Anti-angiogenic properties of plaunotol

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We have investigated a potential anti-angiogenic effect of plaunotol, an extract from the leaves of Plau-noi, in an angiogenesis model consisting of human umbilical vein endothelial cells (HUVECs). Plaunotol inhibited the proliferative activity of HUVECs in a dose-dependent manner. In addition, it caused a remarkable decrease of the ability of HUVECs to adhere and spread on gelatin and vitronectin, but not fibronectin. Tube-like formation in Matrigel was also inhibited in a dose-dependent way. These results strongly suggest the specific inhibition of integrin $\alpha_v\beta_3$ to be the main event of plaunotol-induced suppression of angiogenesis. The $\alpha_v\beta_3$ antagonists are known to be potent inhibitors of tumor angiogenesis and plaunotol, by causing the functional inhibition of $\alpha_v\beta_3$, should be considered a promising new anti-angiogenic drug. *Anti-Cancer Drugs* **16:401–407 © 2005 Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2005, **16**:401–407

Keywords: $\alpha_v\beta_3$, angiogenesis, human umbilical vein endothelial cell, integrin, plaunotol

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Received 26 July 2004 **Revised form accepted** 9 December 2004

Introduction

Most solid tumors are dependent on neovascularization to supply nutrients to the growing tumor and therefore inhibition of angiogenesis is now focused on as a novel target for anti-cancer therapy [1,2]. Several approaches such as the use of endothelial cell-specific antibodies, immunization with endothelial cell vaccines or the development of drugs that selectively inhibit growth of endothelial cells have been tried [3–7]. Among them, selective inhibitors of integrin $\alpha_v\beta_3$, such as tumstatin or angiostatin, have been shown to have strong inhibitory properties on tumor angiogenesis [6,8]. $\alpha_v\beta_3$ belongs to the integrin family of adhesion molecules and is expressed predominantly on endothelial cells [9,10]. $\alpha_v\beta_3$ recognizes and binds to the arginine–glycine–aspartic acid (RGD) sequence in a variety of extracellular matrices (ECMs), such as vitronectin and collagen IV, and therefore plays an important role in ECM-mediated endothelial cell adhesion, proliferation and survival [11–13].

Plaunotol, an isoprenoid, is a component of the extract of a Thai medical plant called Plau-noi, and commercially available as a cytoprotective anti-ulcer agent for gastritis and gastric ulcer in Japan and Thailand (Fig. 1). Several mechanisms of its protective effect on gastric mucosa have been clarified, such as the induction of an increase of prostaglandin E_2 content in gastric tissues and the reduction of the superoxide radicals generated by leukocytes [14–16]. Plaunotol has also been reported to have anti-bacterial activities, especially against *Helicobacter pylori* [17,18]. However, up to now, no reports on its effects on tumor growth or angiogenesis exist in the medical literature.

In the current study, we aimed to investigate a possible anti-tumor effect of plaunotol, especially focusing on angiogenesis. Using human umbilical vein endothelial cells (HUVECs) as a model, we could demonstrate that plaunotol exerted a strong anti-angiogenic activity by inhibiting integrin $\alpha_v\beta_3$ function.

Methods

Reagents and antibodies

Plaunotol was a kind gift from Sankyo (Tokyo, Japan). It was dissolved in dimethylsulfoxide (DMSO), and added to the experimental media to give final concentrations of 10, 20 or 50 μM plaunotol and 0.2% DMSO. Cells cultured with 0.2% DMSO were always used as a negative control. Gelatin, fibronectin and vitronectin were purchased from Sigma (St Louis, MO), and purified or FITC-conjugated anti-$\alpha_5\beta_1$ and anti-$\alpha_v\beta_3$ monoclonal antibodies (mAbs) were from BD PharMingen (San Diego, CA).

HUVECs isolation and culture

Umbilical cords were obtained from normal pregnant women at delivery, after informed consent was obtained. HUVECs were isolated from the umbilical cord blood vessels, as previously described [19]. Briefly, the umbilical vein was cannulated at both edges and 0.2%

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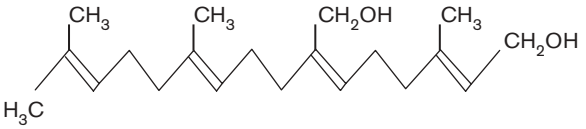
Fig. 1



Chemical structure of plaunotol.

collagenase I in phosphate-buffered saline (PBS) was added to the lumen of the vessels. The umbilical cord was incubated for 20 min at 37°C, and then the PBS containing collagen was recovered and centrifuged to obtain the isolated cells. Cells were washed twice with PBS, suspended in complete medium consisting of MCDB-151 medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 15% fetal calf serum (FCS), 1% antibiotic/antimycotic, acidic fibroblast growth factor (aFGF; PeproTech, Rocky Hill, NJ; 2 ng/ml) and heparin (Sigma; 5 μg/ml), and seeded in culture dishes previously coated with 0.1% gelatin. Cells were routinely cultured in complete medium in an atmosphere of 5% CO_2 at 37°C, passaged by trypsinization and used for up to 10 passages for the experiments.

