

# Platelet-derived adenosine 5 -triphosphate suppresses activation of human hepatic stellate cell: In vitro study

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journal or publication title	Hepatology research
volume	42
number	1
page range	91-102
year	2011
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URL	<a href="http://hdl.handle.net/2241/118506">http://hdl.handle.net/2241/118506</a>

doi: 10.1111/j.1872-034X.2011.00893.x

## **Title page**

### **Platelet-derived adenosine 5'-triphosphate suppresses activation of human hepatic stellate cell - in vitro study -**

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#### **A running title:**

Platelets control hepatic stellate cells

## **ABSTRACT**

**Aim:** Activated hepatic stellate cells (HSC) play a critical role in liver fibrosis.

Suppressing abnormal function of HSC or reversion from activated to quiescent form is a hopeful treatment for liver cirrhosis. The interaction between platelets and HSC remains unknown although platelets go through hepatic sinusoids surrounded by HSC. This study aimed at clarifying hypothesis that platelets control activation of HSC.

**Methods:** We used human platelets, platelet extracts, and primary or immortalized human HSC. We examined the effect of platelets on the activation, DNA synthesis, type I collagen production, and fibrosis-relating gene expressions of HSC. We investigated what suppressed activation of HSC within platelets and examined the mechanism of controlling activation in vitro.

**Results:** Platelets and platelet extracts suppressed activation of HSC. Platelets decreased type I collagen production without affecting DNA synthesis. Platelets increased the expression of matrix metalloproteinase 1. As platelet extracts co-cultured with an enzyme of degrading adenosine 5'-triphosphate (ATP) suppressed activation, we detected adenine nucleotides within platelets or on their surfaces and confirmed the degradation of adenine nucleotides by HSC and the production of adenosine. Adenosine and platelets increased the intracellular cyclic adenosine 5'-monophosphate (cAMP) which is important in quiescent HSC. Much amount of adenosine and ATP also suppressed activation of HSC.

**Conclusion:** Activation of human HSC is suppressed by human platelets or platelet-derived ATP via adenosine-cAMP signaling pathway in vitro. Therefore, platelets have the possibility to be used in the treatment of liver cirrhosis.

**Key words:** adenosine, ATP, cAMP, hepatic stellate cell, and liver fibrosis

## INTRODUCTION

Hepatic fibrosis is often observed under chronic liver damage induced by hepatitis viruses, alcohols, or obstructive jaundice.<sup>1</sup> Since severe liver cirrhosis (LC) impairs liver function and can induce hepatocellular carcinoma, the reduction of fibrosis may eradicate them.<sup>2,3</sup> Unfortunately, the clinical solution of established LC nowadays seems to be only liver transplantation.<sup>4</sup>

Hepatic stellate cells (HSC) play a major role in hepatic fibrogenesis.<sup>5</sup> A lot of experimental studies have focused on suppressing abnormal function of HSC, but they are not applied in clinical use.<sup>6-10</sup> The characteristic features of activated HSC are the spindle-like shape, extensive extracellular matrix production, loss of cytoplasmic fat droplets, and active expression of alpha smooth muscle actin ( $\alpha$ -SMA).<sup>1,11</sup> The quiescent HSC indicates the opposite reactions, and the elevation of intracellular cyclic adenosine 5'-monophosphate (cAMP) has been reported to be an important factor in the anti-proliferative effect in HSC.<sup>12</sup> A cyclic nucleotide phosphodiesterase inhibitor which increases the cytoplasmic cAMP concentration inhibits the transdifferentiation to the activated form of HSC.<sup>13</sup> These previous reports suggest that the elevation of cAMP should be requisite to quiescent HSC.

Platelets have critical roles in hemostasis, and at the same time also contain many kinds of substances which are not essential for hemostasis.<sup>14</sup> The dense granules within platelets have low molecular materials, such as calcium, serotonin, adenosine 5'-diphosphate (ADP), and adenosine 5'-triphosphate (ATP).<sup>15</sup> They are stored in abundance and are released to close spaces.<sup>15,16</sup> Platelets always go through hepatic

sinusoids and adhere there under certain conditions such as ischemia reperfusion injury or inflammation.<sup>17-19</sup> Moreover, platelets can translocate into Disse's space in mice,<sup>20</sup> or can be directly adjacent to collagen fibrils in hepatitis C virus-induced fibrotic human liver.<sup>21</sup> These reports suggest that platelets and HSC can be located closely *in vivo*, and that platelets can provide some substances into Disse's space through multiple fenestrae on liver sinusoidal endothelial cell.<sup>22</sup>

In LC patients, platelet counts are lower and ATP contents are less than those of healthy people.<sup>23,24</sup> We have previously reported that thrombocytosis reduces liver fibrosis in rodent models.<sup>25,26</sup> From these reports, we hypothesized that human platelets released their contents into the environment near HSC and suppressed activation of human HSC in normal liver. In this study, we examined the interaction between human HSC and human platelets and investigated their underlying mechanism to apply platelets to treatment for human LC.

## **Methods**

### **Chemicals or Reagents**

ATP and apyrase, which degrades ATP, were purchased from Sigma-Aldrich Co. (St. Louis, MO). Adenosine, Dulbecco's modified Eagle's medium (D-MEM) and phosphate buffered saline (PBS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acid citrate dextrose formula A is the anticoagulant product used in this study and was purchased from TERUMO Co. (Tokyo, Japan). Anti-human vascular endothelial growth factor (VEGF) antibody MAB293 was purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-human insulin-like growth factor -1 receptor

(IGF-1R) inhibitor II and platelet-derived growth factor-receptor (PDGF-R) tyrosine kinase inhibitor IV were purchased from Calbiochem, Inc. (San Diego, CA). All chemicals are commercially available.

