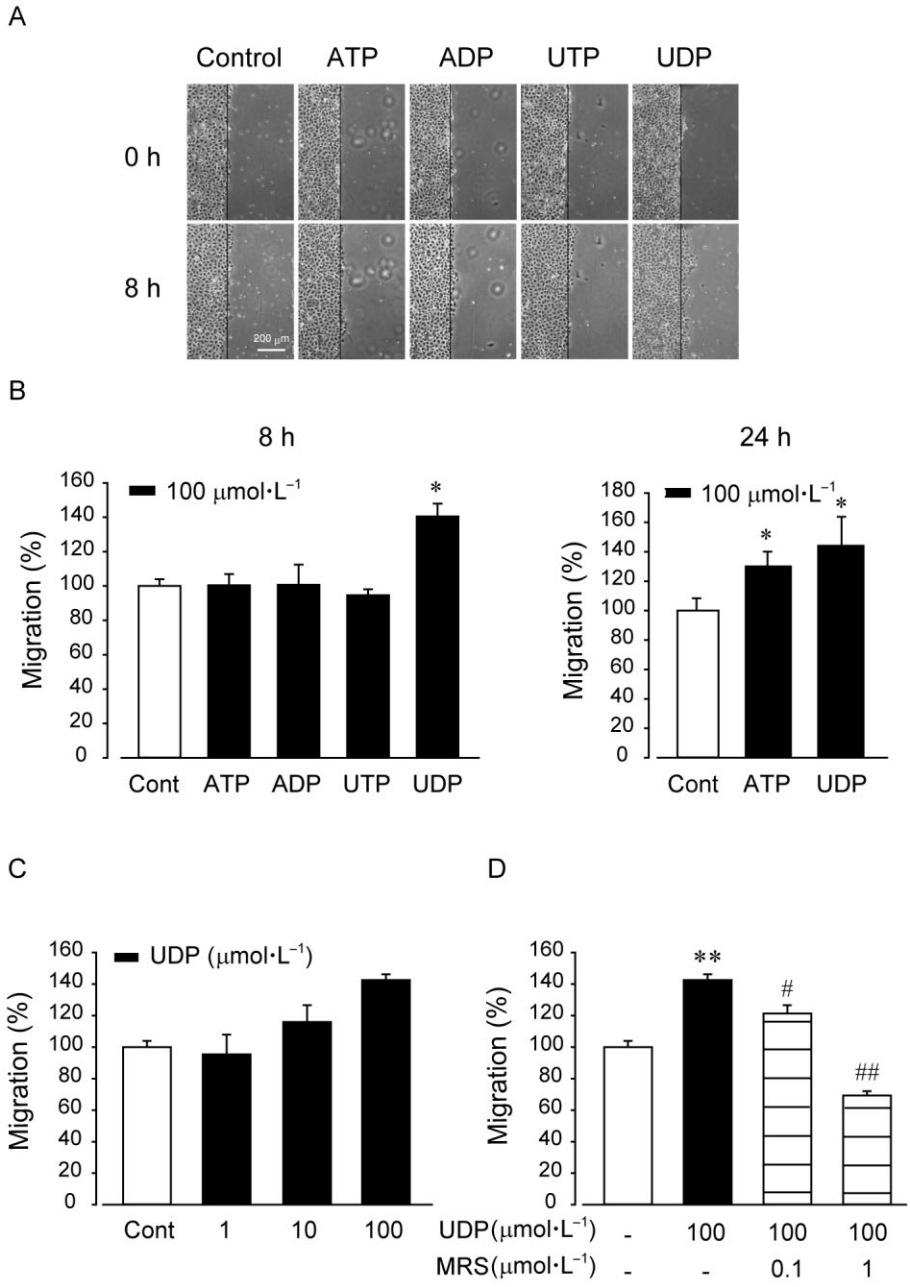

Figure 3

UDP enhances cell migration in both the trans-well and wound-healing assays. (A) A confluent monolayer of IEC-6 cells was scratched with a pipette tip. After being washed three times, each nucleotide (ATP, ADP, UTP and UDP: 100 μmol·L^{-1}) was added to the wounded monolayer. The monolayer was photographed immediately after scratch formation (0 h) (upper panel) and 8 h after scratch formation (lower panel). (B) The number of cells that crossed the wounded area at 8 h (left panel) or 24 h (right panel) was counted at three different places in each experiment. (C) UDP (1–100 μmol·L^{-1}) was added to the scratched monolayer of IEC-6 cells. After 8 h, the number of cells that crossed the wounded area was counted at three different places in each experiment. (D) After incubation with MRS2578 (a P2Y$_6$ antagonist: 0.1–1 μmol·L^{-1}) for 30 min, UDP (100 μmol·L^{-1}) was added to the scratched cell monolayer. Migration was evaluated as a percentage compared with the control. Values are expressed as means ± SEM from four independent experiments. *P < 0.05, **P < 0.01 as compared with the control. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 as compared with 100 μmol·L^{-1} UDP.

