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Thio-Cl-IB-MECA, a novel A_3 adenosine receptor agonist, suppresses angiogenesis by regulating PI3K/AKT/mTOR and ERK signaling in endothelial cells

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ABSTRACT

Although A_3AR agonists exhibit a variety of biological activities including anticancer effects, their possible anti-angiogenic effects have not yet been investigated. In the present study, we assayed the anti-angiogenic activity of thio-Cl-IB-MECA, a novel A_3AR agonist, in cultured HUVECs and mES/EB-derived endothelial cells. Thio-Cl-IB-MECA inhibited migration and tube formation by endothelial cells and dramatically decreased *ex vivo* microvessel sprouting in cultured mouse aortic rings. The anti-angiogenic activity of thio-Cl-IB-MECA was associated with suppression of the expression of the endothelial biomarker PECAM via regulation of PI3K/AKT/mTOR and ERK signaling in mES/EB-derived endothelial cells.

1. Introduction

A_3AR is a subtype of the adenosine receptor family, which includes A_1, A_{2A}, A_{2B}, and A_3 receptors [1]. Based on its expression and signal transduction activity in humans, A_3AR is a clinically relevant therapeutic target for the development of candidate drugs. For example, A_3AR agonists are under investigation for the treatment of myocardial and cerebral ischemia, as well as cancer [2]. Recently, we found that thio-Cl-IB-MECA is a novel selective A_3AR agonist that had anti-proliferative activity in several human cancer cell lines, including leukemia and lung cancers [3–5]. In addition, the expression of A_3AR has been reported in endothelial cells as well as cancer cells [6–8]. Therefore, the A3AR was considered to be essential for inducing angiogenesis in a human umbilical vein endothelial cell (HUVEC) model *in vitro* [9].

Angiogenesis is a complex process that involves sprouting of new capillaries from pre-existing blood vessels. It plays a crucial role in many pathological conditions including tumor growth, diabetic retinopathy, psoriasis, rheumatoid arthritis, and atherosclerosis [2]. In cancer, newly formed vessels not only promote tumor growth but also cause the tumor cells to become more malignant and metastatic [10]. Therefore, angiogenesis is an attractive target for the development of a wide variety of therapies, including antitumor agents [11].

Although A_3AR agonists exhibit anti-proliferative activity against cancer cells, anti-angiogenic effects of A_3AR agonists in endothelial cells have not been investigated. In addition, a recent report suggested one plausible mechanism that might underlie the anti-proliferative effects of A_3AR agonists, namely down-regulation of ERK via activation of the PI3K/AKT pathway in human melanoma cells [12]. In the present study, the potential anti-angiogenic activity and precise mechanism of action of the A_3AR agonist thio-Cl-IB-MECA in endothelial cells were investigated.

2. Materials and methods

2.1. Compound

Thio-Cl-IB-MECA (Fig. 1A) was synthesized as described by Jeong et al. [13] and dissolved in 100% DMSO. A 100 mM stock solution of thio-Cl-IB-MECA was prepared and stored at −20 °C until use.

Abbreviations: A_3AR, A_3 adenosine receptor; thio-Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-4′-thioadenosine-5′-N-methyluronamide; HUVECs, human umbilical vein endothelial cells; mES, mouse embryonic stem; EB, embryoid body; PECAM, platelet/endothelial cell adhesion molecule; IB-MECA, N^6-(3-iodobenzyl)adenosine-5′-N-methyluronamide; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-N-methylcarboxamide; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3-kinase.

