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2-Methoxycinnamaldehyde inhibits tumor angiogenesis by suppressing Tie2 activation

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ABSTRACT

Blood vessels are mainly composed of intraluminal endothelial cells (ECs) and mural cells adhering to the ECs on their basal side. Immature blood vessels lacking mural cells are leaky; thus, the process of mural cell adhesion to ECs is indispensable for stability of the vessels during physiological angiogenesis. However, in the tumor microenvironment, although some blood vessels are well-matured, the majority is immature. Because mural cell adhesion to ECs also has a marked anti-apoptotic effect, angiogenesis inhibitors that destroy immature blood vessels may not affect mature vessels showing more resistance to apoptosis. Activation of Tie2 receptor tyrosine kinase expressed in ECs mediates pro-angiogenic effects via the induction of EC migration but also facilitates vessel maturation via the promotion of cell adhesion between mural cells and ECs. Therefore, inhibition of Tie2 has the advantage of completely inhibiting angiogenesis. Here, we isolated a novel small molecule Tie2 kinase inhibitor, identified as 2-methoxycinnamaldehyde (2-MCA). We found that 2-MCA inhibits both sprouting angiogenesis and maturation of blood vessels, resulting in inhibition of tumor growth. Our results suggest a potent clinical benefit of disrupting these two using Tie2 inhibitors.

1. Introduction

It is well-established that dysregulation of blood vessel formation is involved in several diseases, such as cancer, retinopathy, chronic inflammation and others. To develop optimal strategies for inhibiting angiogenesis and preventing progression of these diseases, the mechanisms controlling blood vessel formation have been extensively analyzed. Of the many growth factors involved in this process, vascular endothelial growth factor (VEGF) or its cognate receptors (VEGFRs) have been targeted by angiogenesis inhibitors which are already utilized clinically, especially in cancer therapy [1]. Indeed, the efficacy of neutralizing antibody to VEGF (bevacizumab) in prolonging survival in patients with malignant colon cancer has been established, and utilization of this drug is currently being extended to other tumor types [2].

VEGF plays a fundamental role in development, tube formation and proliferation of endothelial cells (ECs) [3]. Tubes generated using VEGF alone do not mature; blood vessel stability requires the adherence of mural cells such as pericytes or smooth muscle cells to ECs on the basal side. When Tie2, a receptor tyrosine kinase, expressed predominantly in ECs, is activated by angiopoietin-1 (Ang1), usually released from mural cells, cell adhesion between ECs and mural cells is induced and the structural stability of blood vessels is enhanced [4]. It is widely accepted that mural cell adhesion to ECs induces a transition from the actively elongating status of a new blood vessel to a mature state, resulting in finalization of sprouting angiogenesis. On the other hand, when angiogenesis is ongoing and mural cells are absent near ECs, Tie2 activation induces migration of ECs to support sprouting angiogenesis. Therefore, Tie2 activation plays dual roles as a pro-angiogenic and as an anti-angiogenic factor. Which activity dominates is dependent on intracellular signaling via Tie2 phosphorylation [5]. When it is mainly the Akt pathway that is activated through Tie2, cell adhesion between mural cells and ECs is induced, resulting in vascular quiescence. However, when the Erk rather than Akt pathway is activated via Tie2, the migration of ECs is enhanced.

In the context of therapy with angiogenesis inhibitors, maturation of blood vessels is a key locus for the development of resistance against angiogenesis inhibitors. Mural cells adhere to ECs in mature blood vessels. In this situation, the two cell types engage in cross-talk and stimulate each other by producing several cytokines such as VEGF, Ang1, platelet-derived growth factor (PDGF), transforming growth factor β and others [6]. This results in suppression of apoptosis of both mural cells and ECs. Moreover,

Abbreviations: 2-MCA, 2-methoxycinnamaldehyde; EC, endothelial cell; Ang1, angiopoietin-1; VEGF, vascular endothelial growth factor.

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adhesion molecules responsible for keeping the mural cells and ECs in close proximity also provide signals involved in preventing their apoptosis. Tie2- or Ang1-deficient mice lack EC/mural cell integrity and manifest insufficient vessel outgrowth [7]. Therefore, Tie2 inhibition in tumors may suppress sprouting angiogenesis as well as inhibiting blood vessel maturation.

In the present study, we screened for small molecule Tie2 inhibitors in natural products. Among the molecules investigated, we found that 2-methoxycinnamaldehyde [(2E)-3-(2-methoxyphenyl)acrylaldehyde: 2-MCA] inhibits Ang1-mediated Tie2 phosphorylation. 2-MCA is extracted from the bark of cinnamon trees and other species of the genus *Cinnamomum* and gives those plants their flavor [8]. 2-MCA has been identified as the major active fungitoxic component, especially against Candida albicans [9], and also possesses strong antibacterial activity [10]. Here, we show that 2-MCA abrogates Ang1-mediated endothelial barrier function and tube formation in vitro. Moreover, using 2-MCA we analyzed whether Tie2 inhibition suppresses both the process of sprouting angiogenesis as well as blood vessel maturation in the tumor microenvironment.