(Figure 3A,B, left panel). At 24 h, both UDP and ATP enhanced cell migration (Figure 3B, right panel). The wound-stimulated cell migration at 8 h was dose-dependently increased by UDP (1–100 μmol·L^{-1}; Figure 3C), and the enhanced migration induced by 100 μmol·L^{-1} UDP was inhibited by MRS2578 (0.1–1 μmol·L^{-1}) in a dose-dependent manner (Figure 3D). We next examined the effect of nucleotides exogenously applied to the cell by using a trans-well

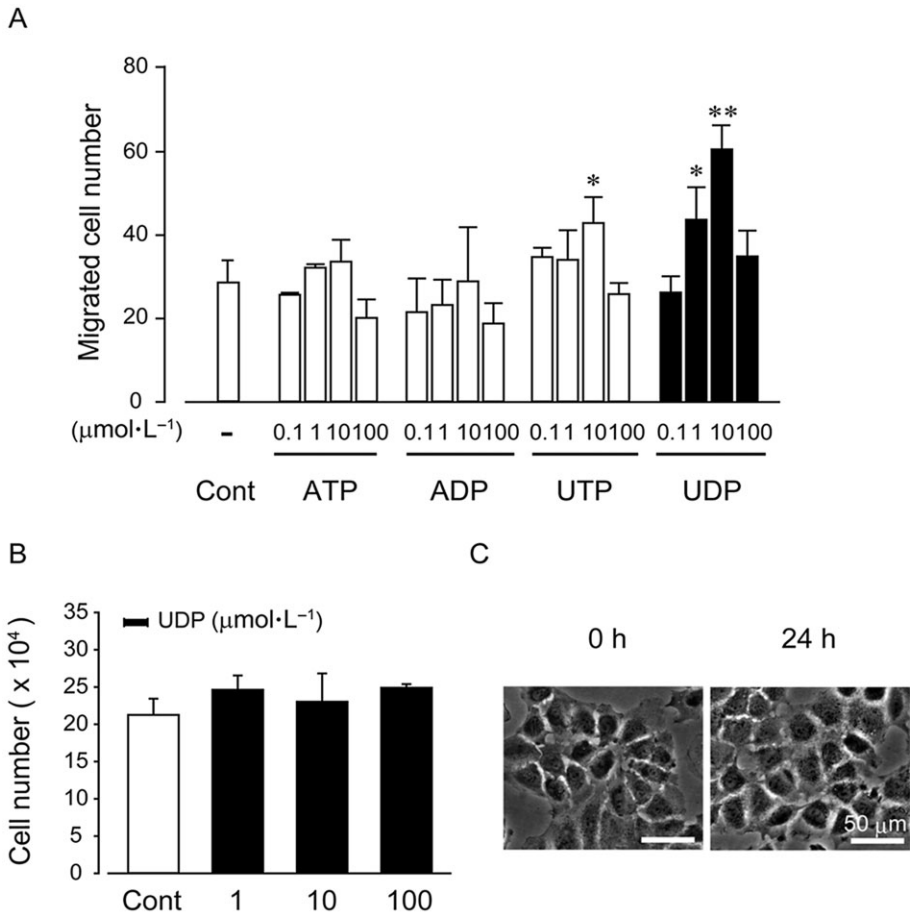

Figure 4

UDP dose-dependently induces intestinal cell migration, but not proliferation, via $P2Y_6$ receptors. (A) A trans-well migration assay was performed by using a Boyden chamber. Serum-starved IEC-6 cells (5×10^4) were seeded on the insert wells, and the indicated concentrations of nucleotides were added to the upper and lower wells. After 8 h, the number of cells that migrated from the upper to the lower wells was counted with the ImageJ software. (B) 1×10^5 cells were seeded onto the 12-well plates and then serum-starved for 24 h after becoming adherent. After 24 h of incubation with 1–100 μmol·L^{-1} UDP, the cell number was counted with a cytometer. (C) Cell morphology before (left) and 24 h after stimulation with UDP (100 μmol·L^{-1}) (right). The data are expressed as means ± SEM from at least four independent experiments. *$P < 0.05$, **$P < 0.01$ as compared with the control.