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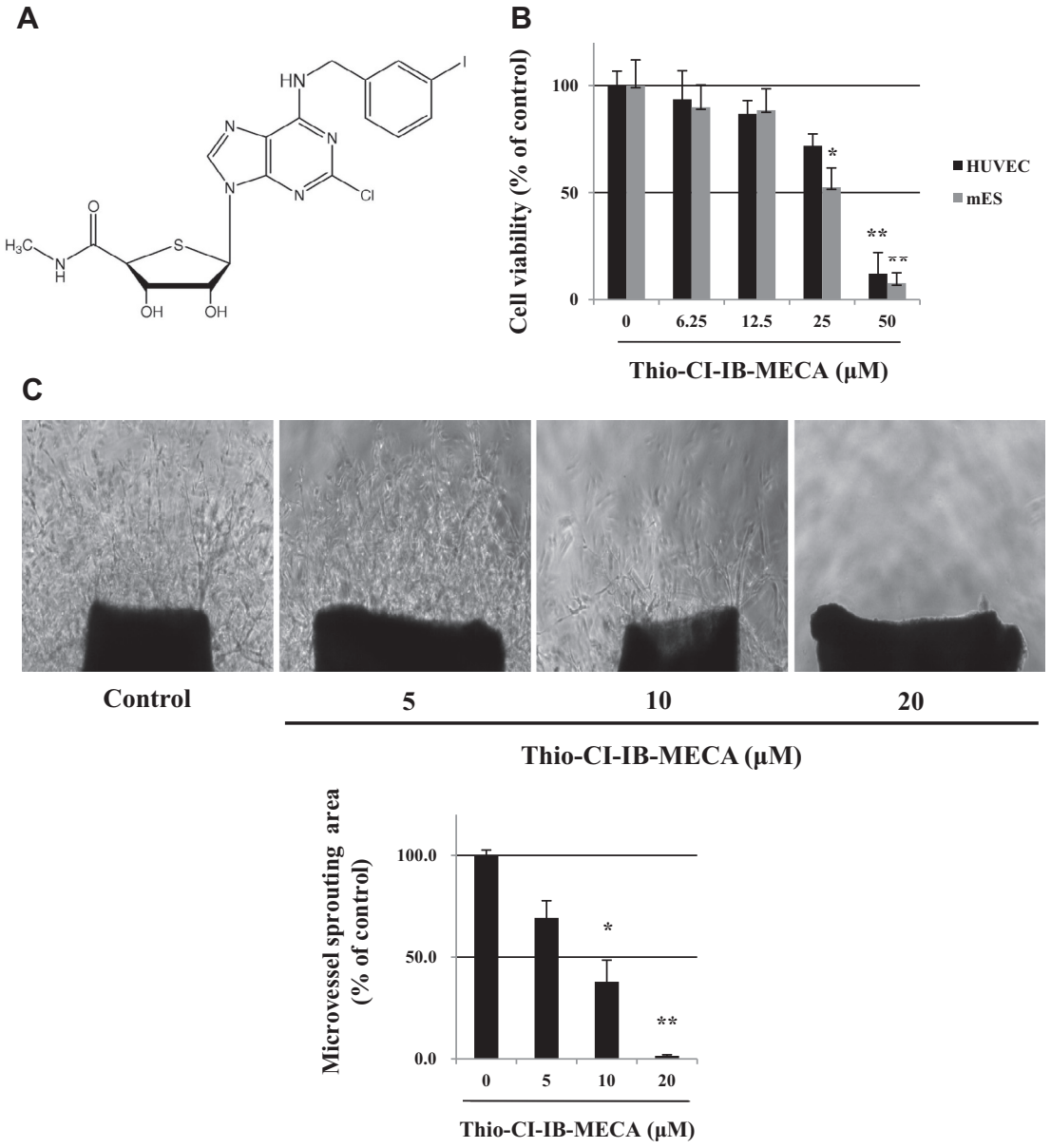

Fig. 1. Chemical structure of thio-Cl-IB-MECA (A) and characterization of endothelial cell exposure to thio-Cl-IB-MECA. (B) HUVECs and mES-derived endothelial cells were treated with thio-Cl-IB-MECA (0–50 μM) for 3 days. Cell viability was expressed as a percentage of the control (cultured in the absence of thio-Cl-IB-MECA). (C) Aortic rings isolated from mice were embedded in Matrigel in 48-well plates and then fed medium containing various concentrations of thio-Cl-IB-MECA for 4 days. The microvessel sprouting area was measured on day 4 of culture. Data are the means ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group.

2.2. Endothelial cell culture

HUVECs were obtained from ATCC (Rockville, MD) and cultured in EGM-2 (Lonza, Walkersville, MD) supplemented with 10% FBS at 37 °C in a 5% CO_2 atmosphere [14,15].

2.3. Culture and differentiation of mouse embryonic stem cells

Mouse D_3 ES cells (ATCC, Rockville, MD) were co-cultured with mitomycin C-treated mouse embryonic fibroblast cells in high glucose DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1000 U/ml of leukemia inhibitory factor (Chemicon, Temecula, CA), and basic ES cell medium components [50 U/ml of penicillin and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA), 1% non-essential amino acids (Invitrogen, Carlsbad, CA) and 0.1 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA)]. The cell culture and endothelial differentiation conditions have been described previously [14,16].

