Inhibition of PDGF Receptor Signaling in Tumor Stroma Enhances Antitumor Effect of Chemotherapy[1]

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ABSTRACT

Lowering of tumor interstitial hypertension, which acts as a barrier for tumor transvascular transport, has been proposed as a general strategy to enhance tumor uptake and therapeutic effects of anticancer drugs. The tyrosine kinase platelet-derived growth factor (PDGF) β-receptor is one mediator of tumor hypertension. The effects of PDGF antagonists on chemotherapy response were investigated in two tumor models that display PDGF receptor expression restricted to the tumor stroma, and in which PDGF antagonists relieve tumor hypertension. Inhibitory PDGF aptamers and the PDGF receptor tyrosine kinase inhibitor STI571 enhanced the antitumor effect of Taxol on s.c. KAT-4 tumors in SCID mice. Treatment with only PDGF antagonists had no effect on tumor growth. Taxol uptake in tumors was increased by treatment with PDGF antagonists. Cotreatment with PDGF antagonists and Taxol was not associated with antiangiogenic effects, and PDGF antagonists did not enhance the Taxol effect on *in vitro* growth of KAT-4 cells. STI571 also increased the antitumor effects of 5-fluorouracil on s.c. PROb tumors in syngeneic BDIX rats, without increasing the effect of 5-fluorouracil on cultured PROb cells. Expression of PDGF receptors in tumor stroma, as well as tumor hypertension, occurs in most common solid tumors. Therefore, our results have implications for treatment regimens for large patient groups and merit clinical testing. In conclusion, our study identifies inhibition of PDGF signaling in tumor stroma as a novel, possibly general strategy for enhancement of the therapeutic effects chemotherapy.

INTRODUCTION

Chemotherapy is a major treatment modality for solid tumors. To potentially reduce toxic side effects and to achieve higher efficacy of chemotherapeutic drugs, strategies to improve the distribution of drugs between normal tissues and tumors are highly warranted. One property of most solid tumors that has been suggested as a potential target for efforts to increase tumor drug uptake is tumor interstitial hypertension (1, 2). Increased tumor IFP[3] acts as a barrier for tumor transvascular transport (3, 4). Reduction of tumor IFP, or modulation of microvascular pressure, has been shown to increase transvascular transport of tumor-targeting antibodies or low molecular weight tracer compounds (5–8). However, until now, experimental evidence has not been presented that demonstrates that selective reduction of tumor IFP, and concomitant increase in tumor drug uptake, represents a possible strategy for enhancement of antitumor effects of chemotherapy.

The etiology of interstitial hypertension in tumors is poorly understood. The scarcity of lymphatic vessels in tumors has been proposed as one factor contributing to the increased tumor IFP (2). Also, the microvasculature and the supporting stroma compartment are likely to be important determinants for tumor IFP (9, 10). Accumulating evidence points toward the transmembrane PDGF β-receptor tyrosine kinase as an interesting candidate target for pharmacological intervention of tumor interstitial hypertension (7, 11).

A role for PDGF β-receptor signaling in the control of IFP was originally demonstrated in a rat model of dextran-induced anaphylaxis, where PDGF β-receptor stimulation was found to normalize the dextran-induced lowering of the IFP (11). In contrast, activation of the structurally related PDGF α-receptor had no effect on loose connective tissue IFP. More recently, a role for PDGF β-receptors in control of tumor IFP was demonstrated in the syngeneic PROb rat colon adenocarcinoma model (7). In this tumor model, where PDGF β-receptor expression is restricted to tumor stroma cells, a significant reduction of tumor IFP was observed after treatment with a DNA aptamer that inhibits the PDGF β-receptor ligands PDGF-AB and -BB. Finally, the well-documented PDGF β-receptor expression in the stromal compartment in many common solid tumors, *e.g.* lung, breast, and colon carcinoma, that also are characterized by tumor interstitial hypertension, is consistent with a role for PDGF β-receptors in the control of tumor IFP (12, 13).

In this study we investigate the effects of PDGF receptor inhibition on the efficacy of two commonly used cytotoxic drugs, Taxol and 5-FU, in tumor models with PDGF receptor expression restricted to the tumor stroma. We also explore whether inhibition of PDGF receptors alters tumor uptake of chemotherapeutical agents. For PDGF receptor inhibition we have used a PDGF-B-specific aptamer (PDGF aptamer; Ref. 14) and STI571, a low molecular weight tyrosine kinase inhibitor, which selectively blocks the PDGF receptor kinase, the c-Kit receptor kinase, and the Abl and Arg nonreceptor kinase (15, 16).

MATERIALS AND METHODS

Animal Care. Fox Chase SCID mice (M&B, Ry, Denmark) and BDIX rats (Harlan, Oxon, United Kingdom) were housed under pathogen-free conditions at the animal facility at the Biomedical Centre, Uppsala, Sweden. All of the animal experiments were approved by the local committee for animal experiments and performed according to United Kingdom Coordinating Committee on Cancer Research guidelines (17).

Tumor Establishment. KAT-4 (18) and PROb tumors were established in Fox Chase SCID mice or BDIX rats, respectively, by s.c. injection of 2×10^6 cells in 50 μl of PBS in the flank.

PDGF Receptor Antagonists. A high affinity DNA-based aptamer to PDGF-B was used as a specific antagonist to PDGF (14). The aptamer was conjugated to M_r 40,000 PEG to improve its pharmacokinetic profile (19). Controls for the aptamer were either a PEG-conjugated sequence-scrambled analogue of the PDGF aptamer with 10,000-fold lower affinity for PDGF-B or M_r 40,000 PEG alone. The aptamer was administered by i.p. injection three times daily at a dose of 12 mg $\times$ kg^{-1} $\times$ day^{-1}. STI571, a PDGF receptor tyrosine kinase inhibitor (15, 16), or PBS as a control, was administered by gavage once daily at a dose of 100 mg $\times$ kg^{-1} $\times$ day^{-1}.

PDGF Binding Assay. KAT-4 cells and porcine aortic endothelial cells expressing the PDGF α-receptor were seeded in 24-well plates and serum starved overnight. On the day of the experiment, samples containing 2 ng/ml [^{125}I]PDGF-BB without or with the addition of 200 ng/ml unlabeled PDGF-BB were added to the cells in triplicate. After incubation for 90 min on ice, cells

were washed three times and lysed, after which the amount of radioactivity in the lysates was measured using a gamma counter.

Measurement of Tumor IFP. Size-matched groups of mice bearing tumors were treated with PDGF receptor antagonists or control for 4 days before the experiment (average tumor weight at the conclusion of the experiment: control for STI571-group, 0.82 g $\pm$ 0.11 g; STI571-group, 0.92 g $\pm$ 0.14 g; scrambled aptamer-group, 0.43 g $\pm$ 0.09 g; and PDGF aptamer-group, 0.48 g $\pm$ 0.10 g). Tumor IFP in KAT-4 tumors was measured 1–2 h after last treatment using the wick-in-needle technique as described (7).