migration assay (without cell wounding). Application of UTP (10 μmol·L^{-1}) or UDP (1 and 10 μmol·L^{-1}) enhanced cell migration (Figure 4A). The effect of 10 μM UTP was comparable to that of 1 μmol·L^{-1} UDP. We also observed that UDP (1–100 μmol·L^{-1}) did not either increase cell proliferation or change the morphology of the cells, even at 24 h (Figure 4B,C). These results suggest that UDP plays a key role in promoting the migration (restitution) of intestinal epithelium via activation of $P2Y_6$ receptors.

ATP, but not UDP, increases intracellular Ca^{2+} concentration

The $P2Y_6$ receptor is coupled to G_q protein (Lazarowski *et al.*, 2001). To investigate whether a change in $[Ca^{2+}]_i$ is involved in the induction of cell migration, we measured the change in $[Ca^{2+}]_i$ in fura-2-loaded IEC-6 cells. As shown in Figure 5, UDP (10 and 100 μmol·L^{-1}) did not significantly increase the $[Ca^{2+}]_i$ (AUC: 0.77 ± 0.25, 566.9 ± 188.4 nmol·L^{-1}·s·10^3), but ATP did significantly increase the $[Ca^{2+}]_i$ (100 μmol·L^{-1}, AUC: 2823.4 ± 939.2). These results suggest that UDP-induced cell migration is unrelated to changes in $[Ca^{2+}]_i$.

UDP/$P2Y_6$-induced migration is mediated by TGF-β

TGF-β and EGF are crucial cytokines for intestinal epithelial migration (Dignass and Podolsky, 1993; Myhre *et al.*, 2004). Consistent with previous findings, TGF-β (10 ng·mL^{-1}) enhanced the wound-induced migration, and this effect of TGF-β was inhibited by ALK5i (10 μmol·L^{-1}), a TGF-β receptor inhibitor. Pretreatment with ALK5i (10 μmol·L^{-1}) also suppressed the wound-induced migration (to 61.3% ± 5.1%; Figure 6A). In the presence of ALK5i, UDP (100 μmol·L^{-1}) failed to enhance migration. These results suggest that the enhanced migration induced by UDP is mediated by TGF-β. In support of this result, we found that the expression of TGF-β mRNA and protein was increased by UDP (100 μmol·L^{-1}) or wound stimu-