### **Cell culture**

TWNT-1, which is the immortalized human HSC cell line and is kindly donated from Dr. Kobayashi, Okayama University, and primary human HSC were cultured in D-MEM supplemented with 10% fetal bovine serum.<sup>27</sup> Cell culture chambers (Becton Dickinson Labware, Franklin Lakes, NJ) whose pore sizes are 0.2  $\mu\text{m}$  were used to separate platelets from TWNT-1. Primary human HSCs were obtained from surgical specimens by a two-step collagenase perfusion method, followed by density gradient centrifugation.<sup>28,29</sup> Surgical specimens were collected under written informed consent and approval by the Ethics Committee and the institutional review board of University of Tsukuba hospital. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### **Platelet preparation**

Blood was obtained from healthy volunteers using an anticoagulant after obtaining informed consents, as previously reported.<sup>30</sup> Washed platelets were suspended in PBS and used as platelet group (PLT). To obtain platelet extracts, platelets were disrupted by freezing and thawing for five cycles and centrifuged at 15000 g, 4°C for 30 min. The supernatant was used as platelet extracts.

### **Light microscopy**

To examine morphologic changes of TWNT-1 after adding platelets, cultured cells were photographed using light microscopy IX71 (OLYMPUS Corp., Tokyo) and the digital camera DP71 (OLYMPUS Corp.) under the high magnification power (x 200) after washing dishes with PBS and removing apoptotic cells or platelets. We divided cells into two groups according their shapes and counted the number of cells. One group consisted of the spindle shaped cells that have a long axis more than twice that of the short axis. The other consisted of the oval shaped cells that have a long axis less than twice that of the short axis. The spindle one was considered as highly-activated status and the oval one was considered as resting status in this study.<sup>11</sup>

### **Western blot**

Cultured cells were washed with PBS, lysated, subjected to 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Millipore Co., Billerica, MA). Primary antibodies were mouse monoclonal anti-human smooth muscle actin (DAKO Co., Grostrup, Denmark) and rabbit polyclonal anti-G3PDH antibody (TREVIGEN, Inc., Gaithersburg, MD). Secondary antibodies were anti-mouse or anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Inc., Danvers, MA). All antibodies were diluted to 1:1000 except for anti-G3PDH (1:5000). Because  $\alpha$ -SMA is a well-known marker of HSC activation,<sup>2</sup> we used the anti-human smooth muscle actin antibody. GAPDH was used as the endogenous control.

### **DNA synthesis assay**

The effect of platelets on DNA synthesis was measured using Cell Proliferation ELISA, 5-bromo-2'-deoxyuridine (BrdU) colorimetric kit (Roche Ltd.). TWNT-1 ( $5 \times 10^3$  cells)

were plated into each well of 96-well dishes. Platelets were added and cultured for 24 hours. The plate was washed before adding BrdU.

### **RNA extraction and reverse transcription – polymerase chain reaction (RT-PCR)**

RNA extraction was carried out using FastPure RNA Kit (Takara BIO Inc., Otsu, Japan), following the procedure recommended by the manufacturer. The synthesis of complimentary DNA was performed using AMV Reverse Transcriptase (Promega Corp., Madison, WI) and random primer (Takara BIO, Inc.). Briefly, the mixture of 1 mM dNTPs (Fermentas LIFE SCIENCES Corp., Ontario, Canada), 0.025 µg/µl random primer, 0.25 U/µl reverse transcriptase, and 500 ng total RNA were incubated at 30°C for 10 min, 37°C for 60 min, 95°C for 5 min, and 4°C before storage in -80°C.

### **Quantitative real-time PCR**

Primers for real-time PCR were designed by Primer Express Software for Real-Time PCR version 3.0 (Applied Biosystems Corp., Foster, CA) using GenBank sequences. Primers were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan) and listed in Table 1. We examined *ACTA2*, *COL1A1*, *MMP-1*, *TIMP-1*, *ADORA1*, *ADORA2A*, *ADORA2B*, *ADORA3* and *GAPDH* (encoding alpha 2 helix of  $\alpha$ -SMA, pro-alpha 1 chain of type I collagen, matrix metalloproteinase-1, tissue inhibitor of metalloproteinases 1, adenosine A1 receptor, adenosine A2A receptor, adenosine A2B receptor, adenosine A3 receptor, and glyceraldehyde-3-phosphate dehydrogenase, respectively). The real-time PCR was performed using SYBR Green Realtime PCR Master Mix-Plus (TOYOBO Co., Ltd., Osaka, Japan) and Applied Biosystems 7300 Real-time PCR system (Applied Biosystems Corp.), following the procedure



recommended by the manufacturer. *GAPDH* was used as the endogenous control.

### **Agarose gel-electrophoresis**

PCR products were subjected to 1% agarose gel containing 0.005% ethidium bromide (Wako Pure Chemical Industries, Ltd.). After the electrophoresis was performed, the gel was pictured when ultraviolet was radiated using Electronic U.V. Transilluminator, FAS-III (TOYOBO Co., Ltd.).

### **Assay of type I collagen in the cultured medium of TWNT-1**

The amount of type I collagen was measured using anti-human procollagen type I C-peptide ELISA kit (Takara BIO, Inc.). The supernatant of cultured medium was separated, centrifuged at 15000 g, 4°C for 30 min to remove the debris, and stored at -80°C until analysis.

### **Analysis of adenine nucleotide concentration**

The concentrations of ATP, ADP, and AMP in platelets were measured by high performance liquid chromatography (HPLC) as previously reported.<sup>31</sup> The HPLC system for adenosine measurement was the GULLIVER HPLC-900 (JASCO Corp., Tokyo, Japan). The column was Wakosil-II 5C18HG (Wako Pure Chemical Industries, Ltd.) and ultraviolet absorbance was measured at 260 nm. The running buffer was 60 mM ammonium phosphate buffer which was adjusted to pH 5.0. In addition, we used Adenosine 5'-triphosphate Bioluminescent Assay kit (Sigma-Aldrich Co.) to assess the ATP concentration on the platelet's surface, referring to the procedure of measuring cellular surface concentration of ATP.<sup>32</sup> Briefly, washed platelets were centrifuged at

1000 g, 4°C for 5 min. The pellet was loosened using a large pipette. After the aliquot of 10 µl pellet and the same amount of Assay Mix in the kit were mixed, the chemical luminescence was measured by luminometer, TD 20 / 20 (Promega Corp.). The same volume of ATP solution was used as control.

