An Angiogenesis Inhibitor E7820 Shows Broad-Spectrum Tumor Growth Inhibition in a Xenograft Model: Possible Value of Integrin α2 on Platelets as a Biological Marker

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ABSTRACT
We reported previously that an angiogenesis inhibitor, E7820, inhibits in vitro tube formation of human umbilical vein endothelial cell through the suppression of integrin α2 expression. Here we describe the antiangiogenic and antitumor effects of E7820 in mice and discuss the feasibility of using platelet integrin α2 expression on platelets as a biological marker of the efficacy of E7820. Oral administration of E7820 significantly inhibited basic fibroblast growth factor-induced angiogenesis in Matrigel implants and human colon WiDr tumor-induced angiogenesis in a dorsal air sac model. Twice-daily treatment with E7820 clearly inhibited the s.c. tumor growth of seven tumor cell lines derived from human colon, breast, pancreas, and kidney, and completely suppressed the growth of human pancreatic KP-1 and human colon LoVo cell lines. Moreover, E7820 significantly inhibited the growth of KP-1 and human colon tumor Colo320DM cells orthotopically implanted in the pancreas and cecum, respectively. The efficacy of E7820 was comparable in the s.c. and orthotopic transplantation models. Immunohistochemical analyses using anti-CD31 antibody showed that E7820 significantly reduced microvessel density in orthotopically implanted KP-1 tumor. E7820 reduced integrin α2 expression on a megakaryocytic cell line, Dami cells, induced by phorbol 12-myristate 13-acetate treatment. It also decreased the expression level of integrin α2 on platelets withdrawn from mice bearing s.c. KP-1 tumor at a dosage close to that affording antitumor activity. These data demonstrate that E7820 showed a broad-spectrum antitumor effect in mice through inhibition of angiogenesis and indicate that the decrease of integrin α2 on platelets might serve as a biological marker for the antitumor efficacy of E7820.

Received 8/28/03; revised 11/10/03; accepted 11/12/03.
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INTRODUCTION
Angiogenesis, the formation of new blood vessels, is essential for growth and malignancy of tumors (1) and an antangiogenesis strategy is expected to provide new therapeutic agents to treat cancer. Angiogenesis is initiated by degradation of extracellular matrix, followed by cell migration, proliferation and capillary tube formation of endothelial cells, and then maturation. Angiogenic factors, such as vascular endothelial growth factor (VEGF; Ref. 2), angiopoietin (3), and fibroblast growth factor (4), regulate the progression of angiogenesis in cooperation with cell adhesion molecules such as integrins (5), cadherin (6), and platelet/endothelial cell adhesion molecule (7). Extracellular matrix-degrading proteolytic enzymes, including matrix metalloproteinases (8) and plasminogen activator (9), also participate in angiogenesis. Several types of inhibitors, such as VEGF inhibitors (10), matrix metalloproteinase inhibitors (11), and integrin antagonists (12), have been reported to inhibit angiogenesis (13) and have already been evaluated clinically (14–16). Among them, anti-VEGF antibody (Ab) is expected to be the new drug for cancer patients (17).

Studies in preclinical models suggested that most angiogenesis inhibitors might cause transition of a growing tumor to a dormant state, rather than tumor regression, although some angiogenesis inhibitors have induced dramatic regression of a few well-established tumors (12, 18). Clinical studies also indicate that angiogenesis inhibitors might stabilize tumor growth (14, 15). Thus, long-term dosing may be necessary to obtain clinical benefits such as disease-free survival and increased life span. Angiogenesis inhibitors appear to have a wide therapeutic ratio, and administration at the maximum-tolerated dose may not be needed (14, 16). Thus, several exploratory clinical investigations to find appropriate dosages have been conducted by using magnetic resonance imaging analysis (19), measurement of endothelial cell apoptosis (20), and DNA microarray analysis (21, 22). Therefore, we thought it would be valuable to identify a surrogate marker in preclinical model study, which would be useful in predicting adequate dosage levels of angiogenesis inhibitors for clinical use.