Proliferation assay

HUVECs were seeded at 1×10^4/well in 96-well flat-bottomed plates in MCDB complete medium containing differing concentrations of plaunotol (0, 10, 20 and 50 μM). Each well was previously coated with 0.1% gelatin. After a 48-h culture, the proliferative activity was determined by the MTS assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI), which monitors the number of viable cells, according to the manufacturer's instructions. Briefly, MTS solution was added at 20 μl/well and, after a 4-h culture, the conversion of MTS to formazan was measured in a plate reader at 490 nm. Results represent the mean value of triplicate wells ± SD.

Detection of apoptosis by flow-cytometry

HUVECs were prepared and treated with plaunotol (50 μM) for 24 or 48 h as described above. Cells were washed twice and incubated with FITC-conjugated Annexin-V and propidium iodide (PI; Biovision, Mountain View, CA) for 5 min at room temperature. The population of Annexin-V^- and PI^- viable cells was evaluated by flow cytometry. Data were collected in a FACSCalibur (Becton Dickinson, Mountain View, CA) and analyzed using CellQuest software (Becton Dickinson).

Adhesion and spreading assays

To clarify the effect of plaunotol on the ability of HUVECs to adhere to and to spread on ECM proteins, adhesion and spreading assays were performed as described previously, with small modifications [20,21]. Briefly, flat-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with either gelatin (0.3 mg/ml), fibronectin (10 μg/ml) or vitronectin (50 μg/ml) overnight at 4°C and blocked with 1% BSA for 1 h at 37°C. HUVECs pre-treated for 24 h with different concentrations of plaunotol were trypsinized and fluoro-labeled by incubation with 1% calcein-AM solution (Dojindo, Kumamoto, Japan) for 30 min. After washing twice with PBS, cells were re-suspended in 0.2% BSA (Sigma)/RPMI and incubated in the presence or absence of anti-$\alpha_5\beta_1$ or anti-$\alpha_v\beta_3$ blocking mAbs (10 μg/ml) for 30 min at 4°C. mAb of unrelated specificity was used as negative control. After incubation with blocking antibodies, cells were re-suspended in 0.2% BSA/RPMI and seeded at 3×10^4/well in the plates coated with ECMs. For the adhesion assay, wells were gently washed to remove the non-adherent cells after 30 min incubation at 37°C and the fluorescence intensity of the adherent cells was measured at 525 nm in a microscope photometer (Terrascan VP; Minervatech, Tokyo, Japan). Adhesion rate was calculated as the ratio to the fluorescence intensity of control unwashed wells. Results represent the mean value of triplicate wells ± SD.

Alternatively, for the detection of cell spreading, mAb-treated cells were suspended in complete medium and seeded at 3×10^4/well in the plates coated with ECMs. Wells were incubated for 1 h at 37°C to allow cells to spread and the micrographs of cells were acquired using a laser confocal microscope (Fluoview; Olympus, Tokyo, Japan).

Migration assay

To evaluate the effect of plaunotol on the migratory capacity of HUVECs, a migration assay was performed using a Boyden chamber as described previously, with small modifications [22]. In brief, the lower surfaces of the polycarbonate filters with 8 μm pores (Neuro Probe, Gaithersburg, MD) were coated with gelatin, fibronectin or vitronectin. HUVECs were pre-treated with differing concentrations of plaunotol for 24 h as described above, trypsinized and re-suspended in complete medium. Filters coated with ECMs were placed on a 96-blind-well chamber (Neuro Probe) and suspended cells were loaded into the upper chamber (1×10^5 cells in 200 μl/well). For the blocking experiments with mAbs, anti-$\alpha_5\beta_1$ or anti-$\alpha_v\beta_3$ blocking mAbs were added to the upper chamber to give 1 concentration of 10 μg/ml. After incubation at 37°C for 3 h, the filters were disassembled, fixed with methanol and stained with a Diff-Quick staining kit (International Reagents, Kobe, Japan). The upper side of the filters was scraped to eliminate the adherent cells. The number of cells that migrated to the lower side of the filter was determined by measuring absorbance at 595 nm using a 96-well plate reader. Results represent the mean value of triplicate wells ± SD.

Tubular formation assay

To investigate the effect of plaunotol on endothelial tubular formation, the Matrigel tube formation assay was performed. HUVECs were suspended in complete medium containing differing concentrations of plaunotol and were seeded at 1.5×10^5cells/well in a 12-well flat-bottomed culture plate previously coated with Matrigel basement membrane matrix (100 µl/well; Becton Dickinson, Bedford, MA). Cells were incubated at 37°C for 24 h and tube formation was examined under phase-contrast microscopy.