### **Measurement of intracellular cAMP**

As cAMP is the second messenger of adenosine signaling pathway, we examined cytoplasmic cAMP of TWNT-1.<sup>33</sup> TWNT-1 ( $7 \times 10^3$  cells) were plated into each well of 96-well dishes and cultured over night. We added stimulants, incubated them for an hour, and washed the plate with PBS before starting measurements. The intracellular cAMP was investigated using cAMP-Screen Assay kit (Applied Biosystems Corp.), following the procedure recommended by the manufacturer.

### **Statistical Analysis**

Data are expressed as means  $\pm$  standard deviation. Student *t* test was performed to assess statistical significance. *P* values less than 0.05 were considered statistically significant.

## **RESULTS**

### **Human platelets suppress the expression of $\alpha$ -SMA**

The expression of  $\alpha$ -SMA was measured by Western blot analysis at 1, 4, or 7 days after incubating platelets with TWNT-1. While  $\alpha$ -SMA expression was increased with or without platelets, platelets reduced their increment (Fig. 1a). We also examined  $\alpha$ -SMA expression after adding platelets to cell culture inserts. It was not suppressed (Fig. 1b).

These findings indicate that human platelets suppress activation of TWNT-1, and that platelets can exhibit it more efficiently when being located close to TWNT-1.

### **Platelets suppress cellular extension of TWNT-1**

TWNT-1 co-cultured with platelets were photographed at random on day 1, 4, and 7, and separated into two groups in terms of their shapes. More oval type cells were shown in the PLT group than the control group. The representative pictures are shown on day 4 (Fig. 1c). The ratio of oval type cells in total decreased in a time-dependent manner in both groups. However, the PLT group showed a significant large ratio on day 4 and 7 (Fig. 1d). This result indicates that platelets also have some effects on keeping TWNT-1 quiescent in terms of their shapes.

### **Platelets do not affect DNA synthesis of TWNT-1**

The BrdU uptake was not changed when incubating platelets with TWNT-1 (Fig. 1e).

### **Platelets decrease the production of type I collagen**

The concentration of type I collagen in cultured medium was significantly decreased in the PLT group on day 4 (Fig. 1f).

### **Platelets have influences on fibrosis-relating gene expressions**

Total RNAs were extracted after incubating platelets with TWNT-1 for 24 hours. After performing real-time PCR, each group was compared with the control group. *ACTA2* and *COL1A1* were significantly decreased in the PLT group (Fig. 2a, b, respectively). *MMP-1* and *TIMP-1* were significantly increased in the PLT group (Fig. 2c, d,

respectively). These findings demonstrate that platelets suppress HSC activation and affect the amount of intrahepatic extracellular matrix. Since TWNT-1 express more  $\alpha$ -SMA in a time-dependent manner (Fig. 1a), we cultured TWNT-1 for 1, 4, or 7 days to prepare activated TWNT-1, incubated platelets with them for another 24 hours, and extracted total RNAs to investigate changes of *ACTA2* in the activated TWNT-1. On day 2, 5, and 8, all PLT groups suppressed *ACTA2* expression (Fig. 2e). This result means that platelets suppress activation of TWNT-1 which has been activated to some degree.

### **Platelet extracts suppress *ACTA2* expression more efficiently when incubated with apyrase**

To examine what contributes to suppression of *ACTA2* expression, we incubated four kinds of reagents before adding platelet extracts as follows: PDGF-R inhibitor, anti-VEGF antibody, IGF-1R inhibitor, and apyrase which degrades ATP to ADP or AMP. Platelet extracts also suppressed *ACTA2* expression as well as platelets (Fig. 3a). PDGF-R inhibitor, anti-VEGF antibody, and IGF-1R inhibitor had no effect on *ACTA2* expression (Fig. 3b, c, d, respectively). On the other hand, apyrase reduced *ACTA2* expression in a dose-dependent manner (Fig. 3e). This result implies that ATP derivatives made by apyrase have more influence on *ACTA2* expression than ATP in platelet extracts.

### **Platelets contain and release large amounts of ATP**

We studied adenine nucleotide concentrations both in platelets by HPLC and on platelet's surfaces by luciferase-mediated chemical luminescence to explain the effect of

derivatives made by apyrase. Platelets contained ATP, ADP, and AMP (Fig. 4a). The number of chemical luminescent counts at platelet's surfaces without ATP administration was significantly higher than that of 100  $\mu$ M ATP solution (Fig. 4b). These results demonstrate that platelets contain large amounts of ATP inside or near their surfaces in vitro.

### **HSC degrades ATP and produces adenosine in vitro**

After culturing TWNT-1 or primary HSC with ATP, we studied the total value of ATP, ADP, and AMP in the cultured medium by HPLC. The values of both the TWNT-1 group and the primary HSC group were significantly decreased in 6 and 24 hours after incubation, compared to control which did not contain any cells (Fig. 4c). After culturing TWNT-1 with ATP or adenosine for 24 hours, we investigated adenosine concentrations in the cultured medium by HPLC as well. The adenosine concentrations after incubating with adenosine or ATP for 24 hours were significantly increased comparing to TWNT-1 alone (Fig. 4d). The adenosine concentration in ATP alone was extremely low (figure not shown). These results suggest that TWNT-1 metabolizes adenosine phosphates and produces adenosine in the medium.

### **Adenosine and platelets increase cytoplasmic cAMP in TWNT-1**

As TWNT-1 produced adenosine in vitro (Fig. 4d), we examined expression of adenosine receptors and changes of their intracellular signaling molecule, i.e., cAMP after incubating adenosine or platelets with TWNT-1. We confirmed four types of mRNA of adenosine receptors by agarose gel-electrophoresis (Fig. 4e). The cytoplasmic cAMP was significantly increased when incubating 1000  $\mu$ M adenosine and platelets

(Fig. 4f). These results suggest that platelets and large amounts of adenosine activate adenosine-cAMP signaling pathway in TWNT-1.

