Growth Factors, Cytokines, Cell Cycle Molecules

Targeting the Expression of Platelet-Derived Growth Factor Receptor by Reactive Stroma Inhibits Growth and Metastasis of Human Colon Carcinoma

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The stromal cells within colon carcinoma express high levels of the platelet-derived growth factor receptor (PDGF-R), whereas colon cancer cells do not. Here, we examined whether blocking PDGF-R could inhibit colon cancer growth *in vivo*. KM12SM human colon cancer cells were injected subcutaneously (ectopic implantation) into the cecal wall (orthotopic implantation) or into the spleen (experimental liver metastasis) of nude mice. In the colon and liver, the tumors induced active stromal reaction, whereas in the subcutis, the stromal reaction was minimal. Groups of mice ($n = 10$) received saline (control), the tyrosine kinase inhibitor imatinib, irinotecan, or a combination of imatinib and irinotecan. Four weeks of treatment with imatinib and irinotecan significantly inhibited tumor growth (relative to control or single-agent therapy) in the cecum and liver but not in the subcutis. The combination therapy completely inhibited lymph node metastasis. Imatinib alone or in combination with irinotecan inhibited phosphorylation of PDGF-R β of tumor-associated stromal cells and pericytes. Combination therapy also significantly decreased stromal reaction, tumor cell proliferation, and pericyte coverage of tumor microvessels and increased apoptosis of tumor cells and tumor-associated stromal cells. These data demonstrate that blockade of PDGF-R signaling pathways in tumor-associated stromal cells and pericytes inhibits the progressive growth and metastasis of colon cancer cells. (Am J Pathol 2006; 169:2054–2065; DOI: 10.2353/ajpath.2006.060653)

metastasis to the liver and lymph nodes.^{1,2} Because progressive and recurrent colon cancers have a low response rate to chemotherapeutic agents, there is a critical need for a better understanding of the biology of colon cancer to allow the development of new approaches to therapy.

Colon carcinoma cells produce various growth factors and cytokines that contribute to progressive growth and metastasis,³ and such molecules represent one possible new therapeutic approach. One example is the family of platelet-derived growth factors (PDGFs), members of a family of dimeric disulfide-bonded growth factors exerting their biological effects through activation of two structurally related tyrosine kinase receptors, the PDGF- α and - β receptors.⁴ PDGF consists of dimeric forms, including PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD.^{5–7} The α -receptor binds all possible forms of PDGF except PDGF-DD, whereas the PDGF- β receptor preferentially binds PDGF-BB. PDGF receptor (PDGF-R) β is expressed on many tumor types, and PDGF-BB is an important autocrine growth factor for many cell types, including gliomas, sarcomas, pancreatic carcinoma, and prostate cancer.⁸ PDGF-R signaling has also been reported to stimulate angiogenesis,⁹ to recruit pericytes,^{10,11} and to control the interstitial fluid pressure in stroma, influencing transvascular transport of chemotherapeutic agents in a paracrine manner.^{12,13} We have recently studied a number of human colon cancer clinical specimens and found PDGF-R β and phosphorylated PDGF-R β predominantly expressed in stromal cells and in pericytes surrounding the tumor microvessels.¹⁴

Although imatinib, a derivative of 2-phenylaminopyrimidine, was originally developed as a competitor for an ATP-binding site of the Abl protein tyrosine kinase,¹⁵ it is

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Colorectal cancer is the second most common cause of cancer death in the United States, in large part due to

also known to be a potent tyrosine kinase inhibitor of c-Kit and PDGF-R.¹⁶ We have reported that imatinib can slow both the progressive growth of human pancreatic carcinoma in nude mice^{17,18} and the growth of experimental bone metastasis of human prostate cancer.^{19,20}

In the present study, we examined the therapeutic effect of imatinib administered as a single agent or in combination with the chemotherapeutic irinotecan against human colon carcinoma cells growing in orthotopic (cecum and liver) and ectopic (subcutis) organs of nude mice.

Materials and Methods

Colon Cancer Cell Line and Culture Conditions

The human colon cancer cell line KM12SM^{21,22} was maintained in minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, a twofold vitamin solution (Life Technologies, Grand Island, NY), and a penicillin/streptomycin mixture (Flow Laboratories, Rockville, MD). Adherent monolayer cultures were maintained on plastic and incubated at 37°C in a mixture of 5% CO₂ and 95% air. The cultures were free of *Mycoplasma* and pathogenic murine viruses (assayed by Science Applications International Co., Frederick, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Reagents