2. Materials and methods

2.1. Screen for Tie2 kinase inhibitors

Human Tie2, cytoplasmic domain [771-1124(end) aminoacids was expressed as an N-terminal GST-fusion protein. First, we selected extracts from several herbs because of their inhibitory effects on proliferation or migration of ECs in the P-Sp culture system that supports vasculogenesis and angiogenesis [11]. Approximately 100 test compounds were isolated from extracts of these herbs by high performance liquid chromatography were evaluated. Compound solution, substrate/ATP/Metal solution, and kinase solution were prepared with assay buffer (15 mM Tris–HCl, 0.01% Tween-20, 2 mM DTT, pH 7.5) and mixed in streptavidin coated 96 well microplates (Perkin Elmer). Plates were incubated for 1 h at room temperature and then washed four times to stop the reaction. Wells were blocked with blocking buffer containing 0.1% BSA and then the detection antibody (HRP-conjugated PY20; Santa Cruz Biotechnology) was added and incubated for 30 min. After washing, TMB solution (MOSS, Inc.) was added to each well and incubated for 5 min. To stop the HRP reaction, 0.1 M sulfuric acid was added. The kinase reaction was evaluated by absorbance at 450 nm. Among compounds tested, we found one with Tie2 kinase inhibitory effects. This compound was identified as 2-MCA by NMR (NM-ECP400; JOEL).

2.2. Reagents

2-MCA (Sigma Aldrich), Recombinant human $VEGF_{165}$ (PEPROTECH), Ang1, and HGF (R&D Systems) were used.

In Western blotting analysis, mouse anti-Tie2 (Ab33) antibodies (Abs) (Upstate), phospho-Tie2 (Tyr992) (R&D Systems, Inc.), rabbit anti-Akt, phospho-Akt (Ser473), p44/42, phospho-p44/42 (Thr202/Tyr204) (Cell Signaling Technology, Inc.) and mouse anti-GAPDH mAb (Chemicon) were used as the first Abs. Anti-phosphoTie2 (Tyr992) Abs were diluted 1:500, other Abs 1:1000. HRP-conjugated anti-rabbit and anti-mouse Ig (Jackson ImmunoResearch) was used as the secondary antibody (diluted 1:1000).

For the immunofluorescence analysis, mouse anti-human ZO-1 (BD Biosciences), rat anti-mouse CD31 (BD Biosciences) and Cy3-conjugated anti-α-smooth muscle actin (1A4; Sigma) mAbs were used as the first Abs (diluted 1:200). Alexa488-conjugated goat anti-mouse and anti-rat Igs (Invitrogen) were used as the secondary Abs (Invitrogen) (dilution; 1:200).

2.3. Cell culture

Ba/F3 cells were grown in RPMI-1640 medium supplemented with 10% FBS and 200 pg/ml IL-3 (GIBCO). Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Kurashiki, Japan) and maintained according to the supplieŕs instructions. For Ang1, VEGF and HGF stimulation, cells were starved in RPMI-1640 medium containing 1% FBS for 3 h. Colon26 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. HCT116 cells were grown in RPMI-1640 medium supplemented with 10% FBS. Platinum-E cells (Plat-E; packaging cells) and stable cell lines transfected with pMRX virus vector were cultured in 10% FBS-containing DMEM [12,13].

2.4. Plasmid construction

Mouse Tie2 or mutant Tie2 (Tie2R848W) was fused to sequences encoding full-length Venus. A Myc epitope was inserted as a tag between Tie2 and Venus. Genes were inserted at the multicloning site of pEGFPN1 vector or pMRX virus vectors.

2.5. Retroviral infection

Plat-E cells were transfected with 1.0 μg of pMRX-Tie2-Myc-Venus or pMRX-Tie2R848W-Myc-Venus vectors using Lipofectamine 2000 (Invitrogen), then incubated for 24 h at 37 °C after which the medium was changed. After 12 h (36 h from transfection) and 24 h (48 h from transfection), conditioned medium was harvested, sterilized by filtration and used to infect Ba/F3 cells. About 8 μg/ml polybrene was added to facilitate infection. Stable cell lines expressing wild-type Tie2 (BaF/WT-Tie2 cells) or constitutively active Tie2 (BaF/R848W-Tie2 cells) were selected by culture in medium containing puromycin (5 μg/ml) or blasticidin (10 μg/ml).

2.6. SDS–PAGE and Western blotting

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lyzed with RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). The cells were incubated on ice for 10 min followed by centrifugation at 15,000 rpm for 5 min at 4 °C. Proteins electrophoretically separated using 7.5% SDS gels were transferred to nylon membranes (Amersham Biosciences) by a wet blotting procedure (140 V, 200 mA, 120 min). The membrane was blocked with 5% skim milk/TBST for 60 min, subsequently incubated with the Abs as indicated in the figures and processed for chemiluminescence detection with ECL solution.

2.7. Tube formation

HUVECs were cultured at 3×10^4 cells/well on 100 μl of growth factor-reduced Matrigel (BD Biosciences) in RPMI-1640 supplemented with 1% FBS. The cells were incubated for 18 h at 37 °C, 5% CO_2. Tube formation was observed in living cells microscopically (Leica AF6000).

2.8. Analysis of cell apoptosis

HUVECs were cultured for 24 h in the presence or absence of 2-MCA (30 μM). The cells were stained with Annexin V and propidium iodide using an Annexin V-FITC apoptosis detection kit (BD Biosciences), and analyzed by flow cytometry (Becton Dickinson).

