Cell surface F$_1$/F$_0$ ATP synthase contributes to interstitial flow-mediated development of the acidic microenvironment in tumor tissues

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Kawai Y, Kaidoh M, Yokoyama Y, Ohhashi T. Cell surface F$_1$/F$_0$ ATP synthase contributes to interstitial flow-mediated development of the acidic microenvironment in tumor tissues. Am J Physiol Cell Physiol 305: C1139–C1150, 2013. First published September 25, 2013; doi:10.1152/ajpcell.00199.2013.—To address pivotal roles of cell surface F$_1$/F$_0$ ATP synthase in the development of acidic microenvironment in tumor tissues, we investigated effects of shear stress stimulation on the cultured human breast cancer cells, MDA-MB-231 and MDA-MB-157, or human melanoma cells, SK-Mel-1. Shear stress stimulation (0.5–5.0 dyn/cm$^2$), the levels of which are similar to those produced by the interstitial flow, induced shear-stress-dependent corelease of ATP and H$^+$ from the cells, which triggered CO$_2$ gas excretion. In contrast, the same level of shear stress stimulation did not induce significant ATP release and CO$_2$ gas excretion from the control human mammary epithelial cells (HMEC). Marked immunocytochemical and mRNA expression of cell surface F$_1$/F$_0$ ATP synthase, vacuolar-ATPase (V-ATPase), carbonic anhydrase type IX, and ectonucleoside triphosphate diphosphohydrolase (ENTPDase) 3 were detected in MDA-MB-231 cells, but little or no expression on the HMEC. Pretreatment with cell surface F$_1$/F$_0$ ATP synthase inhibitors, but not cell surface V-ATPase inhibitors, caused a significant reduction of the shear stress stimulation-mediated ATP release and CO$_2$ gas excretion from MDA-MB-231 cells. The ENTPDase activity in the shear stress-loaded MDA-MB-231 cell culture medium supernatant increased significantly in a time-dependent manner. In addition, MDA-MB-231 cells displayed strong staining for purinergic 2Y1 (P2Y1) receptors on their surfaces, and the receptors partially colocalized with ENTPDase 3. These findings suggest that cell surface F$_1$/F$_0$ ATP synthase, but not V-ATPase, may play key roles in the development of interstitial flow-mediated acidic microenvironment in tumor tissues through the shear stress stimulation-induced ATP and H$^+$ corelease and CO$_2$ gas production.

MDA-MB-231: corelease of ATP and H$^+$; piceatannol; haloflomycin A; CO$_2$ gas excretion; carbonic anhydrase IX, ATPase

IN THE PAST, most cancer research was based on genetics or biochemistry. In such studies, it was found that genes turn stimulatory chemical signals and protein cascades on or off in carcinoma cells (4). However, the crucial roles played by physical factors in the development of the tumor microenvironment were largely ignored. Recently, it was clearly demonstrated that primary tumors influence the tumor tissue microenvironment and microcirculation prior to carcinoma cell metastasis (2, 17, 19). One of the pathophysiological changes observed in the primary tumor microenvironment is the development of acidic tumor tissue. The high glucose consumption and lactic acid production rates of carcinoma cells are known to be key factors for the development of acidic tumor microenvironments (13). On the other hand, several sophisticated molecular mechanisms are responsible for maintaining the alkaline pH and the acidic pH in tumor cells (36). These include proteins that import weak bases such as HCO$_3^-$ into the cells and proteins that export the weak acids generated during metabolism such as carbonic acid or lactic acid out of the cells (9). H$^+$ ions are also directly extruded from the cells by means of the vacuolar ATPase (V-ATPase) (22, 32). Thus, the previous studies demonstrated that the V-ATPase were functionally expressed in cell surfaces of MDA-MB-231 human breast cancer cells and then contributed to the invasion of carcinoma cells in tumor tissues (15). However, as far as we know, no study has evaluated the effects of mechanical forces such as shear stress on the development of acidic tumor microenvironment.

Shear stress is the mechanical force that is physiologically or pathophysiologically generated by the flow of blood, lymph, or interstitial fluid through the cardiovascular system. Upon detecting shear stress, endothelial cells in blood or lymph vessels transmit signals to their interiors, where they trigger responses, including changes in a variety of cell functions (5, 8, 37). Thus initial mechanotransduction responses to shear stress appear to involve calcium influx (28), production of prostaglandins (38), and nitric oxide (NO) (11), and regulation of matrix metalloproteinases (MMPs) (29). In addition, it is also well established that blood vessel and lymphatic endothelial cells release endogenous ATP via the activation of cell surface F$_1$/F$_0$ ATP synthase (20, 37). However, no studies except for our previous study (21) have evaluated the physiological function and relevance of the H$^+$ that is coreleased from endothelial cells through the shear stress stimulation-mediated activation of cell surface F$_1$/F$_0$ ATP synthase. Based on the findings we obtained in the latter study, we proposed a new mechanism for CO$_2$ exchange in the human lung, flow-mediated F$_1$/F$_0$ ATP synthase-dependent H$^+$ secretion, resulting in the facilitation of a dehydration reaction involving HCO$_3^-$ in plasma and the excretion of CO$_2$ gas from human pulmonary arteriolar endothelial cells.

However, the concept has been widely accepted that carcinoma cells in tumor tissues are most responsive to interstitial pressure and rigid matrix-generated stretch, and thus the influences of shear stress would seem to be of little concern in the cells (30). In contrast, if one considers the forces produced by the flow of interstitial fluid outside of the tumor tissues, then shear stress forces may in fact be an important factor in the development of tumor microenvironments.