Imatinib (imatinib mesylate or Gleevec; Novartis Pharma, Basel, Switzerland) is a 2-phenylaminopyrimidine class protein-tyrosine kinase inhibitor of platelet-derived growth factor receptor (PDGF-R), BCR-ABL, and c-Kit.^{15,16} For oral administration, imatinib was diluted in sterile water. Irinotecan (Campotar; Pharmacia, North Peapack, NJ) was kept at room temperature and dissolved in 0.9% NaCl on the day of intraperitoneal injection. Primary antibodies were purchased from the following manufacturers: polyclonal rabbit anti-PDGF-R β , polyclonal rabbit anti-phosphorylated PDGF-R β , polyclonal rabbit anti-PDGF-A subunit, and polyclonal rabbit anti-PDGF-B subunit obtained from Santa Cruz Biotechnology (Santa Cruz, CA); rat anti-mouse CD31 from BD PharMingen (San Diego, CA); mouse anti-desmin from Molecular Probes (Eugene, OR); and α -smooth muscle actin and Ki-67 (MIB-1) both from Dako Cytomation (Carpinteria, CA). The following secondary antibodies were used: Cy3-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-rat IgG, Cy5-conjugated goat anti-rat IgG, Cy5-conjugated goat anti-mouse IgG (all from Jackson ImmunoResearch, West Grove, PA), and peroxidase-conjugated rat anti-mouse IgG1 (BD PharMingen). Other reagents included Sytox Green nucleic acid stain (Molecular Probes) and propyl gallate (ACROS Organics, Morris Plains, NJ). Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was

done using a commercial apoptosis detection kit (Promega, Madison, WI) with modifications.

Animals and Implantation of Tumor Cells

Male athymic Ncr-nu/nu mice were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, the U.S. Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with institutional guidelines when they were 8 to 12 weeks old.

KM12SM cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in Hanks' balanced salt solution (HBSS). Only suspensions consisting of single cells with >90% viability were used. As described previously,²¹ for subcutaneous tumors, 5×10^5 cells in 50 μ l of HBSS were injected into the subcutis, and to produce cecal tumors, 2×10^6 cells in 50 μ l of HBSS were injected into the cecal wall of nude mice. To produce experimental liver metastasis, 1×10^6 KM12SM cells in 50 μ l of HBSS were injected into the spleen of nude mice. Two weeks after the injection, splenectomy was performed as described previously.²²

Treatment of Established Human Colon Carcinoma Tumors Growing in the Cecum, Liver, or Subcutis of Athymic Nude Mice

Seven days after the injection of KM12SM cells into the subcutis or the cecal wall or 21 days after intrasplenic injection, tumor lesions reached the size of 1 to 2 mm. At that time, groups of mice ($n = 10$) were randomly assigned to receive one of the following four treatments: 1) administration of water by daily oral gavage and once per week intraperitoneal injection of phosphate-buffered saline (PBS) (control group); 2) daily oral gavage of imatinib (50 mg/kg, biological optimal dose as determined previously^{18,19}) and once per week intraperitoneal injection of PBS; 3) administration of water by daily oral gavage and once per week intraperitoneal injection of irinotecan (10 or 15 mg/kg); and 4) daily oral imatinib (50 mg/kg) and once per week intraperitoneal injection of irinotecan (10 or 15 mg/kg). The treatments continued for 4 weeks. All therapy experiments were performed twice.

Necropsy Procedures and Histological Studies

The mice bearing orthotopic tumors were euthanized by methophane on day 29 of the treatment. The body weight

was recorded. After necropsy, tumors growing in the cecum were excised and weighed. For immunohistochemical and hematoxylin and eosin staining procedures, respectively, one part of the tumor tissue was fixed in formalin and embedded in paraffin, and the other part was embedded in OCT compound (Miles, Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at -70°C . All macroscopically enlarged mesenteric lymph nodes were harvested, and the presence of metastatic disease was confirmed by histological review. Livers containing human colon cancer colonies were resected, washed, and fixed in Bouin's fixative. In the ectopic (subcutaneous) xenograft model, the tumors were monitored daily until they became necrotic. Tumor volume was calculated with the formula $V = 1/2ab^2$, where a is the longest diameter and b is the shortest diameter of the tumor.