2.4. Cell viability assay

Cell viability was assessed using an MTT assay. HUVECs (5×10^3 cells/well) were seeded into a 96-well plate with EGM-2 medium supplemented with 10% FBS for 24 h. The cells were then cultured in fresh medium containing 2% FBS and various concentrations of thio-Cl-IB-MECA for 3 days. The viability of endothelial cells differentiated from mES cells was determined without LIF, as previously described by Kim et al. [14,16]. After incubation with thio-Cl-IB-MECA, an MTT solution was added, and the plate was incubated for an additional 4 h. The formazan product was dissolved in DMSO, and the absorbance was detected at 570 nm using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

2.5. Scratch-wound migration assay

HUVECs were allowed to grow to full confluence in 6-well plates pre-coated with 0.1% gelatin at 37 °C in a 5% CO_2 atmosphere. The cells were wounded by scratching with a 0.2-ml pipette tip. The medium was then replaced with fresh 0.5% FBS-EGM-2 medium containing various concentrations of thio-Cl-IB-MECA. The cells were incubated for 24 h and images taken under an inverted phase contrast microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The numbers of migrated cells were quantified by manual counting (DMC advanced program), and inhibition was calculated as a percentage relative to control.

2.6. Transwell migration assay

The migration assay was performed using a modified Boyden chamber model (Transwell apparatus, 8-μm pore size; Corning Inc., Corning, NY) as previously described [17]. Images were recorded using an inverted microscope, and the numbers of invasive cells were quantified by manual counting.

2.7. Tube formation assay

A tube formation assay was performed as previously described [18]. Matrigel (70 μl/well) was added to a 96-well plate and polymerized for 30 min at 37 °C. HUVECs (3×10^4 cells/well) and thio-Cl-IB-MECA (0–20 μM) were plated and incubated for 8 h. The formation of endothelial cell tubular structures was visualized under an inverted microscope. Tube formation was quantified by calculating the tube number and was expressed as a percentage of the tubes formed under un-treated control conditions.

2.8. Aortic ring assay

The mouse aortic ring assay was performed as previously described by Baker et al., with slight modifications [19]. Forty-eight-well plates were covered with 150 μl of Matrigel solution (Matrigel:EGM-2 medium = 1:1) and then incubated at 37 °C and 5% CO_2 for 30 min. Aortas were isolated from mice (Central Laboratory Animal Inc., Seoul) and cut into 1 mm rings. After rinsing with PBS, the aortas were placed on pre-coated Matrigel and then covered with an additional 100 μl of Matrigel solution. The aortic rings were cultured in 1 ml of EGM-2 containing supplements for 48 h, and then the medium was replaced with 1 ml of EGM-2 containing supplements with vehicle or thio-Cl-IB-MECA. After 4 days of incubation, microvessel growth was measured by taking photographs under an inverted microscope (40× magnification). The microvessel sprouting area was estimated using a phase-contrast microscope by measuring the distance from the cut end of the aortic segment to the approximate middle point of the capillary (DMC advanced program).

2.9. Immunocytochemistry

mES/EB-derived endothelial cells were incubated with thio-Cl-IB-MECA for 24 h, and then the cells were fixed with 4% paraformaldehyde and incubated overnight at 4 °C. The cells were blocked with blocking solution containing 1% BSA in PBS for 30 min and then incubated with rat anti-mouse PECAM (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After washing, the cells were then incubated with Alexa Fluor 594-labeled chicken anti-rat IgG (1:1000) (Invitrogen, Carlsbad, CA). After staining, the cover slips were mounted with medium containing DAPI (Vector Laboratories, Burlingame, CA). Fluorescence images were observed under a Zeiss Model 710 (Carl Zeiss, Jena, Germany) confocal microscope.

2.10. Three-dimensional collagen type-I sprouting angiogenesis assay

The 3-dimensional tube formation and sprouting angiogenesis assays were performed in type I collagen [20]. Briefly, EBs derived from mES cells were cultured in suspension containing EGM-2 medium for 7 days. The EBs were then plated in a type I collagen solution and incubated in EGM-2 medium. The effects of thio-Cl-IB-MECA on vascular sprouting were determined after incubation with thio-Cl-IB-MECA for 4 days. Vascular sprouting was analyzed using a phase contrast microscope (Nikon, Eclipse TE 2000-U, Tokyo, Japan). Sprouting endothelial cells from EB were grown in type I collagen and stained with Alexa Fluor 594 anti-mouse PECAM antibody (1 μg/ml, Invitrogen) for 10 h at 37 °C and washed 3 times with EGM-2 medium for 15 min each. The morphology and fluorescence were observed under a confocal microscope.

2.11. Western blot analysis

Differentiated endothelial cells were treated with various concentrations of thio-Cl-IB-MECA for 24 h. The harvested cells were lysed in protein extraction solution (Intron Biotechnology, Inc., Seongnam, Kyunggi) containing phosphatase inhibitors for 10 min at 4 °C. Equal amounts (40 μg) of protein samples were subjected to 6–15% SDS–PAGE. The separated proteins were transferred to PVDF membranes (Millipore, Bedford, MA) and then incubated with primary antibodies diluted with 5% BSA in TBST (1:200–1:2000) overnight at 4 °C. The membranes were then washed three times with TBST and incubated with the corresponding secondary antibodies. Protein bands were detected using an enhanced chemiluminescence detection kit (Intron Biotechnology, Inc.) and a LAS-1000 Imager (Fuji Film Corp., Tokyo, Japan).