Tumor Growth Curves. KAT-4 and PROb tumors were allowed to grow to average sizes of ~100 mm³ and 900 mm³, respectively. Subsequently, mice and rats were randomized into four different groups receiving no treatment, PDGF receptor antagonist only, Taxol or 5-FU only, or both PDGF receptor antagonist and Taxol or 5-FU. PDGF receptor antagonists were administered daily throughout the experiment. Taxol (5 mg $\times$ kg^{-1} $\times$ dose^{-1}; Bristol Myers Squibb) was administered s.c. at sites distant from the tumor in 200 μl vehicle (65% PBS, 25% ethanol, and 10% Chremophore EL) on days 4–7, days 11–14, and days 18–21 (STI571 experiment only) and always 1 h after administration of PDGF receptor antagonists. 5-FU (1 mg $\times$ kg^{-1} $\times$ dose^{-1}; Nycomed) was administered i.p. in 2 ml of PBS on day 4, 7, 10, and 13, and always 1 h after administration of STI571. Tumor volume was calculated as described (7).

Extraction of PDGF Receptors from Tumors. Tumors were excised from mice, immediately snap frozen in liquid nitrogen, and stored in −135°C. At the time of analysis, 2 tumors from each treatment group were cut into small pieces and thawed in 3 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, and 200 mM Na$_3$VO$_4$) per gram of tissue. After homogenization using an electrical homogenizer and incubation on ice for 30 min, lysates were cleared by centrifugation at 13,000 $\times$ g for 10 min. To additionally clear the extracts, normal rabbit serum was added and incubated for 60 min in 4°C, followed by collection of the immune-complexes by protein A-Sepharose. Immunoprecipitation of the PDGF β-receptor was performed using the polyclonal rabbit antiserum R3 (20). For Western blot analysis of receptor content and phospho-tyrosine content of the receptors, anti-PDGF receptor antibody 958 (Santa Cruz) and anti-phospho-tyrosine antibody PY-99 (Santa Cruz) were used, respectively. Quantification of the band intensities was performed on a CCD-camera (Fuji Film). The intensity of the phospho-tyrosine signal was divided by the intensity of the receptor signal to yield relative phospho-tyrosine values. The average relative phospho-tyrosine value of control treated tumors was set to 100.

Assessment of Gross Tumor Architecture and Tumor Cell Density. Deparaffinized tumor sections were stained with Mayer's Hematoxylin for 25 s or with Azan's trichrome (Bio-optica) according to the manufacturer's specifications, and subsequently rinsed in tap water, dehydrated, and mounted. Cell density of tumors was quantified by counting all of the cell nuclei within a 0.09 mm² grid ($\times$400 magnification) in three random fields of vision of viable tissue per tumor section.

Uptake of [³H]Taxol. [3″-³H]Taxol was obtained from the Drug Synthesis and Chemistry Branch (National Cancer Institute, Bethesda, MD) at a specific activity of 19.3 Ci/mmol. Size-matched groups of mice bearing tumors (average tumor weight at the conclusion of the experiment: control aptamer-group 8 h, 0.88 g $\pm$ 0.15 g; PDGF aptamer-group 8 h, 0.93 g $\pm$ 0.06 g; control aptamer-group 24 h, 1.34 g $\pm$ 0.12 g; and PDGF aptamer-group 24 h, 1.18 g $\pm$ 0.23 g) were treated with PDGF receptor antagonists or control substances for 4 days before the experiment. One h after last administration, mice were injected s.c. at a site distant from the tumor with 6 μCi of [³H]Taxol in a mixture of 5 mg $\times$ kg^{-1} unlabeled Taxol (Bristol-Myers Squibb), 65% PBS, 25% ethanol, and 10% Chremophore EL (Sigma; total volume 200 μl). After 8 or 24 h, blood samples were taken by heart puncture, mice were sacrificed, and tumors were excised. Tumor tissue was weighed, put into an extraction buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS] and homogenized. The amount of ³H-radioactivity in blood samples and tumor was measured using a scintillation counter. The uptake of [³H]Taxol in each tumor was expressed as dpm/g tumor tissue divided by dpm/ml blood.

Immunohistochemistry. For staining of the PDGF β-receptor, frozen sections of KAT-4 tumors were fixed in acetone, and endogenous peroxidase

activity was quenched with 3% H$_2$O$_2$ for 10 min. Sections were stained with affinity purified, rabbit polyclonal antibody 958 directed against the COOH terminus of the PDGF β-receptor (2 μg/ml; Santa Cruz) or with rabbit IgG (2 μg/ml) as a negative control. For staining of markers for apoptosis and proliferation in KAT-4 tumors, sections from paraformaldehyde-fixed, paraffin-embedded tumors were deparaffinized, immersed in a citrate buffer (pH 6.0) and boiled for 2 $\times$ 6 min at 750 W in a microwave oven. Apoptosis and proliferation were visualized using M-30 CytoDEATH (1:10; Boehringer-Mannheim) and Ki-67 antigen MIB-5 (1:50; Immunotech) antibodies, respectively. All of the stainings of KAT-4 tumor sections were performed on a NexES immunostainer with a diaminobenzidine substrate kit (Ventana Medical Systems, Tucson, AZ). Apoptosis in PROb tumor sections was visualized
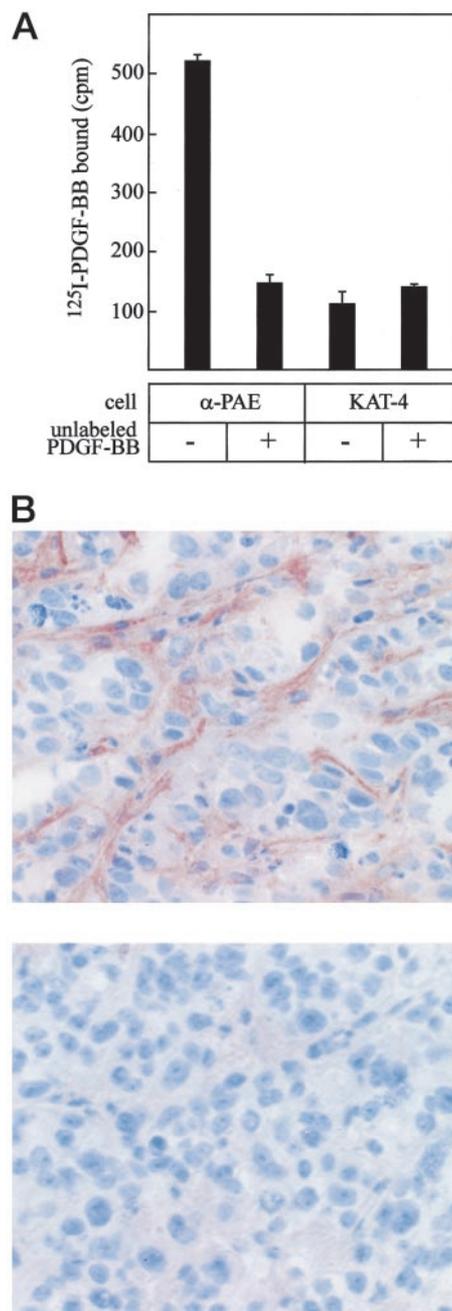