2.9. Mice

Balb/c and KSN nude mice were purchased from Japan SLC (Shizuoka, Japan). Mouse and human colon cancer-derived colon26 and HCT116 cells (3.5×10^6 cells) were inoculated subcutaneously into 8 week-old female mice. Animals were housed in environmentally controlled rooms of the animal experimentation facility at Osaka University. All experiments were carried out following the guidelines of Osaka University Committee for animal and recombinant DNA experiments.

2.10. Immunocytochemistry and immunohistochemistry

For immunocytochemistry, cells on 0.1% gelatin (Sigma Aldrich)-coated glass dishes were rinsed, fixed for 10 min in 4% paraformaldehyde-PBS (pH 7.5) and washed with PBS. Subsequently, the cells were permeabilized with 0.1% Triton X-100 for 30 min. After washing with PBS, cells were blocked with PBS containing 5% normal goat serum and 1% BSA for 30 min and immunostained with first Abs (1:100) for 1 h. Protein reacting with Abs was visualized with secondary Abs (1:200). The cells were observed under a microscope (Leica TCS SP5 Ver1.6) using HCX PL APO lambda blue 63×1.4 oil. Images were processed using Adobe Photoshop CS5 Extended software (Adobe Systems). Immunohistochemical analysis was performed as previously reported [14].

2.11. Statistical analysis

Results were expressed as the mean ± SEM. Student's *t* test was used for statistical analysis. Differences were considered statistically significant when $P < 0.01$.

3. Results

3.1. 2-MCA inhibits phosphorylation of Tie2

Using an ELISA-based in vitro kinase assay to screen for Tie2 inhibitors, we identified 2-MCA as a novel candidate molecule. To confirm that 2-MCA inhibits Ang1-mediated Tie2 phosphorylation, a pro-B lymphocyte cell line (Ba/F3) expressing mouse Tie2 ectopically (BaF/WT-Tie2 cells) was used. When BaF/WT-Tie2 cells were stimulated with Ang1, phosphorylation of Tie2 was observed; this was suppressed by 2-MCA in a dose-dependent manner (Fig. 1A).

It is well known that Tie2 phosphorylation induces activation of downstream signal pathways such as PI3K-Akt and p42/44, Erk. We found that 2-MCA inhibited both of these Ang-1-stimulated, Tie2-dependent signaling cascades (Fig. 1B).

It has been reported that exchange of tryptophan for arginine at position 849 (R849W) of Tie2 by point mutation leads to its constitutive activation and that this mutation is one cause of human hereditary venous malformation [15]. We generated a construct