### **Adenosine and ATP suppress HSC activation**

After incubating adenosine with TWNT-1,  $\alpha$ -SMA expression was examined by Western blot. It was suppressed after incubating 1 mM adenosine with TWNT-1 during 1, 4, and 7 days (Fig. 5a). The  $\alpha$ -SMA expression in primary human HSC was studied as well. It was also suppressed when incubating with 1 mM adenosine, 1 mM ATP, and platelets (Fig. 5b). DNA synthesis was not changed after incubating ATP with TWNT-1 for 24 hours (Fig. 5c). These results indicate that a large amount of ATP or adenosine suppresses HSC activation without affecting DNA synthesis.

## **DISCUSSION**

Activated HSC produces a lot of extracellular matrix and contributes to deteriorate hepatic fibrogenesis.<sup>1,2</sup> Since we have already found that hepatic fibrosis decreases after inducing thrombocytosis in rodents,<sup>25,26</sup> we hypothesized that human platelets would also have an anti-fibrotic effect. In this study, we indicated that human platelets suppress HSC activation and collagen production in human HSC via an adenosine-cAMP signaling pathway in vitro.

It is likely that HSC is physiologically exposed to high concentration of ATP or its derivatives in the microcirculation because ATP is released from red blood cells or intrahepatic cells and hepatocytes are abundant in ATP.<sup>34-36</sup> On the other hand, adenine nucleotides decrease very rapidly in circulating blood through the degradation by

functional enzymes or the uptake from erythrocytes.<sup>37</sup> It seems to be difficult to examine the exact concentration of adenine nucleotides without degradation *in vivo*. This is the limitation of this study.

The DNA synthesis was not changed after incubating platelets or ATP with TWNT-1. These results may indicate that the volume of ATP or platelets in this study is not sufficient to stimulate HSC proliferation *in vitro*.

We clearly indicated that HSC produces adenosine by degrading adenine nucleotides. The nucleotide-hydrolysing pathway mainly comprises four enzymes, ecto-nucleotide triphosphate diphosphohydrolase family (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase family (E-NPP), ecto-5'-nucleotidase (CD73), and alkaline phosphatase. HSC has been also reported to show these activities,<sup>38-40</sup> which demonstrates that the extracellular ATP may be easily broken down to adenosine by HSC *in vivo*. Andrade et al. implied that CD73-dependent adenosine generation might regulate the function of quiescent HSC.<sup>40</sup> Hernández-Muñoz R et al. reported that adenosine administration blocks active hepatic fibrogenesis and increases the collagen degradation in rats.<sup>41,42</sup> These reports together with our results imply that adenosine seems to be important in quiescent HSC and anti-fibrotic effects.

The signal from adenosine is transmitted by four types of adenosine receptors; A<sub>1</sub>R, A<sub>2A</sub>R, A<sub>2B</sub>R, and A<sub>3</sub>R.<sup>33</sup> They are all G protein-coupled receptors whose effectors are adenylyl cyclase (AC) which produces cAMP. We demonstrated the elevation of cAMP in TWNT-1 because it activates cAMP-dependent protein kinase (PKA) and leads to

phosphorylation of cAMP-response element binding protein (CREB) on Ser133.<sup>43</sup> Houglum et al. reported that phosphorylation of CREB on Ser133 might be indispensable for quiescent HSC.<sup>44</sup> From these reports and results of this study, we hypothesize the mechanism of suppressing HSC activation by platelets via adenosine-cAMP signaling pathway in vitro (Fig. 6).

In conclusion, human platelets contribute to suppression of both HSC activation and type I collagen production in vitro. This is mediated by adenosine-cAMP signaling pathway in HSC. We suggest that the effective transport of a high amount of adenine nucleotide into Disse's space should be a key process in the treatment for human LC.

## **ACKNOWLEDGEMENTS**

The authors thank Dr. Kobayashi, Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, for providing the hepatic stellate cell line, TWNT-1. We also thank Chikako Satoh, Department of Surgery, Tohoku University, for technical assistance in HPLC.

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## FIGURE LEGENDS

**Figure 1** Platelets suppress activation of hepatic stellate cells (HSC) and collagen production.

The immortalized human HSC, TWNT-1 ( $1 \times 10^4$  cells), were plated on 6-well dishes and incubated with platelets ( $1 \times 10^8$  cells) for 1, 4, and 7 days. (a) The expression of alpha smooth muscle actin ( $\alpha$ -SMA) which is a well-known marker of HSC activation was examined by Western blot (first row,  $\alpha$ -SMA; second row, GAPDH; third row,  $\alpha$ -SMA in platelets themselves). (b) After incubating platelets with TWNT-1 separately using chambers,  $\alpha$ -SMA expression was examined as well. (c) TWNT-1 incubated with PBS (control) or platelets (PLT) for 1, 4 and 7 days were photographed and separated into two groups ( $n = 10$ ). We determined that the oval shaped cell had a long axis less than twice that of the short axis and was not as activated as the spindle one.<sup>11</sup> The representative figures at day 4 are shown (arrow; oval shaped cell). (d) The ratios of oval shaped cells in total were counted at day 1, 4, and 7. (e) DNA synthesis in TWNT-1 was examined by 5-bromo-2'-deoxyuridine (BrdU) uptake assay after incubating platelets (PLT) for 24 hours ( $n = 8$ ). (f) The supernatant of co-cultured medium with platelets for 1 or 4 days was examined by ELISA for type I collagen ( $n = 6$ ). All results are representative of three independent experiments (mean  $\pm$  SD). \*  $P < 0.01$ .