2.12. Statistical analysis

Data are presented as the means ± SD for the indicated number of independently performed experiments. Statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$) was determined using Student's *t*-test for paired data.

3. Results

3.1. Thio-Cl-IB-MECA decreases proliferation of endothelial cells and suppresses capillary sprouting

To determine whether the possible anti-angiogenic activity of thio-Cl-IB-MECA is related to endothelial cell proliferation, the proliferation rates of HUVECs and mES-derived endothelial cells were evaluated using an MTT assay. Treatment with thio-Cl-IB-MECA for 3 days inhibited the proliferation of both endothelial cell types, with IC_{50} values of 38.5 and 26.0 μM in HUVECs and mES-derived endothelial cells, respectively. In particular, concentrations of thio-Cl-IB-MECA of greater than 25 μM significantly decreased cell viability in the endothelial cells (Fig. 1B). Therefore, further studies of thio-Cl-IB-MECA's biological activities were carried out at less than or equal to 20 μM thio-Cl-IB-MECA to eliminate cytotoxicity in the endothelial cells. In addition, we analyzed the effect of thio-Cl-IB-MECA in an *ex vivo* angiogenesis model, the mouse aortic ring assay, which is widely used to evaluate the anti-angiogenic activities of test compounds [21]. As shown in Fig. 1C, treatment with thio-Cl-IB-MECA significantly and dose-dependently suppressed the outgrowth of microvessels from the aortic rings.

3.2. Inhibition of endothelial cell migration and capillary-like tube formation by thio-Cl-IB-MECA

Because endothelial cell migration is considered a critical step in angiogenesis, we evaluated whether thio-Cl-IB-MECA is able to affect the migration of endothelial cells *in vitro*. As shown in Fig. 2A and A-1, wound healing by migrating HUVECs was almost complete after 24 h of incubation, but thio-Cl-IB-MECA treatment inhibited the migration of the endothelial cells in a concentration-dependent manner. In particular, HUVEC migration was significantly suppressed by treatment with 20 μM thio-Cl-IB-MECA ($^{**}P < 0.01$). In addition, HUVEC invasion in a Matrigel-coated transwell migration assay was also suppressed by treatment with thio-Cl-IB-MECA (Fig. 2B and B-1). Capillary-like tube formation that depends on maturation of migrated endothelial cells is also involved in early steps of angiogenesis. To determine whether thio-Cl-IB-MECA suppresses tube formation, we examined spontaneous tube formation, which occurs upon incubation of HUVECs in Matrigel, in the presence of thio-Cl-IB-MECA. Thio-Cl-IB-MECA inhibited capillary-like network formation by cultured HUVECs in a concentration-dependent manner (Fig. 2C and C-1).

3.3. Suppression of the expression of the endothelial biomarker PECAM and capillary sprouting in cultured EB-derived endothelial cells

To further investigate the relationship between thio-Cl-IB-MECA's inhibitory effects on growth and its suppression of endothelial biomarker expression, we examined the expression of PECAM, a representative endothelial biomarker, in mES/EB-derived endothelial cells. When endothelial cells differentiated from mES/EB were treated with thio-Cl-IB-MECA (0–20 μM) for 24 h, the expression of PECAM was easily detectable by immunofluorescence (control), but thio-Cl-IB-MECA dose-dependently suppressed the expression of PECAM in a 2-dimensional (2D) culture (Fig. 3A). We also