Fig. 1. KAT-4 tumors express PDGF β-receptors in the stroma compartment. *A,* PDGF binding assays on KAT-4 cells and, as a control, porcine aortic endothelial cells expressing the PDGF α-receptor (α-PAE) were performed *in vitro* using ¹²⁵I-PDGF-BB. *B,* frozen sections from KAT-4 tumors were stained immunohistochemically with affinity purified polyclonal antibodies against the PDGF β-receptor (*top panel*) or with rabbit IgG as a control (*bottom panel*). $\times$400 magnification; *bars,* $\pm$SE.
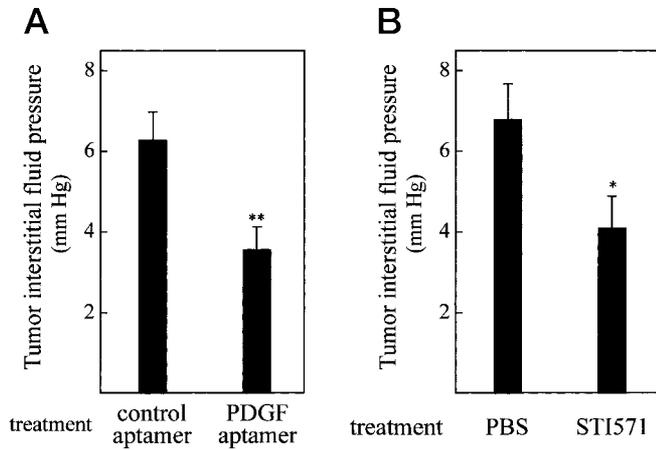
Fig. 2. Treatment with PDGF receptor antagonists lowers IFP in KAT-4 tumors. Tumor IFP was measured 1–2 h after last administration of PDGF receptor inhibitor in KAT-4 tumors grown s.c. in SCID mice. PDGF receptor inhibitors were administered for a total of 4 days. *A,* mice were treated with control aptamer ($n = 8$) or PDGF aptamer ($n = 8$). *B,* mice were treated with PBS ($n = 8$) or STI571 ($n = 9$). ∗$P < 0.05$, Student's t test, ∗∗$P < 0.01$, Student's t test; *bars,* ±SE.

using TUNEL staining (all reagents from Roche) performed as described (21). In each section, the number of positively staining cells in 10 randomly chosen fields of vision (×400 magnification) of viable tissue was quantified. Tumor angiogenesis was assessed by stereological quantification (21, 22) of CD31$^+$ vascular structures counted at ×400 magnification. An eyepiece grid of 10×10 squares (0.3×0.3 mm) was placed at random in the upper left-hand corner of the section and systematically advanced in steps of 2 mm. Morphological parameters of 10–30 fields of vision (×400 magnification) were quantified from each tumor, and length, area, and volumetric density of vessels were calculated using equations in Ref. 21.

In Vitro **Growth Curves.** Cells were cultured under standard conditions and all of the tissue culture medium were supplemented with 10% fetal bovine serum and antibiotics. At day 0, four identical 12-well plates were prepared containing 2×10^4 cells/well and the appropriate addition of drugs. The concentrations used were 20 nM PDGF aptamer, 3 μM STI571, 2.5 nM Taxol (for KAT-4 cells), and 0.75 μM 5-FU (for PROb cells). Cell culture medium and drugs were replaced every day. Duplicate samples were assayed for cell number in a particle counter (Beckman Coulter).

Statistical Analysis. All of the statistical analyses were performed using the unpaired, two-sided Student t test with a significance level of $P < 0.05$. Error bars in figures represent SE.
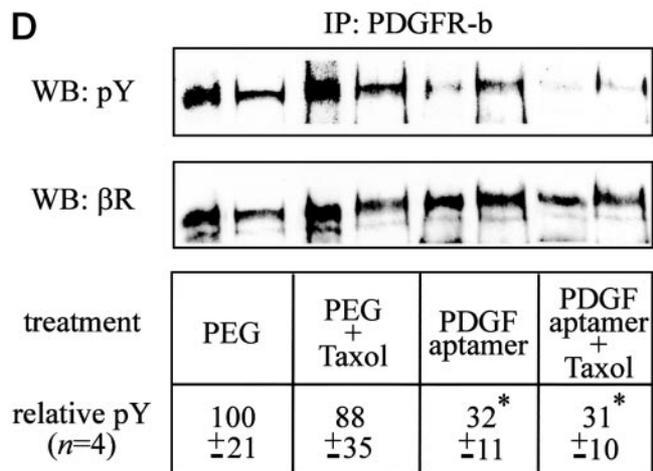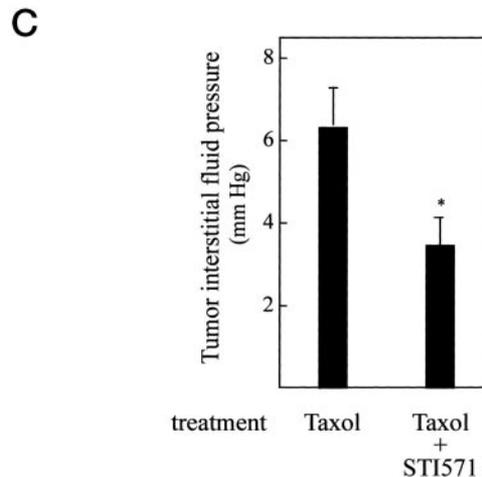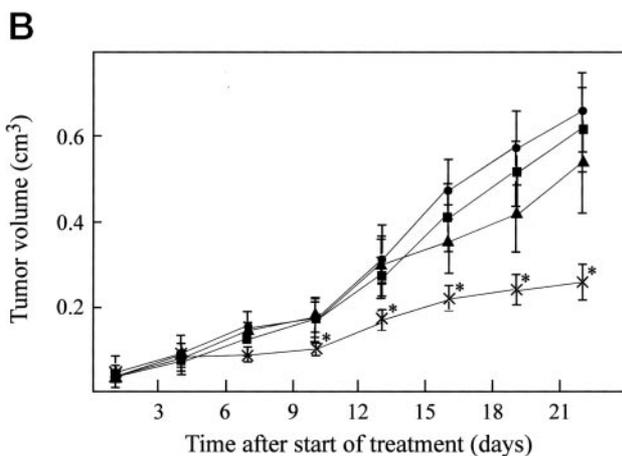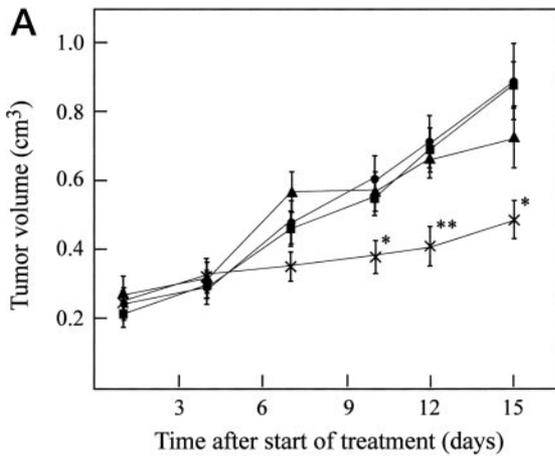