Immunofluorescence Double Staining for PDGF, PDGF-R, p-PDGF-R, or Pericytes (Desmin⁺ Cells) and CD31

Fresh frozen specimens of KM12SM human colon carcinoma cells growing in nude mice were cut into $4\text{-}\mu\text{m}$ sections, mounted on positively charged slides, and stored at -80°C . In preparation for assays, sections were fixed in ice-cold acetone for 10 minutes, followed by three washes with PBS for 3 minutes each. Slides were placed in a humidified chamber and incubated with protein blocking solution (5% normal horse serum and 1% normal goat serum in PBS) for 20 minutes at room temperature. The slides were incubated overnight at 4°C with primary antibody against PDGF-A, PDGF-B, PDGF-R β , p-PDGF-R β , desmin, or α -smooth muscle actin (α -SMA). For desmin and α -SMA staining, the slides were incubated overnight at 4°C with goat anti-mouse IgG, Fab fragment (Jackson ImmunoResearch) to block endogenous immunoglobulins, followed by a short incubation with protein blocking solution and then by incubation with the primary antibody. The slides were then rinsed three times with PBS and incubated for 10 minutes in protein blocking solution. The slides were incubated for 1 hour at room temperature with Cy3-conjugated corresponding secondary antibody. From this step onward, the slides were protected from light. The samples were then rinsed three times in PBS. To identify endothelial cells, slides were incubated overnight at 4°C with an antibody against CD31. The sections were rinsed three times with PBS and incubated for 10 minutes in protein-blocking solution. Slides were incubated for 1 hour at room temperature with Cy5-conjugated goat anti-rat secondary antibody. The samples were then rinsed three times in PBS, and nuclear counterstain with Sytox green was applied for 10 minutes. Samples were then rinsed three times with PBS, and mounting medium was placed on each sample, which was then covered with a glass coverslip (Fischer Scientific, Pittsburgh, PA). Mounting medium consisted of 90% glycerol, 10% PBS, and 0.1 mol/L propyl gallate. Endothelial cells (CD31⁺) were identified by green fluorescence, whereas PDGF, PDGF-R, p-PDGF-R, and

pericytes (desmin⁺ cells) were identified by red fluorescence.

The coverage of pericytes on endothelial cells was determined by counting CD31⁺ cells in direct contact with desmin⁺ cells and CD31⁺ cells without direct association with desmin⁺ cells in five randomly selected microscopic fields (at $\times 100$ magnification).^{17,23}

TUNEL assay was performed using a commercial apoptosis detection kit (Promega) as previously described in detail.²⁴ TUNEL⁺ apoptotic cells were detected by localized green fluorescence within the cell nuclei. The total number of apoptotic cells was quantified in 10 randomly selected microscopic fields and expressed as the ratio of apoptotic stromal cells to the total number of stromal cells (at $\times 400$ magnification).

Immunohistochemical Determination of Ki-67 Antigen and Mean Vessel Density

Paraffin-embedded tissues were used for immunohistochemical identification of Ki-67. Sections were deparaffinized and rehydrated in PBS, microwaved in water for 5 minutes for antigen retrieval, incubated at 4°C with a mouse IgG1 anti-Ki-67 antibody overnight, and incubated for 1 hour at room temperature with a peroxidase-conjugated rat anti-mouse IgG1 antibody. Positive reaction was detected by exposure to stable 3,3'-diaminobenzidine for 5 to 10 minutes. Slides were counterstained with Gill's hematoxylin. For the quantification of mean vessel density (MVD) in sections stained for CD31, 10 random 0.81-mm^2 fields at $\times 100$ magnification were captured for each tumor, and microvessels were quantified according to the method described previously.²⁵ Ki-67 labeling index (LI) was determined by light microscopy at the site of the greatest number of Ki-67⁺ cells. The sites were identified by scanning tumor sections at low power ($\times 40$). For Ki-67 LI, the number of positive cells among approximately 1000 tumor cells was calculated as a percentage. Apoptotic cells were analyzed by using a commercially available TUNEL kit (Promega) as described in detail previously.²⁴ The number of cells undergoing apoptosis was counted in 10 random 0.81-mm^2 fields at $\times 100$ magnification.

Confocal Microscopy

Confocal fluorescence images were collected using $\times 20$ or $\times 40$ objectives on a Zeiss LSM 510 laser scanning microscopy system (Carl Zeiss Inc., Thornwood, NY) equipped with a motorized Axioplan microscope, argon laser (458/477/488/514 nm, 30 mW), HeNe laser (543 nm, 1 mW), HeNe laser (633 nm, 5 mW), LSM 510 control and image acquisition software, and appropriate filters (Chroma Technology Corp., Brattleboro, VT). Confocal images were exported to Adobe Photoshop software, and montages were prepared for publication photos.