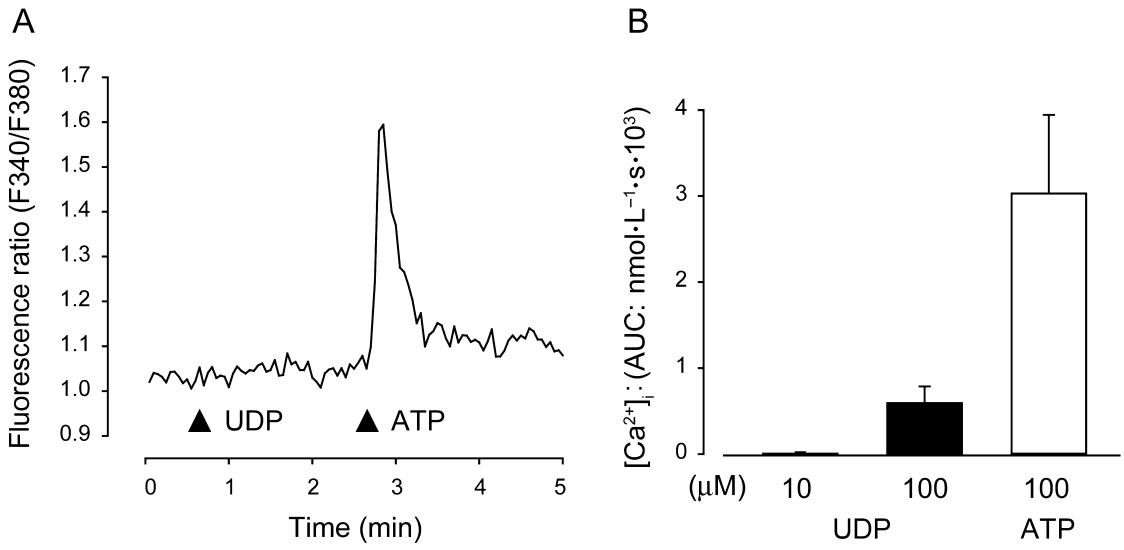

Figure 5

UDP did not increase $[Ca^{2+}]_i$ in IEC-6 cells. (A) Representative tracing of the change in fluorescence ratio (F340/F380) in IEC-6 cells treated with UDP (10 μmol·L^{-1}) followed by ATP (100 μmol·L^{-1}). (B) Analytical data of changes in $[Ca^{2+}]_i$ (AUC: 0–1 min) treated with UDP (10, 100 μmol·L^{-1}) and ATP (100 μmol·L^{-1}). Data are expressed as means ± SEM from three independent experiments.

lation (Figure 6B,C). Moreover, the secretion of TGF-β induced by UDP was inhibited by MRS2578 (1 μmol·L^{-1}) (Figure 6C). Another candidate cytokine for epithelial migration is TGF-α, which is a member of the EGF family (Myhre *et al.*, 2004). Pretreatment with AG1478 (100 nmol·L^{-1}), an EGF receptor inhibitor, inhibited the wound-induced migration (to 80.2% ± 6.7%; Figure 6A), indicating the partial involvement of EGF signalling in wound-stimulated migration. However, this inhibitor did not suppress the UDP-induced migration (114.7% ± 1.3%; Figure 6A). Furthermore, UDP did not affect the mRNA expression of TGF-α (Figure 6B). These results suggest that UDP/P2Y$_6$-induced migration is mediated by a TGF-β-dependent pathway.

The P2Y$_6$ receptor is up-regulated by UDP via TGF-β production

Under resting conditions, P2Y$_6$ receptor mRNA expression in IEC-6 cells was very low. However, the expression of this receptor was significantly increased in wounded cells, and this up-regulation was inhibited by pretreatment with MRS2578 (1 μmol·L^{-1}) and ALK5i (10 μmol·L^{-1}) (Figure 7). These results suggest that the wound-induced up-regulation of P2Y$_6$ receptors is mediated by UDP and also by TGF-β. Consistent with these observations, treatment of the cell with TGF-β (10 ng·mL^{-1}) or UDP (100 μmol·L^{-1}) increased the expression of P2Y$_6$ receptors, and the UDP-enhanced receptor expression was completely inhibited by ALK5i (10 μmol·L^{-1}). These results suggest that UDP up-regulates P2Y$_6$ receptors through *de novo* synthesis of TGF-β in wounded epithelium.

Discussion

Previous reports have shown that P2Y$_2$ signalling mediated by extracellular nucleotides plays an important role in intestinal epithelial wound healing and it was shown that ATP or UTP accelerated the epithelial migration without proliferation within a 24 h observation period (Dignass *et al.*, 1998; Degagne *et al.*, 2013). Consistent with these findings, we confirmed that ATP accelerates epithelial migration without inducing proliferation or any morphological changes after 24 h, which are an indication of cytotoxicity, in a wound-healing assay (Figure 3B,C). In addition to the enhancement of migration at 24 h, we found that UDP, but not ATP, induced intestinal migration after 8 h in the wound-healing assay (Figure 3B), and UDP enhanced cell migration more effectively than ATP in a trans-well migration assay (Figure 4A). We also detected UDP at a higher concentration than ATP after the cell injury (Figure 2), and propose that UDP accelerates cell migration through P2Y$_6$ receptor activation (see Figure 8). These findings suggest that UDP released from injured epithelial cells is another important mediator in intestinal epithelial restitution.

Our preliminary data suggested that the amounts of ATP and UDP within intact cells were almost the same (unpublished data). However, we found that the concentration of the UDP released 5 min after cell injury was higher than that of ATP (Figure 2). This phenomenon can be explained from the previous results showing that the hydrolysis of UDP occurs more slowly than that of ATP (Nicholas *et al.*, 1996).

