Inhibition of Platelet-derived Growth Factor Receptors Reduces Interstitial Hypertension and Increases Transcapillary Transport in Tumors

Kristian Pietras, Arne Östman, Mats Sjöquist, Elisabeth Buchdunger, Rolf K. Reed, Carl-Henrik Heldin, and Kristofer Rubin

ABSTRACT

Most solid malignancies display interstitial hypertension and a poor uptake of anticancer drugs. Platelet-derived growth factor (PDGF) and the cognate tyrosine kinase receptors are expressed in many tumors. Signaling through PDGF-B receptors was shown recently to increase interstitial fluid pressure (IFP) in dermis after anaphylaxis-induced lowering of IFP. In this study, we show that treatment with the selective PDGF receptor kinase inhibitor, STI571, formerly known as CGP57148B, decreased the interstitial hypertension and increased capillary-to-interstitium transport of 51Cr-EDTA in s.c. growing rat PROb colonic carcinomas. Furthermore, treatment with an antagonistic PDGF-B oligonucleotide aptamer decreased interstitial hypertension in these tumors. PDGF-B receptors were expressed in blood vessels and stromal cells but not in the tumor cells of PROb colonic carcinomas. Our study indicates a previously unrecognized role of PDGF receptors in tumor biology, although similar effects of PDGF on IFP have been demonstrated previously in the dermis. The data suggest interference with PDGF receptors, or their ligands, as a novel strategy to increase drug uptake and therapeutic effectiveness of cancer chemotherapy.

INTRODUCTION

One approach to increase the effectiveness of existing anticancer drugs for the treatment of solid malignancies is to augment the uptake of the drugs into tumors and thereby obtain increased therapeutic concentration without elevating the adverse side effects. The stroma of solid tumors differs from normal loose connective tissues in several respects. One major physiological difference is an increased IFP, resulting in a diminished hydrostatic gradient from capillary to interstitium and thereby impaired exchange of solutes over the capillary membrane (1–3). It has been hypothesized that pharmacological intervention to increase the fluid pressure gradient between capillary and tumor interstitium might provide a means to increase uptake of anticancer agents into tumors (4). Several agents that induce a lowering of IFP and thereby increase the transcapillary pressure gradient in experimental murine tumors have been identified, including nicotine (5), tumor necrosis factor-α (6), and dexamethasone (7). It has, however, not been established whether the lowering of tumor IFP induced by these drugs results in an increased uptake of drugs into tumors.

We have reported that loose connective tissues actively control IFP, and as a consequence, fluid content in the tissues (8). During anaphylactic reactions and burn injuries, IFP is lowered by mechanism(s) independent of an intact circulation (8). Available data suggest that connective tissue cells control IFP by exerting a tension on the collagen/microfibrillar network, via the collagen binding integrin α1β1, in rat skin (9–12). This tension would restrain the tendency to swell, intrinsic to the hyaluronan/proteoglycan gel in the ground substance (13). PDGF-BB, but not PDGF-AA or fibroblast growth factor, normalizes dermal IFP, which had been lowered as a consequence of anaphylaxis or inhibition of the α1β1 integrin function (11). Transgenic mice, carrying PDGFβ receptor mutants in the binding site for phosphatidylinositol 3-kinase, do not respond to PDGF-BB by normalizing dermal IFP lowered by mast cell degranulation (14). These data suggest a role for PDGF-activated phosphatidylinositol 3-kinase in the control of dermal IFP.

The evidence for a role of PDGF receptor signaling in control of IFP, together with observations documenting PDGF receptor expression in the stroma of a broad range of solid tumors including colorectal adenocarcinoma (15–17), prompted us to investigate whether PDGF participates in the tumor stroma phenotype characterized by a high IFP and low capillary-to-interstitium transport. The effects of the selective PDGF receptor kinase inhibitor, STI-571, formerly known as CGP57148B (18), on tumor IFP and transcapillary transport in s.c. growing PROb rat colonic carcinoma (19) were investigated. To further study the role of PDGF in generating a high tumoral IFP, we analyzed the effect on PROb tumor IFP by a nuclease-resistant high affinity oligonucleotide aptamer, which specifically inhibits PDGF, both in vitro and in vivo (20, 21).

MATERIALS AND METHODS

Tissue Culture. Cells were cultured under standard conditions, and all tissue culture media were supplemented with 10% fetal bovine serum and antibiotics, unless otherwise stated. PAE cells were maintained in F12 culture medium (Sigma). PROb cells were kept in DMEM (Sigma).

