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Anti-Angiogenic Effect of P2X7 Receptor Antagonist Oxidized ATP as a Mechanism of Anti-Tumor Growth

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Abstract

Extracellular ATP accumulated in tumor microenvironment activates P2X7 receptor on cellular membrane of cancer cells. Recently, an importance of P2X7 receptor in cancer growth or malignancy has been suggested. We have reported an inhibitory effect of oxidized ATP (oxATP), which is an irreversible antagonist of P2X7 receptor, on melanoma growth. However, the mechanism has not yet been established. In this study, we investigated the effect of oxATP on angiogenesis *in vitro* and *in vivo* to reveal the mechanism of anti-tumor growth by oxATP. We found that oxATP strongly suppressed cell migration and wound healing in mouse endothelium b.End3 cells, indicating the suppressive effect of oxATP on angiogenesis *in vitro*. We further investigate the effect of oxATP on angiogenesis *in vivo*. We performed ligation of the femoral artery and vein of BALB/c mouse, and a laser doppler perfusion image analyzer recorded blood flow postoperatively. The blood flow of hind limb was significantly decreased by the operation and recovered within 1-2 weeks, indicating angiogenesis. However, administration of oxATP to mice significantly suppressed the recovery of blood flow. Increase of serum matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) levels contribute to angiogenesis. The serum MMP-2, MMP-9, and VEGF levels were lower in oxATP-treated mice than in control mice. Moreover, production of VEGF in RBL-2H3 mast cells was suppressed by treatment with oxATP. These results suggest that oxATP inhibited angiogenesis *in vivo* via suppression of MMP-2, MMP-9 and VEGF production. We conclude that oxATP has an anti-angiogenic effect, which would contribute to suppression of cancer growth.

Keywords: Cancer; Angiogenesis; P2X7 receptor; VEGF; MMP

Abbreviations: BzATP-2'&3'-O-(4-Benzoyl)Benzoyl ATP; DMEM-Dulbecco's Modified Eagle's Medium; FBS- Fetal Bovine Serum; FITC-Fluorescein Isothiocyanate; MMP- Matrix Metalloproteinase; oxATP-Oxidized ATP; PPADS-Pyridoxal-Phosphate-6-Azophenyl-2',4'-Disulfonate; TNP-ATP-2',3'-O-(2,4,6-Trinitrophenyl)Adenosine-5'-Triphosphate; VEGF- Vascular Endothelial Growth Factor; 5-BDBD-5-(3-Bromophenyl)-1,3-Dihydro-2H-Benzofuro[3,2-E]-1,4-Diazepin-2-One

Introduction

Angiogenesis is a complex manner that is characterized by the 1) migration, 2) degradation of extracellular matrix, 3) proliferation, 4) morphogenesis/capillary tube formation of endothelial cells [1,2]. The growth and metastasis of a neoplasm is also dependent on the formation of adequate vascular support. However, pathological angiogenesis is an abnormal and unregulated manner, and it contributes to the pathogenesis of various diseases. In cancer, active tumor growth and metastasis are highly dependent on angiogenesis [3]. Therefore, angiogenesis is a complex process and that a better understanding of the biology of angiogenesis is critical for designing improved therapies.