Table 1. Therapy of KM12SM Tumors Growing in the Cecal Wall of Nude Mice

Treatment group	Body weight (g) (range)	Tumor incidence	Tumor weight (g) (range)	Lymph node metastasis	Peritoneal metastasis
Control	26.3 (20.8 to 32.0)	10/10	0.25 (0.10 to 0.53)	8/10	4/10
Imatinib (50 mg/kg)	29.1 (22.4 to 32.1)	9/9	0.16 (0.08 to 0.25)*	3/9	0/9
Irinotecan (10 mg/kg)	26.8 (21.6 to 29.3)	10/10	0.20 (0.04 to 0.71)	4/10	3/10
Irinotecan (10 mg/kg) + imatinib (50 mg/kg)	27.1 (23.3 to 31.2)	10/10	0.08 (0.04 to 0.12) ^{†‡}	0/10*	0/10*
Irinotecan (15 mg/kg)	27.6 (23.7 to 31.4)	8/8	0.12 (0.04 to 0.18)	3/8	4/8
Irinotecan (15 mg/kg) + imatinib (50 mg/kg)	27.7 (23.9 to 31.8)	9/9	0.06 (0.01 to 0.09) ^{†‡}	0/9*	0/9

KM12SM cells (2×10^6) were injected into the cecal wall of nude mice. One week later, the mice were randomly assigned ($n = 10$) to receive water daily by oral gavage and intraperitoneal injection of PBS once per week (control); oral gavage of imatinib (50 mg/kg) daily and once-weekly intraperitoneal injections of PBS; irinotecan intraperitoneally once per week and daily administration of water by oral gavage; or oral gavage of imatinib daily and once-weekly intraperitoneal injection of irinotecan. The treatment continued for 4 weeks.

* $P < 0.05$ compared with control group.

[†] $P < 0.001$ compared with control group.

[‡] $P < 0.05$ compared with imatinib alone and irinotecan alone.

Statistical Analysis

Body weight of mice and tumor weight were compared using the Mann-Whitney *U*-test. The differences in Ki-67⁺ cells, MVD (CD31), TUNEL⁺ cells, and ratio of PDGF-R β to TUNEL colocalized cells were determined by the unpaired Student's *t*-test. Incidence of lymph node metastasis and peritoneal metastasis were compared using the Fisher's exact probability test.

Results

Therapy of Human Colon Carcinoma Growing in the Cecum or Subcutis of Nude Mice

We determined the effects of imatinib, irinotecan, or the combination of imatinib and irinotecan on the growth and metastasis of KM12SM human colon carcinoma cells implanted in the cecum of nude mice (Table 1). Tumor incidence was 100% in all treatment groups. The oral administration of imatinib, the intraperitoneal injection of irinotecan, or the combination of the two drugs did not significantly affect body weight. Mice treated with saline had large tumors in the cecum (median 0.25 g) and an 80% incidence of regional (mesenteric) lymph node metastasis. Treatment with imatinib significantly reduced the weight of cecal tumors (0.16 g; $P < 0.05$) and decreased the incidence of lymph node metastasis to 33%. The combination of imatinib and irinotecan enhanced the antitumor effect of irinotecan. The combination of imatinib and irinotecan (15 mg/kg) produced the most significant inhibition of tumor growth (0.06 g, $P < 0.001$ versus control) and completely inhibited lymph node metastasis and tumor growth at the abdominal wall wound site.

Next, we compared the effects of imatinib, irinotecan, and the combination of imatinib and irinotecan on the growth of ectopic (subcutaneous) KM12SM tumors. Treatment with imatinib alone did not inhibit tumor growth compared with control treatment. Treatment with irinotecan alone slightly inhibited tumor growth, but the irinotecan and imatinib combination did not significantly affect

subcutaneous tumor growth compared with irinotecan alone (Figure 1).

Therapy of Human Colon Carcinoma Lesions in the Liver of Nude Mice

Next, we examined the effects of imatinib, irinotecan, or the combination of imatinib and irinotecan on the growth of KM12SM human colon carcinoma cells in the liver of nude mice (Table 2). Representative photographs of each treatment group are shown in Figure 2. Mice treated with saline had large tumor colonies in the liver (median 11 mm). Treatment with imatinib alone did not affect the size of liver tumor colonies. Treatment with irinotecan at

Figure 1. Growth of KM12SM cells implanted in the subcutis (ectopic site) of nude mice. KM12SM cells (5×10^5) were implanted into the subcutis of nude mice. Mean tumor volumes were determined as described in Materials and Methods. The tumor volume in mice treated with irinotecan was smaller than that in control group, but the difference was not statistically significant. Irinotecan and imatinib did not affect tumor growth at the subcutaneous site. Mean $\pm$ SE values.