Therefore, to address the pivotal roles of cell surface F$_1$/F$_0$ ATP synthase on carcinoma cells in the interstitial flow-mediated acidic microenvironment in tumor tissues, we attempted to investigate the effects of shear stress stimulation on ATP release and CO$_2$ gas excretion from human breast cancer cells, MDA-MB-231 and MDA-MB-157, or human...
melanoma cell, SK-Mel-1; 2) to evaluate the protein expression levels of cell surface F1/F0 ATP synthase, cell surface V-ATPase, and cell surface carbonic anhydrase (CA) IX, a cancer cell marker (36), in the carcinoma cells; 3) to study the effects of cell surface F1/F0 ATP synthase inhibitors, piceatannol and angiotatin, or cell surface V-ATPase inhibitors, omeprazole and bafilomycin A, on the shear stress stimulation-induced ATP release and CO2 gas excretion from MDA-MB-231 carcinoma cells; 4) to study the effects of shear stress stimulation on the concentration of lactic acid in the cells’ culture media; and finally 5) to evaluate the effects of shear stress stimulation on the concentration of lactic acid in the cells’ culture media.

**MATERIALS AND METHODS**

**Cell culture.** The human breast adenocarcinoma cell lines MDA-MB-231 and MDA-MB-157, human melanoma cell line SK-Mel-1, and human mammary epithelial cells (HMEC) were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco’s modified Eagle’s medium/Nutrient Mixture F12 Ham (DMEM/F12) culture medium supplemented with 10% fetal bovine serum (FBS). The cells were incubated under normoxic conditions of 21% O2, 5% CO2, and 74% N2 at 37°C.

**Flow-loading experiments.** A parallel plate-type apparatus was used to apply laminar shear stress to the carcinoma cells and HMEC using a slightly modified version of Yamamoto’s method (37). Briefly, one side of the flow chamber consisted of a 1% gelatin-coated glass plate, on which the cultured (37°C, DMEM/F12 supplemented with 1% FBS) carcinoma cells and HMEC rested, and the other side consisted of a polycarbonate plate. These two surfaces were held 200 μm apart with a Teflon gasket. The chamber contained an entrance, which was connected to an upper reservoir by a silicon tube, and an exit for the fluid, which led to a lower reservoir. The flow of the fluid was driven by a peristaltic pump with a flow rate controller. The viscosity of the perfusate (poise), Q is the flow volume (in ml/s), and r the viscosity of the perfusate (poise). Q is the flow volume (in ml/s), and a and b are the cross-sectional dimensions of the flow path (in cm).

**ATP assay.** The ATP concentrations of the carcinoma cells and HMEC culture medium supernatants were determined using a luciferin-luciferase assay based on the Cell Titer-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), as described previously (20). Briefly, to develop a calibration curve for ATP measurement, we first performed the same luciferin-luciferase assay using culture medium containing different concentrations of ATP. Next, 100 μl of each flow-stimulated carcinoma cell supernatant was collected in a 96-well plate, into which 100 μl of luciferin-luciferase solution had been added, and the light emission of each well was recorded using a luminometer (Dainippon Sumitomo Pharma, Osaka, Japan). We used the resultant calibration curve to calculate the ATP concentrations of the flow-stimulated carcinoma cell culture media.

In addition, we investigated the 30-min pretreatment effects of cell surface F1/F0 ATP synthase inhibitors, piceatannol (10–20 μM) and angiotatin (1–10 μM), or cell surfaces V-ATPase inhibitors, omeprazole (10 μM) and bafilomycin A (10 μM), on the shear stress stimulation-mediated release of ATP from the carcinoma cells.

**ATPase activity assay.** To evaluate the hydrolytic activity of ATPases in MDA-MB-231 carcinoma cell and HMEC supernatants, we developed a new method for measuring ATPase activity (21) involving cell supernatants and the luciferin-luciferase assay that was used for the aforementioned ATP measurement. Six hundred microliters of the flow-loaded or unloaded carcinoma cell or HMEC supernatant were collected in a polyethylene tube, into which 600 μl of DMEM/F12 culture medium without FBS containing a constant concentration (10−7 M) of exogenous ATP had been added, and the mixture was kept at 37°C throughout the experiment. At 1, 20, 40, and 60 min of incubation, 100 μl of the resultant solution, which contained no cultured carcinoma cells or HMEC, was collected in a 96-well plate, into which 100 μl of luciferin-luciferase solution had been added, and the light emission values of each well were recorded using a luminometer and used to calculate their ATP concentrations. The decrease in the ratio of the total ATP concentration to the concentration of added ATP enabled us to evaluate the level of ATP hydrolysis in the solution. The viability and reproducibility of our method were established using apyrase, a selective enzyme that hydrolyzes ATP (25). As expected, apyrase dose dependently hydrolyzed the ATP in the solution, suggesting that our new method is capable of precisely evaluating the ATPase activity in the above-mentioned mixtures (21).

**CO2 gas excretion assay.** To evaluate the effects of shear stress stimulation on the excretion of CO2 gas from the carcinoma cells, we shielded a specialized culture chamber and then measured the concentration of CO2 gas using a gas sampling pump kit (GASTEC, Ayase, Japan). The carcinoma cells cultured in the shielded chamber (volume: 250 ml) were subjected to laminar shear stress (1.0 or 5.0 dyn/cm2) for a short period (10 s) using the above-mentioned flow-loading apparatus.

In addition, we evaluated the 30-min pretreatment effects of cell surface F1/F0 ATP synthase inhibitor, piceatannol (10–20 μM), on the shear stress stimulation-induced CO2 gas excretion from the carcinoma cells.

**Lactic acid measurement.** The lactic acid concentrations of MDA-MB-231 carcinoma cell and HMEC culture medium supernatants were measured using a blood lactic acid test meter (ARKLAY).

**Measurement of pH.** To evaluate the effects of shear stress stimulation (1.0 dyn/cm2, 24-h stimulation) with or without a cell surface F1/F0 ATP synthase inhibitor, piceatannol (10 μM), on the changes in the pH of the MDA-MB-231 carcinoma cells culture medium supernatants, we quickly collected 0.2 ml of each supernatant in the culture dishes and then measured its pH using a pH meter (RADIOMETER, Tokyo, Japan).