Cytosolic ATP is released into the extracellular space in response to stress stimuli, such as shear stress, stretching, hypoxia, inflammation, osmotic swelling, and cell death. Extracellular ATP interacts with and activates P2 receptors in an autocrine/paracrine manner. P2 receptors are subdivided into two subfamilies; ionotropic P2X1-7 and metabotropic P2Y1-14 receptors. It is known that a large amounts of ATP accumulate in tumor microenvironment [4], suggesting the activation of P2 receptor in cancer cells. High expression of P2X7 receptor and its role is reported in several types of cancers [5-7]. For examples, the expression of P2X7 receptor is increased in thyroid cancer cells and activation of P2X7 receptor promotes release of interleukin (IL)-6, which may be correlated with malignancy [8,9]. Increased level of P2X7 receptor was recently detected in chronic pancreatitis and pancreas cancer indicating a possible involvement of P2X7 receptors in pancreatic cancer development [10]. Increased expression was also found in B16 melanoma cells, and it was proposed that increase of P2X7 receptor might be a biomarker of cancer [11]. The recent demonstration that P2X7 expression enhances cancer cell invasiveness *in vivo* is in keeping with its angiogenic activity and further stresses the possible role of this receptor in cancer [12]. Recent study has clearly revealed the importance of P2X7 receptor in cancer growth [13]; expression of P2X7 enhanced engraftment ability and *in vivo* growth rate, increased expression of proliferation markers, reduced apoptosis, and enhanced vascular endothelial growth factor (VEGF) release and angiogenesis [13]. Moreover, we have recently reported that P2X7 receptor is involved in ATP release from melanoma in response to decrease of pH in the medium like a tumor microenvironment [14]. Thus, an important role of P2X7 receptor is suggested in cancer cells.

Our feasibility study of melanoma therapy using P2X7 irreversible antagonist oxidized ATP (oxATP) and other investigator's work have reported that administration of oxATP to mouse B16 melanoma-beading mice can suppress melanoma growth *in vivo* [13,14]. In our

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previous study, melanoma growth was significantly suppressed even when oxATP was injected into intra-peritoneal by only 1 time per week in a month (total 4 times/month), suggesting a strong anti-tumor effect of oxATP. Though it is well known that oxATP can block activation of P2X7 receptor [15], other effect of oxATP is also known, such as blockade of other P2X receptor subtypes [16] or unidentified mechanisms [17]. Thus, though oxATP might become a candidate of anti-tumor drug, the mechanism of anti-tumor effect has not been established. In this study, since it is wondering whether oxATP suppress tumor growth via blockade of angiogenesis, we here investigated the effect of oxATP on angiogenesis *in vitro* and *in vivo*.

Materials and Methods

Reagents and antibody

Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Apyrase, ATP, oxATP, 2',3'-O-(2,4,6-Trinitrophenyl) adenosine-5'-triphosphate (TNP-ATP), pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS), suramin and 2'&3'-O-(4-benzoyl) benzoyl ATP (BzATP) were purchased from Sigma-Aldrich (St Louis, MO). 5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD), MRS2211, MRS2578, NF157 and A438079 were purchased from Tocris Bioscience (Ellisville, MO USA). NF449 was purchased from Abcam (Cambridge, UK). Fetal bovine serum (FBS) was purchased from Biowest (Nuaille, France). All other chemicals used were of the highest purity available.

Cell culture

The mouse brain capillary endothelium b.End3 cells and the rat basophilic leukemia RBL-2H3 cells were maintained in DMEM containing 10% fatal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in 5% CO_2/95% air.

Animals

Male BALB/c were purchased from Sankyo Labo Service (Tokyo, Japan) and used at 6 weeks of age. They were housed in plastic cages with paper chip bedding and bred in rooms kept at a temperature of 23 ± 2°C and a relative humidity of 55 ± 10% under a 12 h light-dark cycle. They were allowed free access to tap water and experimental normal diet, CE-2 (CLEA Co, Tokyo, Japan). The mice were treated and handled according to the Guide Principles for the Care and Use Laboratory Animals of the Japanese Pharmacological Society and the protocol was approved by Tokyo University of Science's Institutional Animal Care and Use Committee.

Detection of P2 receptors mRNA

Total RNA was isolated from cells using a Fast pure RNA kit (Takara Bio Inc.). The first-strand cDNA was synthesized from 0.5 mg of total RNA with PrimeScript Reverse Transcriptase (Takara Bio). PCR was carried out by incubating each cDNA sample with the primers (0.5 μM each), Blend Taq polymerase (1.25 U: Toyobo), and dNTP mix (0.2 mM each). Amplification was carried out for 35 cycles (94°C for 30 seconds, annealing at 55°C for 30 seconds, 72°C for 1 minute). The products were then subjected to 2% agarose gel electrophoresis. Bands were stained with ethidium bromide and photographed.