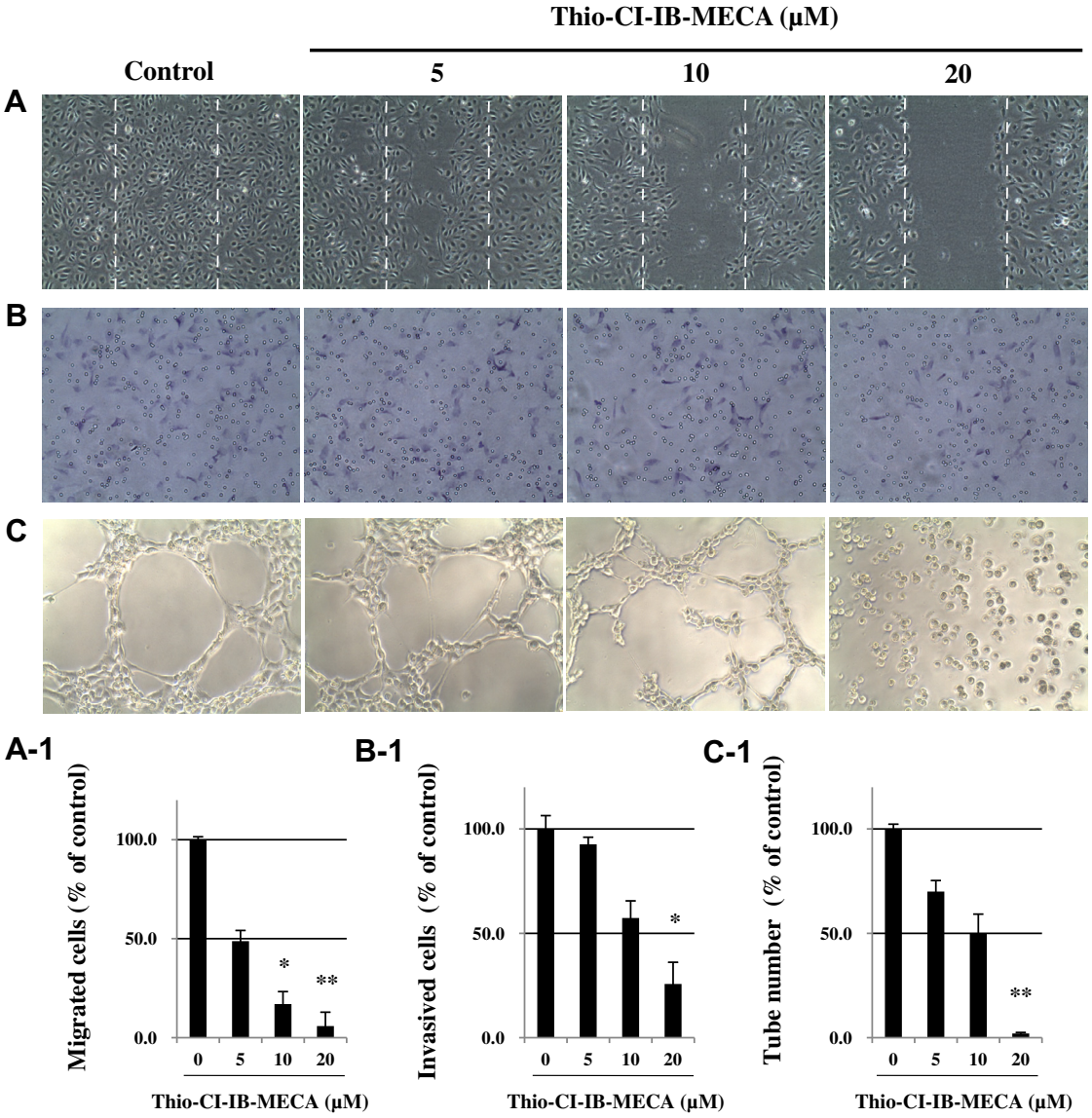

Fig. 2. Effects of thio-Cl-IB-MECA on cell migration and capillary structure formation by endothelial cells. (A, A-1) Cells were grown to confluence in 6-well plates, wounded, and treated with the indicated concentrations of thio-Cl-IB-MECA. (B, B-1) Endothelial cells were seeded in the upper chamber in media containing various concentrations of thio-Cl-IB-MECA, and the bottom chamber was filled with EGM-2 medium containing supplement. The cells with irregular shapes shown in the images are cells that had migrated into the lower chamber. (C, C-1) Cells and thio-Cl-IB-MECA were placed in 96-well plates coated with Matrigel. After 4–8 h in the absence and presence of thio-Cl-IB-MECA, the tubular structures were photographed. The results were reported as the means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control cells.

examined PECAM protein levels by Western blotting and found suppression of PECAM by thio-Cl-IB-MECA that correlated well with the immunofluorescence results (Fig. 3B and B-1). In addition, we also evaluated the effects of thio-Cl-IB-MECA on the formation of vessel-like structures (microvessel sprouting) in a 3-dimensional (3D) culture of mES-derived embryoid bodies. As shown in Fig. 3C, capillary-like microvessel sprouts were produced in cultured mES-derived embryoid bodies, and treatment with thio-Cl-IB-MECA effectively and dose-dependently suppressed this sprouting. In particular, the 20 μM dose of thio-Cl-IB-MECA significantly inhibited the capillary sprouting. When the vascular sprouts were stained using a PECAM antibody, thio-Cl-IB-MECA was also found to reduce the number of PECAM-positive cells (red) in the sprouts derived from mES/EB in 3D culture (Fig. 3C). These findings indicate that thio-Cl-IB-MECA effectively suppresses the formation of capillary-like microvessel sprouts in EB-derived cells.