Table 2. Therapy of KM12SM Tumors Growing in the Liver of Nude Mice

Treatment group	Body weight (g) (range)	Tumor incidence	Liver weight (g) (range)	Number of colonies	Size of colonies (mm)
Control	28.0 (23.8 to 34.3)	9/10	2.5 (1.5 to 6.0)	8 (0 to 22)	11 (2 to 15)
Imatinib (50 mg/kg)	26.3 (20.9 to 29.3)	7/9	2.3 (1.4 to 3.1)	8 (0 to 20)	9 (3 to 13)
Irinotecan (10 mg/kg)	27.2 (22.8 to 32.7)	7/9	1.6 (1.3 to 3.5)*	5 (0 to 18)	6 (1 to 9) [†]
Irinotecan (10 mg/kg) + imatinib (50 mg/kg)	28.5 (24.0 to 31.4)	6/8	1.5 (1.1 to 1.9)*	4 (0 to 10)	3 (1 to 5) ^{†‡}
Irinotecan (15 mg/kg)	27.8 (22.6 to 30.8)	7/8	1.4 (1.1 to 3.5)*	4 (0 to 17)	5 (2 to 7) [†]
Irinotecan (15 mg/kg) + imatinib (50 mg/kg)	26.0 (20.8 to 27.7)	6/9	1.3 (1.1 to 1.5)*	5 (0 to 7)	2 (1 to 4) ^{†‡}

KM12SM cells (1×10^6) were injected into the spleen of nude mice. Two weeks later, splenectomy was performed as described previously.^{21,22} One week later, the mice were randomly assigned ($n = 10$) to receive water daily by oral gavage and intraperitoneal injection of PBS once per week (control); oral gavage of imatinib (50 mg/kg) daily and once-weekly intraperitoneal injections of PBS; irinotecan intraperitoneally once per week and daily administration of water by oral gavage; or oral gavage of imatinib daily and once-weekly intraperitoneal injection of irinotecan. The treatment continued for 4 weeks.

* $P < 0.05$ compared with control group and imatinib alone.

[†] $P < 0.01$ compared with control group and imatinib alone.

[‡] $P < 0.01$ compared with imatinib alone and irinotecan alone.

10 or 15 mg/kg reduced the size of the experimental liver metastasis ($P < 0.05$). The combination of imatinib and irinotecan enhanced the antitumor effect of irinotecan ($P < 0.01$) (Table 2; Figure 2).

Histopathological Analysis of KM12SM Tumors

At the periphery of KM12SM cecal tumors in control and irinotecan-treated mice, tumor cells invaded the stroma containing abundant extracellular matrix (ECM). In contrast, cecal tumors in mice treated with imatinib alone or imatinib with irinotecan were

surrounded by a fibrous capsule with lesser ECM (Figure 3A).

Tumor sections from orthotopic (cecum or liver) or ectopic (subcutaneous) sites were analyzed immunohistochemically for the expression of PDGF-A, PDGF-B, PDGF-R β , and pPDGF-R β . PDGF-A and PDGF-B were expressed by tumor cells, and treatment with imatinib, irinotecan, or the combination did not alter the expression level of the ligands or the receptors (data not shown). Orthotopic KM12SM tumors had abundant stroma in which the stroma cells expressed PDGF-R β or phosphorylated PDGF-R β (Figure 3, A and B), whereas subcuta-

Figure 2. Experimental liver metastasis. Liver tumor colonies were produced by the intrasplenic injection of KM12SM cells, followed by splenectomy. The mice were treated for 4 weeks with saline (control), imatinib alone, irinotecan alone, or the combination of imatinib and irinotecan. The livers were fixed in Bouin's fixative.

Figure 3. Double immunofluorescence staining for CD31 and PDGF-R β or pPDGF-R β in KM12SM cecal (A), liver (B), and subcutaneous (C) tumors. Tumor sections were stained with H&E, anti-CD31 antibody (green), and anti-PDGF-R β or pPDGF-R β (in red fluorescence) as described in Materials and Methods. A and B: Expression of PDGF-R β by tumor-associated stromal cells was found in cecal and liver tumors from all treatment groups. Phosphorylation of PDGF-R β of tumor-associated stromal cells was inhibited by treatment with imatinib and imatinib and irinotecan. C: In subcutaneous tumors, the expression of PDGF-R β by perivascular stroma was low, and the PDGF-R β was not phosphorylated. T, tumor nest; S, stroma.

Orthotopic sites

A cecum

B liver

Ectopic site

C s.c.

Figure 4. Fluorescence double-labeled immunohistochemistry of KM12SM human colon cancer cells growing in the cecum or subcutis of nude mice. Representative images show immunohistochemistry for CD31 (endothelial marker), desmin (pericyte marker), and α -SMA (marker for myofibroblast and pericyte) in green and PDGF-R β in red. Orthotopic KM12SM tumors (**A** and **B**) had abundant stroma in which α -SMA⁺ and desmin⁺ stromal cells (myofibroblasts and pericytes) expressed PDGF-R β (yellow), whereas ectopic tumors did not (**C**).

neous ectopic tumors had little stroma with unphosphorylated PDGF-R β (Figure 3C). PDGF-R β protein colocalized with desmin and α -SMA proteins, indicating myofibroblasts and pericytes expressed PDGF-R β in orthotopic sites (Figure 4, A and B) but not in ectopic sites (Figure 4C). The level of PDGF-R β expressed by the stromal cells in cecal tumors and liver tumor colonies was unchanged by any of the treatments. In contrast, the phosphorylation of PDGF-R β was significantly inhibited in orthotopic tumors of mice treated with imatinib alone or imatinib and irinotecan (Figure 3, A and B).