We reported previously that E7820, an aromatic sulfonamide derivative, is a novel angiogenesis inhibitor that inhibits both proliferation and tube formation of human umbilical vein endothelial cell (HUVEC) induced by either basic fibroblast growth factor (bFGF) or VEGF (23). E7820 decreased integrin α2, 3, 5, and β1 in confluent cultures of HUVEC. In particular, E7820 reduced only integrin α2 mRNA as an early event. Furthermore, the suppression of integrin α2 on HUVEC by E7820 treatment contributed to the inhibition of tube formation in a type I collagen matrix culture model.

In the present study, we first examined the in vivo pharmacological efficacy of E7820 against both angiogenesis and tumor growth. In vivo antiangiogenic effects of E7820 were
examined by using angiogenic factor-induced and tumor-induced angiogenesis models. We examined the antitumor effect of E7820 on growth of orthotopically implanted tumors in addition to s.c. inoculated tumors to determine the effect of E7820 on organ-specific endothelial cells. Secondly, we examined the alteration of integrin α2 expression on a megakaryocytic cell line *in vitro* and on platelets in a xenograft model. E7820 showed antitumor activity in various human tumor xenograft models and suppressed integrin α2 expression on platelets at a dosage similar to that inhibiting tumor growth in the xenograft models.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture.** The tumor cell lines were purchased from the American Type Culture Collection (Rockville, MD), except for KP-1, which was obtained from National Kyusyu Cancer Center (Fukuoka, Japan); WiDr, which was obtained from Dainippon Seiyaku (Osaka, Japan); and KCC-1, which was obtained from Central Institute for Experimental Animals (Kawasaki, Japan). The tumor cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (50 μM) and sodium pyruvate (1 mM). HUVECs were isolated from human umbilical vein by the method described previously (24), amplified in epithelial growth medium (2% FCS; Cambrex, Walkerville, MD) on type I collagen-coated flasks and used at passages 4 to 6.

**Monoclonal Antibody.** Mouse monoclonal antihuman integrin α2 Ab (A2-IIE10) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). FITC-conjugated F(ab')2 fragment of rabbit antiangiome IgG was purchased from Dako (Glostrup, Denmark). FITC-conjugated hamster monoclonal antiamo2 Ab, rat monoclonal antiamo2 CD31 Ab (MEC13.3), FITC-conjugated rat monomeric IgG and FITC-conjugated rat IgG were purchased from Pharmingen (San Diego, CA).

**Preparation of E7820.** For *in vitro* assay, E7820 was first dissolved in DMSO and diluted further in medium. For *in vivo* studies, E7820 was suspended in 5% methylcellulose. In dorsal air sac (DAS) model, E7820 was dissolved in 35% methylcellulose. In vivo first dissolved in DMSO and diluted further in medium. For *in vivo* assay, E7820 was dissolved in DSMSO and diluted further in medium. For *in vivo* assay, E7820 was dissolved in 35% methylcellulose. In vivo first dissolved in DMSO and diluted further in medium.

**Sandwich Tube Formation Assay.** The tube formation assay was performed as described previously (23). Briefly, HUVEC were plated on the collagen gel at a concentration of 1 to 1.2 x 10⁶ cells/well (24-well plate) in serum-free medium (human endothelial-serum-free basal growth medium; Life Technologies, Inc., Grand Island, NY) with EGF (Life Technologies, Inc., Grand Island, NY) at 10 ng/ml and either bFGF (Life Technologies, Inc., Grand Island, NY) or VEGF (Wako Pure Chemical Industries, Osaka, Japan) at 20 ng/ml. Then, HUVEC were covered with a second collagen gel and cultured with or without E7820 in serum-free medium supplemented with angiogenic factors. Tube length of capillaries was quantified by calculating the pixel density of outline images based on images obtained with a microscope. All experiments were done at least in duplicate and were repeated three times.

**Cell Growth Assay.** Cell growth assay for endothelial cells was performed as described previously (23). Tumor cells were plated at 1 to 2 x 10⁵ cells/well on 96-well plates in 0.1 ml of RPMI 1640 containing 10% fetal bovine serum. After 24 h, either E7820 or vehicle was added to duplicate cultures of cells, and at 2 or 3 days after addition of E7820, the ratios of surviving cells were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All experiments were done at least in duplicate and repeated twice.