PDGF/PDGF Receptor Inhibitors. The selective protein tyrosine kinase inhibitor STI-571 inhibits PDGF receptor kinase, Abl kinase, and the Kit receptor (18, 22). This inhibitor has a low toxicity, and in humans, it has a plasma half-life of between 12 and 14 h (22). DNA aptamers were produced by the systemic evolution of ligands by exponential enrichment (SELEX) method (23). The PDGF-B-specific aptamer used in the present study has a high affinity for PDGF-B with a Kd~0.1 nM (20, 21). The aptamer was linked to polyethylene glycol (Mw~40,000), yielding a product with a plasma half-life in rats of ~8 h (21). As a control, an aptamer with a scrambled central portion was used; this aptamer has a Kd for PDGF-B in the micromolar range (21).

Specificity Test of STI-571. PAE cells, stably transfected with PDGFβ receptor (PAE/PDGF-β), VEGFR-1 (PAE/VEGFR-1), and VEGFR-2 (PAE/VEGFR-2) cDNAs (24), were seeded in 60-mm dishes (Nunc) and cultured (23). The PDGF-B-specific aptamer used in the present study has a high affinity for PDGF-B with a Kd~0.1 nM (20, 21). The aptamer was linked to polyethylene glycol (Mw~40,000), yielding a product with a plasma half-life in rats of ~8 h (21). As a control, an aptamer with a scrambled central portion was used; this aptamer has a Kd for PDGF-B in the micromolar range (21).

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4 The abbreviations used are: IFP, interstitial fluid pressure; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PAE, porcine aortic endothelial.
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Na3VO4 (lysis buffer A), and lysates were precleared by centrifugation 10,000 × g for 15 min. Receptors were subsequently precipitated using WGA-Sepharose (Amersham Pharmacia Biotech). The WGA-Sepharose fractions were washed three times in lysis buffer A and once in 20 mM Tris-HCl (pH 7.5), heated for 3 min at 95°C in SDS-sample buffer, and subjected to SDS-gel electrophoresis using 7.5% polyacrylamide gels. After semidry transfer to nitrocellulose filters and blocking of the filters in 5% BSA in Tris-buffered saline (TBS), immunoblotting using phosphotyrosine antibody PY99 (Santa Cruz Biotechnology) was performed. Tyrosine phosphorylated PDGF and VEGF receptors were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech) after incubation with appropriate horseradish peroxidase-conjugated secondary antibodies.

Tumor Establishment and Treatment with PDGF Inhibitors. s.c. growing PROb tumors (19) were established in BDIX rats by injection of 5 × 106 tumor cells in 50 μl of PBS in the flank. The rats were kept under pathogen-free conditions and were fed ad libitum. They were monitored regularly for tumor growth, and experiments were performed 8–12 weeks after tumor cell implantation on rats bearing tumors ranging in size between 0.6 and 7.6 cm3. For STI-571 treatment, the rats received 50 mg/kg of STI-571 in PBS administered by gavage once a day for 4 consecutive days. The PDGF-B-specific SELEX aptamers and a control aptamer (see above) were given as i.p. injections in 2 ml of PBS twice daily for 4 consecutive days at a dose of 7 mg/kg × day−1. All animal experiments described in the present report were approved by the Ethical Committee for Animal Experiments (Uppsala, Sweden).

Measurement of Tumor IFP. Tumor IFP was measured using the wink-in-needle technique (25). Briefly, rats were anesthetized using isoflurane in a mixture of O2 and air. A standard 23-gauge needle filled with nylon floss and saline, supplemented with 50 IE/ml of heparin, was inserted into the center of the tumor and connected to a pressure transducer. This makeup enables continuous and stable recordings of fluid pressure. Fluid communication between the needle and the transducer was confirmed by compression and decompression of the tubing during each measurement. The recorded pressure should return rapidly to the pressure prior to clamping the catheter and should not deviate from this pressure by more than 0.5–1 mm Hg to be an acceptable reading. Tumor IFP was measured once before treatment with STI-571 and again 2–3 h after the last administration of STI-571 or vehicle alone. The change in tumor IFP was calculated for each tumor. After the second IFP measurement, the rats were sacrificed, and the tumors were excised and snap frozen in liquid nitrogen for further analyses. In the case of treatment with PDGF-B aptamers, tumor IFP was measured 1–2 h after the last administration of the aptamers.

Measurement of Blood Pressure. BDIX rats received treatment with STI-571 (n = 8) or PBS (n = 7) as described previously. Two to 3 h after the last administration of STI-571, rats were anesthetized, and a catheter connected to a pressure transducer was inserted into the right arteria femoralis. Blood pressure was recorded, and the mean arterial blood pressure was calculated.