**Figure 2** Platelets affect fibrosis-relating gene expressions.

TWNT-1 ( $1 \times 10^6$  cells) were plated on 6 cm dishes and incubated with PBS (control) and platelets ( $2 \times 10^8$  cells, PLT) for 24 hours. Total RNAs were extracted and real-time PCR was performed ( $n = 3$ ). (a-d) *ACTA2*, *COL1A1*, *MMP-1*, and *TIMP-1* were

examined, respectively. (e) In *ACTA2*, after TWNT-1 ( $1 \times 10^6$ ,  $5 \times 10^5$ , and  $1 \times 10^5$  cells) were cultured for 1, 4, and 7 days to obtain subconfluent dishes at the same time, respectively, TWNT-1 were incubated with platelets for another 24 hours and real-time PCR was performed as well. All results are representative of three independent experiments (mean  $\pm$  SD). \*  $P < 0.01$ , \*\*  $P < 0.05$  versus control. *ACTA2*, alpha 2 helix of  $\alpha$ -SMA; COL1A1, pro-alpha 1 chain of type I collagen; MMP-1, matrix metalloproteinase-1; TIMP-1, tissue inhibitor of metalloproteinases-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Figure 3** Platelet extracts suppress *ACTA2* expression more efficiently when incubated with apyrase.

(a) TWNT-1 ( $1 \times 10^6$  cells) were plated on 6 cm dishes and incubated with PBS (control) and platelet extracts (equivalent to  $2 \times 10^8$  cells) for 24 hours. Total RNAs were extracted and real-time PCR for *ACTA2* was performed ( $n = 3$ , \*  $P < 0.01$  versus control). (b-e) TWNT-1 ( $1 \times 10^6$  cells) were incubated with platelet-derived growth factor receptor (PDGF-R) inhibitor, anti-vascular endothelial growth factor (VEGF) antibody, insulin-like growth factor-1 receptor (IGF-1R) inhibitor, or apyrase which degrades adenosine 5'-triphosphate (ATP) for 30 minutes, respectively. Then, platelet extracts (equivalent to  $2 \times 10^8$  cells) were incubated for 24 hours. Real-time PCR for *ACTA2* was performed as well ( $n = 3$ , \*  $P < 0.01$ , \*\*  $P < 0.05$  versus 0 U / ml). All results are representative of three independent experiments (mean  $\pm$  SD).

**Figure 4** HSC dephosphorylates platelet-derived ATP and produces adenosine which activates adenosine-cyclic adenosine 5'-monophosphate (cAMP) signaling pathway.

(a) Adenine nucleotide concentrations of platelets were measured by high performance liquid chromatography (HPLC) (n = 5). (b) The ATP concentration of platelet's surfaces was examined by luciferase reaction (n = 5, \*P < 0.01 versus 100  $\mu$ M). (c) After TWNT-1 or primary human HSC ( $3 \times 10^4$  cells) were incubated with 1 mM ATP, the total amount of ATP, adenosine 5'-diphosphate (ADP), and AMP in the medium was measured at 0, 6, and 24 hours by HPLC (n = 5, \* P < 0.01). (d) After culturing TWNT-1 with 1 mM adenosine or 1 mM ATP for 24 hours, the adenosine concentration in the medium was investigated by HPLC (n = 5, \* P < 0.01). (e) After PCR for four types of adenosine receptors in TWNT-1, we examined their expressions by agarose gel-electrophoresis. (f) TWNT-1 ( $7 \times 10^3$  cells) were plated on 96-well dishes and incubated with adenosine or platelets ( $1 \times 10^7$  cells) for an hour. After washing, the intracellular cAMP was measured (n = 8, \*\* P < 0.05 versus 0  $\mu$ M). All results are representative of three independent experiments (mean  $\pm$  SD). A1-R, adenosine A1 receptor; A2A-R, adenosine A2A receptor; A2B-R, adenosine A2B receptor; A3-R, adenosine A3 receptor.

**Figure 5** Much amount of adenine nucleotides suppresses activation of HSC.

(a) TWNT-1 ( $1 \times 10^4$  cells) were plated on 6-well dishes and incubated with adenosine for 1, 4, and 7 days. The  $\alpha$ -SMA expression was examined by Western blot. (b) Primary human HSC ( $5 \times 10^3$  cells) were plated and incubated with PBS (control), platelets ( $1 \times 10^8$  cells, PLT), 1 mM ATP, or 1 mM adenosine during 1, 4, and 7 days. The  $\alpha$ -SMA expression was investigated as well. (c) After incubating TWNT-1 ( $5 \times 10^3$  cells) with ATP for 24 hours, the BrdU uptake was measured (n = 8). All results are representative of three independent experiments (mean  $\pm$  SD).



**Figure 6** Scheme for inactivation of human HSC by human platelets. Platelets contact with HSC. Platelets release adenine nucleotides into a close environment. They are hydrolyzed by ecto-nucleotide triphosphate diphosphohydrolase (E-NTPDase), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP), or ecto-5'-nucleotidase (CD73) on HSC plasma membrane, which produces large amounts of adenosine around HSC. Adenosine receptors transmit signals into cytoplasm by activating adenylyl cyclase (AC). AC increases intracellular cAMP. The elevation of cAMP activates cAMP-dependent protein kinase (PKA). The cAMP-response element binding protein (CREB) is phosphorylated by PKA. This phosphorylation is an important factor in quiescent HSC that expresses less  $\alpha$ -SMA and produces less extracellular matrix.