Cell migration assay

Cell migration was tested in wound-healing based assay using Culture-Inserts (ibidi, Martinsried, Germany). After cell adherence, Culture-Inserts were removed. Twenty-four hours after removal of Culture-Inserts, pictures of the remaining gaps were taken at ten random points using a fluorescence microscope (BIOREVO; KEYENCE, Osaka, Japan).

Wound-healing assay

b.End3 cells were cultured to confluence. Cells were scratched manually with a sterile 20-200 μL pipette tip, and the detached cells were removed by washing with medium. The cells were incubated with P2 receptor antagonists for 24 h. Phase contrast images of ten random points in the wound area were taken at 24 h after scratching using a microscope with a fluorescence microscope (BIOREVO; KEYENCE, Osaka, Japan).

Hindlimb ischemia model

The right femoral artery and vein and cutaneous vessels branching from the caudal femoral artery side branch were performed. OxATP (0.5 mg/head) was intraperitoneal injected on the 0th, 4th, 7th and 11th days after ligature. A laser doppler perfusion image analyzer (Moor LDI2-IR; Moor Instruments) recorded blood flow postoperatively.

FITC-labeling gelatin zymography

Gelatin was dissolved in a 0.1 M sodium carbonate buffer at a final concentration of 10 mg/ml. A fluorescein isothiocyanate (FITC) solution (24 μL) (dissolved in dimethyl sulfoxide at 5 mg/ml) was added to the gelatin solution. Labeling was carried out for 24 hours at 4°C. The FITC gelatin was purified by gel filtration (Sephadex G-25). The supernatant was mixed with the sample buffer and the proteins ware separated on 10% SDS-PAGE containing 0.1% FITC gelatin. After electrophoresis under a constant voltage of 100 V, the gels were washed three times for 10 min each in 2.5% Triton X-100, followed by incubation at 37°C for 24 hours in an incubation buffer (50 mM Tris [tris (hydroxymethyl) aminomethane], 0.2 M NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and pH 7.5) in a light-shielded box. The gel was placed on a transilluminator and the activity of matrix metalloproteinase (MMP)-9 was measured.

Determination of VEGF, MMP-2 and MMP-9 in mouse serum

Mouse serum VEGF, MMP-2 and MMP-9 concentration were measured by ELISA according to the manufacturer's instructions (Quantikine; R&D Systems).

Determination of VEGF production from RBL-2H3 cells

RBL-2H3 cell (1×10^5 cells/mL) were plated into 48-well plates in complete medium and allowed to adhere for 48 h. After this time, cells were transferred to fresh medium and incubated for further 24 h in the absence or presence of ATP, BzATP, oxATP or A438079. The conditioned medium was harvested and centrifuged. VEGF release into the supernatants was measured with Quantikine Immunoassay for rat VEGF (R&D Systems) as described by the manufacturer.

Statistics

Results are expressed as mean ± SE. The statistical significance of differences between two groups was calculated by using Student's t-test, with the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA). The criterion of significance was set at $p<0.05$.

Results and Discussion

OxATP strongly suppressed migration and wound-healing of endothelium b.End3 cells

Cell migration of endothelium cells is important for a mechanism of angiogenesis. It is known that various P2 receptor subtypes including P2X7 receptor play important roles in cell migration or chemotaxis [18,19]. First, we confirmed the expression pattern of P2X and P2Y receptor subtypes in mouse brain capillary endothelium b.End3 cells. Figure 1 shows mRNA expression of P2X1, P2X4, P2X5, P2X7, P2Y2, P2Y5, P2Y6 and P2Y13 receptors in b.End3 cells. The faint expression was observed in P2X2, P2X3, P2Y4, P2Y12 and P2Y14 receptors.