**Immunocytochemistry.** The cultured carcinoma cells and HMEC were subjected to immunocytochemical analysis. In brief, the carcinoma cells or HMEC were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then were or were not permeabilized. Next, the cells were washed three times with PBS and then incubated overnight at 4°C in primary polyclonal mouse antiserum to F1/F0 ATP synthase (dilution 1:50; Millipore, Billerica, MA), V-ATPase (dilution 1:50; Enzo Life Sciences, New York, NY), carbonic anhydrase type IX (CA IX; dilution 1:50; R&D Systems, Minneapolis, MN), the purinergic 2Y1 receptor (P2Y1; dilution 1:50; Almone Labs, Jerusalem, Israel), or ectonucleoside triphosphate diphosphohydrolase 3 (ENTPDase 3; dilution 1:50; Lifespan Biosciences). After being washed three times in PBS, the cells were incubated for 1 h at room temperature with 1:100 diluted Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 488 chicken anti-rabbit IgG, Alexa Fluor 488 donkey anti-mouse IgG, or Alexa Fluor 594 donkey anti-mouse IgG (Invitrogen, Carlsbad, CA). Next, the nuclei of the cultured cells were counterstained and mounted with Pro Long Gold antifade reagent and DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen), examined with a
fluorescent or phase-contrast microscope (Leica Microsystems, Wetzlar, Germany), and photographed.

For non-specific staining, the primary antibodies were omitted as a negative control.

**Quantitative RT-PCR.** The mRNA expression levels of CA IX, ENTPDase 3, and P2Y1 were evaluated using quantitative RT-PCR. Total RNA was extracted from the cultured carcinoma cells using the ISOGEN reagent (Nippon Gene, Toyama, Japan), according to the manufacturer’s instructions. Then, the concentration of each RNA was calculated from its absorbance at 260 nm, which was measured with a spectrophotometer. The extracted RNA was then reverse-transcribed with M-MLV reverse transcriptase (Ambion, Austin, TX). For RT-PCR analysis, each superscript first-strand synthesis kit (Invitrogen) was used together with 1.0 μg of total RNA. The following forward and reverse primers were used to produce the probes for CA IX (NM_001216.2; 149), ENTPDase 3 (NM_001248.2; 184), and P2Y1 receptor (NM_002563.2; 83) (TAKARA, Tokyo, Japan), and the cyclophilin A probe was produced using the following primers: 5'-TTCGTGCTCTGAGCACTGGAG-3' (forward) and 5'-GGACCCGTATGCCTTAGATGAA-3' (reverse). The cDNA was diluted fivefold before amplification, and quantitative RT-PCR was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics, Burgess Hill, UK). The reactions were performed in a 20-μl volume containing 0.5 μM primers, Taq DNA polymerase, and the buffer included in the SYBR Premix Ex Taq kit (TAKARA). The PCR protocol included a 10-s denaturation step followed by 45 cycles of denaturation for 5 s at 95°C and annealing for 20 s at 60°C. The fluorescent products were detected at the end of the 60°C extension period. Negative controls including the PCR reaction products produced using each primer pair were subjected to melting curve analysis. Data were analyzed with the LightCycler analysis software. The results are presented as the ratio of the expression level of each mRNA to that of cyclophilin A.

**Western blot analysis.** Western blot analysis was performed to quantitatively evaluate cell surface Fv/Fo ATP synthase or CA IX protein expression in the cultured MDA-MB-231 carcinoma cells. In the case of evaluating cell surface Fv/Fo ATP synthase or fibronectin, we used the Thermo Scientific Cell Surface Isolation Kit (Thermo Fisher Scientific, Rockford, IL) to isolate selectively cell surface proteins using a cell-impermeable, cleavable biotinylation reagent. The isolated cell membranes or the carcinoma cells were dissolved in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) and then centrifuged at 14,000 g for 10 min. A 15-μg sample of total lysate was dissolved in SDS sample buffer for SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Atto, Tokyo, Japan), where it was incubated for 45 min. The membrane was then probed with anti-Fv/Fo ATP synthase antisera (dilution 1:50, Millipore), anti-fibronectin antisera (Dilution 1:100, R&D Systems), or anti CA IX antisera (dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and incubated with anti-rabbit or anti-mouse IgG horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology). The same membranes were reprobed with monoclonal anti-actin antibody (Santa Cruz Biotechnology). All membranes were visualized with an enhanced chemiluminescence-Western blotting detection system (Amersham Biosciences, Cambridge, UK).

**Proliferation assay.** The MDA-MB-231 carcinoma cells were seeded at a density of 1.0 × 10^4 cells/96-multifwell plate and cultured using DMEM/F12 medium containing 10% FBS. After 1 day incubation, the medium was exchanged for DMEM/F12 medium containing 1% FBS (pH, 7.35 ± 0.01, n = 4) in the absence or presence of 1.0 dyn/cm^2 shear stress stimulation with or without 10^-6 M ATP, 10 μM or 20 μM piceatannol. After 24 h, the carcinoma cells were counted using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). This assay employs a colorimetric method for determining the number of viable cells. The One Solution reagent contains a novel tetrazolium (MTS) and an electron-coupling reagent (PES). The MTS tetrazolium compound is bioreduced by the cells into a colored formazan product that is soluble in culture medium. This conversion is presumably accomplished by the NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The assays were performed by adding a small amount of the reagent to the culture wells, incubating the cultures for 2 h, and then recording their absorbance at 490 nm with a 96-well plate reader. The absorbance score was strongly correlated with number of cells; i.e., the correlation coefficient for the relationship between the absorbance score and cell number in the two cell lines was 0.993.

**Drugs.** All salts were obtained from Wako (Tokyo, Japan). Apyrase, ATP, angiotatin, piceatannol, acetazolamide, omeprazole (a proton pump inhibitor), and bafilomycin A (a V-ATPase inhibitor) were purchased from Sigma (St Louis, MO). The piceatannol was diluted with ethanol. The concentration of ethanol used did not affect the biological viability of the cultured carcinoma cells. Drug concentrations are expressed as the final concentration in the culture plate.