Fig. 3. Treatment with PDGF receptor antagonists enhances the effect of Taxol on KAT-4 tumors *in vivo*. Growth curves of KAT-4 tumors grown s.c. in SCID mice. *A,* mice received PEG (■, $n = 8$), PEG-conjugated PDGF aptamer (●, $n = 8$), PEG and Taxol (▲, $n = 8$), or PDGF aptamer and Taxol (×, $n = 8$). *B,* mice received no treatment (■, $n = 8$), STI571 (●, $n = 6$), Taxol (▲, $n = 4$), or STI571 and Taxol (×, $n = 8$). ∗P 0.05, PDGF-receptor antagonist + Taxol *versus* Taxol alone, Student's t test, ∗∗$P < 0.01$ PDGF-receptor antagonist + Taxol *versus* Taxol alone, Student's t test. *C,* measurement of tumor IFP in tumors at the conclusion of the treatment study. Mice received Taxol ($n = 4$) or STI571 and Taxol ($n = 8$). ∗$P < 0.05$, STI571 + Taxol *versus* Taxol alone, Student's t test. *D,* Western blot analysis of the levels of PDGF β-receptor (βR) and the phospho-tyrosine content of the receptors (pY) in homogenates of tumors from each treatment group ($n = 4$ in each group). Two representative tumors from each treatment group are shown. The intensity of the signals for pY was normalized with the intensity of the signals for βR to yield the relative pY value. The average relative pY value of control treated tumors was set to 100. ∗$P < 0.05$, *versus* PEG-treated tumors, Student's t test; *bars,* ±SE.
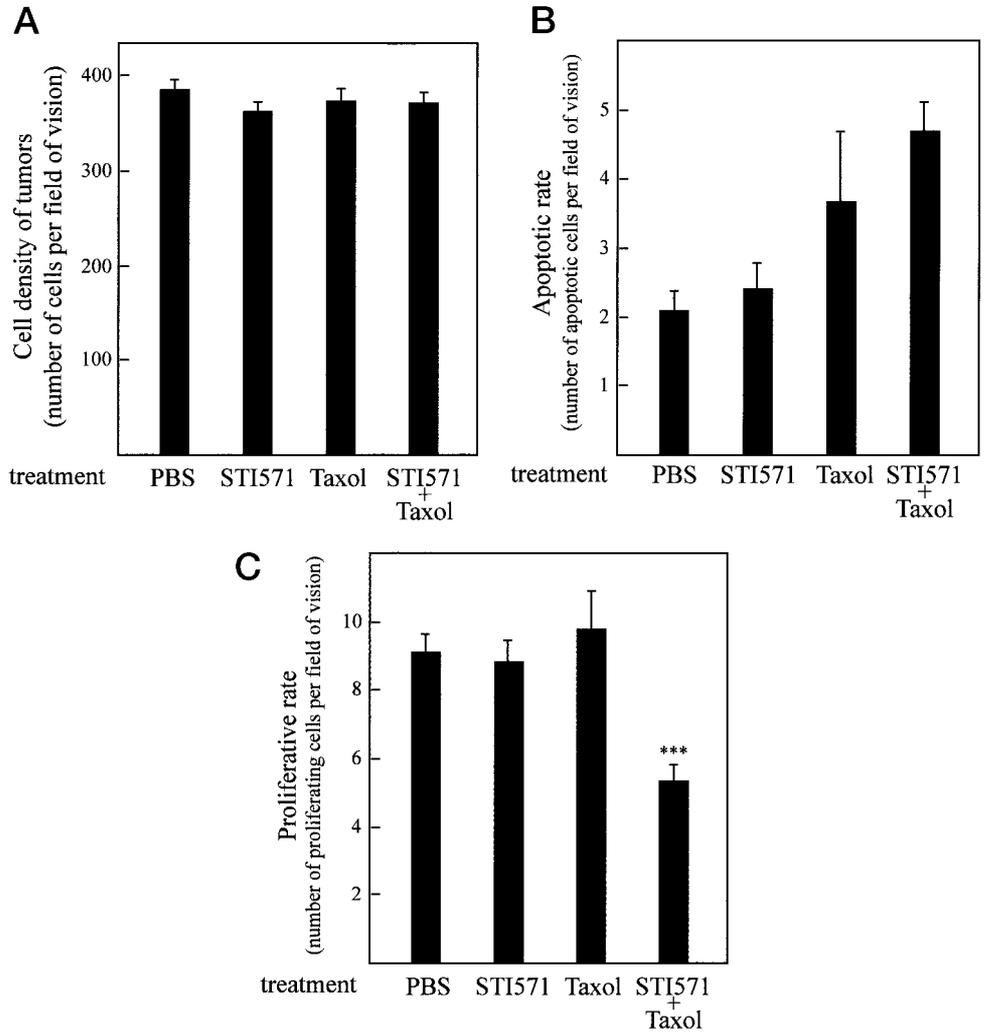
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Fig. 4. Treatment with PDGF receptor antagonists does not alter tumor cell density, but enhances the effect of Taxol on KAT-4 tumor cell apoptosis and proliferation *in vivo*. *A,* cell density of KAT-4 tumors was calculated by counting the number of cells in three random fields of vision of viable tissue for each tumor. *B* and *C,* the number of positively staining cells per field of vision of viable tissue, quantified from immunohistochemical stainings of apoptosis (*B*) and proliferation (*C*) in sections from all of the KAT-4 tumors in the treatment study using STI571. ∗∗∗$P < 0.001$, STI571 and Taxol *versus* Taxol alone, Student's *t* test; *bars,* ±SE.

RESULTS

Inhibition of PDGF Receptor Signaling in Tumor Stroma Reduces Tumor IFP in KAT-4 Tumors. The KAT-4 cell line is derived from a human anaplastic thyroid carcinoma. When cultured *in vitro*, KAT-4 cells express PDGF-BB[4] but do not express either of the PDGF receptors (Fig. 1*A*). KAT-4 cells form stroma-rich s.c. tumors with PDGF-receptor expression restricted to the tumor stroma (Fig. 1*B*). To validate KAT-4 as a model system for studying the effects of PDGF receptor antagonists on the efficacy and uptake of cytotoxic drugs, we investigated whether the IFP of KAT-4 tumors is sensitive to PDGF receptor antagonists. We treated size-matched groups of SCID mice carrying s.c. KAT-4 tumors with PDGF aptamer or control aptamer. The mean tumor IFP in animals that received control aptamer was 6.2 ± 0.7 mm Hg (±SE), whereas mean tumor IFP in PDGF aptamer-treated animals was reduced to 3.5 ± 0.5 mm Hg (±SE; Fig. 2*A*). In a parallel experiment, the selective PDGF receptor tyrosine kinase inhibitor STI571 also significantly reduced KAT-4 tumor IFP (Fig. 2*B*).