Cytosolic-free Ca^{2+} is an important factor in regulating epithelial migration (Rao *et al.*, 2008). As UDP activates G_q-protein-coupled P2Y$_6$ receptors, we investigated whether UDP induces cell migration by increasing $[Ca^{2+}]_i$ (Alexander *et al.*, 2011). Contrary to our expectations, 10–100 μmol·L^{-1} UDP did not significantly increase $[Ca^{2+}]_i$, but markedly induced cell migration (Figures 3B, 4A and 5B), indicating that the P2Y$_6$ receptor in intestinal epithelial cells may not be coupled to G_q protein. These results are consistent with

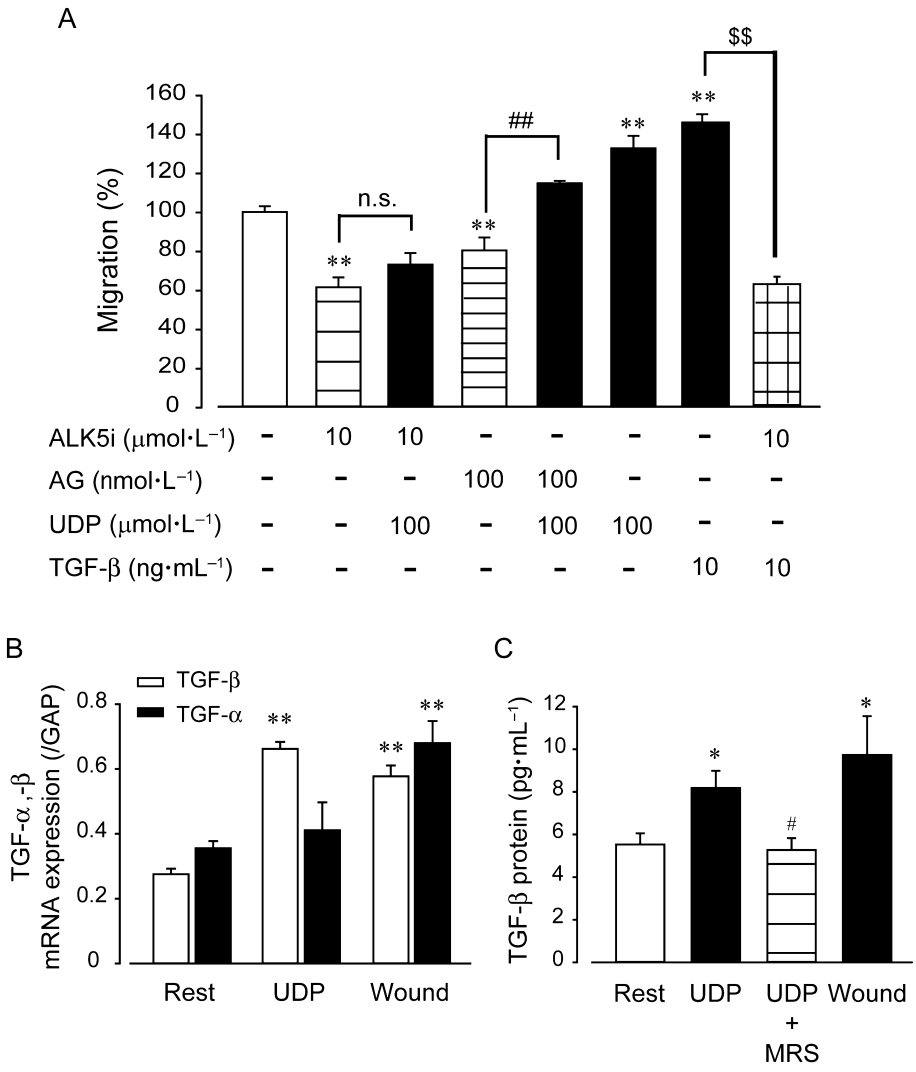

Figure 6

UDP/P2Y$_6$-induced cell migration is mediated by a TGF-β-dependent pathway. (A) Confluent IEC-6 cells were incubated with an ALK5Inhibitor (ALK5i) (a TGF-β receptor blocker: 10 μmol·L^{-1}) or AG1478 (an EGF receptor blocker: 100 nmol·L^{-1}) for 30 min. The monolayer was photographed immediately after scratch formation (0 h) and 8 h after scratch formation, with or without 100 μmol·L^{-1} UDP or TGF-β (10 ng·mL^{-1}). Migration was evaluated as a percentage by counting the number of cells across the wounded area. Values are expressed as means ± SEM from four independent experiments. $^{**}P < 0.01$ as compared with the control. $^{\#\#}P < 0.01$ as compared with 100 nmol·L^{-1} AG1478. $^{\#\#}P < 0.01$ as compared with 100 μmol·L^{-1} UDP. $^{\$\$}P < 0.01$ as compared with 10 ng·mL^{-1} TGF-β. (B) Monolayer of IEC-6 cells was stimulated by 100 μmol·L^{-1} UDP or cell wounding for 4 h. TGF-β or TGF-α mRNA expression levels are expressed as ratios to GAPDH mRNA. Values are expressed as mean ± SEM from four independent experiments. $^{**}P < 0.01$ as compared with resting cells. (C) A monolayer of IEC-6 cells was stimulated by 100 μmol·L^{-1} UDP or cell wounding, and the samples were collected after 6 h. TGF-β levels were measured with ELISA. Values are expressed as mean ± SEM from four independent experiments. $^{**}P < 0.01$ as compared with resting cells. $^{\#}P < 0.05$ as compared with UDP (100 μmol·L^{-1}).