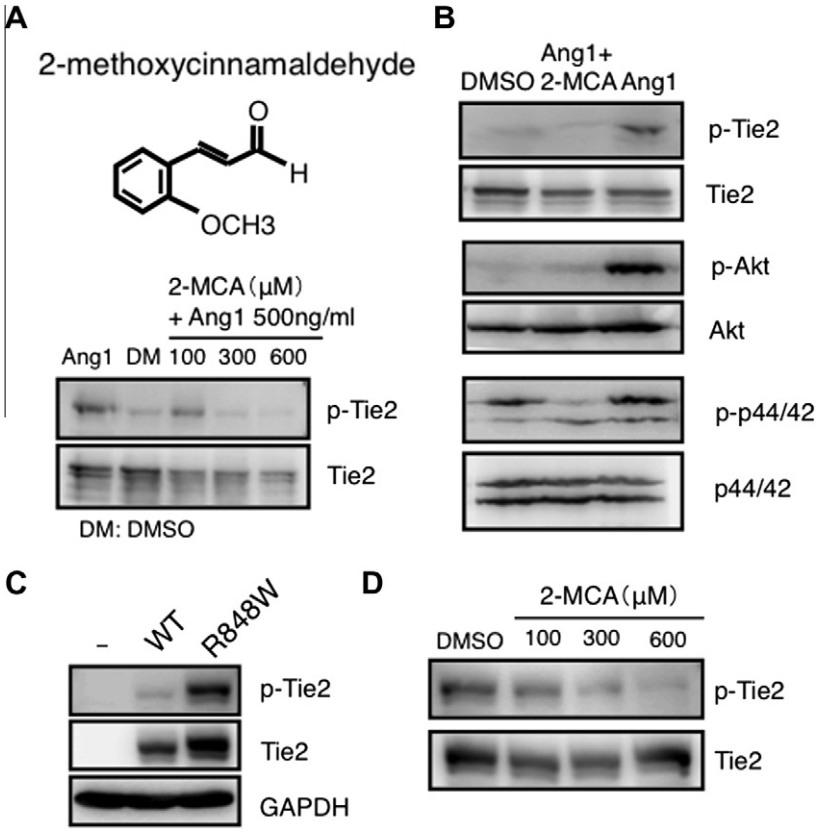

Fig. 1. 2-MCA inhibits phosphorylation of Tie2. (A) Chemical structural formula of 2-methoxycinnamaldehyde (2-MCA) (upper). Western blotting was performed using BaF/WT-Tie2 cells (bottom). BaF/WT-Tie2 cells were stimulated with or without Ang1 for 15 min in the presence or absence of titrated doses of 2-MCA. (B) Effect of 2-MCA on downstream signals of Tie2. BaF/WT-Tie2 cells were stimulated with or without Ang1 (500 ng/ml) for 15 min in the presence or absence of 2-MCA (600 μM). Phosphorylation of Tie2, Akt and p44/42 was assessed. (C) Western blotting of Ba/F3, BaF/WT-Tie2 (WT), and BaF/R848W-Tie2 cells (R848W). GAPDH was used as the internal control. (D) Inhibitory effect of 2-MCA on phosphorylation of constitutively active Tie2. BaF/R848W-Tie2 cells were cultured with titrated doses of 2-MCA as indicated for 15 min. Tie2 phosphorylation was then assessed.

coding for this mutant Tie2 in mice (R848W) and transfected it into Ba/F3 cells. Wild-type Tie2 was only weakly phosphorylated without Ang1 on overexpression, but mutant Tie2 was strongly phosphorylated (Fig. 1C). This phosphorylation of constitutively active Tie2 was also inhibited by 2-MCA in a dose-dependent manner (Fig. 1D).

3.2. 2-MCA inhibits Ang1-mediated stabilization of cell-cell junctions and tube formation

Activation of Tie2 in ECs enhances the stability of blood vessels and supports angiogenesis mainly via Akt or Erk activation, respectively, as described above [5]. Therefore, we next investigated whether 2-MCA affects these functions of Tie2 using human umbilical vein endothelial cells (HUVECs). We confirmed that activation of Akt and Erk mediated by stimulation with Ang1 was abrogated by 2-MCA in HUVECs (Fig. 2A), as was observed using BaF/Tie2 cells. When HUVECs reach confluence, tight junctions marked by ZO-1 appear (Fig. 2B). ZO-1 expression was not observed on EC–EC contact on stimulation with VEGF, consistent with its well known action to disturb EC–EC junction formation and facilitate hyperpermeability [16]. However, Ang1 inhibited VEGF-mediated disruption of junction formation. When 2-MCA was added at the same time as VEGF and Ang1, the effect of Ang1 on stabilization of EC-EC contact was annulled (Fig. 2B). This finding suggested that 2-MCA blocks maturation processes during blood vessel formation. When HUVECs were cultured on the Matrigels, Ang1 was seen to induce cord-like structures involved in tube formation, as previously reported [17] (Fig. 2C). However, 2-MCA suppressed this, consistent with its inhibitory effects on angiogenesis.