PDGF Receptor Inhibition Increases the Therapeutic Effect of Taxol on KAT-4 Tumors. Next, we determined whether the lowered tumor IFP in KAT-4 tumors after treatment with PDGF receptor antagonists leads to a better antitumor effect of Taxol. It has been shown previously that KAT-4 tumors are sensitive to treatment with
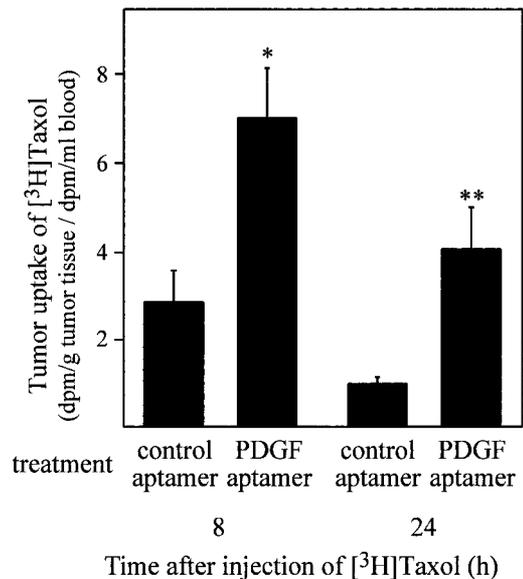

Fig. 5. Treatment with PDGF receptor antagonists increases uptake of [³H]Taxol in KAT-4 tumors. After treatment with PDGF-receptor antagonists or control, mice with KAT-4 tumors were injected s.c. with [³H]Taxol. Radioactivity was measured in homogenates of tumors and in blood samples 8 or 24 h after s.c. injection of radiolabelled compound. Mice were treated with control aptamer (8 h, $n = 6$; 24 h, $n = 7$) or PDGF aptamer (8 h, $n = 6$; 24 h, $n = 7$); ∗$P < 0.05$, Student's *t* test; ∗∗$P < 0.01$, Student's *t* test; *bars,* ±SE.

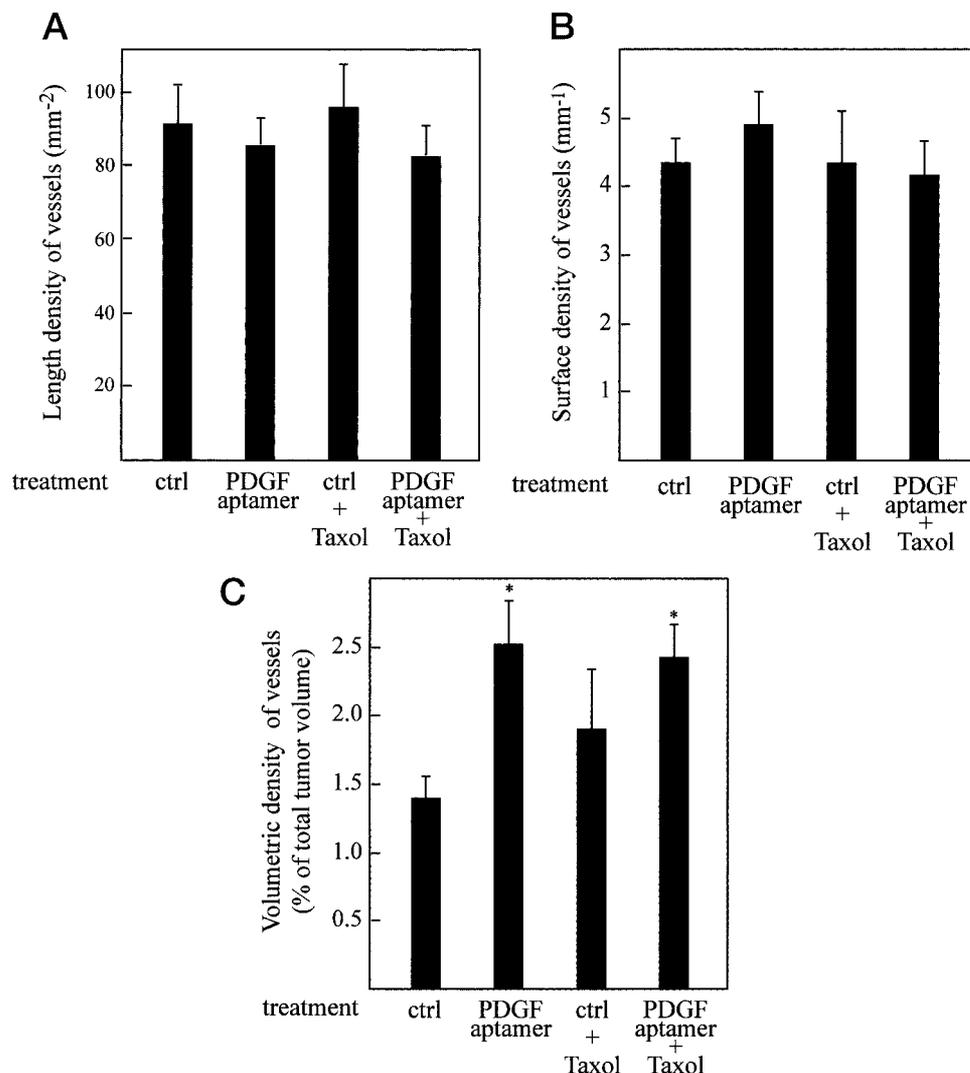[4] Simon Fredriksson, personal communication.

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Fig. 6. Treatment with PDGF receptor antagonists in combination with Taxol is not antiangiogenic. Stereological analysis of blood vessel density in KAT-4 tumors treated with control (*ctrl*; $n = 8$), PEG-conjugated PDGF aptamer ($n = 8$), control and Taxol ($n = 8$), or PDGF aptamer and Taxol ($n = 8$). Control treatment consisted of PEG. *A*, length of blood vessels per tumor volume. *B*, surface area of blood vessels per tumor volume. *C*, volume of blood vessels per tumor volume. ∗$P < 0.05$, Student's *t* test *versus* PEG only; *bars*, ±SE.

Taxol (18). In the first experiment, Taxol was combined with PDGF aptamer or with PEG as a control. At the low dose administered ($5 \text{ mg} \times \text{kg}^{-1} \times \text{dose}^{-1}$), Taxol by itself only had a slight effect on tumor volume (Fig. 3*A*). In contrast, when Taxol was given in combination with PDGF aptamer, tumor growth was retarded. At the conclusion of the experiment, tumors from mice treated with both Taxol and PDGF aptamer had a significantly smaller volume, and tumor size was reduced by 35% compared with tumors from mice treated with Taxol only (Fig. 3*A*). In an analogous experiment, a treatment study was performed using STI571 to block PDGF receptor signaling (Fig. 3*B*). Tumor volume was reduced by 52% after treatment with STI571 in combination with Taxol compared with treatment with Taxol only (Fig. 3*B*). In neither experiment did the PDGF antagonists display any antitumor effects by themselves (Fig. 3, *A* and *B*). No increased toxicity, as judged by weight loss or morbidity, was observed in the treatment groups receiving PDGF receptor antagonists and Taxol as compared with other treatment groups (data not shown).

