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ORIGINAL ARTICLE

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Targeting stromal cells for the treatment of platelet-derived growth factor C-induced hepatocellular carcinogenesis

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Abstract Non-invasive therapies for the treatment of hepatocellular carcinoma (HCC) would be of great benefit to public health. To this end, we have developed a platelet-derived growth factor-C (PDGF-C) transgenic (Tg) mouse model, which mimics many aspects of human liver carcinogenesis. Specifically, overexpression of PDGF-C results in liver fibrosis, which is preceded by activation and proliferation of hepatic stellate cells, and is followed by the development of dysplastic lesions and angiogenesis, and progression to HCCs by 8 months of age. Here, we show that PDGF-C overexpression induces the proliferation of endothelial-like cells that are present in tumors and adjacent non-neoplastic parenchyma. The protein tyrosine kinase inhibitor, imatinib (Gleevec), decreases the proliferation of non-parenchymal cells (NPC) *in vitro* and *in vivo*, with concomitant inhibition of Akt. *In vivo* treatment with imatinib also blocks the expression of CD34 in PDGF-C Tg mice. Decreased NPC proliferation and CD34 expression correlated with lower levels of active ERK1/2 and total levels of PDGF receptor α (PDGFRα). In summary, the small molecule inhibitor imatinib attenuates stromal cell proliferation in PDGF-C-induced HCC, which coincides with decreased expression of both CD34 and PDGFRα, and activated Akt. Our findings suggest that imatinib may be efficacious in the treatment of hepatocarcinogenesis, particularly when neovascularization is present.

Key words hepatocellular carcinoma · imatinib mesylate · tumor stroma · fibrosis · liver · angiogenesis · CD34

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Introduction

Hepatocellular carcinoma (HCC) is the world's fifth most frequent cancer, with an increasing incidence worldwide (Bruix et al., 2004; Llovet et al., 2004). HCC most commonly arises in a setting where chronic injury and inflammation lead to cell death resulting in a persistent stimulus for liver regeneration (Thorgeirsson and Grisham, 2002; Suriawinata and Xu, 2004). Chronic liver injury can be caused by a variety of insults including viral hepatitis (e.g., hepatitis B and C), alcoholic liver disease, non-alcoholic steatohepatitis, and inherited liver disorders such as hemochromatosis (Schuppan and Porov, 2002; Friedman, 2003; Iredale, 2003; Llovet et al., 2003; Bataller and Brenner, 2005). Recent studies suggest that HCC develops from precursor dysplastic nodules, particularly small-cell high-grade nodules (Kojiro and Roskams, 2005; Libbrecht et al., 2005; Llovet et al., 2006). Despite this knowledge of risk

factors for, and progression of HCC, curative therapies are limited (Llovet et al., 2004; Avila et al., 2006; Forner et al., 2006; Giannelli and Antonaci, 2006).

Once HCC occurs, invasive techniques such as radiofrequency ablation, resection, and liver transplantation are the only therapeutic options currently available (Llovet et al., 2004; Bruix and Sherman, 2005; Bruix et al., 2006; Forner et al., 2006). The development of new therapeutic strategies for the various stages of hepatocarcinogenesis included (i) treatment of the underlying cause of fibrosis, (ii) regression or reversal of fibrosis or cirrhosis, (iii) prevention of progression of precursor lesions to tumors, and (iv) treatment of HCC. Because liver tumorigenesis progresses sequentially in a step-wise manner, early intervention therapies that prevent the progression of early fibrosis to end-stage liver disease and HCC might offer the best chance for patient survival, and is an area of on-going research (Albanis et al., 2003; Rockey, 2005; Friedman et al., 2007; Iredale, 2007). Unfortunately, many HCCs are diagnosed when advanced disease leads to clinical symptoms, and for these patients even surgical options are limited. Non-invasive therapies, such as small-molecule inhibitors or other chemotherapeutic agents, may provide the best option for the treatment of these patients.

Development of small-molecule inhibitors directed toward protein tyrosine kinases involved in carcinogenesis is a focus of many academic labs and pharmaceutical companies (Fabbro et al., 2002; Baselga, 2006; Steeghs et al., 2007). Despite these efforts, few inhibitors are currently available for the treatment of HCC or fibrosis (Schuppan and Porov, 2002; Avila et al., 2006; Giannelli and Antonaci, 2006; Friedman et al., 2007). Although HCC ultimately results from the abnormal proliferation of hepatocytes, it has long been known that other liver cell types such as activated hepatic stellate cells (myofibroblasts), liver endothelial cells, and natural killer cells contribute to disease progression (Bouwens et al., 1992; Kmiec, 2001). Collectively, these different liver cell types contribute to the development of the stromal composition of hepatic tumors. In other cancers, the role of stromal cells in carcinogenesis has been appreciated, and is a current focus of treatment strategies (Bhowmick et al., 2004; Ostman, 2004; Zalatnai, 2006; Chu et al., 2007; Radisky and Radisky, 2007). The specific contributions of these different non-hepatocyte cell types depend on the etiology of the liver disease, and their specific contributions to hepatocarcinogenesis are under investigation (Safadi et al., 2004; Bataller and Brenner, 2005; Russo et al., 2006; Ryschich et al., 2006).