Detection of cell surface integrins by flow cytometry

HUVECs were treated without or with plaunotol for 24 h as described above, trypsinized, washed twice with PBS and suspended in 0.1% BSA/PBS. Cell surface integrins were stained using purified anti-$\alpha_5\beta_1$ mAb and goat polyclonal anti-mouse Ig secondary antibody or FITC-conjugated anti-$\alpha_v\beta_3$ mAb. FITC-conjugated mouse IgG mAb of unrelated specificity was always used as a control. Data were collected and analyzed using a FACSCalibur and CellQuest software.

Statistical analysis

The unpaired Student's *t*-test was used to determine statistical significance. Differences at $p < 0.05$ were considered statistically significant.

Results

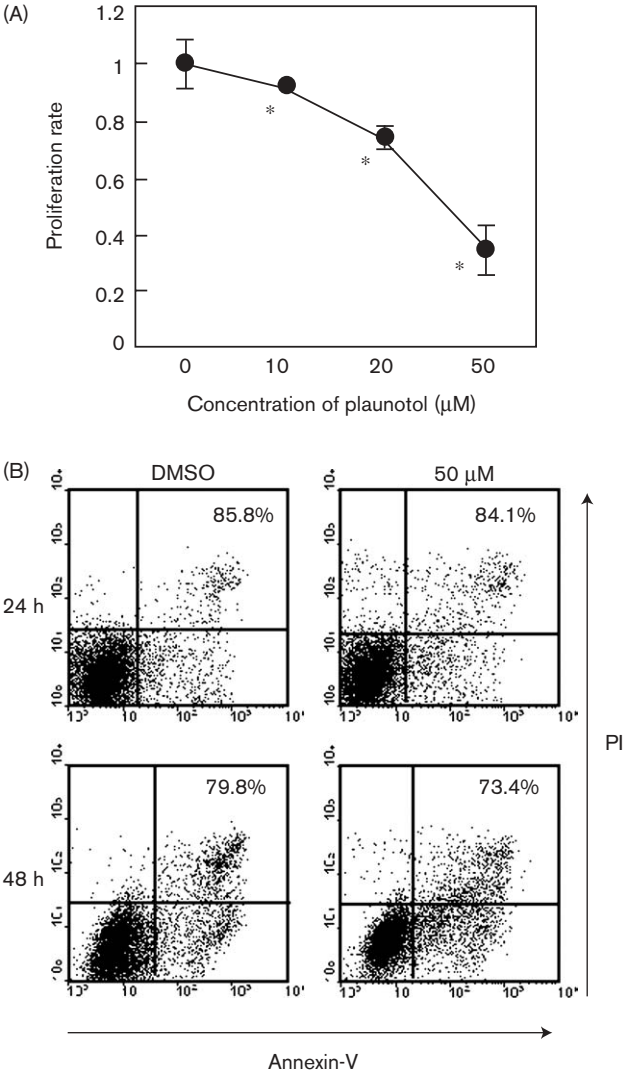
Suppression of HUVEC proliferation induced by plaunotol

As shown in Fig. 2(A), a 48-h incubation in the presence of plaunotol resulted in a significant inhibition of HUVEC proliferation, in a dose-dependent manner. The number of viable cells was reduced to 34.2% of control, with the highest dose tested (50 µM). To evaluate whether this suppression was apoptosis dependent, Annexin-V/PI staining was performed. Although incubation with plaunotol for 24 h also caused small inhibition of proliferation (data not shown), even treatment with the highest concentration (50 µM) of plaunotol caused no alteration in the percentage of Annexin-V$^-$/PI$^-$ viable cells population (85.8% in control versus 84.1% in plaunotol treatment, Fig. 2B). Treatment for periods as long as 48 h resulted in a small decrease in the percentage of viable HUVECs (79.8% in control versus 73.4% in plaunotol treatment), but the effect is not enough to explain the decreased number of cells measured in the MTS assay. It suggests that induction of apoptosis is not the main mechanism. The Trypan blue exclusion test was also performed to evaluate cell viability, yielding similar results (data not shown).