3.4. Suppression of PI3K/AKT/mTOR and ERK signaling in cultured mES/EB-derived endothelial cells by thio-Cl-IB-MECA

mES/EB-derived endothelial cell systems have been previously suggested as a novel *in vitro* model for the study of anti-angiogenic activity [22]. To further understand the molecular mechanism by which thio-Cl-IB-MECA mediates its anti-angiogenic activity, we

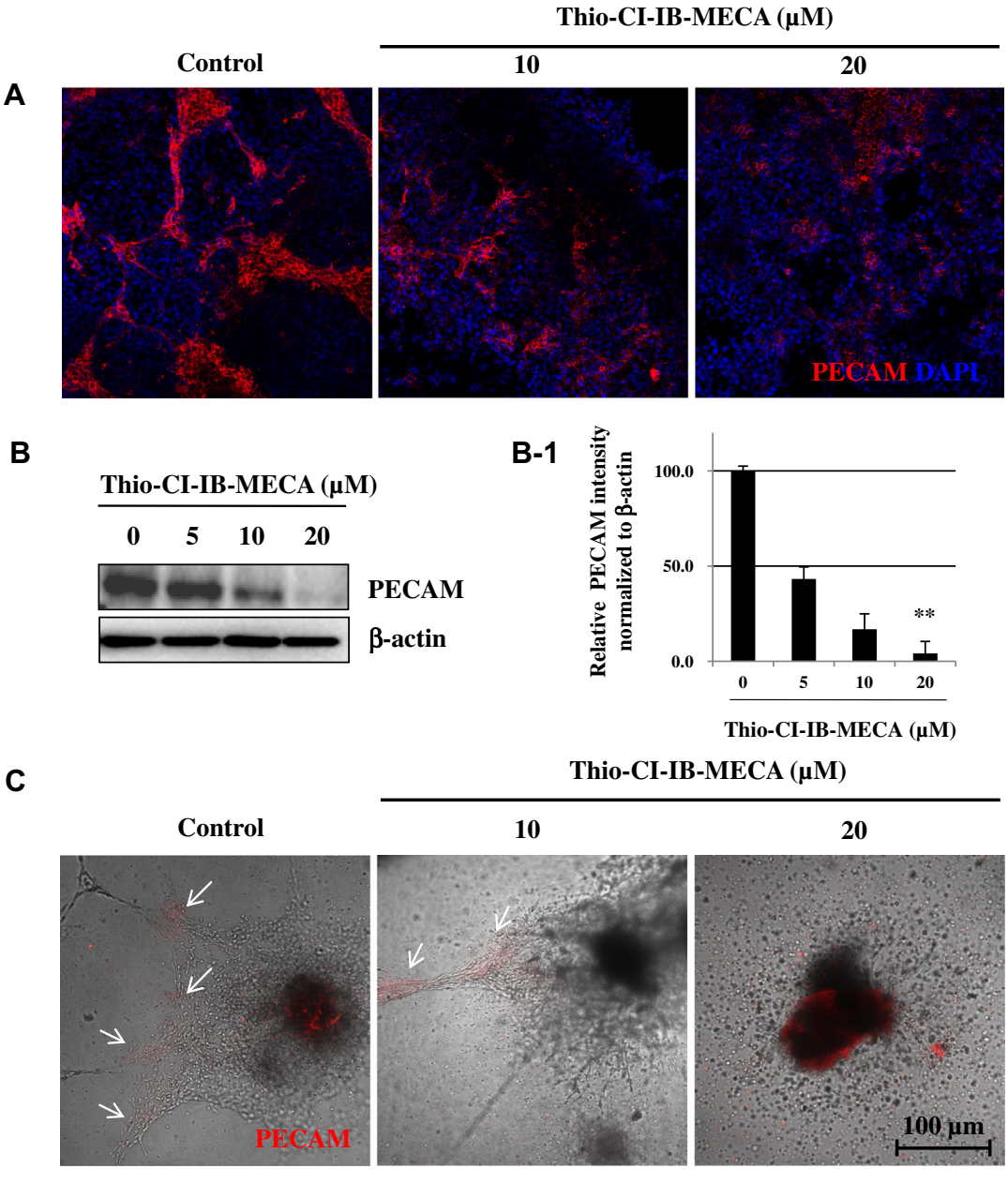

Fig. 3. Effects of thio-Cl-IB-MECA on vascularization of endothelial cells derived from mES/EBs. (A) mES/EB-derived cells were differentiated for 10 days and then exposed to thio-Cl-IB-MECA (0, 10, or 20 μM) for 24 h. The mES/EB-derived endothelial cells were stained with an antibody directed against the endothelial cell biomarker PECAM. Nuclei were stained with DAPI. Magnification×100. (B, B-1) PECAM protein levels in cell lysates from mES/EB-derived endothelial cells after 24 h of incubation with thio-Cl-IB-MECA (0–20 μM) were measured by Western blot analysis. β-actin was used as an internal control. The statistical analysis was performed with Student's *t*-test. $^{**}P < 0.01$ compared to control. (C) EBs derived from mES were cultured in suspension containing EGM-2 medium for 7 days. EBs embedded in collagen gel were treated with thio-Cl-IB-MECA for 4 days. Changes in the sprouting activity of EB-derived endothelial were observed upon treatment with thio-Cl-IB-MECA (0, 10, or 20 μM). PECAM-positive cells were observed in sprouting EBs in 3D culture under a confocal microscope. White arrows highlight the sprouted PECAM-positive cells (red). Magnification× 40.