Cell Proliferation (Ki-67), Apoptosis (TUNEL), and Microvessel Density

Cell proliferation was evaluated by staining for Ki-67 (Figure 5A). In orthotopic tumors from control mice, the Ki-67 labeling index (LI) was 13.5 ± 1.0 in cecal tumors and 14.2 ± 1.3 in liver tumor colonies. As shown in Table 3, treatment with imatinib alone or irinotecan alone did not decrease Ki-67 LI, but their combination produced a significant decrease (4.9 ± 0.5 in cecal tumors, 4.3 ± 0.4 in liver tumor colonies, $P < 0.001$).

The induction of apoptosis in orthotopic tumors was evaluated by the TUNEL assay (Table 3). In tumors from control and imatinib-treated mice, the median number of apoptotic tumor cells was minimal (1 ± 1). The number of apoptotic cells rose to 11 ± 2 in cecal tumors and 12 ± 2 in liver tumor colonies from mice treated with irinotecan ($P < 0.01$) and 14 ± 3 in cecal tumors and 10 ± 3 in liver tumor colonies from mice treated with irinotecan and imatinib ($P < 0.01$).

The MVD in the tumors as determined by immunohistochemical staining with antibodies against CD31 was 56 ± 2 in cecal tumors and 45 ± 3 in liver tumor colonies of control mice (Table 3). Treatment with irinotecan alone, imatinib alone, or the combination did not affect the MVD.

Immunofluorescence Double Staining for TUNEL and CD31 or PDGF-R β

Next, we determined whether therapy was associated with apoptosis of stromal cells and pericytes by using the fluorescence double-labeling technique for PDGF-R β /TUNEL or CD31/TUNEL (Figure 5B). Tumors from control mice had no TUNEL⁺ tumor-associated stromal cells. In tumors from mice treated with imatinib alone or imatinib and irinotecan, the level of apoptosis in PDGF-R β ⁺ stromal cells was significantly increased ($P < 0.01$) (Table 3).

Pericyte Coverage of Microvessels

The effects of the various treatments on pericyte coverage of tumor-associated microvessels was evaluated by using the double immunofluorescence staining technique of anti-CD31 antibody and anti-desmin antibody (Figure 5C). In cecal and liver tumor colonies from control or irinotecan-treated mice, the pericytes were enlarged and formed multilayer coverage on the endothelial cells. Peri-

cytes in tumors from mice treated with imatinib alone or with the imatinib and irinotecan were thin and scattered. Treatment with irinotecan did not affect the number of pericytes, but treatment with imatinib or with imatinib and irinotecan significantly reduced the number of pericytes ($P < 0.01$) (Table 3).

Discussion

We demonstrate that blockade of PDGF-R β signaling by oral administration of the PDGF-R tyrosine kinase inhibitor imatinib or imatinib combined with irinotecan significantly inhibited the growth of orthotopic tumors and the incidence of lymph node metastasis in nude mice. Histopathological analysis of the human KM12SM colon carcinoma growing in the cecum and liver of mice treated with imatinib alone or with imatinib combined with irinotecan demonstrated decreased stromal reaction and inhibition of phosphorylated PDGF-R β in the tumor-associated stromal cells. These effects were associated with the inhibition of tumor cell proliferation (Ki-67⁺ cells), an increase of apoptosis in stromal cells (TUNEL⁺/PDGF-R β ⁺ cells), and a decrease in the number of pericytes (desmin⁺ cells) surrounding the tumor-associated microvessels. In contrast, treatment of mice with imatinib alone or imatinib combined with irinotecan did not affect the growth of KM12SM tumors in the subcutaneous space, demonstrating once again that the biology of tumors differs with the organ microenvironment and confirming that models for experimental therapeutic studies should focus on orthotopic models.^{26,27}