**In Vivo Growth Factor-Induced Angiogenesis.** The method described by Passaniti et al. (25) was used, with some modification. Briefly, bFGF (300 ng/pellet) was embedded in a pellet of Matrigel (300 μl; Becton Dickinson, Bedford, MA) and injected s.c. in C57BL/6N mice (Charles River Japan, Inc., Kanagawa, Japan). Vehicle or E7820 was given orally, by gavage, for 7 days (twice daily) after Matrigel implantation. At autopsy, the pellet was removed, and the hemoglobin content was measured by Drabkin’s procedure (Drabkin reagent kit; Sigma, St. Louis, MO), according to the manufacturer’s instructions.

**Mouse DAS Model.** The mouse DAS method was performed as described previously (26). Briefly, a suspension of 1.5 x 10⁷ WiDr cells in collagen gel was injected into a chamber. This chamber was implanted into a DAS produced by injecting 10 ml of air into a C57BL/6N mouse. Administration of E7820 was started at 6 h after the transplantation (day 1). Vehicle or E7820 was given orally, by gavage, for 4 days (once daily). On day 5, 51Cr-labeled (Amersham Pharmacia Biotech, Tokyo, Japan) blood cells were injected into the tail vein of mice. Vascular volume was calculated from the radioactivity of the skin attached to the chamber.

**Xenograft Model.** Six-week-old female nude mice (KSN mice; SLC, Shizuoka, Japan) underwent s.c. transplantation of human tumors (5–10 x 10⁶ cells/mouse, except for RCC-1; 2- to 3-mm tumor fragments). Administration was started when the tumor volume reached 60–100 mm³ (except for AsPC-1; one day after transplantation). E7820 was administered orally on a schedule of twice daily every day for 3–6 weeks. Tumor volumes were checked twice a week during the experiment by direct measurement of the diameter of tumors with calipers. Tumor volume was calculated using the formula (a x b x b)/2, where a is the largest diameter and b is the diameter perpendicular to a. Values of ΔT/C (% of control for Δgrowth) were calculated from the formula, (ΔT/ΔC x 100), where ΔT and ΔC are changes in tumor volume (Δgrowth) for each treated and vehicle control group. In the case of reduction of tumor volume, ΔT/C values were calculated according to the following formula, ΔT/C (%) = (TVn-TV1)/TV1 x 100, where TVn is the tumor volume of treated mice on day n.

**Orthotopic Transplantation Model.** Seven-week-old female KSN mice were anesthetized and an incision was made at the abdomen. The tail of the pancreas or cecum was gently exposed. Seven million human pancreatic cancer cells (KP-1) were injected into the parenchyma of the pancreatic gland. A 20-ng block of human colon cancer tumor tissue (Colo320DM) was implanted on the cecum. The administration of E7820 was started 7 or 8 days (day 1) after transplantation. In the pancreatic orthotopic transplantation model, E7820 was orally administered twice daily for 21 days. On day 22, the locally grown
tumors were resected, weighed, and then used for immunohistochemistry. In colon orthotopic transplantation model, E7820 was administered twice daily for 2 weeks. On day 15, the locally grown tumors were weighed and photographed.

**Histological Analysis of Tumor Blood Vessels.** The resected tumors were mounted in OCT compound (Miles Scientific, Naperville, IL), frozen, and stored at −80°C until required. Frozen tumor sections were cut and stained by indirect immunoperoxidase with rat antimouse CD31 monoclonal Ab, which detects vascular endothelial cells in the tumor. Immune staining with anti-CD31 Ab was performed according to the method described previously (26). Tumor vessels were counted by microscopy in $\times 33$ fields (2–5 fields/tumor) and were calculated as vessel density (/mm$^2$).