One of the difficulties in conducting experimental studies on the pathogenesis of HCC is the lack of good animal models that incorporate fibrosis and/or cirrhosis in the progression to cancer, as is seen in the human disease. We have developed a novel model of liver fibrosis that involves the hepatic overexpression of platelet-derived growth factor C (PDGF-C) in transgenic mice. The development of HCCs in PDGF-C Tg mice is preceded by liver fibrosis, angiogenesis, and the emergence of pre-neoplastic nodules (Campbell et al., 2005). Here, we show that the proliferation of liver endothelial-like cells is seen in HCCs and adjacent non-neoplastic parenchyma in PDGF-C Tg mice. Imatinib (also known as Gleevec) treatment blocks the proliferation of non-parenchymal cells (NPC) and decreases the expression of CD34-positive sinusoidal cells in the livers of PDGF-C Tg mice. Imatinib treatment also blocks the activation of Akt, and decreases the expression of PDGFRα. These results suggest that imatinib may be a useful therapeutic agent for blocking the activation of CD34-positive, endothelial-like cells during hepatoceullular carcinogenesis.

Methods

Animal studies

The generation and characterization of PDGF-C Tg mice have been described previously (Campbell et al., 2005). For imatinib inhibitor experiments, stock solutions of 10 mM imatinib mesylate (5.88 mg/ml (w:v), Novartis Pharmaceuticals, East Hanover, NJ) were prepared by dissolving the inhibitor in sterile saline and allowing it to settle before use. Two-treatment protocols were used. Imatinib was injected: (a) 2 hr necropsy (50 mg/kg), or (b) once a day for 7 days (100 mg/kg) via an intra-peritoneal (i.p.) injection. Bromodeoxyuridine (BrdU, 50 mg/kg body weight) was injected i.p. 2 hr before necropsy as described (Campbell et al., 2005, 2006). At the indicated time points, mice were killed by CO_2 inhalation. All animal studies were carried out under approved IACUC protocols at the University of Washington.

Histology and immunohistochemistry (IHC)

Mouse livers were fixed in 10% neutral-buffered formalin or Methacarn (60% methanol, 30% chloroform, and 10% acetic acid: v/v/v) overnight, processed to paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E) or Masson's trichrome using standard techniques. IHC was performed as described (Hudkins et al., 2004; Campbell et al., 2005) or as indicated by the manufacturer using antibodies specific for BrdU (anti-mouse Dako, Carpinteria, CA), CD34 (anti-rat CD34, Cedar Lanes, Laboratories, Burlington, NC), and α smooth muscle actin (αSMA, clone 1A4, Sigma). Detection of the primary antibody was carried out using the appropriate biotinylated antibody (Vectastain, Burlingame, CA) and the peroxidase DAB kit (Ventana, Tucson, AZ). Nuclear incorporation of BrdU into liver cells was used to measure cell proliferation. The mouse on mouse (MOM) kit (Vectastain) was used to detect labeling of both NPC and hepatocytes. Data are represented as the number of BrdU positive nuclei present in thirty $\times 40$ fields (1.3 mm^2; approximately 3,000 hepatocytes). Morphometry for CD34 staining was performed as described (Wen et al., 2002). In brief, 8–10 $\times 20$ fields that did not contain portal tracts or central veins were analyzed per liver section using digital photography (Olympus DP11: Olympus America, Melville, NY) and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). For the simultaneous detection of αSMA and BrdU epitopes, specific primary antibodies were used together with purple and brown secondary reagent substrate kits (Vector VIP, Vectastain), and DAB (Ventana, Tucson, AZ) respectively. For CD34 and BrdU double staining, specific primary antibodies were used together with a DAB (Ventana, Tucson, AZ) and a blue secondary reagent substrate kit (Vector BCIP/NPT, Vectastain), respectively.

Protein extraction and immunoblotting

Whole liver protein lysates were prepared in a Triton-X 100 lysis buffer and quantified using the Bradford method with bovine serum albumin as a standard as described previously (Campbell et al., 2005, 2006). Equal amounts of each sample (35 μg) were separated using SDS-PAGE, and immunoblotting was performed using standard procedures with the following primary antibodies: phospho-S473-Akt (Cell Signaling, Beverly, MA), total Akt (Cell Signaling), phospho-ERK1/2 (NEB) and total ERK1/2 (Seger et al., 1994), β-actin (Sigma A5441), PDGFRβ (Santa Cruz), and PDGFRα (R&D Systems). The monoclonal antibody for osteopontin ($MPIIIB10_1$) was obtained from the Developmental Studies Hybridoma Bank maintained at the University of Iowa.