Adhesion, spreading and migration assay

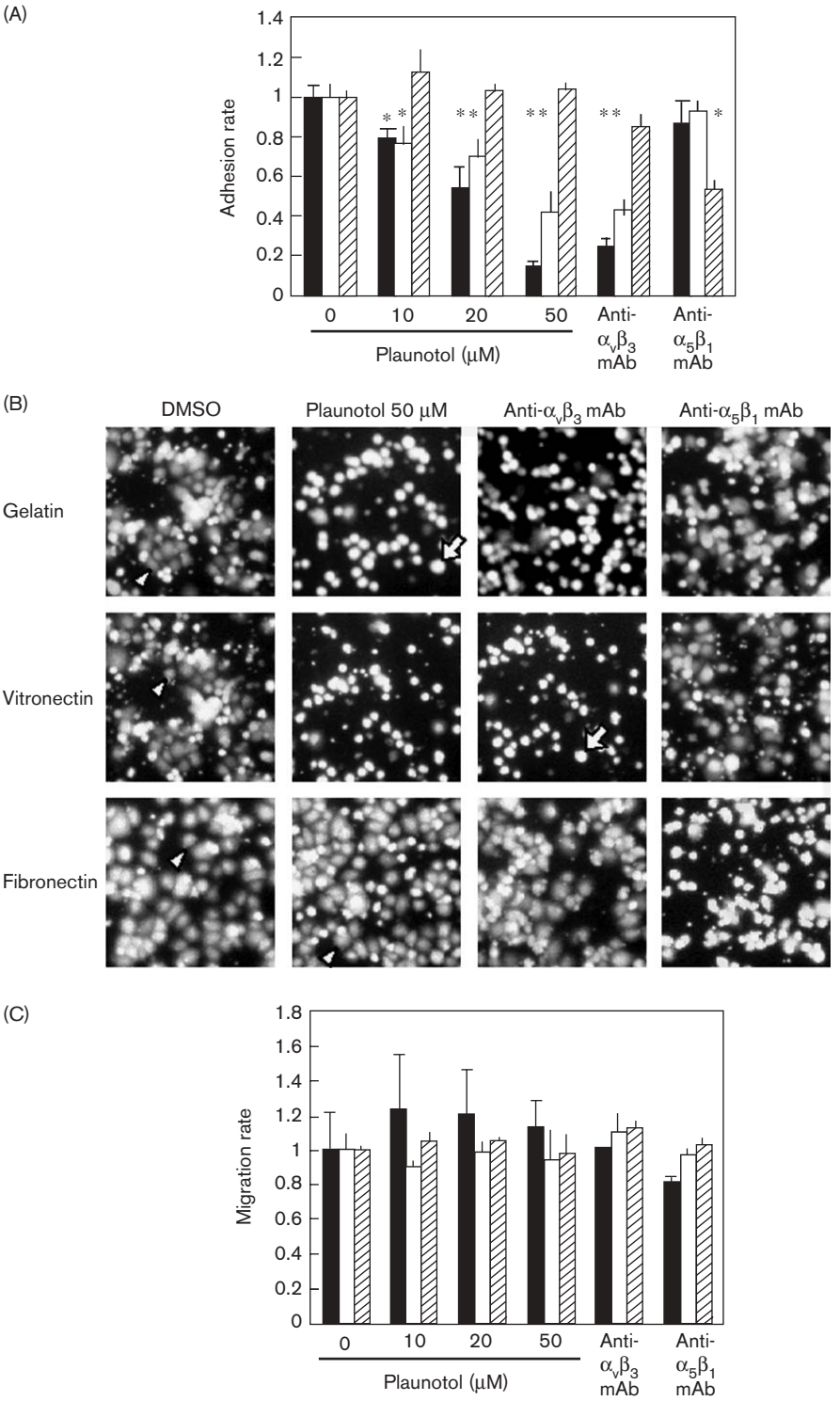
Because the decreased proliferation of HUVECs was not dependent on the direct cytotoxicity of plaunotol, next we evaluated the effect of plaunotol on the adhesion, spreading and migration of HUVECs on ECM proteins, i.e. gelatin, fibronectin and vitronectin. As shown in Fig. 3(A), a 24-h incubation in the presence of plaunotol significantly and dose-dependently decreased the ability of HUVECs to adhere gelatin- and vitronectin-coated microplates, whereas the adhesion to fibronectin was not affected (Fig. 3A). Blocking of integrin $\alpha_v\beta_3$ with the specific mAb resulted in inhibition of adhesion to gelatin and vitronectin, but not fibronectin, resembling the effect of plaunotol. On the other hand, blocking of integrin $\alpha_5\beta_1$ resulted in the suppression of adhesion to fibronectin, but not to gelatin or vitronectin. Then, the effect of plaunotol

Fig. 2



Effect of plaunotol on proliferation and survival of HUVECs. (A) HUVECs cultured with different concentrations of plaunotol for 48 h. The proliferation rate was determined using MTS assay. The data are expressed as mean ± SD of results from three independent experiments and an asterisk indicates statistical significance. (B) Alternatively, HUVECs were cultured without or with plaunotol (50 µM) for 24 or 48 h, and double-stained by Annexin-V (*x*-axis) and PI (*y*-axis). The percentage of Annexin-V$^-$/PI$^-$ viable cells is shown.