examined PI3K/AKT/mTOR and ERK signaling in mES/EB-derived endothelial cells. It is well known that activation of the PI3K/AKT/mTOR pathway contributes to proliferation and migration in endothelial cells [23]. We found that thio-Cl-IB-MECA treatment suppressed PI3K/AKT activation, leading to suppression of the activation of mTOR and its downstream effector p70S6K (Fig. 4A). We also found that thio-Cl-IB-MECA effectively down-regulated the activation of ERK and cyclin D1 in mES/EB-derived endothelial cells (Fig. 4B). The effects of thio-Cl-IB-MECA on the AKT and ERK signaling pathways were further confirmed using co-treatment with thio-C1-IB-MECA and the AKT inhibitor LY294002 or the ERK inhibitor PD98059. As shown in Fig. 4C and D, co-treatment with thio-Cl-IB-MECA and the AKT or ERK inhibitor resulted in greater suppression of AKT and ERK activities than either inhibitor alone. These data suggest that the decreases in proliferation and PECAM expression induced by thio-Cl-IB-MECA treatment of mES/EB-derived endothelial cells might be partially due to down-regulation of PI3K/AKT/mTOR and ERK signaling.

4. Discussion

Recent findings suggested that A_3AR plays key roles in a variety of physiological functions, including cell proliferation. On this note, A_3AR agonists such as IB-MECA and Cl-IB-MECA have been found