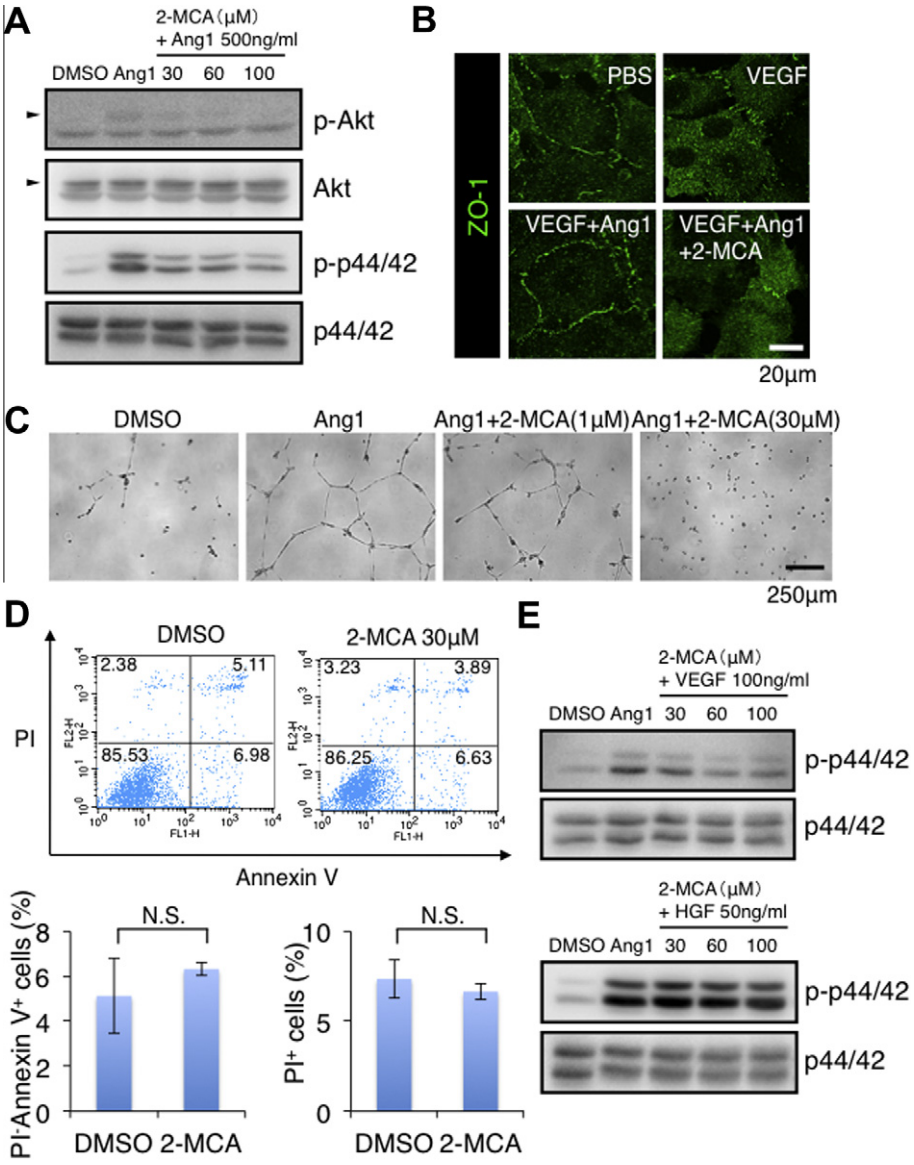

Fig. 2. Effects of 2-MCA on Ang1-mediated HUVEC junction and tube formation. (A) Effect of 2-MCA on Ang1-mediated Tie2 downstream signals, Akt and p42/44 in HUVECs. HUVECs were cultured with or without Ang1 (500 ng/ml) in the presence of titrated doses of 2-MCA as indicated for 15 min. (B) Effect of 2-MCA on Ang1-mediated membrane stability of the tight junction protein ZO-1. HUVECs were cultured with VEGF (10 ng/ml) with or without Ang1 (100 ng/ml) in the presence or absence of 2-MCA (60 μM) for 24 h, after which ZO-1 expression was assessed immunocytochemically. The bar indicates 20 μm. (C) Tube formation analysis. HUVECs were cultured on Matrigels with or without of Ang1 (500 ng/ml) in the presence or absence of titrated doses of 2-MCA as indicated. Bar indicates 250 μm. (D) HUVECs were cultured in the presence or absence of 2-MCA (30 μM) for 24 h and cell death and apoptosis was then evaluated by the expression of Annexin V and PI staining by FACS (upper). Numbers in each quadrant indicate the percentage of cells among total cells. Quantitative evaluation of $PI^{-}AnnexinV^{+}$ apoptotic cells and PI^{+} dead cells (lower two graphs) ($n = 3$). (E) Effect of 2-MCA on VEGF- or HGF-mediated p42/44 activation in HUVECs. HUVECs were cultured for 15 min. with or without VEGF (100 ng/ml) or HGF (50 ng/ml) in the presence of titrated doses of 2-MCA as indicated.