Microdialysis Technique. Transcapillary transport of 51Cr-EDTA (M, 341,30; NEN, Sollentuna, Sweden) in PROb tumors was measured by microdialysis 1 h after the final treatment with STI-571. Rats were anesthetized and tracheotomized, and implantable CMA 20 microdialysis probes, 10 mm in length and with a molecular mass cutoff of 20,000 Da (CMA/Microdialysis, Solna, Sweden), were inserted into the tumors and in the left jugular vein. Eighty μl of 51Cr-EDTA were injected in the right jugular vein. The dialysis probes were perfused at a rate of 2.5 μl/min, and fractions of 25 μl were collected in a Microfraction Collector CMA140 (CMA/Microdialysis). Dialysates were sampled for a total of 120 min after the administration of 51Cr-EDTA, and radioactivity was quantified by scintillation counting using a LKB Wallac gamma counter. Capillary-to-interstitium transport was expressed as the relative area under the curve of the tumor and the plasma curves. This was obtained by dividing the total amount of counts in the fractions collected from the tumor and the plasma perfusate, respectively.

Immunohistochemistry. For routine morphology, paraffin-embedded, 4-μm sections were stained with van Gieson staining. Immunohistochemistry was performed on 6-μm cryosections from PROb tumors. Sections were fixed in acetone and blocked with 0.3% hydrogen peroxide in methanol for 15 min, rinsed, and further incubated in a solution containing 20% goat normal serum in a buffer containing 2% rat serum, 3% BSA, 0.01% NP40 in PBS (RM buffer) for 5 h at 4°C. Primary antibodies dissolved in RM buffer were added, either 4 μg/ml affinity-purified rabbit anti-PDGFβ receptor IgG (26) for 5 h at 4°C or overnight at 4°C with 1.3 μg/ml of the monoclonal mouse anti-PDGF-AB/BB IgG (PDGF 007; Mochida Pharmaceutical Company, Tokyo, Japan). Sections were rinsed in PBS with 0.01% NP40. Bound IgG was detected with biotinylated goat antirabbit or biotinylated rabbit antimouse antibodies, respectively. Sections were developed with a Vectastain ABC elite kit (Vector, Burlingame, CA) using amino-ethyl-carbazole as a chromophore. Sections were counterstained with Mayer’s hematoxylin for 30 s.

Extraction of PDGFβ Receptor from Tumors. Tumors excised from rats treated with STI-571 as described above or with PBS alone for 4 days were cut into small pieces, snap frozen in liquid nitrogen, and submerged into lysis buffer B (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 μM Na3VO4). Samples were homogenized, incubated 20 min at 4°C, and precleared by centrifugation at 10,000 × g for 20 min. After incubation for 2 h at 4°C with preimmune serum and recovery of the immune complexes using Protein A-Sepharose, PDGFβ receptors in the samples were immunoprecipitated using a rabbit polyclonal antiserum raised against the COOH-terminal part of the PDGFβ receptor (26). The precipitated immune complexes were subsequently washed three times in lysis buffer B and once in 20 mM Tris-HCl, heated for 3 min at 95°C in SDS-sample buffer, and subjected to SDS-gel electrophoresis using 7.5% polyacrylamide gels. After semidry transfer to nitrocellulose filters and blocking of the filters in 5% BSA in TBS, immunoblotting, using the anti-phosphotyrosine antibody PY-99 or the anti-PDGFβ receptor antibody P-20 (Santa Cruz Biotechnology), was performed. Western blot signals were quantified using the public domain NIH Image program (developed at the NIH), and the intensities of the phosphotyrosine signals were correlated to the intensities of the receptor signals and expressed as relative pY signal.

Statistical Analysis. Statistical analysis was performed using the paired (Fig. 2A) or unpaired (Figs. 2B and 3, and Table 1), two-sided Student’s t test. P < 0.05 was considered statistically significant.

RESULTS

To extend previous studies on the selectivity of STI-571, the effect of this inhibitor on ligand-induced tyrosine phosphorylation of VEGF receptor-1 (Flt-1) and VEGFR-2 (KDR) was compared with the effect on PDGFβ receptor. In all cases, stably transfected PAE cells were used. No decrease in VEGFR-1 and VEGFR-2 tyrosine phosphorylation was observed in the presence of 10 μM STI-571, whereas PDGFβ receptor tyrosine phosphorylation was completely blocked by 3 μM STI-571 of the inhibitor (Fig. 1).