Fig. 3



Adhesion, spreading and migration assays on ECMs. (A) Adhesion of HUVECs to gelatin (filled bars), vitronectin (open bars) or fibronectin (shaded bars). (B) Spreading of HUVECs seeded on each ECM. Spread cells, indicated by open arrowheads, appear as wide cells with a dim margin. Non-spread cells, indicated by open arrows, are round and bright cells. (C) Migration through gelatin (filled bars)-, vitronectin (open bars)- or fibronectin (shaded bars)-coated membranes. The data are expressed as mean ± SD of results from three independent experiments and an asterisk indicates statistical significance.

on the ability of HUVECs to spread on ECMs was investigated. Control untreated HUVECs spread on gelatin and vitronectin, but plaunotol treatment resulted in a significant loss of this ability, resulting in cell–cell aggregation (Fig. 3B). The ability to spread on fibronectin, however, was not affected by plaunotol. Similar to the results of the adhesion assay, blocking of $\alpha_v\beta_3$ with the specific mAb, resembling the effect of plaunotol, resulted in inhibition of spreading on gelatin and vitronectin, but not fibronectin. $\alpha_5\beta_1$ blocking had a distinct effect, suppressing the spreading on fibronectin, but not on gelatin and vitronectin. Although plaunotol treatment significantly affected the ability of HUVECs to adhere to and spread on gelatin and vitronectin, the ability of HUVECs to migrate, as evaluated in the Boyden chamber, did not change significantly (Fig. 3C). Treatment with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin-specific blocking mAbs also showed no alteration on the migratory property of HUVECs.

Tubular formation assay

The ability of HUVECs to form tube-like structures on Matrigel was significantly and dose-dependently inhibited by plaunotol (Fig. 4). Treatment for 24 h with 50 μM of plaunotol resulted in the complete disruption of tube-like structure formation by HUVECs.

Integrin expression

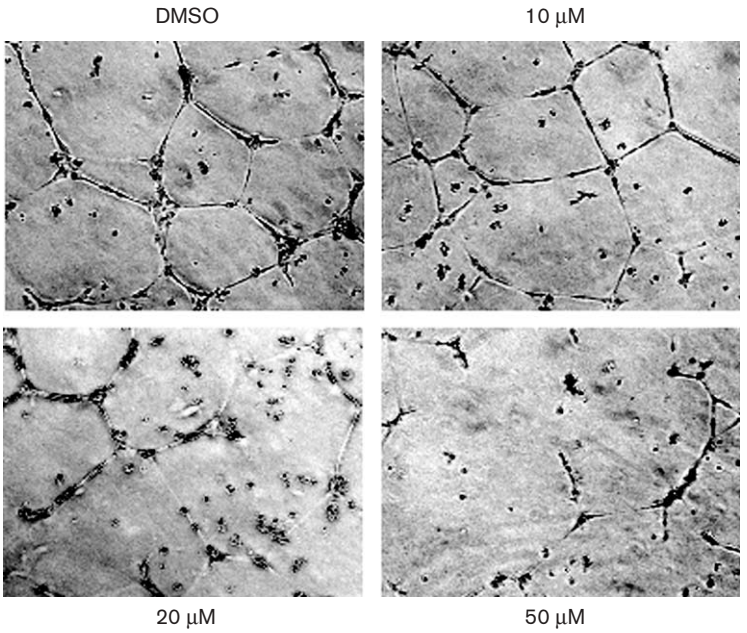
Both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins are known to play a pivotal role in the adhesion and survival of ECs [23,24]. Using flow cytometry, we evaluated the changes in the expression of these integrins on ECs treated with plaunotol. Plaunotol treatment resulted in a significant inhibition of the ability of HUVECs to adhere to and to spread on gelatin and vitronectin, but not fibronectin, suggestive of $\alpha_v\beta_3$-specific inhibition. However, the expression levels of these integrins were not affected even with the highest dose of plaunotol tested (50 μM, Fig. 5), suggesting that the effect of plaunotol is dependent on a functional down-regulation of $\alpha_v\beta_3$ on HUVECs.

Discussion

Angiogenesis is characterized by invasion, migration and proliferation of endothelial cells, and integrin $\alpha_v\beta_3$-mediated cell interactions with ECMs have been shown to be key players in these processes [11–13]. In recent years, several selective $\alpha_v\beta_3$ inhibitors, such as tumstatin and angiostatin, have been developed as specific angiogenesis inhibitors, but most of these drugs are peptide antagonists which contain the RGD motif and exert the inhibitory effect by binding to the cell surface integrin molecules [6,8,25]. Because of the unstable nature of these peptide drugs when administered *in vivo*, non-peptide selective $\alpha_v\beta_3$ inhibitors need to be developed.