Second, we investigated the effect of P2 receptor antagonists including oxATP on migration of b.End3 cells. The cell migration was analyzed by using culture insert system. In this assay system, cells not stimulated or scratched were moving on gap area made by culture insert system, resulting in reduction of gap area. We treated b.End3 cells with apyrase (ecto-ATPase), PPADS (broad P2X receptors antagonist), oxATP (irreversible antagonist of P2X7 receptor), A438079 (competitive P2X7 receptor antagonist), suramin (broad P2Y receptors antagonist), MRS2578 (selective P2Y6 receptor antagonist), NF157 (selective P2Y11 receptor antagonist) and MRS2211 (competitive P2Y13 receptor antagonist). The degree of cell migration was determined by reduction of gap area made by culture insert. As shown in Figure 2, the gap area in control group was decreased and filled by migrated cells. The remained gap area was wider in cells treated with apyrase, suggesting that extracellular nucleotides is involved in cell migration. Treatment with PPADS, A438079, suramin, MRS2578 or MRS2211 did not affect cell migration, and NF157 or MRS2211 was partially suppressed cell migration. Among these antagonists, the suppressive effect on cell migration was strongest in oxATP-treated cells. These results suggested that suppression of migration by oxATP would be mediated by a mechanism except for blockade of P2X7 receptor.

Third, we further examined the effect of P2 antagonists on wound healing of b.End3 cells. After cells were scratched and washed, cells were treated apyrase, PPADS, NF449 (highly selective P2X1 receptor antagonist), TNP-ATP, 5-BDBD (P2X4 receptor antagonist), oxATP, A438079, suramin, MRS2578, NF157 or MRS2211. As shown in Figure 3, the inhibition of wound healing was strongest in oxATP-treated cells. The wound healing was partially suppressed by treatment with apyrase, 5-BDBD and NF157-treated cells. On the other hand, other

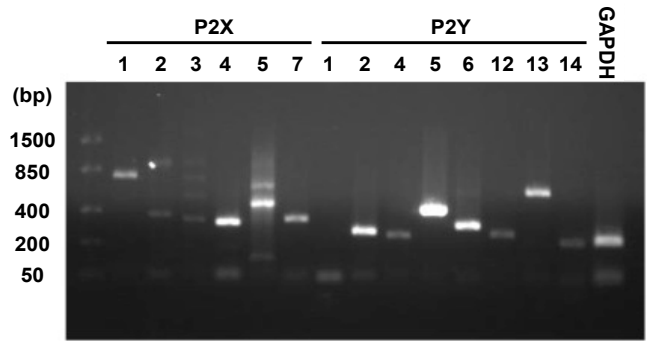

Figure 1: Expression of P2X and P2Y receptors mRNA in b.End3 cells. Total RNA was isolated from b.End3 cells and the mRNA expression of P2X and P2Y receptor subtypes was analyzed by RT-PCR using the specific primers for each P2 receptor subtype.