In general, tumor cells in a neoplasm are biologically heterogeneous, and their phenotype can be modified by the organ microenvironment.²⁸ Histologically, human carcinoma tissues are composed of both parenchyma and stroma. Tumor stroma consists of fibroblasts, smooth muscle cells, inflammatory cells, microvessels, and abundant ECM.²⁹ Tumor-associated stroma at both primary sites and metastatic sites are thought to be functionally organized to generate a favorable microenvironment and promote the survival of cancer cells.³⁰ Fibroblasts are activated by various growth factors and cytokines that are released by cancer cells, for example, transforming growth factor- β , PDGF, and fibroblast growth factor 2.³¹ Activated fibroblasts express α -SMA, leading to the term "myofibroblasts."³² Stromal reaction (desmoplasia), such as myodifferentiation of fibroblasts and accumulation of ECM,^{33,34} clearly alters the stromal phenotype. In the liver, hepatic stellate cells are the only mesenchymal cells present in the liver parenchyma. They are also activated by various stimuli from tumor cells and undergo transformation into myofibroblasts, which are characterized by expression of α -SMA.³⁵ Shimizu et al³⁶ examined interaction between human colon carcinoma cells and hepatic stellate cells by *in vitro* co-culture system. They found that LM-H3 colon cancer cells expressed PDGF-AB and activated hepatic stellate cells. In turn, activated hepatic stellate cells produced PDGF-AB and hepatocyte growth factor, which stimulate proliferation and migration of colon cancer cells. In gen-

Figure 5. Analysis of cell proliferation (Ki-67) (**A**), apoptosis (TUNEL/PDGF-R β and TUNEL/CD31) (**B**), and pericyte coverage of microvessels (desmin/CD31) (**C**). Mice with cecal KM12SM tumors were treated with control, imatinib, irinotecan, or imatinib and irinotecan. **A:** KM12SM tumors were resected and processed for immunohistochemical evaluation of Ki-67 as described in Materials and Methods. **B:** Double immunofluorescence staining of TUNEL and PDGF-R β or CD31. Stromal cells (PDGF-R β ⁺) stained with red fluorescence, and apoptotic cells (TUNEL⁺) stained with green fluorescence. Colocalization of stromal cells undergoing apoptosis yielded yellow images. Peri-endothelial cells (pericytes and stromal fibroblasts) undergoing apoptosis. **C:** Pericyte coverage on tumor-associated endothelial cells in the KM12SM cecal tumors. Tumor sections were stained with anti-CD31 antibody in green fluorescence and anti-desmin antibody (pericyte marker) in red fluorescence. T, tumor nest; S, stroma.

Table 3. Immunohistochemical Analysis of KM12SM Human Colon Carcinoma Cells Growing in the Cecum and Subcutis of Nude Mice

Primary site	Treatment groups	Tumor cells		Endothelial cells*		Stromal cells	
		Ki67 LI	TUNEL	MVD	Pericyte coverage (%)	TUNEL (%)	Range
Cecum (orthotopic)	Control	13.5 ± 1.0	1 ± 1	56 ± 2	86 ± 6	0	0–1
	Imatinib (50 mg/kg)	12.0 ± 0.8	4 ± 2	50 ± 4	19 ± 2 [†]	8 [‡]	3–11
	Irinotecan (10 mg/kg)	11.3 ± 0.6	11 ± 2 [†]	53 ± 3	75 ± 7	1	0 to 2
	Imatinib (50 mg/kg) + irinotecan (10 mg/kg)	4.9 ± 0.5 [†]	14 ± 3 [‡]	50 ± 3	15 ± 2 [†]	12 [‡]	5 to 22
Liver (orthotopic)	Control	14.2 ± 1.3	1 ± 1	45 ± 3	42 ± 4	0	0–1
	Imatinib (50 mg/kg)	13.1 ± 1.1	2 ± 2	42 ± 4	13 ± 3 [†]	6 [‡]	2 to 12
	Irinotecan (10 mg/kg)	13.8 ± 0.9	12 ± 2 [†]	46 ± 5	38 ± 5	0	0 to 2
	Imatinib (50 mg/kg) + irinotecan (10 mg/kg)	4.3 ± 0.4 [†]	10 ± 3 [‡]	43 ± 4	14 ± 3 [†]	11 [‡]	3 to 17
Subcutaneous (ectopic)	Control	13.4 ± 1.2	2 ± 1	29 ± 3	18 ± 3	0	0 to 1
	Imatinib (50 mg/kg)	13.0 ± 1.1	2 ± 1	32 ± 2	15 ± 2	0	0 to 1
	Irinotecan (10 mg/kg)	11.0 ± 0.5	5 ± 2	30 ± 4	16 ± 4	1	0 to 2
	Imatinib (50 mg/kg) + irinotecan (10 mg/kg)	10.7 ± 0.7	4 ± 3	27 ± 3	14 ± 2	1	0 to 2

The mice were randomly assigned ($n = 10$) to receive water daily by oral gavage and intraperitoneal injection of PBS once per week (control); oral gavage of imatinib (50 mg/kg) daily and once-weekly intraperitoneal injections of PBS; irinotecan (10 mg/kg) intraperitoneally once per week and daily administration of water by oral gavage; or oral gavage of imatinib daily and once-weekly intraperitoneal injection of irinotecan. The treatment continued for 4 weeks.