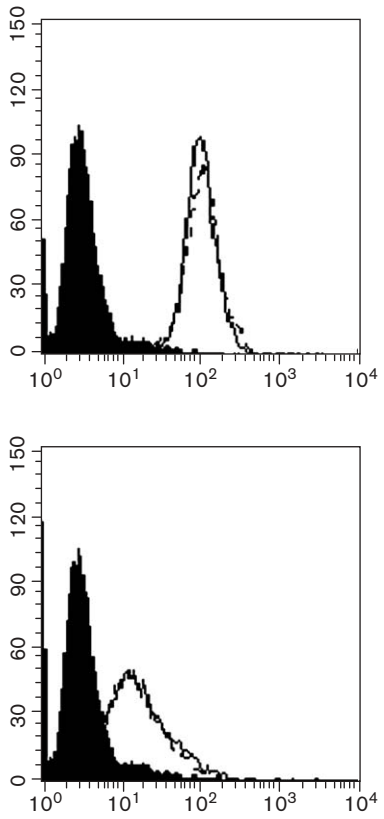
Plaunotol is a non-peptide isoprenoid derived from the tree oil extracted from the leaf of Plau-noi and is widely used as an anti-ulcer drug in Japan. Although several

Fig. 4



Tubular formation assay. HUVECs were seeded on the Matrigel in the presence of different concentrations of plaunotol. After a 24-h incubation, the tubular formation of cells was examined under phase-contrast microscopy. Representative data from three independent experiments are presented.

Fig. 5



Flow-cytometric analysis of integrin expression. HUVECs were incubated in the absence (solid line) or presence (50 μM, dotted line) of plaunotol for 24 h, and the expression of integrin $\alpha_5\beta_1$ (upper) and $\alpha_v\beta_3$ (lower) was evaluated by flow cytometry. The filled line represents the negative control. Representative data from three independent examinations are presented.

mechanisms of its anti-ulcer properties have been clarified, no reports of its effects on cancer are found in the medical literature [14–16,26]. Here, we aimed to investigate the effect of plaunotol on tumor angiogenesis and, for this purpose, we used FGF-activated HUVECs as a model. HUVECs, especially those activated by FGF, have characteristics that resemble tumor vascular endothelium and are frequently used as an *in vitro* model of angiogenesis [27–29].

Initially, the effect of plaunotol on EC proliferation and survival was investigated. Plaunotol inhibited the proliferation of ECs in a dose-dependent manner, but no evidence of apoptosis was observed even after a 24-h treatment at the highest dose (50 μM). After a 48-h treatment, only a small decrease of Annexin-V^-/PI^- viable cells was observed, leading us to conclude that the cytotoxic effect of plaunotol on ECs is not the main event.

Next we evaluated the effect of plaunotol on the integrin-related functions of ECs, i.e. adhesion, spreading and migration on ECM proteins. Several previous reports have shown that adhesion and spreading of ECs on vitronectin and gelatin depend on integrin $\alpha_v\beta_3$, whereas those on fibronectin is predominantly mediated by integrin $\alpha_5\beta_1$ [21,24,30]. Incubation of ECs with plaunotol for 24 h significantly suppressed the adhesion to gelatin and vitronectin, but not the ability to adhere to fibronectin. Blocking $\alpha_v\beta_3$ with the specific mAb also resulted in the same pattern of inhibition, whereas blocking of $\alpha_5\beta_1$ did not affect the adhesion to gelatin and vitronectin. This result suggested that the inhibition of EC proliferation induced by plaunotol would be dependent on the selective inhibition of $\alpha_v\beta_3$, with consequent decreased ability to bind its ligands. In addition, plaunotol also affected the ability of ECs to spread on $\alpha_v\beta_3$ ligands, corroborating this hypothesis. However, the migratory ability of ECs was not affected by plaunotol, regardless of the ECM protein tested.

To confirm the anti-angiogenic property of plaunotol, the ability of ECs to form tube-like structures in Matrigel was investigated. ECs form a tube-like network when seeded on Matrigel and this process is known to be inhibited by selective inhibition of integrin $\alpha_v\beta_3$ [31]. As expected, and compatible with the results of adhesion and spreading assays, plaunotol disrupted the tubular formation of ECs in a dose-dependent manner. Although all the data shown here are suggestive of plaunotol-induced $\alpha_v\beta_3$ inhibition, no evident alteration of the flow-cytometric expression of $\alpha_v\beta_3$ or $\alpha_5\beta_1$ could be found. Thus, the plaunotol-induced $\alpha_v\beta_3$ inhibition seems be more functional than structural.

Plaunotol is an isoprenoid and its structure resembles that of geranylgeraniol. Geranylgeraniol is an intermediate product in the mevalonate pathway and functions as an essential compound for cell proliferation [32–34]. Recent studies have shown that geranylgeraniol has apoptosis-inducing activity in a variety of cancer cell lines, and activation of caspase-8 and suppression of bcl-x_L are essential for geranylgeraniol-mediated apoptosis [35–38]. In the present study, plaunotol exerted only a small apoptosis-inducing effect in proliferating endothelial cells, despite its structure resembling that of geranylgeranyol. Although plaunotol showed a strong inhibitory effect on angiogenesis, it was mediated by the selective suppression of $\alpha_v\beta_3$ function and not by the direct induction of apoptosis. Both plaunotol and geranylgeraniol have anti-cancer potential, but the mechanism of tumor growth suppression seems to be different.