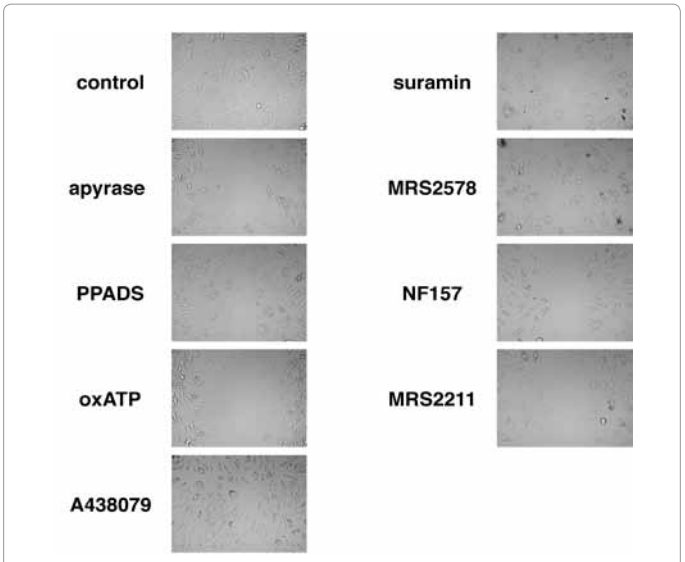

Figure 2: Effect of P2 receptor antagonists on cell migration of b.End3 cells. After Culture-Inserts were removed, cells were pretreated with apyrase (20 U/mL), PPADS (100 μM), oxATP (300 μM), A438079 (100 μM), suramin (100 μM), MRS2578 (10 μM), NF157 (50 μM) or MRS2211 (100 μM) and incubated for 24 h. Pictures of the remaining gaps were taken with using a microscope.

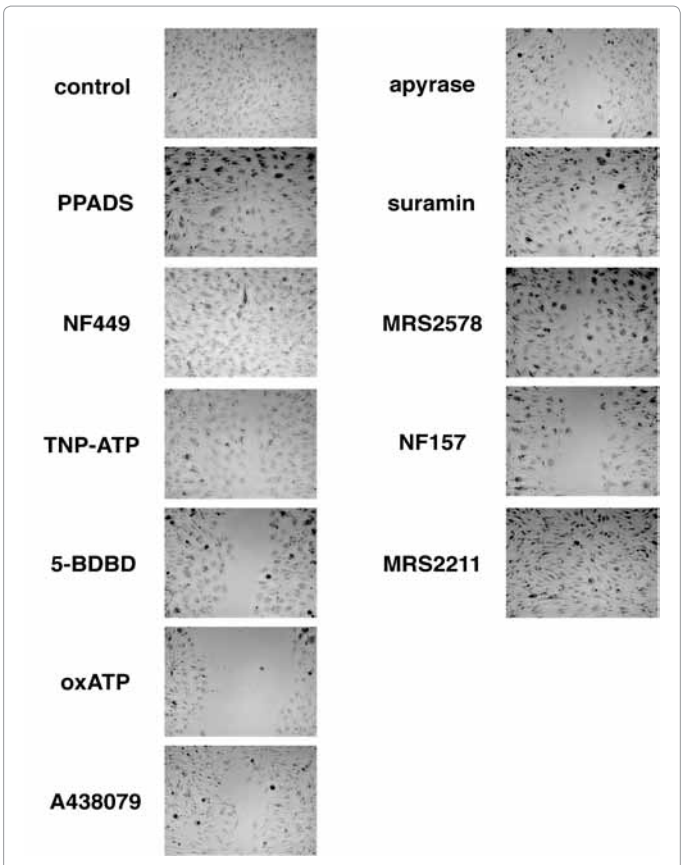

Figure 3: Effect of P2 receptor antagonists on wound-healing of b.End3 cells. Cells were scratched and washed with medium. Scratched cells were treated with PPADS (100 μM), NF449 (10 μM), TNP-ATP (10 μM), 5-BDBD (10 μM), oxATP (300 μM), A438079 (100 μM), apyrase (20 U/mL), suramin (100 μM), MRS2578 (10 μM), NF157 (50 μM) or MRS2211 (100 μM), and incubated for 24 h. Pictures of the remaining gaps were taken with using a microscope.

antagonists did not block the wound healing of b.End3 cells. These results indicate that oxATP has a strongest suppressive effect of wound healing, and involvement of P2X7 or P2X4 receptor in wound healing was suggested. Although oxATP is potent antagonist of P2X7, oxATP is also known to inhibit other P2X receptors. These results suggest that oxATP might inhibit not only P2X7 receptor but also other P2X receptor subtypes such as P2X4 receptor, resulting in stronger inhibition of wound healing than other inhibitors.