**Statistical analysis.** All results are expressed as means ± SE. Statistical significance was analyzed using the Student’s t-test for unpaired observations or one-way ANOVA followed by Duncan’s post hoc test, as appropriate. P < 0.05 was considered statistically significant.

**RESULTS**

Shear stress-induced cell surface Fv/Fo ATP synthase-dependent ATP release from carcinoma cells. We measured the ATP concentrations of the supernatants of cultured MDA-MB-231 carcinoma cells subjected to various levels of shear stress (1.0 or 5.0 dyn/cm^2), all of which were compatible with the levels produced by the interstitial flow in tumor tissues (18) or the physiological flow rates of lymph (11). The results are summarized in Fig. 1A. Ten seconds’ stimulation of shear stress with 1.0 or 5.0 dyn/cm^2 produced a strength-dependent increase in the ATP concentration of the MDA-MB-231 cell supernatant.

To evaluate whether or not the shear stress-induced ATP release is a universal mechanism for carcinoma cells, we next examined the effects of shear stress stimulation (5.0 dyn/cm^2, 10 s) on the release of ATP in the supernatants of cultured human breast cancer cells, MDA-MB-157 and human melanoma cells, SK-Mel-1. The results are also summarized in Fig. 1A. Similar to the responses of MDA-MB-231 carcinoma cells, the shear stress stimulation caused a significant release of ATP in the MDA-MB-157 and SK-Mel-1 cell supernatants. In agreement with these findings, immunocytochemical staining demonstrated that the MDA-MB-231, MDA-MB-157, and SK-Mel-1 carcinoma cells were positive for cell surface Fv/Fo ATP synthase (Fig. 1B).

To confirm again the cell surface Fv/Fo ATP synthase protein expression, we examined quantitatively the presence of cell surface Fv/Fo ATP synthase protein on the MDA-MB-231 carcinoma cells using the selective isolation kit of cell surface protein and Western blot analysis. Figure 1D shows the representative immunoblot result of cell surface Fv/Fo ATP synthase protein expression on the MDA-MB-231 carcinoma cells. The marked immunoblot result of fibronectin expression demonstrates a representative cell surface protein of the carcinoma cells as a positive control in the kit used (Fig. 1D).

In contrast, the same level of shear stress stimulation (5.0 dyn/cm^2, 10 s) did not produce any significant increase in the ATP concentration of the cultured human mammary epithelial cell (HMEC) supernatant (Fig. 1A). In agreement with these
findings, no or little immunocytochemical staining and protein expression of cell surface F₁/FO ATP synthase was detected in the HMEC (Fig. 1, B and D).

To confirm the involvement of cell surface F₁/FO ATP synthase in shear stress stimulation-induced ATP release from MDA-MB-231 carcinoma cells, we next investigated the effects of the cell surface ATP synthase inhibitors, angiotatin (26) and piceatannol (37), on the shear stress stimulation (1.0 dyn/cm², 10 s)-mediated ATP release from the carcinoma cells. Thirty minute pretreatment with 1–10 μM angiotatin or 10–20 μM piceatannol caused a significant dose-related reduction in the shear stress stimulation-mediated ATP release from the cells (Fig. 1E). We also confirmed the pretreatment with the concentrations of inhibitors produced no significant effect on the viability of the MDA-MB-231 carcinoma cells (no treatment, 99.7 ± 0.3%; treatment, 99.7 ± 0.3%).

Next, we examined whether or not the V-ATPase activity of the carcinoma cells is involved in the shear stress stimulation-mediated ATP release from the carcinoma cells. The possibility may be supported by the immunocytochemical findings of V-ATPase in the carcinoma cells. Thus marked immunocytochemical staining of V-ATPase are observed in the MDA-MB-231, MDA-MB-157, and SK-Mel-1 carcinoma cells, but not in the HMEC (Fig. 1C). However, 30-min pretreatment with the
V-ATPase inhibitors, 10 μM omeprazole or 10 μM bafilomycin A, the concentrations of which are known to inhibit selectivity the V-ATPase in the cells (10, 27), caused no significant effect on the shear stress stimulation (1.0 dyn/cm², 10-s stimulation)-mediated ATP release from the MDA-MB-231 carcinoma cells (Fig. 1E).

Shear stress induced cell surface F₁/F₀ ATP synthase-dependent CO₂ gas excretion from the carcinoma cells. Next, we evaluated whether the H⁺ that is coreleased by the shear stress stimulation-induced activation of cell surface F₁/F₀ ATP synthase is responsible for the observed CO₂ gas excretion from the carcinoma cells. Ten seconds’ shear stress stimulation (1.0 or 5.0 dyn/cm²) induced the strength-dependent excretion of CO₂ gas in a shielded culture chamber containing MDA-MB-231, MDA-MB-157, and SK-Mel-1 carcinoma cells (Fig. 2A). In contrast, no significant CO₂ gas excretion from the HMEC was observed at the same level (5.0 dyn/cm², 10 s stimulation) of shear stress stimulation (Fig. 2A).

To evaluate the mechanisms responsible for the shear stress stimulation-induced excretion of CO₂ gas from the carcinoma cells, we examined the effects of the cell surface F₁/F₀ ATP synthase inhibitor, piceatannol; a carbonic anhydrase inhibitor, acetazolamide (6); a proton pump inhibitor, omeprazole (10); and a V-ATPase inhibitor, bafilomycin A (27), on the shear stress stimulation-induced excretion of CO₂ gas from the MDA-MB-231 carcinoma cells. Pretreatment with 10–20 μM piceatannol or 0.1–1.0 μM acetazolamide, but not 10 μM omeprazole or 10 μM bafilomycin A, caused a significant dose-related reduction in the shear stress stimulation (5.0 dyn/cm², 10 s)-induced excretion of CO₂ gas from the carcinoma cells (Fig. 2B).