**Analysis by Flow Cytometry.** Dami cells, a megakaryocytic cell line, were stimulated with 2 ng phosphor 12-myristate 13-acetate (Sigma, St. Louis, MO) and cultured either with or without E7820 at the indicated doses for 72 h. The cells were harvested and suspended at $2 \times 10^5$ cells in 100 μl of PBS containing 0.1% BSA, then incubated with 1 μg of primary antibodies (antihuman integrin α2 Ab) for 30 min at 4°C. Cells were washed with PBS and incubated in FITC-conjugated secondary Ab diluted 1:40 in PBS for 30 min at 4°C. The control sample (for background) was incubated with control IgG in PBS containing 0.1% BSA. Fluorescence signals from $1 \times 10^6$ cells were acquired using a fluorescence-activated cell sorter (Calibur; Becton Dickinson, Mountain View, CA) to quantify staining intensity. The expression of each molecule was calculated using the mean fluorescence of each sample as determined by flow cytometry: Relative expression (relative mean fluorescence intensity; RMFI) = mean fluorescence intensity (MFI) of sample/MFI of background.

**Evaluation of the Expression Level of Integrin α2 on Platelets in KP-1 Xenograft Model.** Six-week-old female nude mice (KSN mice) underwent s.c. transplantation of KP-1 human pancreatic tumor. Administration was started (day 1) at 7 days after transplantation. E7820 was administered orally twice daily for 3 weeks. The expression level of integrin α2 on platelets of mice treated with either vehicle or E7820 was measured once a week. Blood was withdrawn from the eye of anesthetized mice in PBS containing 0.004% sodium citrate and measured once a week. Blood was collected from the aorta and counted with a Total Hematometry Management System (Technicon H*1; Bayer Co.).

**RESULTS**

**Antiangiogenic Effect of E7820.** We first examined the effects of E7820 on proliferation of endothelial cells and tumor cells and also on tube formation of endothelial cells. Proliferation of HUVEC induced by either bFGF and VEGF in serum-free medium was inhibited by E7820 treatment and IC$_{50}$ values of 0.10 and 0.081 μg/ml, respectively (Table 1). However, the in vitro antiproliferative activity of E7820 against tumor cells was approximately 100-fold less potent than that against endothelial cells (Table 1). E7820 also inhibited both bFGF- and VEGF-driven tube formation of HUVEC in this assay. The IC$_{50}$ values were 0.20 and 0.24 μg/ml, respectively (Table 1).

To examine the in vivo efficacy of E7820, we first evaluated the antiangiogenic activity of E7820 against in vivo angiogenesis induced by bFGF in the Matrigel plug assay. The mean hemoglobin content of bFGF-containing pellets was 21.8 ± 7.9 g/dl in vehicle-treated mice. The mean hemoglobin contents of bFGF-containing pellets in mice treated with E7820 at the doses of 6.25, 12.5, and 25 mg/kg, twice daily for 7 days, were 16.5 ± 8.2 (71%), 13.7 ± 4.7 (55%), and 10.1 ± 3.3 g/dl (35%), respectively (Fig. 1A). Thus, oral administration of E7820 inhibited angiogenesis induced by bFGF in a dose-dependent manner, and the effect was significant at 25 mg/kg ($P < 0.01$). Next, we evaluated the in vivo antiangiogenic efficacy of E7820 against human colorectal tumor (WiDr)-induced angiogenesis in a modified DAS model, which was reported to be dependent on VEGF secreted from tumor cells (26). WiDr-induced angiogenesis was quantified as the vascular volume in the skin attached to those chambers. The mean vascular volume in the group of tumor-containing chambers was 2.56 ± 0.50 μl, whereas that of vehicle chambers was 1.27 ± 0.29 μl. The vascular volumes in E7820-treated groups were 2.32 ± 0.48 (100 mg/kg, once daily), 2.14 ± 0.46 (200 mg/kg), and 2.00 ± 0.35 μl (400 mg/kg). E7820 significantly inhibited WiDr tumor-induced angiogenesis at 200 ($P < 0.05$) and 400 mg/kg ($P < 0.01$; Fig. 1B).