Since plaunotol is already commercially available in Japan and Thailand, and a previous report has shown that 120 mg of plaunotol, when orally administered to a normal healthy human adult, could achieve an approximate dose of 16.3 μM in plasma [39], the development of clinical

trials to confirm our present data is feasible. Furthermore, the oral administration of doses as high as 240 mg has proved to cause no significant side-effects, as confirmed by physical findings and laboratory data (phase I study, unpublished data), and therefore concentrations higher than 16.3 μM may be clinically achievable. In the present study, a significant suppression of EC proliferation could be achieved even with the lowest dose (10 μM) tested and, therefore, plaunotol should be a promising novel angiogenesis inhibitor. Further investigation is needed to elucidate the potential *in vivo* anti-angiogenic effect of plaunotol.

References

1 Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; **86**:353–364.
2 Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* 1992; **3**:65–71.
3 Matsuno F, Haruta Y, Kondo M, Tsai H, Barcos M, Seon BK. Induction of lasting complete regression of preformed distinct solid tumors by targeting the tumor vasculature using two new anti-endoglin monoclonal antibodies. *Clin Cancer Res* 1999; **5**:371–382.
4 Takahashi N, Haba A, Matsuno F, Seon BK. Antiangiogenic therapy of established tumors in human skin/severe combined immunodeficiency mouse chimeras by anti-endoglin (CD105) monoclonal antibodies, and synergy between anti-endoglin antibody and cyclophosphamide. *Cancer Res* 2001; **61**:7846–7854.
5 Okaji Y, Tsuno NH, Kitayama J, Saito S, Takahashi T, Kawai K, *et al.* Vaccination with autologous endothelium inhibits angiogenesis and metastasis of colon cancer through autoimmunity. *Cancer Sci* 2004; **95**:85–90.
6 O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, *et al.* Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997; **88**:277–285.
7 Klohs WD, Hamby JM. Antiangiogenic agents. *Curr Opin Biotechnol* 1999; **10**:544–549.
8 Maeshima Y, Colorado PC, Kalluri R. Two RGD-independent alpha vbeta 3 integrin binding sites on tumstatin regulate distinct anti-tumor properties. *J Biol Chem* 2000; **275**:23745–23750.
9 Enenstein J, Kramer RH. Confocal microscopic analysis of integrin expression on the microvasculature and its sprouts in the neonatal foreskin. *J Invest Dermatol* 1994; **103**:381–386.
10 Max R, Gerritsen RR, Nooijen PT, Goodman SL, Sutter A, Keilholz U, *et al.* Immunohistochemical analysis of integrin alpha vbeta3 expression on tumor-associated vessels of human carcinomas. *Int J Cancer* 1997; **71**:320–324.
11 Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, *et al.* Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994; **79**:1157–1164.
12 Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 1994; **264**:569–571.
13 Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA. Definition of two angiogenic pathways by distinct alpha v integrins. *Science* 1995; **270**:1500–1502.
14 Ushiyama S, Matsuda K, Asai F, Yamazaki M. Stimulation of prostaglandin production by (2*E*,6*Z*,10*E*)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-+ + + hexadecatetraen-1-ol (plaunotol), a new anti-ulcer drug, *in vitro* and *in vivo*. *Biochem Pharmacol* 1987; **36**:369–375.
15 Kohda I, Nagai H, Iwai M, Watanabe M, Yokoyama K, Tsujikawa K, *et al.* Further studies on the anti-ulcerogenic effects of compound, 2-(*E*-2-decenoylamino)ethyl 2-(cyclohexylethyl) sulfide. *Chem Pharm Bull (Tokyo)* 1991; **39**:1832–1836.
16 Okabe N, Okada M, Sakai T, Kuroiwa A. Effect of plaunotol on superoxide production activity *in vivo*. *Dig Dis Sci* 1995; **40**:2321–2322.
17 Matsumoto Y, Hamashima H, Masuda K, Shiojima K, Sasatsu M, Arai T. The antibacterial activity of plaunotol against *Staphylococcus aureus* isolated from the skin of patients with atopic dermatitis. *Microbios* 1998; **96**:149–155.
18 Karita M, Li Q, Okita K. Evaluation of new therapy for eradication of *H. pylori* infection in nude mouse model. *Am J Gastroenterol* 1993; **88**:1366–1372.