To identify the type of carbonic anhydrase (CA) involved in the shear stress stimulation-induced excretion of CO₂ gas, we investigated the immunocytochemical, mRNA, and protein expression of CA IX, which is known to be a selective CA that is expressed on the surfaces of carcinoma cells (36), in the

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**Fig. 2.** Shear stress-induced cell surface F₁/F₀ ATP synthase-dependent CO₂ gas excretion from the carcinoma cells. A: effects of 10-s shear stress stimulation (1.0 or 5.0 dyn/cm²; – no stimulation) on CO₂ gas excretion from cultured MDA-MB-231, MDA-MB-157, and SK-Mel-1 carcinoma cells and cultured HMEC. The ordinate is the concentration of CO₂ gas (ppm) in the shielded culture chamber (n = 4). The negative control shows the concentration of CO₂ gas (ppm) in the room air. **P < 0.01, significant difference between each column. NS, not significant. B: effects of the cell surface F₁/F₀ ATP synthase inhibitor, 10–20 μM piceatannol (PIC), a carbonic anhydrase inhibitor, 0.1–1.0 μM acetazolamide (ACZ), and a proton pump and V-ATPase inhibitor, 10 μM omeprazole (OME), and a selective V-ATPase inhibitor, 10 μM bafilomycin A (BFM), on the 10-s shear stress stimulation (5.0 dyn/cm²)-induced CO₂ gas excretion from the MDA-MB-231 carcinoma cells. The ordinate (n = 4) and negative control are the same items as those shown in A, respectively. **P < 0.01, significant difference between each column. NS, not significant. C: representative photomicrographs of immunocytochemical stainings of cell surface carbonic anhydrase (CA) IX on the nonpermeabilized MDA-MB-231 carcinoma cells (1) and HMEC (2). The bottom photomicrograph shows the negative control for the MDA-MB-231 cells (3). The marker is 50 μm. D: the CA IX mRNA expression in the MDA-MB-231 carcinoma cells and HMEC, as evaluated by RT-PCR. The ordinate is the ratio of CA IX mRNA signal to the cyclophilin A signal (n = 4). **P < 0.01, significant difference between each column. E: representative immunoblot results of CA IX and actin protein expression in the MDA-MB-231 carcinoma cells and HMEC, as evaluated by Western blot analysis.
cultured MDA-MB-231 carcinoma cells. Marked immunocytochemical staining of cell surface CA IX was detected on the MDA-MB-231 carcinoma cells (Fig. 2C), but only weak staining was observed on the control HMEC (Fig. 2C). Similar results were obtained for the MDA-MB-231 and HMEC cells with regard to CA IX mRNA and protein expression (Fig. 2, D and E).

Shear stress did not have a significant effect on the lactic acid concentration of the carcinoma cell culture medium. Next, to evaluate whether shear stress stimulation-induced lactic acid secretion was involved in the observed CO₂ excretion from the carcinoma cells, we measured the changes in the concentrations of lactic acid in the MDA-MB-231 and HMEC cell supernatants before and after shear stress stimulation (5.0 dyn/cm², 10 s stimulation). The concentration of lactic acid in the MDA-MB-231 carcinoma cell supernatant was ~10.0 mmol/l, which was about ten times higher than that observed in the control HMEC supernatant. However, no significant changes in the lactic acid concentrations of the MDA-MB-231 or HMEC cell supernatants were observed after shear stress stimulation (Fig. 3A). In agreement with the different lactic acid concentrations displayed by the MDA-MB-231 and HMEC cells, markedly different mitochondrial F₁/F₀ ATP synthase distribution patterns were observed between the MDA-MB-231 and HMEC cells (Fig. 3B). Namely, in the HMEC the characteristic mitochondrial reticulum network was apparent, as is observed in normal endothelial cells in blood or lymph vessels (21). In contrast, the mitochondrial reticulum network had completely broken down in the MDA-MB-231 carcinoma cells (Fig. 3B). Thus the mean length of the mitochondrial network in the MDA-MB-231 carcinoma cells was significantly shorter than that in the HMEC. Quantitative data related to the above immunocytochemical findings are summarized in Fig. 3C.

Shear stress increased the enzymatic hydrolysis of ATP. To assess the pathophysiological roles of the ATP released from the carcinoma cells in response to shear stress stimulation, we first investigated the effects of shear stress stimulation on the activity of the cell surface enzymes involved in the hydrolysis of ATP in the MDA-MB-231 and HMEC cell culture media. Thus we measured the cell surface ATPase activity in the culture medium supernatants of cells that had been subjected to a shear stress of 1.0 or 5.0 dyn/cm² (60 s stimulation). In the shear stress-loaded MDA-MB-231 cell supernatants (Fig. 4A, horizontal-lined and hatched columns), the ATPase activity was significantly increased at 20 min after the shear stress stimulation and then increased in a time- and strength-dependent manner. In addition, in the shear stress-unloaded MDA-MB-231 cell supernatants (Fig. 4A, black columns), the ATPase activity also increased in a time-dependent manner, although the increases were smaller than those observed in the shear stress-loaded carcinoma cells. In contrast, no significant change in ATPase activity was observed in the shear stress-loaded or -unloaded HMEC supernatants (Fig. 4B).

In agreement with the shear stress-induced increase in ATPase activity, significant immunocytochemical staining of

![Fig. 3. Shear stress-induced no significant change in the concentration of lactic acid in the cultured medium supernatant of MDA-MB-231 cells. A: effects of 10-s shear stress stimulation (5.0 dyn/cm²; - : no stimulation) on the concentration of lactic acid in the culture medium supernatants of MDA-MB-231 carcinoma cells and HMEC. The ordinate is the concentration of lactic acid (mmol/l) in the culture medium supernatant (n = 4). **P < 0.01, significant difference between each column. NS, not significant. B: representative photomicrographs (×1,000) of immunocytochemical stainings of mitochondrial F₁/F₀ ATP synthase in the permeabilized MDA-MB-231 carcinoma cells (1) and HMEC (2). The bottom photomicrograph shows the negative control for the MDA-MB-231 cells (3). The markers represent 10 μm. C: mean length of the mitochondrial network in the MDA-MB-231 carcinoma cells and HMEC. The ordinate shows the mean length of the mitochondrial network measured using each photomicrograph (n = 8). **P < 0.01, significant difference between each column.](http://ajpcell.physiology.org/doi/figure/10.1152/ajpcell.00199.2013)
ENTPDase 3 was observed on the surfaces of the MDA-MB-231 carcinoma cells (Fig. 4C). In contrast, no or little immunocytochemical staining of ENTPDase 3 was observed on the HMEC (Fig. 4C). Similarly, the ENTPDase 3 mRNA expression of the MDA-MB-231 carcinoma cells was significantly higher than that of the HMEC (Fig. 4D). However, the shear stress stimulation (5.0 dyn/cm², 60 s stimulation) caused no significant effect on the immunocytochemical and mRNA expression of ENTPDase 3 in the carcinoma cells.