**Table 1**

The list of primers used in PCR

Gene name	GenBank accession number	forward (5' to 3')	reverse (5' to 3')
ACTA2	NM_001613	CAGGGCTGTTTTCCCATCCA	CCTCTTTTGCTCTGTGCTTCGT
COL1A1	Z74615	GGGCTTGCCTTCCATTCCT	GGAAGTAGAAAGGTTTGCGGTATAA
MMP1	NM_002421	CACAGCTTCCCAGCGACTCT	GCCACTATTTCTCCGCTTTTCA
TIMP1	NM_003254	ACTGTTGGCTGTGAGGAATGC	GGTCCGTCCACAAGCAATG
ADORA1	NM_000674	CCTCACGCACGGCAACTC	TGGCAGCGGAAATGGTCAT
ADORA2A	NM_000675	CTGCTGGCTGCCCCTACAC	GAAGGGATTACAAACCGAATTG
ADORA2B	M97759	GCTCTTCGCCATCCCCTTT	CACGAAGCAGGCGAGGAA
ADORA3	NM_000677	GTCAGCCTGGGCATCACAAT	CACAGCGATGGCCAGCAA
GAPDH	NM_002046	GGAGTCCACTGGCGTCTTCA	TTCACACCCATGACGAACATG

Figure 1

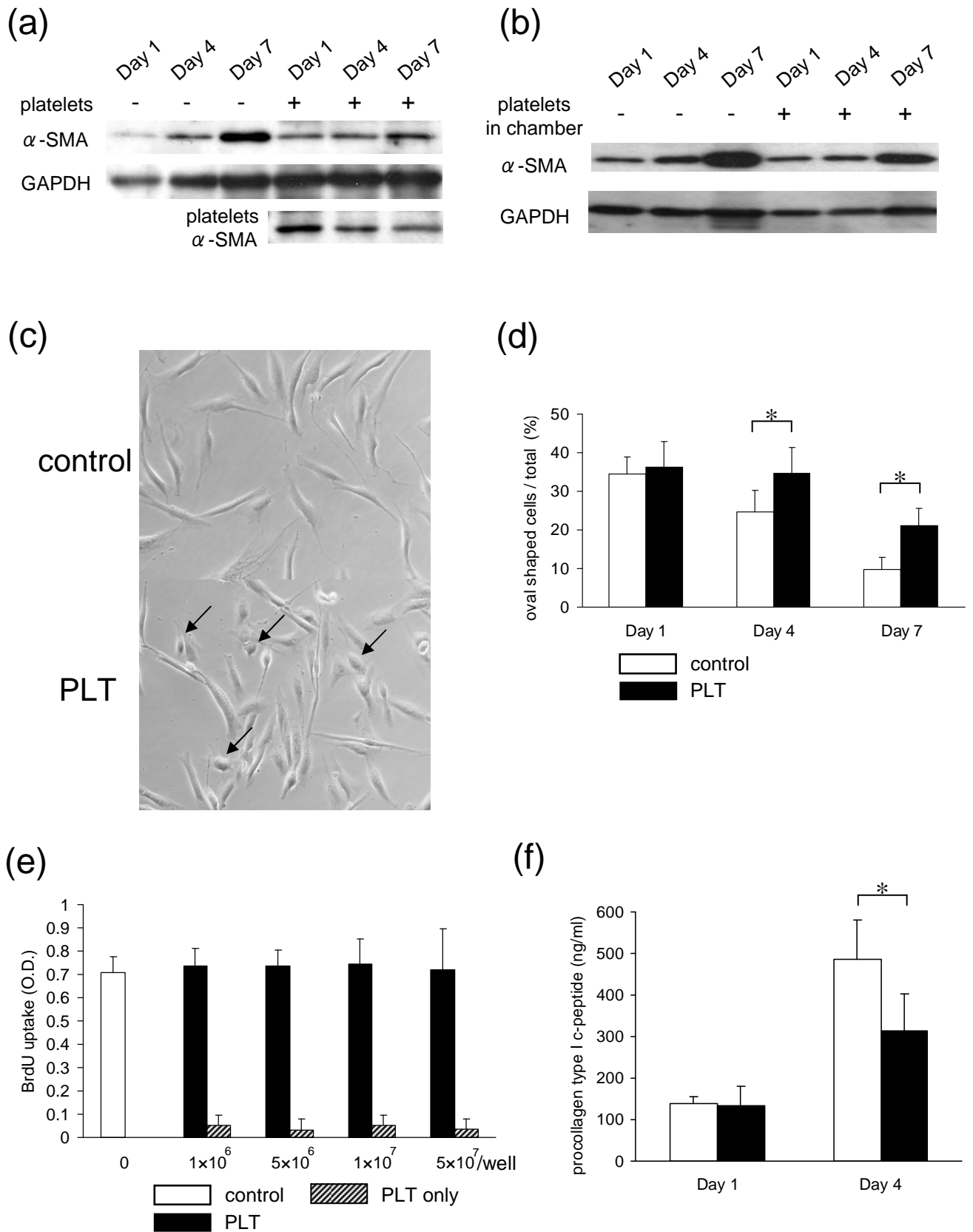


Figure 2

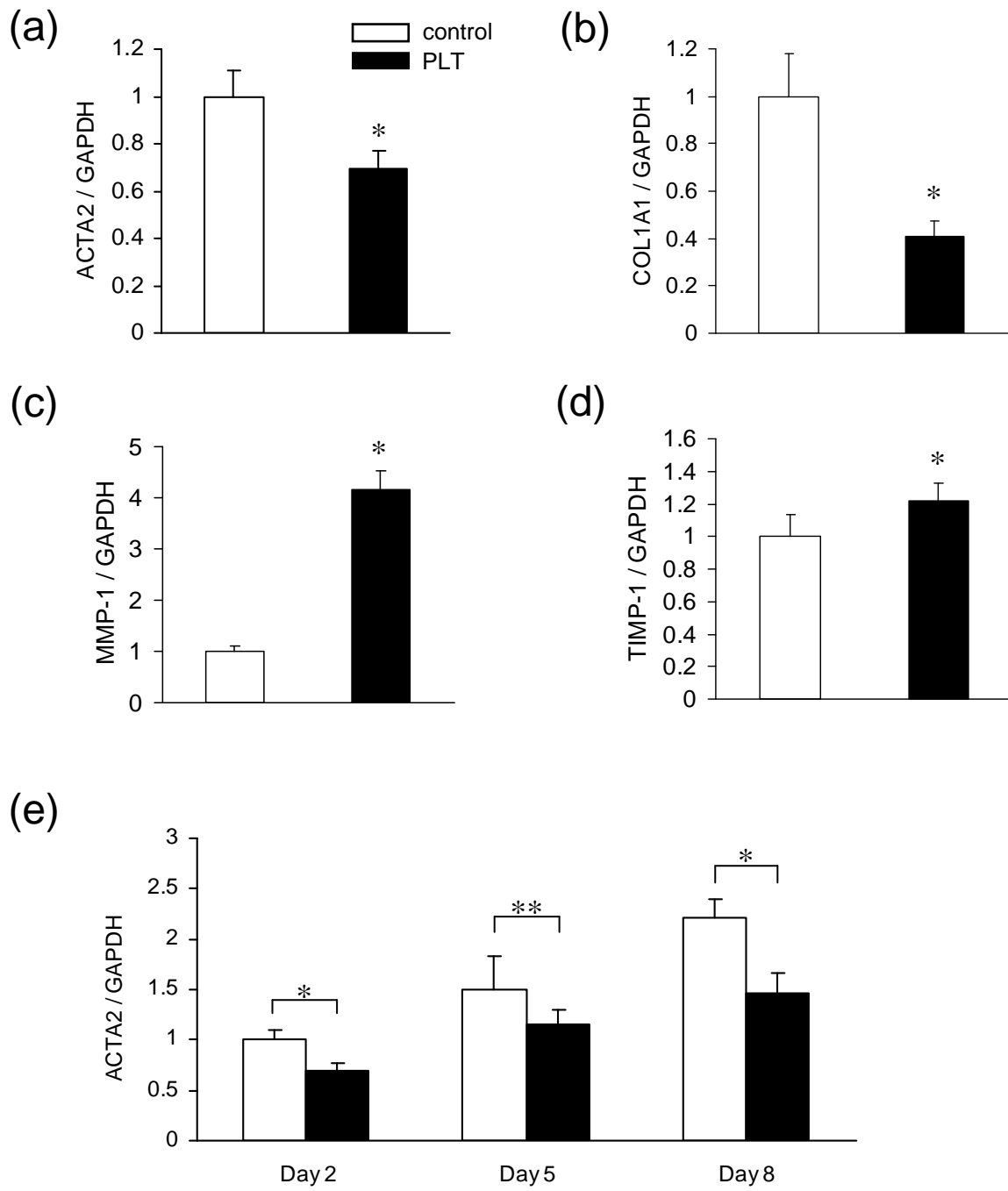
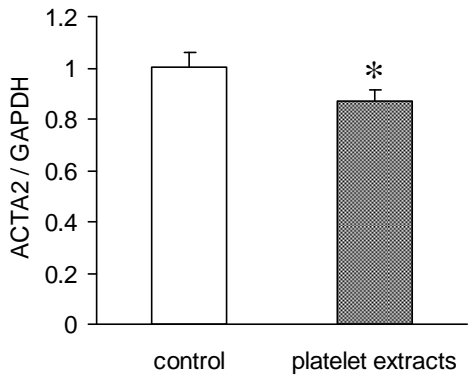
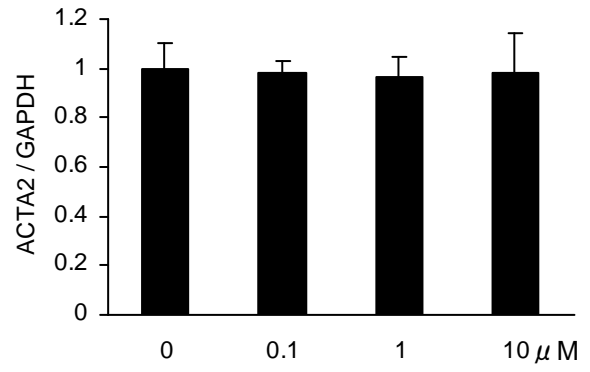


Figure 3

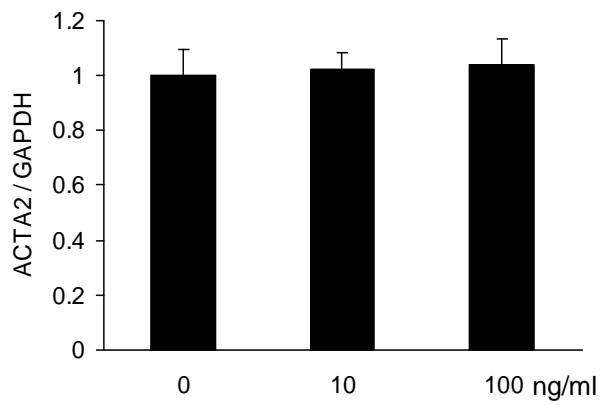
(a)