previous findings showing that P2Y$_6$ activation does not mediate $[Ca^{2+}]_i$ elevation but is linked to cAMP elevation in colonic epithelium (Kottgen *et al.*, 2003). In this study, we further examined the effect of a PKA inhibitor (H89) on UDP-induced migration, but we failed to observe any effects (unpublished data). It has been reported that cAMP-elevating agents decrease rather than increase intestinal epithelial cell migration (Zimmerman *et al.*, 2012). In cardiomyocytes, P2Y$_6$ signalling activates G$\alpha_{12/13}$, which triggers the up-regulation of TGF-β expression (Nishida *et al.*, 2008). Thus, intestinal epithelial P2Y$_6$ may also be a G$_{12/13}$-protein-coupled receptor. Further investigations are needed to clarify this mechanism.

ATP released from injured corneal epithelial cells enhances migration via indirect activation of the EGF receptor (Klepeis *et al.*, 2004; Yin *et al.*, 2007). Contrary to this, we observed that the migration induced by UDP/P2Y$_6$-receptors was not inhibited by an EGF receptor inhibitor (AG1478) (Figure 6A), and UDP stimulation did not increase the expression of TGF-α mRNA (Figure 6B). In intestinal epithelial cell migration, TGF-β is a major cytokine that promotes migra-

tion (Xian *et al.*, 2002; Myhre *et al.*, 2004). In agreement with these reports, TGF-β enhanced the wound-induced migration and this effect was suppressed by ALK5i (TGF-β receptor inhibitor) (Figure 6A). We also found that the wound-induced migration was inhibited by ALK5i and that UDP failed to enhance wound-induced migration in the presence of ALK5i. These results suggest that UDP may activate cell migration indirectly through TGF-β signalling.

Another important finding of this study is that the expression of P2Y$_6$ receptors was increased in the wounded cells.

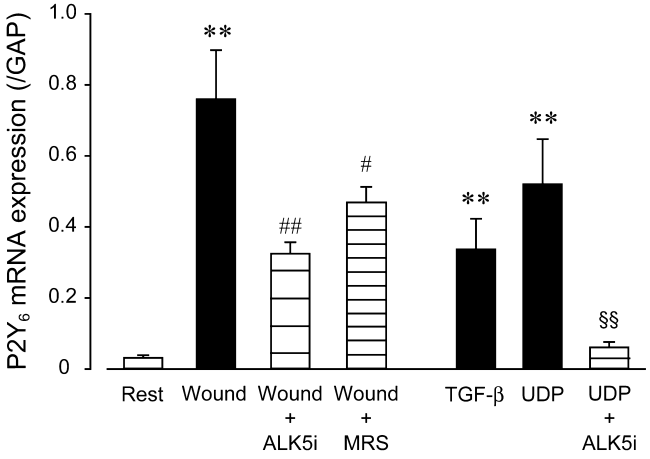

Figure 7

P2Y$_6$ receptor expression is increased by UDP/TGF-β signalling. Confluent IEC-6 cells were incubated with or without ALK5i (a TGF-β receptor blocker: 10 μmol·L^{-1}) or MRS2578 (a P2Y$_6$ antagonist: 1 μmol·L^{-1}) for 30 min. Cells were then stimulated with UDP (100 μmol·L^{-1}), TGF-β (10 ng·mL^{-1}) or scratching. After 4 h, the mRNA of the cells was extracted and translated into cDNA. The mRNA expression level is expressed as a percentage of the GAPDH mRNA level. Values are expressed as means ± SEM from four independent experiments. $^{**}P < 0.01$ as compared with resting cells. $^{\#\#}P < 0.01$ as compared with wounded cells. $^{\S\S}P < 0.01$ as compared with UDP (100 μmol·L^{-1}).