**Antitumor Effects of E7820 in s.c. and Orthotopic Xenograft Models.** We investigated the antitumor efficacy of E7820 against s.c. growth of seven human tumor cells derived from colon (WiDr, Colo320DM, LoVo), breast (BT-20), pancreas (KP-1, AsPC-1), and kidney (RCC-1) in xenograft models. E7820 was administered orally at 50, 100, and 200 mg/kg twice daily for 3–6 weeks from the initial day. Tumor growth was inhibited by E7820 treatment in a dose-dependent manner in all s.c. xenograft models we examined, and administration of E7820 even at 50 mg/kg showed significant antitumor activity against seven tumors (AsPC-1 by $P < 0.05$, others by $P < 0.01$; Table 2). E7820 completely inhibited s.c. tumor growth of LoVo tumor cells and also regressed the tumor mass of KP-1 tumor cells at the dosages of both 100 and 200 mg/kg (Fig. 2, A and B).

<table>
<thead>
<tr>
<th>Table 1 Antiangiogenic activities of E7820 in vitro</th>
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<tr>
<td><strong>Assay</strong></td>
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<tr>
<td>Sandwich TF$^a$ assay</td>
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<td>Cell growth assay</td>
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<tr>
<td>Endothelial cell</td>
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<tr>
<td>HUVEC</td>
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<tr>
<td>Tumor cell</td>
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<tr>
<td>Colo320DM</td>
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<td>WiDr</td>
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<tr>
<td>KP-1</td>
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<td>AsPC-1</td>
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<td>BT-20</td>
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$^a$ TF, tube formation; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cells.
E7820 treatment was well tolerated in all experiments and did not change the body weight of mice compared with vehicle-treated mice (data not shown).

Subsequently, the antitumor effects of E7820 on the local growth of either orthotopically implanted pancreatic tumor or colon tumor at the pancreas and cecum, respectively, was evaluated. Human pancreatic tumor cells, KP-1 cells, were inoculated into the pancreas, and E7820 was administered orally at 50, 100, and 200 mg/kg from 7 days after implantation (day 1, tumor weight = 54 ± 4 mg), twice daily for 3 weeks. At autopsy, the mean tumor weight of vehicle-treated mice was 406 ± 116 mg, whereas the corresponding values for the E7820-treated groups were 217 ± 64, 80 ± 16, and 41 ± 4 mg, respectively. E7820 significantly inhibited tumor growth in the pancreas at all of the dosages examined, and the tumor weight of mice treated at 200 mg/kg was comparable with that on day 1 (Fig. 3A). Immunohistochemical analysis of KP-1 tumors by staining endothelial cells with anti-CD31 Ab showed fewer stained endothelial cells and reduced microvessel density in a cross-section of tumor from mice treated with E7820 compared with the vehicle control (Fig. 3, C-E), demonstrating antiangiogenic efficacy of E7820 against endothelial cells in the pancreas. Pieces of human colon Colo320DM tumor tissue were implanted onto the cecum, and E7820 was administered orally at 50, 100, and 200 mg/kg from 8 days after implantation (day 1), twice daily for 2 weeks. The mean tumor weight of mice treated with vehicle was 762 ± 605 mg, whereas the corresponding values in the E7820-treated groups were 285 ± 100, 254 ± 90, and 132 ± 113 mg, respectively. Thus, E7820 inhibited tumor growth significantly at the cecum at all dosages examined (Fig. 3B). Furthermore, Colo320DM tumors growing on the cecum were reddish in vehicle-treated mice, whereas those in E7820-treated mice were pale in color (Fig. 3F).