*Mean ± SE.

[†] $P < 0.001$ compared with control group.

[‡] $P < 0.01$ compared with control group.

eral, tumor-associated stroma is an abundant source of tumor-promoting growth factors and cytokines, and stromal cells activated by cancer cells can in turn regulate the growth and progression of carcinoma cells.^{37,38} The progressive growth of colorectal tumors in experimental animals has been correlated with proliferation of myofibroblasts, whereas regression of tumors has been linked to a fibrous capsule, suggesting that the presence of reactive stromal cells (myofibroblasts) may contribute to the growth of tumor cells.³⁹ Wounding has been associated with tumor-promoting effects from clinical, chemical carcinogenesis, and transgenic animal studies.⁴⁰ Functionally, tumor-associated stromal cells are similar to stromal cells in healing wounds insofar as they are involved in expression of myodifferentiation markers, production of ECM, expression of growth factors and cytokines, and neovascularization.^{31,40}

We previously reported that PDGF-R β is predominantly expressed and phosphorylated in tumor-associated stroma of clinical specimens of human colon carcinomas, and its expression level was associated with invasion and metastasis.¹⁴ In the present study, we demonstrate that PDGF-R β is highly expressed and phosphorylated by tumor-associated stromal cells of human KM12SM colon carcinoma cells growing in the cecum and liver but not the subcutis. The combination of imatinib and irinotecan completely inhibited tumor cell growth at the abdominal wound healing site induced at the time of tumor cell injection into the cecum. Imatinib may inhibit stromal reaction at the orthotopic primary site and the surgical wound. PDGF-R signaling pathway also plays an important role in increasing tumor interstitial hypertension, which may block the accumulation of antitumor

drugs.^{12,13} We found that imatinib impaired PDGF-R signaling in the orthotopic sites and enhanced the antitumor effects of irinotecan. Therefore, blocking the PDGF-R signaling pathway by imatinib can modify tumor-stromal interaction and enhance antitumor effects of irinotecan.

The microvasculature in both tumor tissue and normal colon mucosa consists of endothelial cells, pericytes (mural cells or smooth muscle cells), and basement membranes. All of these components are thought to be abnormal in tumor vessels. Pericytes are key cells in vascular development, stabilization, maturation, and remodeling.^{41,42} Functional-blocking antibodies that target PDGF-R β block pericyte recruitment during vascular development.⁴³ In our study, desmin⁺ pericytes were found on microvessels in both normal organs and colon cancers, albeit with different morphological characteristics. Specifically, pericytes in tumor-associated vessels, but not those in vessels of normal colon mucosa, were enlarged and overexpressed PDGF-R β and p-PDGF-R β .¹⁴ In agreement with earlier reports,^{11,17} we found that treatment with imatinib decreased pericyte coverage on tumor-associated endothelial cells. The inhibition of PDGF-R signaling by a protein tyrosine kinase inhibitor decreased pericyte recruitment and attachment to endothelial cells and destabilized tumor vasculature by killing pericytes. In the absence of decreased vessel density in tumors treated with imatinib combined with irinotecan, functional rather than quantitative changes of the tumor vasculature by imatinib may diminish tumor growth.⁴⁴

Recently, targeting the vascular endothelial growth factor (VEGF) pathway to inhibit tumor angiogenesis is attracting attention as a novel cancer therapy⁴⁵; however, it has been suggested that VEGF-targeting therapies are

mostly active against immature vessels,⁴⁶ ie, normalization of the tumor vasculature.⁴⁷ Because imatinib inhibits pericyte coverage on tumor vasculature, it will be very interesting to determine whether the combined inhibition of PDGF-R and VEGF receptor signaling may produce synergistic antivasculature effects.^{17,48,49} Multitarget tyrosine kinase inhibitors are under current investigation in clinical trials. Sorafenib and sunitinib target not only multiple VEGF receptors but also PDGF-R β , and phase II studies show promising activity of these molecules in renal cell carcinoma,⁵⁰ likely due to inhibition of angiogenesis.⁵¹

In summary, the present results recommend the administration of imatinib to inhibit the phosphorylation of the PDGF-R β on colon cancer-associated stromal cells and microvessel pericytes in combination with an anticycling drug such as irinotecan. This approach could provide a new approach to target the reactive stroma of colon cancer growing in its primary and metastatic sites.

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