Colocalization of cell surface ENTPDase 3 and purinergic P2Y1 receptor on the MDA-MB-231 carcinoma cells. To address pivotal roles of ATP produced by the shear stress stimulation in the regulation of cell surface ENTPDase 3 activity, we examined significant immunocytochemical staining of the purinergic receptors on the surfaces of the MDA-MB-231 and HMEC cells. These studies demonstrated that marked immunocytochemical staining of purinergic 2Y1 (P2Y1) receptors were confirmed on the cell surfaces of MDA-MB-231 carcinoma cells but little or...
no staining on the HMEC (Fig. 4E). A similar finding was obtained for the P2Y1 receptor mRNA expression in the carcinoma and HMEC cells (Fig. 4F). In addition, the high-magnification photomicrograph at the bottom panel of Fig. 4G, which shows a merged image of DAPI counterstaining of ENTPDase 3 and the P2Y1 receptor, clearly demonstrates the colocalization (yellow color) of ENTPDase 3 and the P2Y1 receptor in the MDA-MB-231 carcinoma cell.

A 24-h shear stress stimulation develops acidic microenvironment in the MDA-MB-231 cell culture medium supernatant. To confirm the hypothesis that the long-period shear stress stimulation on the carcinoma cells develops the cell surface F$_1$/F$_0$ ATP synthase-mediated acidic microenvironment, we finally measured changes in the pH of culture medium supernatants of the MDA-MB-231 carcinoma cells in the presence or absence 24-h shear stress stimulation (1.0 dyn/cm$^2$) with or without the treatment with 10 μM piceatannol, a cell surface F$_1$/F$_0$ ATP synthase inhibitor. The results are summarized in Fig. 5A. Thus 24-h shear stress stimulation (1.0 dyn/cm$^2$) caused a significant decrease of the pH in culture medium supernatants of the MDA-MB-231. The treatment with piceatannol (10 μM) also produced a significant reduction of the shear stress stimulation-induced decrease of the pH in the supernatants of MDA-MB-231 carcinoma cells. In contrast, the same treatment with piceatannol did not affect the pH in the supernatants of MDA-MB-231 carcinoma cells unloaded with the 24-h shear stress stimulation. In the control experiment without the shear stress stimulation, the pH in the culture medium supernatants of MDA-MB-231 cells at 24-h after cultured at high concentration of carbon dioxide (5%) was about 7.11 (the pH before the experiment is about 7.35). The lowering pH in the supernatants may be related to not only the cultured environment of high concentrations of carbon dioxide but also the high concentration of lactic acid released from the MDA-MB-231 cells unloaded with the shear stress stimulation (Fig. 3A).

A 24-h shear stress stimulation or exogenous ATP inhibits the proliferation of MDA-MB-231 carcinoma cells. To assess pathophysiological roles of ATP released in response to long-period shear stress stimulation on the proliferation of MDA-MB-231 carcinoma cells, we investigated the effects of 24-h shear stress stimulation (1.0 dyn/cm$^2$) with or without the treatment with 10–20 μM piceatannol or exogenous ATP (10$^{-6}$ M) with 10$^{-6}$ M suramin [a nonselective purinergic X/Y receptors inhibitor, (20, 21)] on the proliferation of MDA-MB-231 carcinoma cells using a proliferation assay. These results are summarized in Fig. 5B. Thus the 24-h shear stress stimulation decreased significantly the proliferation activity of MDA-MB-231 carcinoma cells, the decrease of which was significantly reduced by the simultaneous treatment with 10 or 20 μM piceatannol. In similar to the shear stress stimulation, exogenous ATP (10$^{-6}$ M) caused a significant reduction of the proliferation activity of MDA-MB-231 carcinoma cells, the reduction of which was significantly inhibited by the simultaneous treatment with 10$^{-6}$ M suramin.

**DISCUSSION**

Recently, it has become known that primary tumors influence the tumor tissue microenvironment prior to metastasis (1, 12, 16). During this process, primary tumor cells activate matrix metalloproteinase 9 via the release of vascular endothelial growth factor A/B; alter matrix rigidity; and hence, facilitate carcinoma cell metastasis. Solid tumors also form organ-like entities comprised of neoplastic cells and nontransformed host stromal cells embedded in an extracellular matrix (16). Similar to normal tissues, blood vessels nourish tumor cells. However, unlike normal blood vessels the tumor vasculature displays organizational, structural, and functional abnormalities (12). In addition, tumor vessels are leaky, and their blood flow is heterogeneous and often compromised. Vascular hyperpermeability and the lack of functional lymph vessels inside tumor tissues increase the interstitial fluid pressure in solid tumors (12). Furthermore, the elevated interstitial fluid pressure in tumors increases fluid flow from the tumor margin into the peritumor area (1). Therefore, we have hypothesized that carcinoma cells in the primary microenvironment are constantly subjected to high levels of shear stress due to the increased interstitial fluid flow rate. Thus we attempted to
address the pathophysiological roles of the shear stress stimulation of carcinoma cells in the development of the tumor tissue microenvironment.