Table 2. Antitumor effects of E7820 on s.c. tumor growth

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Evaluation day</th>
<th>Dose (mg/kg)</th>
<th>T/C% (a) (Δ growth)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LoVo (colon)</td>
<td>Day 43</td>
<td>50</td>
<td>0.17 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.05 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.01 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>0.01 (c)</td>
</tr>
<tr>
<td>Colo320DM (colon)</td>
<td>Day 22</td>
<td>50</td>
<td>0.56 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.44 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.19 (b)</td>
</tr>
<tr>
<td>WiDr (colon)</td>
<td>Day 22</td>
<td>50</td>
<td>0.52 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.49 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.27 (b)</td>
</tr>
<tr>
<td>KP-1 (pancreatic)</td>
<td>Day 22</td>
<td>50</td>
<td>0.21 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.15 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.08 (b)</td>
</tr>
<tr>
<td>AsPC-1 (pancreatic)</td>
<td>Day 22</td>
<td>50</td>
<td>0.38 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.33 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.25 (b)</td>
</tr>
<tr>
<td>BT-20 (breast)</td>
<td>Day 36</td>
<td>50</td>
<td>0.41 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.24 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.20 (b)</td>
</tr>
<tr>
<td>RCC-1 (renal)</td>
<td>Day 40</td>
<td>50</td>
<td>0.49 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.24 (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>0.20 (b)</td>
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\(a\) T/C% was calculated using the following formula, except for AsPC-1: \((\Delta T/\Delta C) \times 100\). \(\Delta T\) and \(\Delta C\) are changes in tumor volume (Δ growth) for each treated and control group. In the case of reduction of tumor volume, \(\Delta T/C\) values were calculated using the following formula: \(\Delta T/C(\%) = (TVn - TV1)/TV1 \times 100\), where \(TVn\) is the tumor volume of treated mice on day \(n\).

\(b\), \(c\), \(d\), \(e\) indicate statistical significance at \(P < 0.01\), \(P < 0.05\), \(P < 0.01\), respectively.
Antitumor Effects of E7820 and a Biological Marker

Administration of E7820 was started when the tumor volume reached 100 mm$^3$ and continued for 42 days (Fig. 5). These effects of E7820 were detected from 7 days after initial administration and were significant at all dosages examined on day 22. The integrin α2 expression on platelets in E7820-treated mice was restored after withdrawal of E7820 treatment for 7 days, whereas the antitumor activity remained at 100 and 200 mg/kg (Fig. 5, A and B). The expression level of CD31 was less affected by E7820 treatment (Fig. 5C). Decrease of CD31 expression on platelets was observed only at the highest dose (200 mg/kg). Decrease of α2 integrin expression on platelets was detected at doses close to those showing antitumor activity. The number of platelets was not changed by administration of E7820 for 3 weeks (data not shown).

DISCUSSION

We reported previously that E7820, an aromatic sulfonamide derivative, showed antiangiogenic activity through inhibition of proliferation and tube formation of endothelial cells (23). Additionally, we confirmed that E7820 inhibited tube formation through suppression of integrin α2. In the present report, the in vivo pharmacological profile of E7820 was examined. Furthermore, we suggested that alteration of integrin α2 expression on platelets might serve as a biological marker for efficacy of E7820.

E7820 inhibited both bFGF-induced angiogenesis in a Matrigel plug model and tumor (human colorectal WiDr tumor cell)-induced vascularization in the DAS model after oral administration. In addition, we reported previously that angiogenesis induced by WiDr cells was dependent on VEGF in the DAS model (26), suggesting that E7820 is effective against both bFGF- and VEGF-dependent angiogenesis in vivo as well as in vitro. E7820 showed broad-spectrum tumor inhibition at doses from 50 to 200 mg/kg in s.c. xenograft models involving human colon, breast, pancreas, and kidney tumor. It might be important to inhibit multiple factors for effective inhibition of tumor angiogenesis, because it was reported that multiple angiogenic factors are secreted from tumor cells, and expression of angiogenic factors was increased in late-stage tumors (27). The in vitro growth of tumor cells we examined was extremely insensitive to E7820 compared with that of HUVEC, as shown in Table 1, except for RCC-1 (in vivo passing tumor line). The microvessel density in tumor tissue was also reduced after E7820 treatment. These results suggested E7820 inhibits tumor growth through angiogenesis inhibition in mice. E7820 was well tolerated in all of the in vivo experiments. Its profile was consistent with that of an angiogenesis inhibitor.