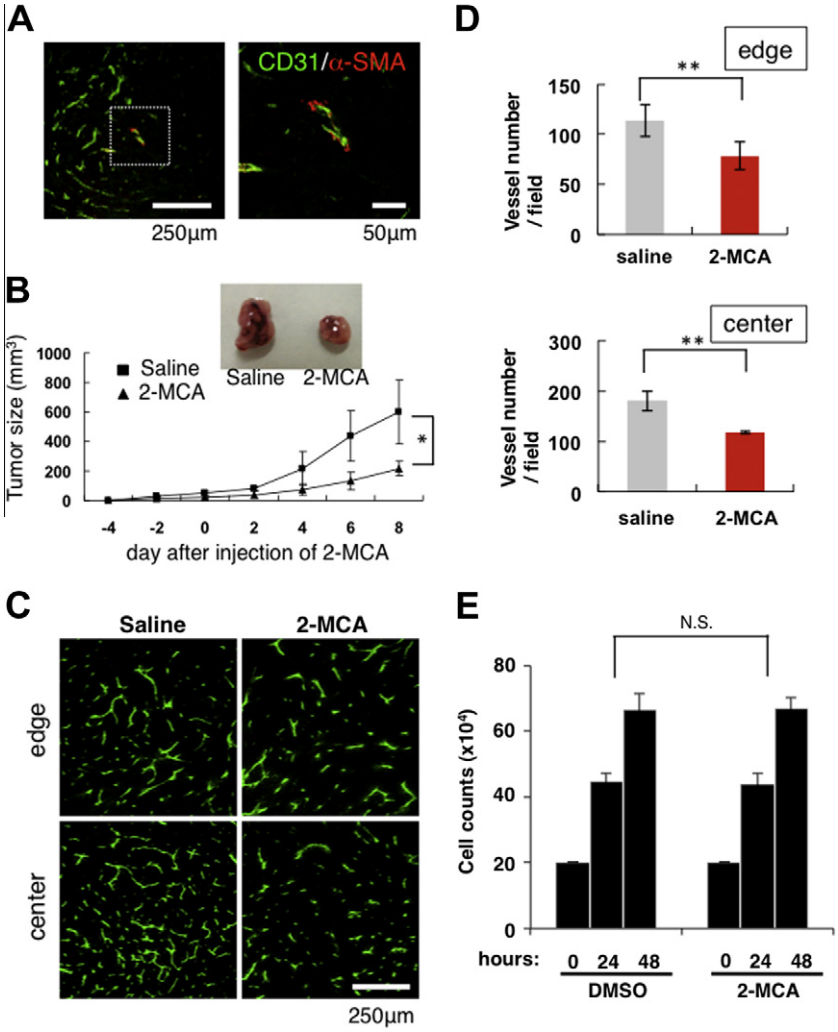

Fig. 3. Effect of 2-MCA on tumor angiogenesis. (A) Blood vessel formation observed in tumors established by inoculating colon26 mouse colon cancer cells. Section of tumor was stained with anti-CD31 (green) and anti-α-SMA (red) antibodies. Right panel shows higher magnification of area indicated by dashed box in the left panel. Bars indicate 250 μm (left) and 50 μm (right). (B) Effect of 2-MCA on tumor growth. 2-MCA (0.5 mg/kg) was injected intraperitoneally every day from day 0 to day 7, starting 4 days after inoculation of tumor cells. Tumor growth was monitored by measuring tumor size. Inset shows gross appearance of tumors dissected on day 8. $^{*}P < 0.05$ ($n = 3$). (C) Suppression of the formation of tumor vasculature by injecting 2-MCA. Sections from the edge and center lesions of the tumor were stained with anti-CD31 antibody. Bar indicates 250 μm. (D) Quantitative evaluation of vascular density calculated for the edge (upper) and center (bottom) of the tumor. The number of blood vessels in 5 random fields was counted. $^{**}P < 0.01$. (E) In vitro proliferation assay using colon26 cells with DMSO or 30 μM 2-MCA.

To investigate direct effects of 2-MCA on ECs, HUVECs were cultured in its presence or absence. Results suggested that the effects of 2-MCA did not depend on enhancement of HUVEC death or apoptosis (Fig. 2D). To further investigate whether the inhibitory effects of 2-MCA were mediated specifically via the angiopoietin/Tie2 pathway, cellular assays were performed to monitor Erk activation by two other angiogenesis-related receptor tyrosine kinases, VEGFR and c-Met, induced by their cognate ligands, VEGF and HGF, respectively. Similar to the results from stimulation by Ang1, both VEGF and HGF were capable of inducing the phosphorylation of Erk. However, no inhibitory effects of 2-MCA were observed on Erk activation by these factors (Fig. 2E). These results indicate that the observed effect of 2-MCA was most likely mediated through specific inhibition of the Ang1-triggered Tie2 activation pathway.

3.3. 2-MCA suppresses tumor growth via inhibition of angiogenesis

Using tumor xenograft models, we previously reported that there are two types of tumor associations with mural cell coverage of ECs in the tumor vasculature [14]. In one type, most blood vessels are not covered with mural cells and greater numbers of vessels develop in the tumor microenvironment. In the other type, mature blood vessels in which ECs are covered with mural cells are observed especially at the edge of the tumor, and the number of vessels is low relative to the first type. We have now tested the effect of 2-MCA on tumor angiogenesis in the first type using the mouse colon cancer cell line, colon26. As shown in Fig. 3A, blood vessels covered with mural cells are rare and angiogenesis is robustly induced in this tumor. Four days after cell inoculation into mice, we started to inject 2-MCA daily for 8 days and monitored tumor growth by evaluating tumor volume. The results documented a clear tumor growth inhibitory action of 2-MCA (Fig. 3B). Blood vessel formation was evaluated by dividing the area into edge and center of tumor; vascular density was found to be decreased in both areas (Fig. 3C and D). 2-MCA did not show any suppressive effects on proliferation of colon26 cells themselves in vitro (Fig. 3E). These data suggest that 2-MCA inhibited tumor growth by suppressing tumor angiogenesis.