Activation of cell surface $F_1/F_0$ ATP synthase on the MDA-MB-231 carcinoma cells contributes to the interstitial flow-mediated development of acidic microenvironment in tumor tissues. We evaluated the pathophysiological relevance of the shear stress stimulation-mediated corelease of ATP and $H^+$ in the development of acidic tumor microenvironment. The high glucose consumption and high lactic acid production of tumor tissues are known to be important for the development of the acidic tumor microenvironment (13). Carbonic anhydrase IX (CA IX) is also overexpressed in several types of solid tumors including the MDA-MB-231 carcinoma cells (15, 27), predominantly owing to strong transcription factor 1 (HIF-1) (33). Interestingly, CA IX is the most strongly overexpressed gene in response to hypoxia in human cancer cells (34), and it is the most active isoform of carbonic anhydrase for the carbon dioxide hydration reaction (14). In addition, it has been demonstrated that V-ATPase, which is involved in the acidification of intracellular compartments and the extension of protons through the cytoplasmic membrane of the cell (10), has a crucial role in regulating the pH of normal cells and tumor cells. The V-ATPase consists of multiple subunits with both cytosolic and transmembrane domains (10). With the proton transfer processes, the V-ATPase is involved in the creation of acidic tumor microenvironment. It is also known that one of the most selective V-ATPase inhibitors is the macrolcyclic antibiotic bafilomycin A (27). However, no paper exists to evaluate the involvement of cell surface $F_1/F_0$ ATP synthase in the acidic tumor microenvironment.

In the present study, in agreement with the previous studies, marked immunocytochemical stainings of cell surfaces CA IX and cytosolic and transmembrane V-ATPase were confirmed on the MDA-MB-231 carcinoma cells. In addition, the concentration of lactic acid in the cultured MDA-MB-231 cell supernatant was ~10.0 mmol/l, which was about ten times higher than that observed in the control HMEC supernatant. This finding might be compatible with the mitochondrial $F_1/F_0$ ATP synthase immunocytochemical staining results obtained with the MDA-MB-231 and HMEC cells. Namely, in the carcinoma cells the mitochondrial reticulum network had completely broken down, which may be compatible with the high concentration of lactic acid in the cultured supernatant of MDA-MB-231 carcinoma cells. However, the shear stress stimulation of the carcinoma cells had no significant effect on the concentration of lactic acid in the cultured supernatant. In addition, the pretreatment with V-ATPase inhibitors, omeprazole and bafilomycin A, caused no significant effect on the shear stress stimulation-mediated $CO_2$ excretion from the carcinoma cells. On the other hand, the pretreatment with the cell surface, but not mitochondrial (37) $F_1/F_0$ ATP synthase inhibitors, piceatannol and angiostatin, only produced a significant reduction of the shear stress stimulation-mediated $CO_2$ excretion from the carcinoma cells. In addition, the treatment with piceatannol also inhibited significantly the shear stress stimulation-mediated decrease of the pH in the culture medium supernatants of MDA-MB-231 carcinoma cells. These findings suggest that activation of cell surface $F_1/F_0$ ATP synthase induced by the interstitial flow-dependent shear stress stimulation contributes to the interstitial flow-mediated development of acidic microenvironment in tumor tissues. The wide distribution of cell surface CA IX on the MDA-MB-231 carcinoma cells may also play an accelerated role in the interstitial flow-mediated development of acidic microenvironment in tumor tissues. The conclusion may be supported by the finding of the present study is that the stimulation with shear stress ranging from 1.0 to 5.0 dyn/cm$^2$, the levels of which are similar to those produced by interstitial flow in tumor tissues (18), resulted in the cell surface $F_1/F_0$ ATP synthase-mediated release of $H^+$ from MDA-MB-231 carcinoma cells, which subsequently triggered $CO_2$ gas excretion from the cells. The shear stress stimulation-mediated $CO_2$ gas excretion might be accelerated by the cell surface carbonic anhydrase (CA) type IX because the $CO_2$ gas excretion was significantly reduced by pretreatment with 1.0 $\mu$M acetazolamide (6). Thus the present study might be the first to demonstrate that the shear stress stimulation-mediated corelease of ATP and $H^+$ via the activation of cell surface $F_1/F_0$ ATP synthase on carcinoma cells contributes, in part, to the interstitial flow-mediated development of acidic microenvironment in tumor tissues. In addition, the shear stress stimulation-mediated ATP release and $CO_2$ gas excretion were observed with not only MDA-MB-231 carcinoma cells but also the other carcinoma cells such as human breast cancer cell, MDA-MB-157 and human melanoma cell, SK-Mel-1. Thus the fact that interstitial flow-mediated cell surface $F_1/F_0$ ATP synthase-dependent development of acidic microenvironment in tumor tissues may be one of the universal mechanisms of human carcinoma cells.

Next, we evaluated the pathophysiological relevance of the shear stress stimulation-mediated corelease of ATP and $H^+$, and $CO_2$ gas excretion in the development of tumor microenvironment. How does the shear stress stimulation-mediated excretion of $CO_2$ gas from carcinoma cells contribute to the development of the tumor tissue microenvironment? The answer might be offered by a previous study (35) in which elevated tissue $CO_2$ levels selectively inhibited interleukin-6 and tumor necrosis factor expression in macrophages and decreased phagocytosis. Thus one of the pathophysiological roles of the shear stress stimulation-mediated $CO_2$ gas excretion in the development of the tumor microenvironment might be to protect the carcinoma cells from attack by activated macrophages in tumor tissues.