Blockade of blood flow recovery by oxATP *in vivo* via suppression of VEGF and MMPs release

Since our results indicate that oxATP might have a potency of anti-angiogenesis, we investigated whether oxATP inhibits angiogenesis *in vivo*. Next, we investigated the effect of oxATP on angiogenesis *in vivo* by analyzing a blood flow recovery of ischemic hind limb. Administration of oxATP (0.5 mg/head) was performed 2 times/week. As shown in Figure 4, the blood flow rate of hind limb was significantly decreased to less than 10% by the ischemic operation, but recovered within 2 weeks, indicating angiogenesis. However, administration of oxATP to mice significantly suppressed the recovery of blood flow rate compared with control group.

It is known that the angiogenesis is caused by MMP-2, MMP-9 and VEGF, which are produced by mast cells or stromal cells. Matrix metalloproteinases are family of zinc dependent endopeptidases. Activity of MMPs is commonly thought to be involved in the process of angiogenesis, because a number of studies have shown that various kinds of MMPs were up-regulated in ischemia-induced angiogenesis [20]. It is also reported that activation of P2X7 receptor is also involved in release of MMP-9 from monocytic cells [21]. Thus, activity of MMPs plays an important role in angiogenesis.

We measured the activity of MMP-9 and concentration of MMP-2, MMP-9 and VEGF in serum of hind limb ischemia mice treated with or without oxATP. The serum was obtained from the operated mice at 14th day. Figure 5A shows that serum MMP-9 activity in oxATP-treated group was lower than that in control group. Serum concentrations of MMP-2, MMP-9 and VEGF in oxATP-treated group were also lower (Figures 5B-D). These results indicate that oxATP would suppress blood flow recovery via blockade of MMP-2, 9 and VEGF release in hind limb ischemia mice.

Finally, we investigated whether oxATP can suppress VEGF production from mast cells, which is known to contribute to blood flow recovery of ischemic hind limb. We measured concentration of

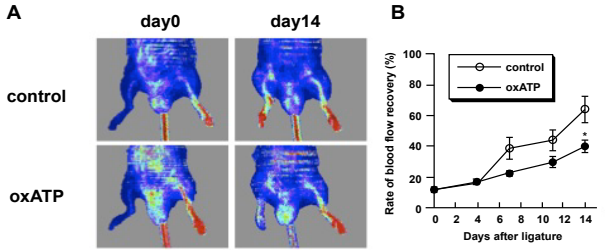

Figure 4: Inhibitory effect of oxATP on blood-flow recovery. Hind limb ischemia was induced in BALB/c mice (n=5/group). Mice were administrated with oxATP (0.5 mg/head, i.p.) on the 0th, 4th, 7th and 11th days after ligature. Mice were analyzed a laser doppler perfusion image analyzer for blood perfusion in the ligated limb at 14th day after vessel ligation. Quantification of blood flow after vessel ligation was shown in B.