19 Yoshida M, Szente BE, Kiely JM, Rosenzweig A, Gimbrone Jr MA. Phosphorylation of the cytoplasmic domain of E-selectin is regulated during leukocyte–endothelial adhesion. *J Immunol* 1998; **161**:933–941.
20 Kawai K, Tsuno NH, Kitayama J, Okaji Y, Yazawa K, Asakage M, *et al.* Epigallocatechin gallate attenuates adhesion and migration of CD8 T cells by binding to CD11b. *J Allergy Clin Immunol* 2004; **113**:1211–1217.
21 Dormond O, Foletti A, Paroz C, Ruegg C. NSAIDs inhibit alpha V beta 3 integrin-mediated and Cdc42/Rac-dependent endothelial-cell spreading, migration and angiogenesis. *Nat Med* 2001; **7**:1041–1047.
22 Shida D, Kitayama J, Yamaguchi H, Okaji Y, Tsuno NH, Watanabe T, *et al.* Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1. *Cancer Res* 2003; **63**:1706–1711.
23 Matter ML, Ruoslahti E. A signaling pathway from the $alpha_5beta_1$ and $alpha_vbeta_3$ integrins that elevates bcl-2 transcription. *J Biol Chem* 2001; **276**:27757–27763.
24 Ruegg C, Yilmaz A, Bieler G, Bamat J, Chaubert P, Lejeune FJ. Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat Med* 1998; **4**:408–414.
25 Yeh CH, Peng HC, Yang RS, Huang TF. Rhodostomin, a snake venom disintegrin, inhibits angiogenesis elicited by basic fibroblast growth factor and suppresses tumor growth by a selective $alpha_vbeta_3$ blockade of endothelial cells. *Mol Pharmacol* 2001; **59**:1333–1342.
26 Shiratori K, Watanabe S, Takeuchi T, Chang JH, Moriyoshi Y. Effect of plaunotol on release of plasma secretin and pancreatic exocrine secretion in humans. *Pancreas* 1989; **4**:323–328.
27 Zimrin AB, Villeponteau B, Maciag T. Models of *in vitro* angiogenesis: endothelial cell differentiation on fibrin but not Matrigel is transcriptionally dependent. *Biochem Biophys Res Commun* 1995; **213**:630–638.
28 Bagley RG, Walter-Yohrling J, Cao X, Weber W, Simons B, Cook BP, *et al.* Endothelial precursor cells as a model of tumor endothelium: characterization and comparison with mature endothelial cells. *Cancer Res* 2003; **63**:5866–5873.
29 Eliceiri BP, Cheresh DA. The role of alphav integrins during angiogenesis. *Mol Med* 1998; **4**:741–750.
30 Dormond O, Bezzi M, Mariotti A, Ruegg C. Prostaglandin E_2 promotes integrin alpha Vbeta 3-dependent endothelial cell adhesion, rac-activation, and spreading through cAMP/PKA-dependent signaling. *J Biol Chem* 2002; **277**:45838–45846.
31 Yeh CH, Peng HC, Huang TF. Accutin, a new disintegrin, inhibits angiogenesis *in vitro* and *in vivo* by acting as integrin alphavbeta3 antagonist and inducing apoptosis. *Blood* 1998; **92**:3268–3276.
32 Crick DC, Waechter CJ, Andres DA. Geranylgeraniol restores cell proliferation to lovastatin treated C6 glial cells. *SAAS Bull Biochem Biotechnol* 1996; **9**:37–42.
33 Edwards PA, Ericsson J. Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway. *Annu Rev Biochem* 1999; **68**:157–185.
34 Crick DC, Andres DA, Waechter CJ. Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. *Biochem Biophys Res Commun* 1997; **237**:483–487.
35 Ohizumi H, Masuda Y, Nakajo S, Sakai I, Ohsawa S, Nakaya K. Geranylgeraniol is a potent inducer of apoptosis in tumor cells. *J Biochem (Tokyo)* 1995; **117**:11–13.
36 Burke YD, Stark MJ, Roach SL, Sen SE, Crowell PL. Inhibition of pancreatic cancer growth by the dietary isoprenoids farnesol and geraniol. *Lipids* 1997; **32**:151–156.
37 Ohizumi H, Masuda Y, Yoda M, Hashimoto S, Aiuchi T, Nakajo S, *et al.* Induction of apoptosis in various tumor cell lines by geranylgeraniol. *Anticancer Res* 1997; **17**:1051–1057.
38 Takeda Y, Nakao K, Nakata K, Kawakami A, Ida H, Ichikawa T, *et al.* Geranylgeraniol, an intermediate product in mevalonate pathway, induces apoptotic cell death in human hepatoma cells: death receptor-independent activation of caspase-8 with down-regulation of Bcl-x_L expression. *Jpn J Cancer Res* 2001; **92**:918–925.
39 Elsom LFC, Hawkins DR, Darragh A, Lambe RF. The pharmacokinetics of ^{14}C-plaunotol in human subjects after oral administration. *Prog Med* 1985; **5**:863–868.