Pathophysiological relevance of the shear stress stimulation-mediated ATP release from the carcinoma cells in the development of tumor microenvironment. Another important aspect of the present study is that the shear stress stimulation, being produced by the interstitial flow, produces a cell surface $F_1/F_0$ ATP synthase-dependent ATP release from the MDA-MB-231 carcinoma cells. This evidence is derived from the fact that treating the carcinoma cells with selective cell surface $F_1/F_0$ ATP synthase inhibitors [10–20 $\mu$M piceatannol or 1–10 $\mu$M angiostatin; these concentrations are known to selectively inhibit the cell surface enzyme (11, 37)], significantly decreased their shear stress stimulation-induced ATP release and that the immunocytochemical staining and protein expression of cell surface $F_1/F_0$ ATP synthase was clearly observed on the surfaces of the MDA-MB-231 cells, but not the HMEC. In addition, the concentration of ATP released from the MDA-MB-231 carcinoma cells subjected to 5.0 dyn/cm$^2$ shear stress stimulation was around 5.0 x 10$^{-7}$ M, which was significantly higher than that released from human pulmonary arteriolar
endothelial cells (the human blood vessel endothelial cells that are most sensitive to shear stress stimulation) in response to the same level of shear stress (21). In conclusion, MDA-MB-231 carcinoma cells might act as a major source of ATP release into the tumor tissue microenvironment in response to interstitial tissue fluid flow.

Next, we evaluated the pathophysiological relevance of shear stress stimulation-induced ATP release from MDA-MB-231 carcinoma cells. The present study showed that in the shear stress (1.0–5.0 dyn/cm², 60 s stimulation)-loaded culture medium supernatant, the hydrolytic activity of ATPases was increased at 20 min after the shear stress stimulation and then increased in a time- and strength-dependent manner. In agreement with these findings, the immunocytochemical staining of the ATPase, ENTPDase 3 and its mRNA expression were significantly observed in the MDA-MB-231 carcinoma cells. The enzymatic activity of ENTPDase 3 is known to be produced by several conserved extracellular amino acid residues (7). Therefore, shear stress stimulation might disconnect the extracellular regions of ENTPDase 3 from the carcinoma cell surface, resulting in the production of ATPase activity in the carcinoma cell culture medium supernatant. The possibility may be supported by the present finding that the shear stress stimulation (5.0 dyn/cm², 60 s stimulation) caused no significant effect on the immunocytochemical and mRNA expression of ENTPDase 3 in MDA-MB-231 carcinoma cells. In conclusion, the ENTPDase 3 activity occurring at the carcinoma cell surface might contribute to the selective hydrolysis of the ATP secreted in response to the shear stress stimulation-induced activation of cell surface F1/F0 ATP synthase. In addition, the immunocytochemical colocalization of ENTPDase 3 and the P2Y1 receptor was clearly demonstrated on the surfaces of the MDA-MB-231 carcinoma cells. However, more convincing of the colocalization of ENTPDase 3 and P2Y1 receptor will be in the future needed to demonstrate the coimmunoprecipitation of interaction of the two proteins. These findings suggest that the ATP secreted by the shear stress stimulation of the carcinoma cells may be degraded by the activation of cell surface ENTPDase 3 through the binding of the secreted ATP with the P2Y1 receptors. Namely, the ATP secreted from the MDA-MB-231 carcinoma cells under shear stress stimulation binds to cell surface P2Y1 receptors, which increases cell surface ATPase activity; and hence, causes the ATP to be broken down, which might be related to the observed reduction in the direct inhibitory effects of ATP on the proliferation of the MDA-MB-231 carcinoma cells. The conclusion may be also related to the present experimental findings that the 24-h shear stress stimulation (1.0 dyn/cm²) caused a significant inhibition of proliferation activity of MDA-MB-231 carcinoma cells, and that exogenous ATP reduced significantly the proliferation activity of the MDA-MB-231 cells via activation of purinergic 2X/Y receptors on the cells.

It is also worth investigating how shear stress stimulation-induced ATP release from carcinoma cells contributes to the development of the tumor microenvironment. Previous studies of the roles of ATP or its metabolites in macrophage polarization (23), inflammasome formation (31), neutrophil function (24), or the secretory responses of mast cells (3) in the tumor microenvironment offer answers to this question. Namely, macrophage priming might be rapidly affected by signals from the surrounding microenvironment. Recently, novel macrophage 2-associated markers were characterized and identified as genes that control the extracellular metabolism of ATP to generate pyrophosphates (PPi). Extracellular ATP induces the expression of nucleotide-binding domain and leucine-rich repeat-containing receptors on macrophages, which are emerging as key regulators of innate immunity and are involved in

![Proposed hypothesis of interstitial flow-mediated development of acidic microenvironment in tumor tissues](http://ajpcell.physiology.org/)

Fig. 6. Proposed hypothesis that shear stress, the mechanical force produced by interstitial fluid flow, in tumor tissues plays a key role in the producing an acidic microenvironment for carcinoma cells by the cell surface F1/F0 ATP synthase-activated extracellular secretion of high amounts of ATP and high CO2 gas excretion from the carcinoma cells.
inflammasome formation via the activation of purinergic 2X7 (P2X7) receptors (23, 31). In addition, extracellular ATP enhances the respiratory burst responses of neutrophils (24) and amplifies the secretory responses of mast cells (3) during antigen stimulation-released ATP from carcinoma cells and then might contribute to the development of the tumor microenvironment by controlling the functions of macrophages, neutrophils, and leukocytes, as well as the migration of mast cells into tumor tissues.

Therefore, taking all of the findings obtained in the present study and previous reports into consideration, we propose that shear stress stimulation-mediated activation of the cell surface Fv/F0 ATP synthase on carcinoma cells plays key roles in the development of the tumor microenvironment (Fig. 6). In conclusion, shear stress, the mechanical force produced by interstitial fluid flow, in tumor tissues plays pathophysiological roles in producing a suitable microenvironment for carcinoma cells by adjusting the functions of attacking macrophages, activated neutrophil leukocytes, and mast cells by inducing the extracellular secretion of high amounts of ATP and/or high CO2 gas excretion in tumor tissues.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Y.K. and T.O. conceived and design of research; Y.K., M.K., and Y.Y. performed experiments; Y.K. and T.O. analyzed data; Y.K. and T.O. prepared figures; Y.K. and T.O. drafted manuscript; Y.K. and T.O. edited and revised manuscript; Y.K., M.K., Y.Y., and T.O. approved final version of manuscript.

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