Additionally, exogenously applied UDP and TGF-β increased the expression of P2Y$_6$ receptor mRNA, and the UDP-induced response was suppressed by a TGF-β inhibitor (Figure 7). These results suggest that UDP increases P2Y$_6$ receptor expression through TGF-β secretion in wounded epithelial cells, creating a positive-feedback loop between UDP and TGF-β signalling.

In the trans-well migration assay, the concentration–response curve for the nucleotides was bell-shaped (Figure 4A). A similar phenomenon has been observed previously in a trans-well migration assay but not in the wound-migration assay (Klepeis *et al.*, 2004). P2Y$_6$-receptor activation has been reported to exhibit desensitization after long-term treatment with UDP (Brinson and Harden, 2001). Such receptor down-regulation is also well known for other purine receptors (Burnstock, 1990). Thus, the highest concentration of UDP induced less migration in the trans-well migration assay in this study.

Recently, the results from several studies have suggested that the activation of P2Y$_6$ by UDP plays an important role in inflammatory reactions. For instance, the UDP/P2Y$_6$ receptor system has been shown to act as a sensor to initiate phagocytosis by microglia in the CNS (Koizumi *et al.*, 2007). UDP was also found to enhance MCP-1 secretion from the intestinal epithelium, which modulates the recruitment of monocytes/macrophages (Zhang *et al.*, 2011). In addition, P2Y$_6$ receptors are up-regulated in activated macrophages, while their expression is kept at a very low level in resident macrophages (Bar *et al.*, 2008). Moreover, P2Y$_6$ receptors are up-regulated in intestinal tissue from patients with inflammatory bowel disease (Grbic *et al.*, 2008). These data and our present findings suggest that UDP/P2Y$_6$ signalling is important for the homeostasis of the intestinal epithelial barrier in both physiological and inflammatory conditions.

In summary, we demonstrated that UDP released from injured cells enhances the intestinal epithelial restitution through the activation of P2Y$_6$ receptors and this effect is mediated by *de novo* synthesis of TGF-β. In addition, UDP-induced production of TGF-β increased the expression of P2Y$_6$ receptors, indicating the presence of a positive-feedback loop mechanism for mucosal repair (Figure 8).

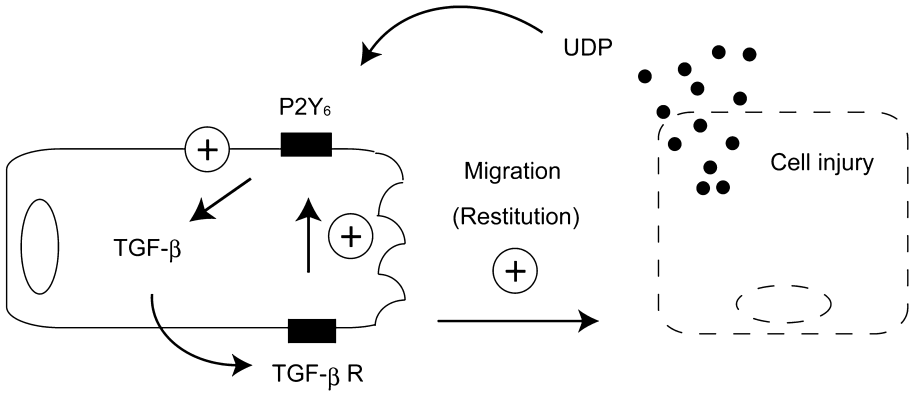

Figure 8

Proposed pathway for the cell restitution induced by UDP/TGF-β signalling. UDP released from wounded cells affects neighbouring cells to induce *de novo* synthesis of TGF-β through activation of the P2Y$_6$ receptor. Up-regulated P2Y$_6$ receptors promote UDP-induced responses (positive-feedback loop). UDP and TGF-β cooperate with each other to induce migration and contribute to the intestinal epithelial wound healing.

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Conflict of interest

The authors have no conflicts of interest.

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