At the conclusion of the treatment study, tumor IFP was measured in the groups that received Taxol alone or Taxol in combination with STI571 to investigate whether Taxol alone was able to relieve interstitial hypertension. Tumors in mice that only received Taxol displayed a tumor IFP of 6.3 ± 1.0 mm Hg, which closely resembles the tumor IFP in control-treated tumors (Fig. 2*A* and *B*; Fig 3*C*). The IFP of tumors in mice that received Taxol in combination with STI571

was lowered to 3.4 ± 0.7 mm Hg (Fig. 3*C*), confirming that a long-term treatment (21 days) with PDGF receptor inhibitors lowers tumor IFP.

To confirm that the PDGF receptor activity was suppressed in tumors from mice treated with PDGF receptor inhibitors, we prepared homogenates from tumors included in the PDGF aptamer and Taxol treatment study. Immunoprecipitation of the PDGF receptor, followed by Western blot analysis of receptor levels and phosphorylation, revealed that PDGF receptors from tumors treated with PDGF aptamer display a significantly lower activation grade compared with receptors from control-treated tumors (Fig. 3*D*).

To explore whether tumors from the various treatment groups differ with regard to gross tumor architecture, we examined Azan's trichrome stainings of sections from the tumors. No change in overall histology or in collagen distribution was noted (data not shown). Also, treatment with PDGF receptor antagonists did not change the cell density of the tumors (Fig. 4*A*), excluding changes in cell density as a cause for the observed effects of PDGF receptor inhibitors. Given the difference in cell density between the stromal compartment and the tumor cell-dominated parts of the tumor (Fig. 1*B*), we also conclude that in the absence of variations in cell density, there is no evidence for a change in the proportion of normal and tumor cells.

We stained KAT-4 tumor sections from all of the mice included in the STI571 and Taxol treatment study to analyze tumor cell apoptosis
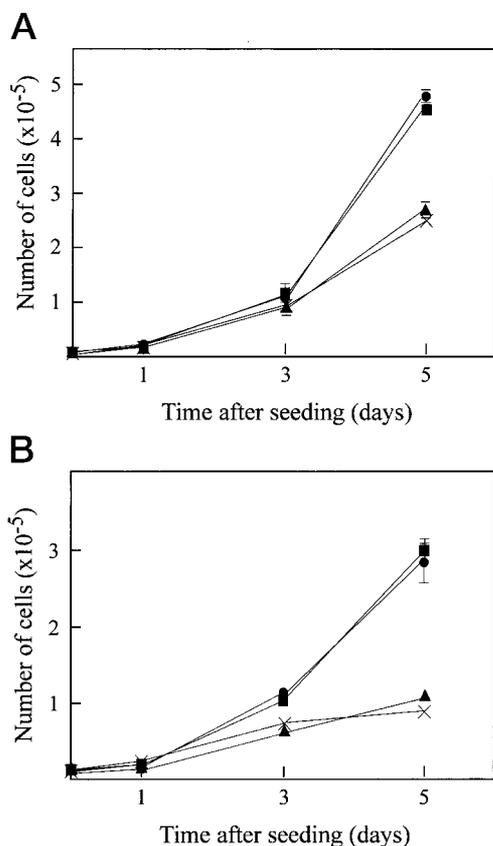
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A

B

Fig. 7. PDGF receptor antagonists do not potentiate the effect of Taxol on cultured KAT-4 cells. *A,* growth curves of cultured KAT-4 cells grown with no addition (■), or in the presence of PDGF aptamer (●), Taxol (▲), or PDGF aptamer and Taxol (×). *B,* growth curves of cultured KAT-4 cells grown with no addition (■), or in the presence of STI571 (●), Taxol (▲), or STI571 and Taxol (×). The data in *A* and *B* are representative of two and three repeated experiments, respectively. Data points are average from duplicate wells; *bars,* ±SE.

(Fig. 4*B*) and proliferation (Fig. 4*C*). Tumors from mice treated with both STI571 and Taxol showed a higher rate of apoptosis and a significantly lower rate of proliferation compared with all of the other treatment groups. These results are in good agreement with the fact that KAT-4 tumor growth was slower in mice treated with both STI571 and Taxol compared with mice treated with Taxol only.

PDGF Receptor Inhibition Increases Tumor Uptake of Taxol. To investigate whether the enhanced effect of Taxol induced by PDGF antagonists was attributable to increased tumor uptake of Taxol, we injected size-matched groups of PDGF aptamer- or control aptamer-treated mice with [³H]Taxol s.c. at sites distant from the tumor. After 8 or 24 h, we excised and homogenized the tumors, and measured the blood and tumor concentrations of [³H]Taxol. As seen in Fig. 5, significantly increased tumor uptake of [³H]Taxol was observed after treatment with PDGF aptamer, most notably at the 24 h time point where mean tumor uptake was 4.1-fold higher in the PDGF aptamer-treated mice compared with control. Similarly, treatment with STI571 led to an increased tumor uptake of [³H]Taxol (data not shown). These results show that the lowering of IFP in KAT-4 tumors by PDGF receptor antagonists is paralleled by an increase in tumor uptake of [³H]Taxol.

PDGF Antagonists Alone or in Combination with Taxol Do Not Induce Antiangiogenic Effects in KAT-4 Tumors. Recent reports have demonstrated that a continuous low-dose treatment regimen with chemotherapeutic agents is antiangiogenic and, when combined with other angiogenesis inhibitors, results in eradication of established tumors in mice (23, 24). To investigate whether the combination of

PDGF receptor antagonists and Taxol is antiangiogenic, we performed a stereological assessment of the blood vessel density. This method is based on characterization of the vasculature on regular tissue sections but allows estimations of changes not only in vessel density but also in morphological parameters such as cross-section areas of vessels and vessel lengths. Previous use of this method has readily detected changes in the tumor vasculature after antiangiogenic treatments (22, 25). No antiangiogenic effects, as judged by blood vessel length density, surface area density, or volumetric density, could be detected in our study (Fig. 6, *A–C*). Interestingly, the volumetric density of blood vessels in tumors treated with PDGF aptamer, either alone or in combination with Taxol, was significantly increased compared with control-treated tumors (Fig. 6*C*).

PDGF Antagonists Do Not Sensitize KAT-4 Cells to *in Vitro* Effects of Taxol. To investigate whether the improved therapeutic effect of Taxol on KAT-4 tumors, when administered in combination with PDGF receptor antagonists, involved sensitization of KAT-4 cells to Taxol, we analyzed the effects of PDGF antagonists on Taxol sensitivity *in vitro*. Neither PDGF aptamer nor STI571 increased the cytotoxic effect of Taxol on cultured KAT-4 cells (Fig. 7, *A* and *B*).

Thus, no evidence was observed that indicated antiangiogenic effects by PDGF antagonists. Neither was sensitization of PDGF antagonists on the *in vitro* effects of Taxol observed. These findings, together with the evidence for PDGF antagonist-mediated increase in tumor uptake of Taxol, support the notion that the *in vivo* enhancement by PDGF inhibitors on Taxol antitumor effects occurs through a mechanism that involves reduction in tumor IFP and increased tumor uptake of Taxol.