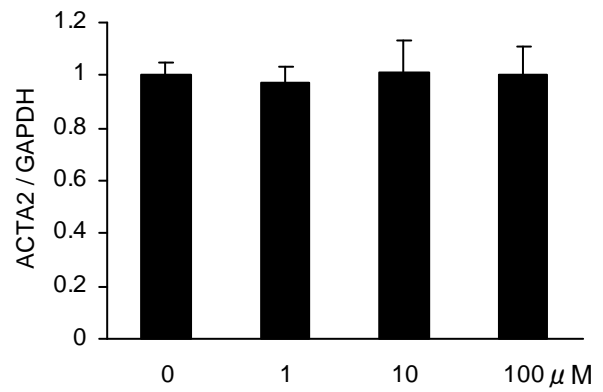
(b) PDGF-R inhibitor IV



(c) Anti-VEGF antibody



(d) IGF-1R inhibitor



(e) Apyrase

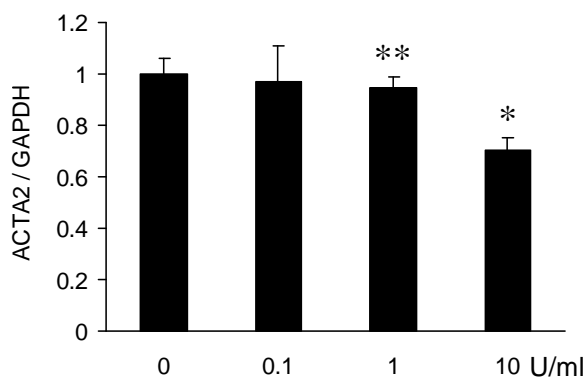


Figure 4

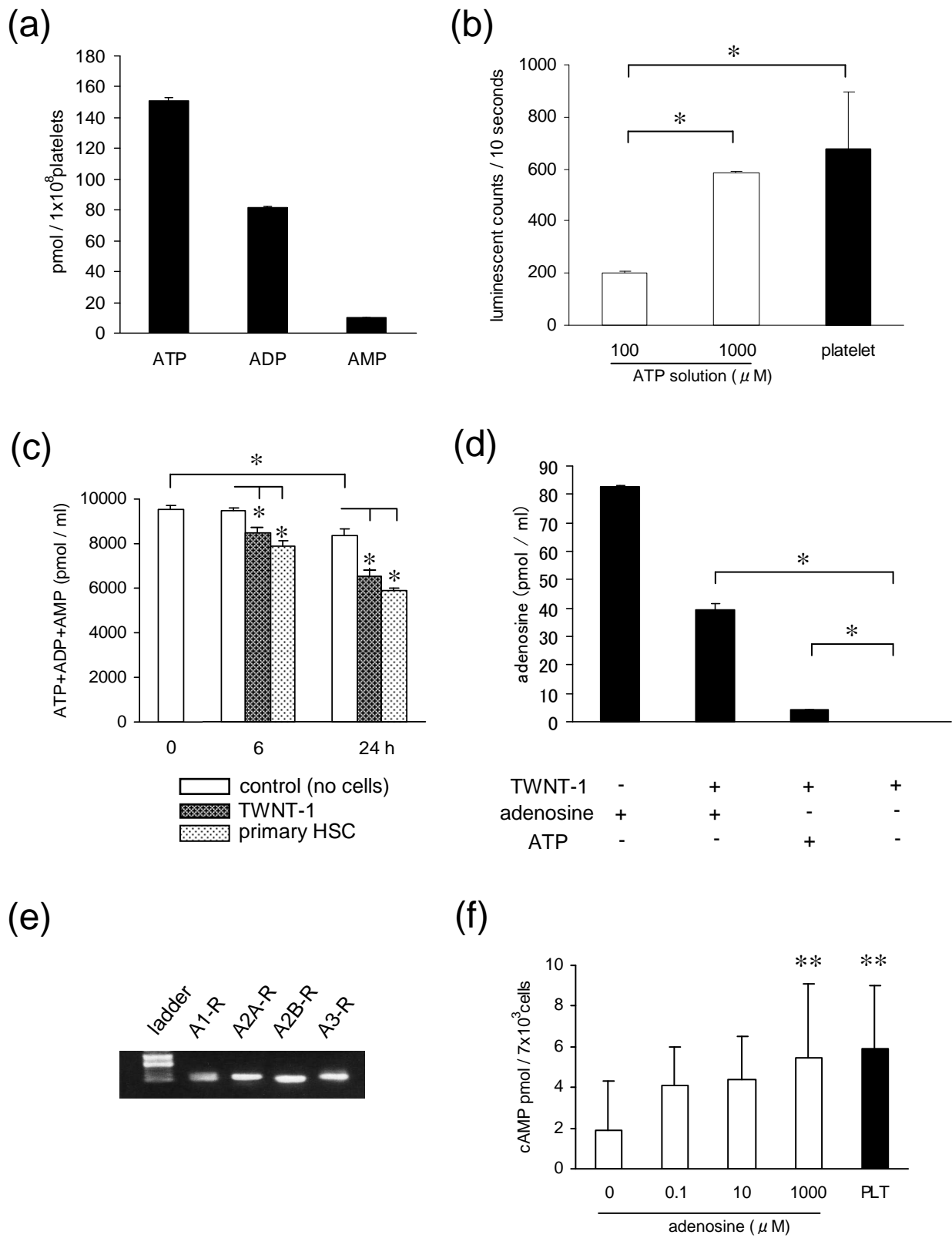
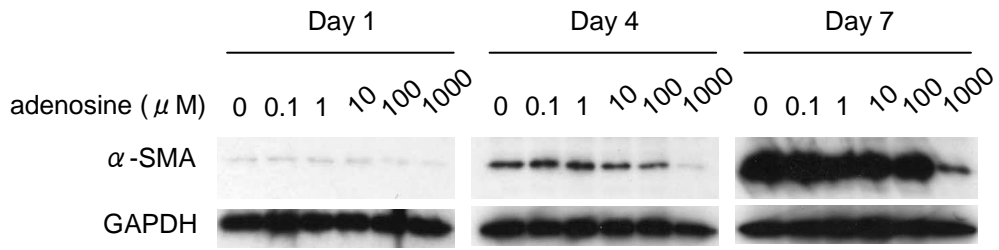
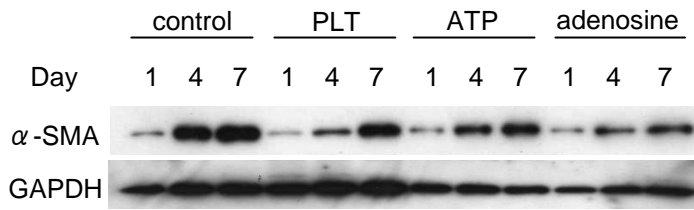


Figure 5

(a)



(b)



(c)

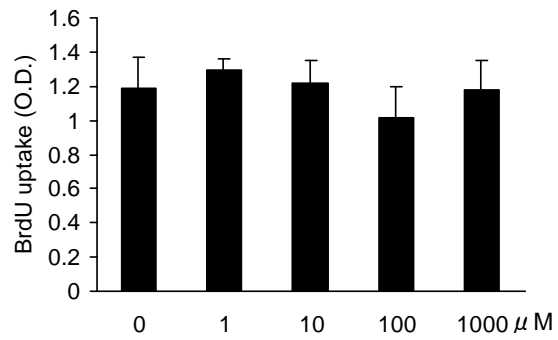


Figure 6