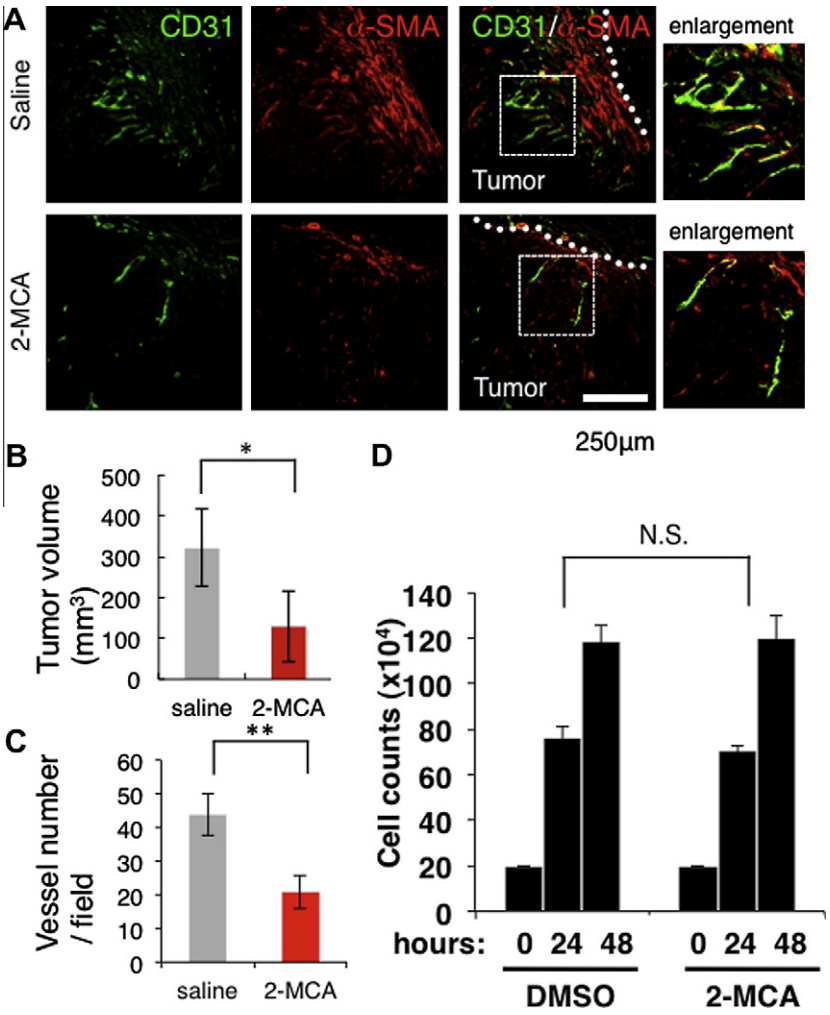

Fig. 4. Effect of 2-MCA on tumor angiogenesis. (A) Blood vessel formation observed in tumors established by inoculation of HCT116 human colon cancer cells. Schedule and doses of 2-MCA were the same as in Fig. 3. Tumors were dissected on day 8 (12 days after tumor cell inoculation) and sections were stained with anti-CD31 (green) and anti-α-SMA (red) antibodies. Right panels show higher magnification of area indicated by dashed box in the left-hand side. Bars indicate 250 μm. (B) Effect of 2-MCA on tumor growth. $^{*}P < 0.05$ ($n = 3$). (C) Quantitative evaluation of vascular density. The number of blood vessels in 5 random fields was counted. $^{**}P < 0.01$. (D) In vitro proliferation assay using HCT116 cells with DMSO or 30 μM 2-MCA.

3.4. 2-MCA inhibits maturation of the tumor vasculature

Using the human colon cancer cell line HCT116 in a xenograft model, we observed very few blood vessels in the center of the tumor but mature vessels covered with mural cells were plentiful at the periphery (Fig. 4A). To investigate whether 2-MCA affects maturation of blood vessels in the tumor environment, we injected it using the same schedule as described in Fig. 3B. Twelve days inoculation of tumor cells, the tumor volume was significantly smaller in the 2-MCA-injected group than in controls (Fig. 4B). Moreover, vascular density was also significantly reduced in the 2-MCA-injected group (Fig. 4A and C). The number of mature blood vessels that were still present in 2-MCA-treated tumors was reduced. 2-MCA did not mediate any direct suppressive effects on HCT116 cell proliferation in vitro (Fig. 4D). These data suggest that 2-MCA affects the maturation of blood vessels by inhibiting mural cell attachment.

4. Discussion

In this work, we screened natural products for Tie2 inhibitory activity and isolated 2-MCA, a small molecule which inhibited tumor growth by suppressing tumor angiogenesis. We cannot completely exclude the possibility that 2-MCA also affected angiogenesis by mechanisms other than Tie2 inhibition, but the finding that 2-MCA blocked Ang1-mediated tube formation and barrier formation implies that its inhibitory action on Tie2 must be at least partly responsible for its suppression of tumor angiogenesis.

One of the objectives of the present study was to identify inhibitors that can block maturation of blood vessels as well as progression of sprouting angiogenesis. This aim arose from the evidence that tumor repopulation is observed from the edge of the tumor even under circumstances where tumor growth seemed to be completely inhibited by treatment with vascular disrupting agents [18]. Similar evidence was also reported that invasion of cancer cells is induced from the edge of the tumor after treatment with angiogenesis inhibitors [19]. We previously reported that blood vessels are fully mature at the edge of the tumor, unlike in its center [14]. Therefore, invasion of cancer cells and repopulation of the tumor after treatment with angiogenesis inhibitors seems to be caused by the resistance of mature blood vessels in the tumor rim to the action of anti-angiogenic drugs. In our present studies using HT116 cells, the number of mature blood vessels in which ECs were covered with mural cells at the edge of the tumor was

reduced by 2-MCA treatment. Therefore, this agent is useful for inhibiting maturation processes of the tumor vasculature during tumor growth.

Constitutively active mutant Tie2 is seen in patients with hereditary venous malformation [15]. Recently, it has been reported that Tie2 constitutively active due to somatic mutation is also found in sporadic venous malformation [20]. Histopathological examination revealed that blood vessels are dilated in a disorderly manner and mural cell attachment is essentially absent in vascular lesions of such patients. Akt activation via Tie2 activation induces maturation of blood vessels. On the other hand, Erk activation via Tie2 activation induces angiogenesis. In the patients with constitutively active Tie2, it is likely that the Erk pathway is strongly activated. As reported here, 2-MCA could inhibit both Erk as well as Akt activation through Tie2 phosphorylation. Therefore, agents like 2-MCA should also be effective for treating lesions in venous malformation as well as tumor angiogenesis.

In terms of Tie2 inhibition, several lines of evidence from studies using soluble Tie2 receptors or aptamers have already suggested that Tie2 inhibition could be effective for suppressing tumor growth [21–23]. However, there is little data on using small molecule Tie2 inhibitors to prevent tumor growth. Although many kinase inhibitors for the VEGF receptor pathway have been developed, Tie2 inhibitors still require more developmental work for expansion of their application to different therapeutic approaches.