Treatment with STI571 Enhances the Effects of 5-FU on PRObb Tumors *in Vivo* but Does Not Sensitize PROb Tumor Cells *in Vitro* to the Action of 5-FU. To extend our findings on enhancement of chemotherapeutic effects by PDGF antagonists to another tumor model, we investigated whether STI571 could increase the effects of 5-FU in the PROb syngeneic colon adenocarcinoma model in BDIX rats. Cultured PROb cells do not express PDGF ligands or receptors (data not shown). After s.c. injection, PROb cells form stroma-rich tumors with PDGF receptor expression restricted to the tumor stroma and with infiltrating cells, morphologically identified as macrophages, producing PDGF-BB and/or -AB (7). Treatment of mice carrying PROb tumors with STI571 leads to inhibition of PDGF receptor signaling, a lowering of tumor IFP, and increased tumor uptake of the low molecular weight compound ⁵¹Cr-EDTA (7).

Treatment of PROb tumors in BDIX rats with STI571 alone or with 5-FU alone (1 mg × kg⁻¹ × dose⁻¹) had no substantial effect on tumor volume (Fig. 8*A*). However, treatment with 5-FU in combination with STI571 led to a significant reduction in growth rate of PROb tumors compared with treatment with 5-FU only (Fig. 8*A*). At the end of the study, tumor volume in the combination treatment group was reduced by 44% compared with tumors treated with 5-FU only (Fig. 8*A*). No increased toxicity, as judged by weight loss or morbidity, was observed in the treatment group receiving STI571 and 5-FU as compared with other treatment groups (data not shown). Moreover, IFP measurements confirmed that the tumor IFP was lowered after a long-term treatment (15 days) with STI571 (data not shown).

Subsequently, all of the PROb tumors from the treatment study were sectioned to assess the histology of the tumors. No change in overall histology or in collagen distribution was observed (data not shown), and the average cell density of the tumors from all of the treatment groups was similar (Fig. 8*B*).

To visualize apoptosis in sections from PROb tumors, we used TUNEL-staining. The apoptotic rate was found to be significantly higher (67% higher than in tumors treated only with 5-FU) in tumors from rats treated with 5-FU in combination with STI571 (Fig. 8*C*).
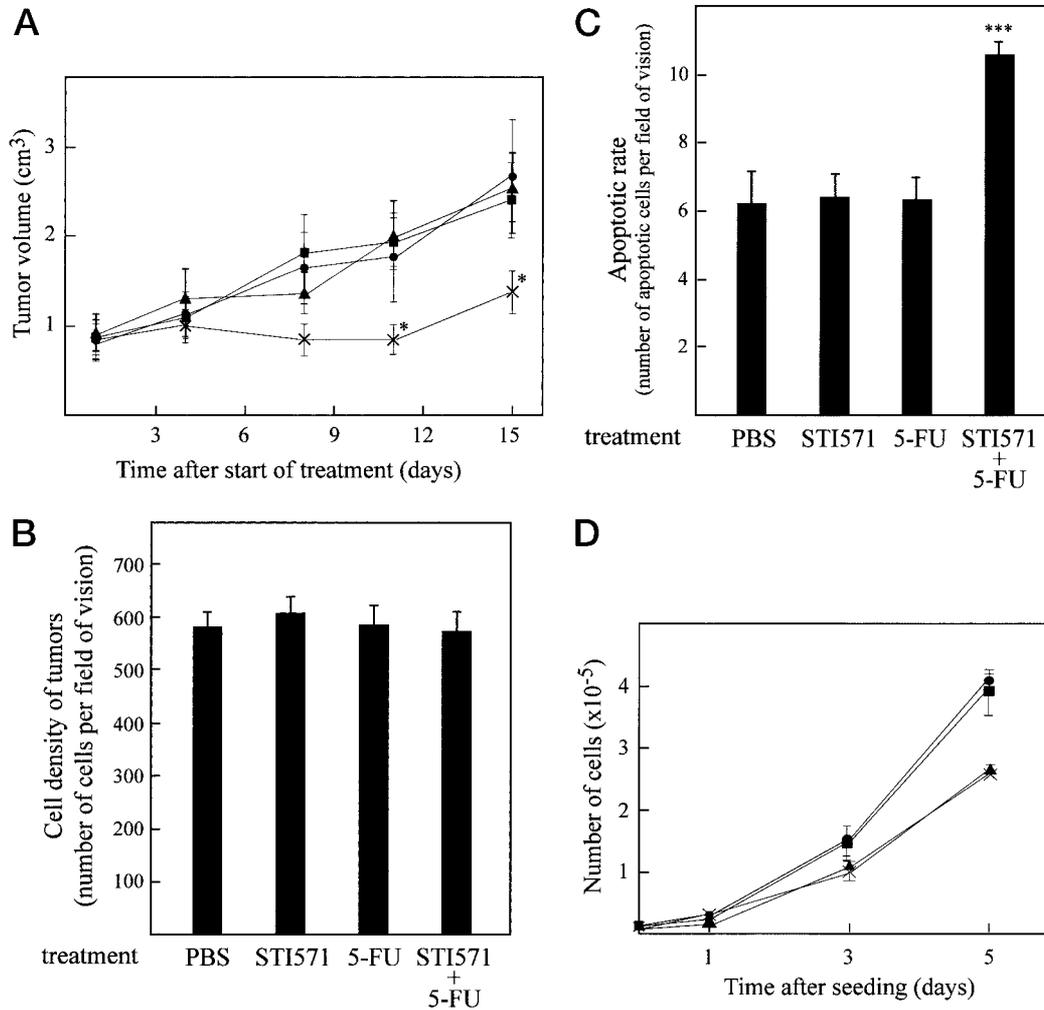
Fig. 8. Treatment with STI571 enhances the effect of 5-FU on PROb tumors. *A,* growth curves of PROb tumors grown s.c. in BDIX rats. Rats received no treatment (■, *n* = 5), STI571 (●, *n* = 5), 5-FU (▲, *n* = 5), or STI571 + 5-FU (×, *n* = 5). *P < 0.05 STI571 and 5-FU *versus* 5-FU alone, Student's *t* test. *B,* cell density of PROb tumors was calculated by counting the number of cells in three random fields of vision of viable tissue for each tumor. *C,* the number of positively staining cells per field of vision of viable tissue, quantified from TUNEL-staining for apoptosis in sections from PROb tumors grown s.c. in BD IX-rats. Rats received no treatment (*n* = 5), STI571 (*n* = 5), 5-FU (*n* = 5), or STI571 and 5-FU (*n* = 5). ***P < 0.001 STI571 and 5-FU *versus* 5-FU alone, Student's *t* test. *D,* growth curves of cultured PROb cells grown with no addition (■), in the presence of STI571 (●), 5-FU (▲), or STI571 and 5-FU (×). The data shown are representative of three repeated experiments. Data points are average from duplicate wells; *bars,* ±SE.

The *in vitro* growth of PROb cells was also analyzed. Cells were cultured in the absence of drugs or in the presence of STI571 alone, 5-FU alone, or both drugs in combination. STI571 did not potentiate the effect of 5-FU on cultured PROb cells (Fig. 8*D*). Therefore, we conclude that the effects of STI571 *in vivo* are not caused by direct sensitization of tumor cells to the action of 5-FU.