Blood vessels in different organs are known to be functionally and structurally different from each other. Furthermore, it was reported that angiogenic heterogeneity was regulated by the organ microenvironment of the tumor. Histopathological analysis in a human renal carcinoma xenograft model showed that tumors growing in the subcutis of mice had few blood vessels compared with those in the kidney, which had many vessels (28). In a human colon carcinoma xenograft model, angiogenic factors such as bFGF and interleukin-8 in tumor tissues were increased in the cecl wall region as compared with s.c. tissue (29). These findings prompted us to consider the

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**Effect of E7820 on Expression Level of Integrin α2 on Platelet.** Flow cytometric analysis showed that the expression level of integrin α2 on Dami cells was up-regulated by phorbol 12-myristate 13-acetate treatment for 3 days. This up-regulation of integrin α2 was inhibited in the presence of E7820 in a dose-dependent manner. Relative mean fluorescence intensity of integrin α2 on Dami cells without E7820 treatment was 30.8 ± 4.8, whereas the corresponding values for cells treated with E7820 at 5, 50, and 500 ng/ml were 19.6 ± 10.2, 13.0 ± 2.5, and 6.7 ± 2.3, respectively (Fig. 4). To investigate whether the expression level of integrin α2 on platelets is altered by E7820 treatment in vivo, we measured sequentially the expression level of integrin α2 on platelets from mice treated with E7820 in the KP-1 xenograft model, accompanied with measurement of the tumor volumes. E7820 was administered twice daily for 3 weeks orally from 7 days after transplantation. E7820 induced a dose-dependent reduction of integrin α2 expression on platelets (Fig. 5B). These effects of E7820 were detected from 7 days after initial administration and were significant at all dosages examined on day 22. The integrin α2 expression on platelets in E7820-treated mice was restored after withdrawal of E7820 treatment for 7 days, whereas the antitumor activity remained at 100 and 200 mg/kg (Fig. 5, A and B). The expression level of CD31 was less affected by E7820 treatment (Fig. 5C). Decrease of CD31 expression on platelets was observed only at the highest dose (200 mg/kg). Decrease of α2 integrin expression on platelets was detected at doses close to those showing antitumor activity. The number of platelets was not changed by administration of E7820 for 3 weeks (data not shown).
Fig. 3  Effect of E7820 on local growth of orthotopically transplanted tumor. A, antitumor effect against human pancreatic KP-1 tumor inoculated into the pancreas. The administration of E7820 was started 7 days (day 1) after transplantation. E7820 was orally administered twice daily for 21 days. On day 1 and day 22, the locally growing tumor was resected and weighed. The vehicle control group and E7820-treated group consisted of 10 and 8 mice, respectively. B and F, antitumor effect against human colorectal Colo320DM tumor implanted on the cecum. Vehicle and E7820 were administered by gavage twice daily for 14 days from 8 days after implantation. F, on day 15, the locally growing tumor was weighed and photographed. The vehicle control group consisted of 12 animals. The E7820-treated group consisted of seven or eight mice. C and D, immunohistochemical analysis of intratumoral blood vessels in the KP-1 orthotopic transplantation model. The KP-1 tumors growing at the pancreas from mice treated with either 200 mg/kg E7820 or vehicle were excised, embedded in OCT compound (Miles Scientific), frozen and stained with a rat antirat CD31 primary antibody. Brown staining indicates endothelium. C, vehicle. D, E7820 at 200 mg/kg. E, measurement of vessel density (㎜²). The data represent means; bars, ±SD. **, P < 0.01 versus the vehicle group by Dunnett-type multiple comparison test.
possibility that the activity of angiogenesis inhibitors might vary in different organ-specific endothelial cells. The effect of E7820 on organ-specific endothelial cells was examined in orthotopically inoculated KP-1 pancreatic and Colo320DM colon cancer cells. E7820 had a similar antitumor effect against both local growth at orthotopic sites and s.c. growth of KP-1 and Colo320DM tumors (Table 2; Fig. 3). These results suggested that E7820 was able to affect endothelial cells of skin, pancreas, and colon. E7820 also showed an antitumor effect against tumor growth at the mammary fat pad (data not shown) and seems to be effective on endothelial cells specific for various organs.