Tumor IFP was determined before and after a 4-day treatment with STI-571 of BDIX rats bearing PROb tumors. As shown in Fig. 2A, treatment with STI-571 significantly reduced tumor IFP. Untreated tumors showed a mean IFP of 16.8 ± 0.9 mm Hg (± SE), whereas the treated group displayed a mean tumor IFP of 11.3 ± 1.1 mm Hg (± SE). The change in tumor IFP, induced by the STI-571 treatment, for each individual tumor (ΔIFP) was also calculated (Fig. 2B). A mean ΔIFP of −4.3 mm Hg was recorded in STI-571-treated animals, whereas the ΔIFP in control animals was 1.9 mm Hg. The 4-day

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PBS</th>
<th>STI-571</th>
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<tbody>
<tr>
<td>MABP (mm Hg)</td>
<td>98 ± 6</td>
<td>93 ± 8a</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td>2.6 ± 0.6</td>
<td>2.5 ± 0.3a</td>
</tr>
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*No significant difference.
Treatment with STI-571 did not affect mean arterial blood pressure or tumor weight (Table 1).

Treatment of PROb tumor-bearing rats with the PDGF-B-specific aptamer also resulted in a decrease in tumor IFP when compared with rats treated with a control aptamer (Fig. 3). Mean IFP in control aptamer–treated tumors was 14.6 ± 1.2 mm Hg (± SE) and 9.7 ± 1.6 mm Hg (± SE) in tumors treated with the PDGF-B-specific aptamer.

The technique of microdialysis enables continuous recording of capillary-to-interstitium transport in tumors (27–30). Using this technique, we investigated whether systemic treatment of rats with STI-571 increased transcapillary transport of $^{51}$Cr-EDTA into PROb tumors. $^{51}$Cr-EDTA was chosen as a tracer because this compound has a low molecular weight, is not bound by plasma proteins, and is, for these reasons, commonly used as a marker for fluid flow in tissues (31). Capillary-to-interstitium transport was expressed as the ratio of total radioactivity recovered from tumors and plasma during a 2-h period. The average ratio was 0.32 ± 0.04 (± SE) in control animals and 0.59 ± 0.12 (± SE) in STI-571-treated animals. Thus, treatment with STI-571 led to an 82% increase in capillary-to-interstitium transport of the low molecular mass compound $^{51}$Cr-EDTA.

The average ratio was 0.32 ± 0.04 (± SE) in control animals and 0.59 ± 0.12 (± SE) in STI-571-treated animals. Thus, treatment with STI-571 led to an 82% increase in capillary-to-interstitium transport of the low molecular mass compound $^{51}$Cr-EDTA.
To investigate whether STI-571-sensitive PDGF receptors could be demonstrated in PROb tumors, tumor tissue was homogenized, lysed, and subjected to immunoprecipitation with a PDGF receptor antiserum. After SDS gel electrophoresis and transfer to nitrocellulose membranes, PDGF receptors were visualized by PDGF receptor immunoblotting. PDGF receptors could be immunoprecipitated from both treated and untreated PROb tumors (Fig. 5). The activation state of PDGF receptors was determined by immunoblotting with phosphotyrosine antibodies of the same filter. Whereas PDGF receptors extracted from control-treated tumors yielded a strong phosphotyrosine signal, indicative of the presence of ligand-activated receptors in this tumor model, STI-571-treated tumors contained PDGF receptors with specific tyrosine phosphorylation reduced by 40% (Fig. 5).

PROb tumors were analyzed with regard to morphology, as well as distribution of PDGF-AB/BB and PDGFβ receptors. The tumors displayed a heterogeneous morphology. At the tumor periphery, tumor cells were arranged in glandular structures, whereas more centrally, tumor cells were less abundant and less well organized (Fig. 6A). The central part was basically acellular (Fig. 6A). Expression of PDGF-AB/BB in PROb tumors was found in blood vessels and...
possibly in extravascular stromal cells surrounding tumor glands (Fig. 6B). In the central part of the tumors, few if any tumor cells were present, but strongly PDGF-AB/BB positive cells were seen (Fig. 6C). In no part of the tumors could PDGF-AB/BB positive tumor cells be clearly discerned. PDGFB receptors were found in vascular cells of larger vessels and in unidentified, possibly microvascular, cells in the stroma (Fig. 6D). The absence of PDGF-AB/BB and β-receptor expression by carcinoma cells in PROb tumors is in agreement with the characteristics of cultured PROb cells (data not shown).