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References

[1] S.P. Ivy, J.Y. Wick, B.M. Kaufman, An overview of small-molecule inhibitors of VEGFR signaling, Nat. Rev. Clin. Oncol. 6 (2009) 569–579.
[2] H. Hurwitz, L. Fehrenbacher, W. Novotny, et al., Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer, N. Engl. J. Med. 350 (2004) 2335–2342.
[3] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669–676.
[4] H.G. Augustin, G.Y. Koh, G. Thurston, K. Alitalo, Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system, Nat. Rev. Mol. Cell Biol. 10 (2009) 165–177.
[5] S. Fukuhara, K. Sako, T. Minami, K. Noda, H.Z. Kim, T. Kodama, M. Shibuya, N. Takakura, G.Y. Koh, N. Mochizuki, Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1, Nat. Cell Biol. 10 (2008) 513–526.
[6] R.K. Jain, Molecular regulation of vessel maturation, Nat. Med. 9 (2003) 685–693.
[7] D.J. Dumont, G. Gradwohl, G.H. Fong, M.C. Puri, M. Gerstenstein, A. Auerbach, M.L. Breitman, Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo, Genes Dev. 8 (1994) 1897–1909.
[8] S. Morozumi, Isolation purification and antibiotic activity of *o*-methoxycinnamaldehyde from *Cinnamon*, Appl. Environ. Microbiol. 36 (1978) 577–583.
[9] H.B. Singh, M. Srivastava, A.B. Singh, A.K. Srivastava, Cinnamon bark oil a potent fungitoxicant against fungi causing respiratory tract mycoses, Allergy 50 (1995) 995–999.
[10] S.T. Chang, P.F. Chen, S.C. Chang, Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*, J. Ethnopharmacol. 77 (2001) 123–127.
[11] N. Takakura, T. Watanabe, S. Suenobu, Y. Yamada, T. Noda, Y. Ito, M. Satake, T. Suda, A role for hematopoietic stem cells in promoting angiogenesis, Cell 102 (2000) 199–209.
[12] S. Morita, T. Kojima, T. Kitamura, Plat-E: an efficient and stable system for transient packaging of retroviruses, Gene Ther. 7 (2000) 1063–1066.
[13] T. Saitoh, H. Nakano, N. Yamamoto, S. Yamaoka, Lymphotoxin-L receptor mediates NEMO-independent NF-UB activation, FEBS Lett. 532 (2002) 45–51.
[14] N. Satoh, Y. Yamada, Y. Kinugasa, N. Takakura, Angiopoietin-1 alters tumor growth by stabilizing blood vessels or by promoting angiogenesis, Cancer Sci. 99 (2008) 2373–2379.
[15] M. Vikkula, L.M. Boon, K.L. Carraway, J.T. Calvert, A.J. Diamonti, B. Goumnerov, K.A. Pasyk, D.A. Marchuk, M.L. Warman, L.C. Cantley, J.B. Mulliken, B.R. Olsen, Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2, Cell 87 (1996) 1181–1190.
[16] S.W. Lee, W.J. Kim, Y.K. Choi, H.S. Song, M.J. Son, I.H. Gelman, Y.J. Kim, K.W. Kim, SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier, Nat. Med. 9 (2003) 900–906.
[17] I. Kim, H.G. Kim, S.O. Moon, S.W. Chae, J.N. So, K.N. Koh, B.C. Ahn, G.Y. Koh, Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion, Cir. Res. 86 (2000) 952–959.
[18] G.M. Tozer, C. Kanthou, B.C. Baguley, Disrupting tumour blood vessels, Nat. Rev. Cancer 5 (2005) 423–435.
[19] M.P. Ribes, E. Allen, J. Hudock, T. Takeda, H. Okuyama, F. Vinals, M. Inoue, G. Bergers, D. Hanahan, O. Casanovas, Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis, Cancer Cell 15 (2009) 220–231.
[20] N. Limaye, V. Wouters, M. Uebelhoer, M. Tuominen, R. Wirkkala, J.B. Mulliken, L. Eklund, L.M. Boon, M. Vikkula, Somatic mutations in angiopoietin receptor gene TEK cause solitary and multiple sporadic venous malformations, Nat. Genet. 41 (2009) 118–124.
[21] P. Lin, P. Polverini, M. Dewhirst, S. Shan, P.S. Rao, K. Peters, Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth, J. Clin. Invest. 100 (1997) 2072–2078.
[22] R. Tournaire, M.P. Simon, F.I. Noble, A. Eichmann, P. England, J. Pouyssegur, A short synthetic peptide inhibits signal transduction, migration and angiogenesis mediated by Tie2 receptor, EMBO Rep. 5 (2004) 262–267.
[23] Y.J. Koh, H.Z. Kim, S.I. Hwang, J.E. Lee, N. Oh, K. Jung, M. Kim, K.E. Kim, H. Kim, N.K. Lim, C.J. Jeon, G.M. Lee, B.H. Jeon, D.H. Nam, H.K. Sung, A. Nagy, O.J. Yoo, G.Y. Koh, Double antiangiogenic protein, DAAP, targeting VEGF-A and angiopoietins in tumor angiogenesis, metastasis, and vascular leakage, Cancer Cell 18 (2010) 171–184.