Taken together, these data, and results from our previous studies, show that in the PROb syngeneic tumor model STI571 reduces PDGF receptor activation, lowers tumor IFP, increases tumor uptake of low molecular weight compounds, and enhances the therapeutic effect of 5-FU.

DISCUSSION

In the present study, we demonstrate that STI571 enhances the therapeutic effects of Taxol and 5-FU in the KAT-4 and the PROb tumor models, respectively (Figs. 3 and 8). Similar results were obtained when PDGF aptamers were used together with Taxol for treatment of KAT-4 tumors. Treatment with PDGF receptor antagonists only did not affect tumor growth. No potentiation of the effects of 5-FU or Taxol by PDGF antagonists could be observed *in vitro*, indicating that the effect of PDGF aptamers and STI571 does not

occur by sensitization of tumor cells to the effects of the cytotoxic drugs (Fig. 7; Fig. 8*D*). We also show that the PDGF aptamer increases the tumor uptake of [^{3}H]Taxol and lowers IFP in KAT-4 tumors (Figs. 2 and 5). We have demonstrated previously that STI571 increases tumor uptake of ^{51}Cr-EDTA and lowers IFP in PROb tumors (7). Stereological analysis of the vessel density in KAT-4 tumors gave no support for the notion that the beneficial effect of cotreatment with PDGF aptamer involved an antiangiogenic effect (Fig. 6). An increase in vessel volume density was observed in KAT-4 tumors treated with PDGF aptamers (Fig. 6*C*). Whether the change in vessel architecture is secondary to the reduction in tumor IFP or represents an independent effect of PDGF inhibition presently remains unclear. Interestingly, an increased diameter of blood vessels has also been observed concomitant to a lowering of tumor IFP induced by taxanes (26). It remains to be established whether the increase in vessel volume density contributes to the increased Taxol uptake in KAT-4 tumors.

The PDGF aptamer is an oligonucleotide that binds with high specificity to the PDGF B chain and neutralizes the biological activity of PDGF-AB and -BB, but not PDGF-AA (14). In the case of STI571, other tyrosine kinases than PDGF receptors are known to also be

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inhibited, *e.g.,* Abl and the stem cell factor receptor (c-Kit; Refs. 15, 16). STI571 and the PDGF aptamer exerted similar effects in the KAT-4 tumors with regard to IFP reduction and increase in the therapeutic effect of Taxol (Figs. 2 and 3). Also, both STI571 and PDGF aptamer reduced tumor IFP to a similar degree in PROb tumors (7). Therefore, we conclude that not only the effects of the PDGF aptamer, but also those of STI571, are caused by PDGF receptor inhibition. This is also supported by the absence of data implicating other known STI571 targets in control of tumor IFP.

Together our findings establish inhibition of PDGF receptor signaling as a novel, possibly general, strategy for enhancement of chemotherapeutic effects on solid tumors. Our data also strongly suggest that the mechanism underlying the beneficial effects of co-treatment involves increased tumor uptake of chemotherapeutic agents that most likely occur as a consequence of reduction in tumor IFP. It is notable that the effects of PDGF antagonists was achieved by interference with processes controlled by tumor stroma cells, and thus illustrates the potential of these cells as targets for novel cancer therapies.

Tumor interstitial hypertension has been documented in many types of clinical tumors, *e.g.* breast carcinoma (27, 28), metastatic melanoma (27, 29, 30), head and neck carcinoma (31), and liver metastases of colorectal carcinoma (27). In agreement with the notion that interstitial hypertension in tumors acts as a barrier to drug delivery, high-tumor IFP in patients has, in some instances, been shown to be associated with a poor response to treatment. In a study of patients with melanoma, all of the responders displayed a low tumor IFP compared with the nonresponders (30).

In addition to PDGF antagonists, several other compounds have been used to lower tumor IFP in experimental tumor models. These agents include nicotinamide (32), dexamethasone (33), tumor necrosis factor α (34), prostaglandin E_1 (8), hyaluronidase (35), a bradykinin B_2 receptor agonist (36), and vascular endothelial growth factor antibodies (37). Periodic modulation of mean arterial blood pressure by angiotensin II has also been used to increase the tumor transvascular pressure gradient (6). Some of these strategies have subsequently been used to test whether an increase of the tumor transvascular pressure gradient leads to improved tumor transvascular transport. Pretreatment with hyaluronidase or intermittent treatment with angiotensin II enhances tumor uptake of monoclonal antibodies (5, 6). Prostaglandin E_1 augments capillary-to-tumor interstitium transport of the low molecular weight compound ^{51}Cr-EDTA (8), and, finally, treatment with vascular endothelial growth factor antibodies elevates tumor oxygen pressure (37). Thus, taken together, there is mounting experimental evidence that the transport from blood vessels into the tumor interstitium can be improved by modulating the transvascular pressure gradient. Our finding of increased therapeutic effects of anticancer drugs after reduction of tumor IFP by PDGF receptor antagonists should thus encourage studies addressing the possibility that other IFP-reducing agents can increase the efficacy of anticancer drugs. Furthermore, a recent study identifies IFP as a prognostic marker for radiation therapy in cervix cancer (38). Therefore, whether or not lowering of IFP will sensitize cervix tumors or other tumors to radiation therapy is an interesting and valid question for future studies.

Studies on the regulation of IFP in connective tissue have shed some light on the mechanism whereby PDGF receptor signaling in stromal fibroblasts regulates IFP. Experimental evidence has demonstrated that the IFP of loose connective tissue is actively controlled through regulation by fibroblasts, or pericytes, of the tension of an extracellular collagen/microfibrillar network (39, 40). Moreover, control of IFP also involves PDGF receptor modulation of the function of integrins, which are the predominant mediators of interactions between fibroblasts and extracellular matrix (7, 11). Concerning the question of which of the PDGF β-receptor activated signaling pathways that mediate IFP regulation, studies in mice deficient in PDGF β-receptor triggered phosphatidylinositol-3-kinase activation have identified this pathway as a mediator of PDGF β-receptor control of IFP (41).

Our findings have important clinical implications. Many common solid tumors, *e.g.,* lung, colon, and breast cancer, which presently are treated with chemotherapy, display tumor interstitial hypertension, PDGF receptor expression in the tumor stroma, and PDGF production (12, 13). Our observations suggest that inclusion of PDGF antagonists in these treatments could improve the therapeutic effects or, in other cases, reduce toxic side effects by allowing reductions of the systemic doses of cytotoxic drugs. The recently completed clinical studies using STI571 to inhibit Bcr-Abl or c-Kit tyrosine kinases in chronic myeloid leukemia or gastrointestinal stromal tumors, respectively, have not given any evidence for major toxic effects of PDGF receptor inhibition (42, 43). Clinical trials investigating the effects of STI571, or other PDGF antagonists, on tumor IFP, drug uptake in tumors, and response to chemotherapy are, therefore, highly warranted.

ACKNOWLEDGMENTS

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Inhibition of PDGF Receptor Signaling in Tumor Stroma Enhances Antitumor Effect of Chemotherapy

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