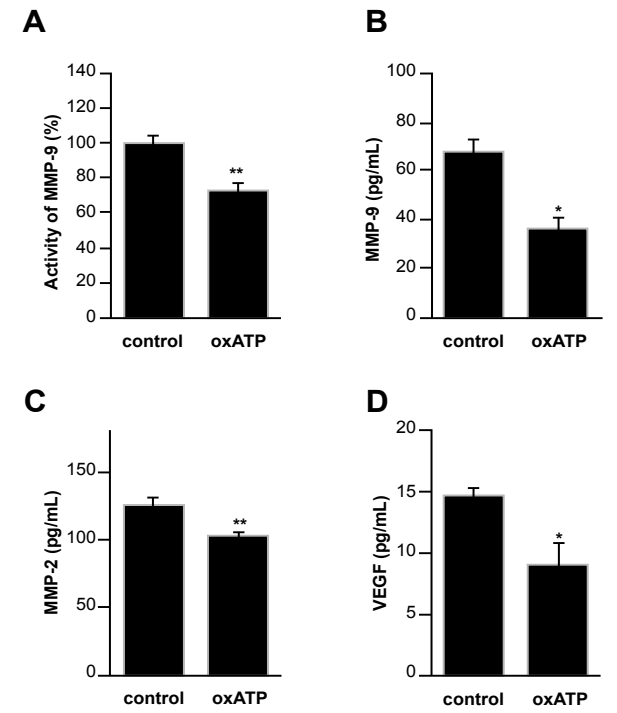

Figure 5: Effect of oxATP on serum MMP-9, MMP-2 or VEGF levels in hind limb ischemic mice. Blood of hind limb mice were collected at 14th day after ligation. (A) Activity of MMP-9 were detected by gelatin zymography (n=5/group). (B-D) Serum VEGF (n=5), MMP-2 (n=5), and MMP-9 (n=5) levels of hind limb mice were measured by ELISA. The data represent the means ± SE. Statistically significant differences are indicated by *(p<0.05) or **(p<0.01).

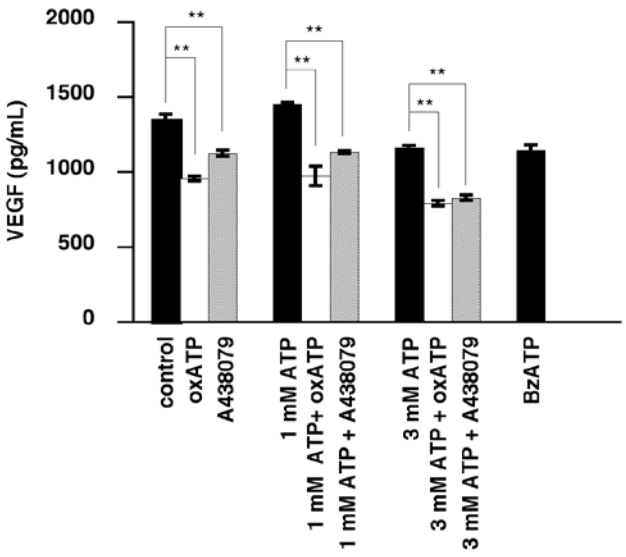

Figure 6: Effect of P2X7 agonists and P2X7 antagonists on VEGF production in RBL-2H3. RBL-2H3 cells were pre-incubated with vehicle, oxATP (300 μM) or A438079 (100 μM), and incubated with vehicle, ATP (1-3 mM), BzATP (100 μM) for 24 h. The concentration of VEGF in culture medium was measured by ELISA. The data represent the means ± SE. Statistically significant differences are indicated by **(p<0.01).

VEGF in culture medium of rat mast cell RBL-2H3 cells. We found the production of VEGF in RBL-2H3 mast cells was suppressed by treatment with oxATP. Another P2X7 antagonist A438079 also suppressed the VEGF production. However, stimulation of P2X7 receptor by treatment with 1-3 mM ATP or P2X7 agonist BzATP did not affect the production of VEGF. These results indicate that P2X7 receptor would be constitutively activated to produce VEGF in mast cells, and that suppression of VEGF production by oxATP would be involved in anti-angiogenetic effect *in vivo*. Considering these observations, it is suggested that suppression of VEGF production would be a mechanism of oxATP-induced anti-angiogenesis *in vivo* (Figure 6).

Conclusion

This is a first report showing that oxATP suppresses angiogenesis *in vivo*. Our data also suggest that the oxATP-induced anti-angiogenesis is mediated by various mechanisms such as suppression of the migration of endothelium cells, wound healing of endothelium cells, MMP-2/9 release, and VEGF production from mast cells. Further, inhibitory effects of oxATP might be mediated by blockade of not only P2X7 receptor but also other target such as other P2 receptor subtypes. Considering these data of anti-angiogenesis and previous report about anti-tumor effect of oxATP on melanoma growth, it is suggested that oxATP would be a good candidate of anti-tumor drug for melanoma.

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