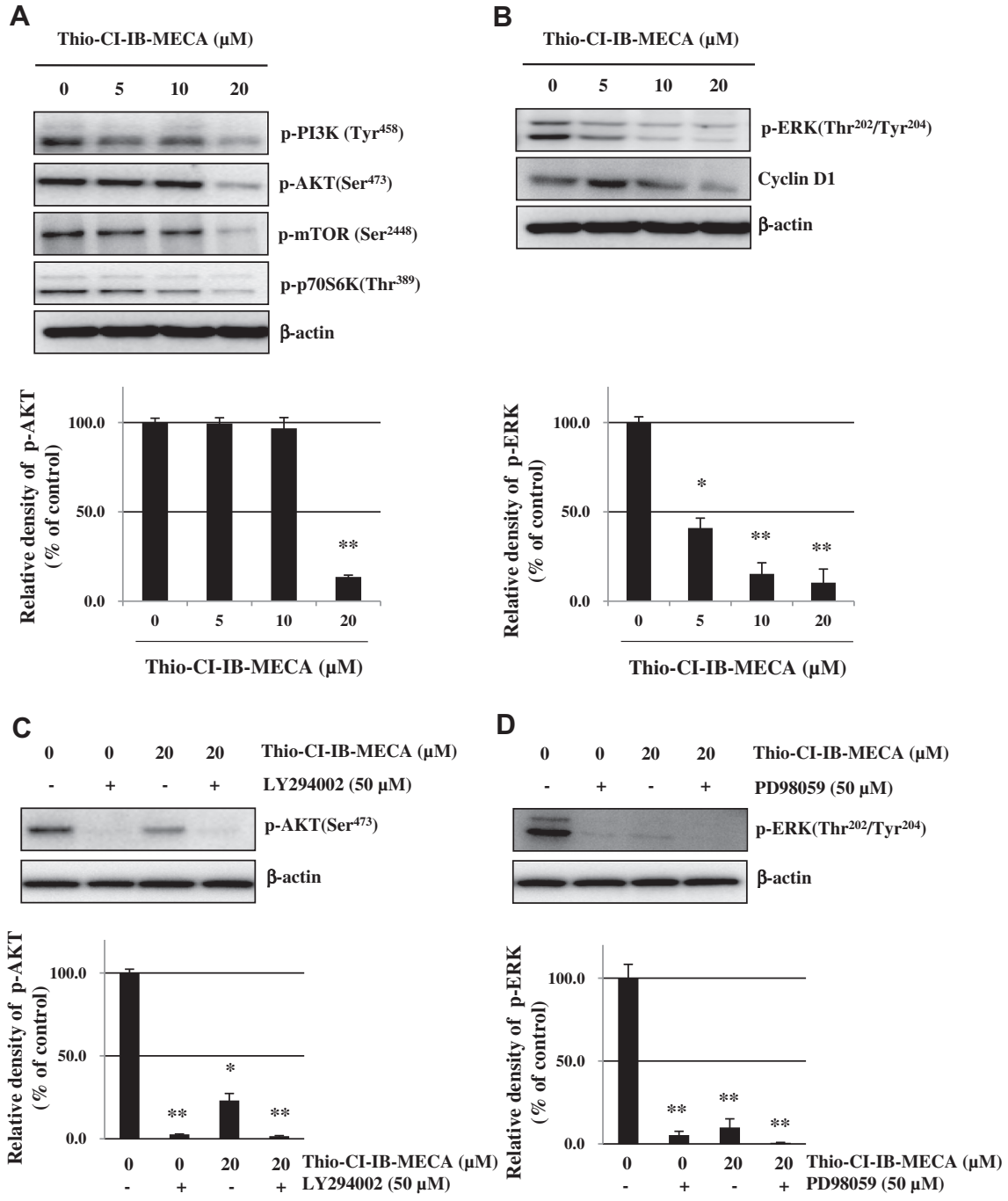

Fig. 4. Effects of thio-Cl-IB-MECA on PI3K/AKT/mTOR and ERK activation in mES/EB-derived endothelial cells. (A and B) Cells were treated with the indicated concentrations of thio-Cl-IB-MECA for 24 h and the protein expression analyzed by Western blotting. (C and D) The cells were treated with 50 μM of the AKT inhibitor LY294002 and 50 μM of the ERK inhibitor PD98059 with or without thio-Cl-IB-MECA (at 0 or 20 μM). β-Actin was used as an internal control. The statistical analysis was performed with Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control.

to act as anti-tumor agents *in vitro* and *in vivo* [24,25]. Recently, we developed thio-Cl-IB-MECA for use as a A_3AR agonist and found that its selectivity and affinity for A_3AR were superior to those of IB-MECA and Cl-IB-MECA [13]. Thio-Cl-IB-MECA exhibits anti-proliferative and anti-tumor effects in several human cancer cell lines in both *in vitro* and *in vivo* models [3,5,26]. However, possible anti-angiogenic activities of thio-Cl-IB-MECA are as yet unreported. Therefore, we investigated, for the first time, the effect of thio-Cl-IB-MECA on angiogenesis and investigated a possible mechanism of action in endothelial cells. In our previous study, we also effectively developed a method to differentiate mES cells into the endothelial lineage using EGM-2 medium [15]. In this study, we used this mES/EB-derived endothelial cell system to evaluate thio-Cl-IB-MECA's anti-angiogenic activity. Thio-Cl-IB-MECA effectively inhibited migration and tube formation in cultured endothelial cells, including HUVECs and mES-derived cells. More evidence for anti-angiogenic effects of thio-Cl-IB-MECA was supplied by its inhibition of capillary sprouting by endothelial cells in an *ex vivo* model and in the mES/EB-derived endothelial model system (2-D and 3-D culture systems). These inhibitory activities were also well correlated with suppression of the expression of the endothelial biomarker PECAM.

Anti-angiogenic therapy is currently considered one of the most promising and efficient therapeutic strategies for the treatment of cancer [27,28]. Previous studies have shown that PI3K and AKT are activated by various stimuli, including VEGF, in endothelial cells and go on regulate multiple critical steps of angiogenesis by phosphorylating downstream molecules such as mTOR [29]. Indeed, activation of the PI3K/AKT/mTOR signaling pathway in endothelial cells in culture promotes cell proliferation, angiogenesis, invasion, and metastasis [30]. In particular, AKT regulates cell proliferation, survival and migration. We found that the activation of AKT was significantly suppressed by thio-Cl-IB-MECA in mES/EB-derived endothelial cells. In addition, the ERK pathway is known to be activated by various stimuli including mitogens and cell survival factors [31,32], and ERK [33] also regulates cyclin D1 expression in somatic cells [34]. A_3AR agonists such as IB-MECA and Cl-IB-MECA cause cell cycle arrest and decreased proliferation in cancer cells by regulating ERK signaling [33]. The data presented here suggest that thio-Cl-IB-MECA effectively suppresses the activation of AKT and ERK, thus causing decreased PECAM expression via inhibition of cyclin D1 in endothelial cells.

In summary, the present study shows that thio-Cl-IB-MECA, a novel class of A_3AR agonist, inhibits migration and tube formation by endothelial cells. One plausible mechanism underlying this action is regulation of the PI3K/AKT/mTOR and ERK signaling pathways.

Acknowledgments

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