The results of recent clinical studies suggested that angiogenesis inhibitors have modest toxicity and induce stabilization of disease (14, 15). The induction of tumor dormancy is considered to be a clinically useful outcome that might increase disease-free survival and survival time. Compared with standard evaluation in terms of response rate, long-term dosing may be required for evaluation of this type of drug. Therefore, a surrogate marker other than response rate is needed to allow evaluation of the biological effects of angiogenesis inhibitors in early phase studies of short duration and to identify appropriate dosages, which are not close to the maximum-tolerated dose but are sufficient to show antiangiogenic activity. Because the inhibitory effect of E7820 on tube formation of HUVEC within type I collagen gels resulted from a decrement of integrin α2 expression (23), we examined whether this unique biological profile could be applied as a marker for the biological effect of E7820. Integrin α2 was reported to be a collagen receptor on platelets and to be involved in platelet aggregation (30). Platelets are derived from megakaryocytes, and integrin α2 is up-regulated in the process of megakaryocyte differentiation toward platelets (31). In an in vitro model of megakaryocyte differentiation using human megakaryocytic Dami cells (32), E7820 suppressed
phorol 12-myristate 13-acetate-induced expression of integrin α2 at a similar concentration to that at which it was effective on HUVEC (Fig. 4). Furthermore, oral administration of E7820 decreased integrin α2 expression on platelets during treatment in the KP-1 s.c. xenograft model. The effective dose for decreasing integrin α2 expression on platelets was comparable with that for inhibiting s.c. growth of KP-1 tumor cells. Because the number of platelets was not changed in mice treated with E7820, E7820 might suppress integrin α2 expression in megakaryocytes without affecting platelet differentiation. It was unclear whether the mechanism by which expression of integrin α2 subunits is regulated in endothelial cells is the same as that in megakaryocytes. However, the comparable effects of E7820 on integrin α2 expression on platelets and growth of KP-1 tumor cells suggested that the decrement of integrin α2 expression on platelets might be useful as a marker for biological effect of E7820. Expression levels of integrin α2 on platelets of individual humans differ. However, in each healthy volunteer examined, the level of integrin α2 expression on platelets was constant over 2 weeks [RFMI ratio to day 1: 0.94 ± 0.01 (Day 8), 1.01 ± 0.02 (day 15), n = 3]. Therefore, integrin α2 on platelets could be used as a biological marker by monitoring its expression level before and after E7820 treatment in clinical studies. E7820 was highly effective against s.c. growth of LoVo and KP-1 cells among the in vivo tumor panel. Moreover, E7820 regressed the tumor mass of KP-1 tumor cells in the s.c. xenograft model. Some angiogenesis inhibitors, such as VEGF receptor kinase inhibitor, an integrin antagonist (12, 18), have been shown to promote tumor regression by inducing apoptosis of tumor vasculature. It is possible that E7820 might induce apoptosis of endothelial cells in KP-1 tumor. Because it was reported that the survival of endothelial cells was dependent on a survival signal via integrin (12), the difference of integrin expression on vascular endothelial cells of tumor tissues might be involved. Integrin αvβ3 was reported to have a critical role in tumor angiogenesis. Expression of both α1β1 and α2β1 was reported to be induced by VEGF, and an inhibitory Ab against α1β1 and α2β1 inhibited not only angiogenesis by VEGF-overexpressing tumor cells but also tumor growth in mice (33, 34). Therefore, it is of interest to examine integrin expression on intratumoral endothelial cells in KP-1 tumor.

E7820 is a novel angiogenesis inhibitor that has a unique biological activity suppressing an expression of integrin α2 subunit. Although many angiogenesis inhibitors are now being clinically evaluated, combination therapy with angiogenesis inhibitors that act on different processes of angiogenesis might be a worthwhile approach to potentiate the antitumor effect. In summary, we have shown in this study that E7820 has promising antitumor effects in a preclinical model through inhibition of angiogenesis and that monitoring the expression level of integrin α2 on platelets might be a valuable predictive marker for the biological effect of E7820. A clinical evaluation of E7820 seems warranted.

ACKNOWLEDGMENTS

We thank Dr. Funakoshi (National Kyusyu Cancer Center, Fukuoka, Japan) for providing KP-1 human pancreatic cancer cell line. The technology of surgical orthotopic implantation is licensed from AntiCancer Inc. (San Diego, CA).

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