DISCUSSION

The present data show that systemic treatment with the selective PDGF receptor tyrosine kinase inhibitor STI-571 or with an antagonistic PDGF-B oligonucleotide aptamer reduces tumor IFP in an experimental rat colonic carcinoma in which PDGF receptor expression is restricted to the stromal and perivascular cells. Furthermore, STI-571 increases the tumor capillary-to-interstitium transport of the low molecular mass compound 51Cr-EDTA in the same tumor model. These findings demonstrate for the first time that stromally expressed PDGF receptors contribute to the hydrodynamic properties of a tumor interstitium and that STI-571 treatment leads to a more effective exchange of low molecular mass solutes from plasma into the interstitium of a solid tumor. An increased capillary-to-interstitium transport in solid tumors is highly desirable to improve therapy based on currently used anticancer drugs, e.g., cytostatics. STI-571 is well tolerated, it can be administered p.o., and clinical trials to investigate its effectiveness for treatment of chronic myelogenous leukemia are ongoing (reviewed in Ref. 22). The present data suggest that STI-571, in addition, can be of clinical relevance as an adjuvant to chemotherapeutics for the treatment of solid malignancies.

As with other tyrosine kinase inhibitors, the precise specificity of STI-571 remains to be documented. It has been shown that STI-571, in addition to PDGF receptor tyrosine kinases, also inhibits the Abl nonreceptor and Kit receptor tyrosine kinases (18, 22). It can therefore not formally be excluded that targets, other than the PDGF receptor, mediate the effect of STI-571 on transcapillary transport. However, recent observations demonstrating increased tumor uptake of antibodies or low molecular weight compounds after modulation of capillary-to-tumor pressure gradients (32, 33) strongly suggest that the effect of STI-571 on transcapillary transport is linked to its effect on PDGF receptor controlled tumor IFP. The expression pattern of PDGF receptors in PROb tumors was similar to that of human colorectal adenocarcinoma, in which a particularly high expression of PDGFβ receptors is seen in vascular smooth muscle cells and microvascular pericytes (15, 16, 34). In PROb tumors, PDGF-AB/BB was not detected in tumor cells but was present in the stroma and/or vasculature of the tumors. The findings that both PDGF AB/BB and PDGFB receptors were expressed in the PROb tumors and that STI-571 treatment induced a lowering of the level of tyrosine phosphorylation of PDGFB receptors recovered from PROb tumors also indicate that PDGF receptors are the target of STI-571.

The permeability of the microvasculature of normal tissues is selective, and transport of solutes over the capillary walls occurs by diffusion and convection (8). Low molecular mass compounds are in general transported across the capillary wall by diffusion, which in turn depends on concentration gradients, whereas macromolecules to a large extent are thought to be transported by convection, which depends on the net capillary filtration pressure (35). In contrast, capillary-to-interstitium transport in tumors is believed to occur by convection (3, 36). The convective, i.e., flow driven, transport over a nonselective microvasculature can be increased either by raising the capillary pressure or by lowering the IFP. In experimental murine tumors, an increased fluid pressure gradient between tumor vessels and interstitium, achieved by systemic and intermittent angiotensin II infusions, results in an enhanced uptake of tumor-specific antibodies into the tumors (32). In a previous report, we demonstrated that an acute lowering of tumor IFP induced by administration of proスタグ004ndlandin E1 around experimental rat PROb colonic and mammary carcinomas is paralleled by an increase in capillary-to-interstitium transport of 51Cr-EDTA in both tumor models (33). Our present data, showing that a chronic lowering of tumor IFP results in an increased capillary-to-interstitium transport of 51Cr-EDTA in rat colonic carcinomas, is strongly suggestive of a direct relation between tumor IFP and transport of low molecular mass solutes into tumor tissue.

The pathophysiological mechanisms generating a high IFP in tumors have been suggested to involve functionally underdeveloped tumor blood vessels, highly permeable to plasma proteins, and lack of lymphatic drainage (3, 37). We have proposed a model in which stromal fibroblastoid cells, or pericytes, control IFP in normal rat skin by regulating their tension on a collagen/microfibrillar network, which in turn restrains a glycosaminoglycan/hyaluronan gel from swelling (9, 10). Hence, IFP is a dynamic parameter that can be controlled via cellular activity. Furthermore, previous studies in our laboratories have shown that PDGF-BB counteracts a lowering of IFP induced by anaphylaxis or by infusion of anti-αβ1 integrin antibodies in rat and mouse skin (11, 14). The present data, showing PDGF to be involved in the build up and/or maintenance of a high tumoral IFP, indicate a role of tumor stromal fibroblastoid cells, or pericytes, in the control also of tumoral IFP.

In conclusion, the present study suggests that treatment with PDGF inhibitors might provide a way to increase uptake, and thereby therapeutically effective, of anticancer drugs in solid tumors, including the low molecular weight chemotherapeutic agents currently in use.

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