Culture of activated stellate cells, ^{3}H-thymidine incorporation, and imatinib treatment

Rat stellate cell lines (2G) were maintained as described (Greenwel et al., 1993). To measure DNA replication, all cell lines were switched to serum-free media overnight and then treated with PDGF-CC (10 ng/ml), PDGF-BB (10 ng/ml), or serum (2%) as indicated. ^{3}H-thymidine was added to media at a final concentration of 1 μCi/ml for 4 hr, and incorporation was measured as described (Argast et al., 2004). For inhibitor experiments, cells were plated as above, except that imatinib was added 30 min before the addition of growth factor or serum. PDGF-CC and PDGF-BB were obtained from ZymoGenetics Inc. (Seattle, WA) (Gilbertson et al., 2001) and R&D Systems, respectively.

Statistical analyses

Statistical analysis was performed by non-parametric analysis, using the Mann–Whitney *U*-test using GraphPad Prizm software (GraphPad for Science Inc., San Diego, CA). Data are presented as average ± SEM., with $p < 0.05$ considered to be statistically significant.

Results

PDGF-C Tg mice develop severe fibrosis and HCC

We showed previously that hepatic overexpression of PDGF-C results in pericellular and perivenular fibrosis by 6 weeks of age, which is accompanied by increased peri-sinusoidal αSMA and glial fibrillary acidic protein (GFAP) expression (Campbell et al., 2005). As PDGF-C Tg mice age, they display hepatomegaly with a doubling of their liver weights by 6 months of age independent of their gender (Fig. 1A). The increased ratio of liver weight to body weight is due in part to increased proliferation of NPC and the development of multifocal hepatic tumors (Fig. 1B and [Campbell et al., 2005]). Multifocal tumors (Fig. 1B, black arrows) are surrounded by non-neoplastic tissue (Fig. 1B, white asterisks) with neo-angiogenesis frequently surrounding macroscopic tumors (Fig. 1B, white arrowheads). By 12 months, over 85% of PDGF-C Tg mice develop HCCs ([Campbell et al., 2005] and data not shown).

Angiogenesis often accompanies the development of human HCC (Semela and Dufour, 2004; Sun and Tang, 2004) and is correlated with metastatic capacity in other solid tumors. Extensive sinusoidal collagen deposition is apparent in PDGF-C Tg mice livers (Fig. 2A) and HCCs develop between the ages of 8 and 9 months (Fig. 2B and [Campbell et al., 2005]). Histological examination of these HCCs demonstrates proliferation of endothelial-like cells in the tumors (Figs. 2C,2D) as well as in adjacent non-neoplastic tissue (data not shown). Neoplastic hepatocytes within these tumors show frequent mitotic figures (Fig. 2, black arrows). The extent of endothelial cell proliferation varies between PDGF-C Tg mice, with heterogeneous expression throughout the parenchyma, although proliferation of endothelial cells is observed in all PDGF-C Tg mice. Some areas with extensive endothelial cell proliferation have a telangiectatic appearance with large vascular spaces and a villiform architecture (Fig. 2C), while in other regions neoplastic hepatocellular cords are lined by sinusoidal endothelial cells without sinusoidal dilatation (Fig. 2D). No endothelial cell proliferation was observed in the livers of WT littermates (data not shown). As can be seen in Figure 1B, most tumors display neo-angiogenesis.

Proliferation of NPC in PDGF-C Tg mice

The observation of endothelial cell proliferation in HCCs from PDGF-C Tg mice prompted a more in-depth investigation of the non-parenchymal cell populations that are proliferating when PDGF-C is overexpressed in the liver. We hypothesized that overexpression of PDGF-C stimulates *both* hepatic stellate cell (Campbell et al., 2005) and endothelial cell activation and proliferation, resulting in fibrogenesis and neo-angiogenesis, respectively. We wished to determine whether myofibroblast proliferation solely accounts for the proliferating NPC in the livers of PDGF-C Tg mice. We performed double staining for αSMA, as a marker of activated stellate cells, and BrdU, as a marker of proliferation. As determined by BrdU labeling, proliferation of both hepatocytes and NPC is observed in the livers of PDGF-C Tg mice (Fig. 3, black arrows and black arrowheads, respectively). Some of the BrdU-positive cells also co-stain with αSMA (Figs. 3A,3B, black arrowheads). These results indicate that activated stellate cells are proliferating in PDGF-C Tg mice, a conclusion that is supported by increased GFAP staining and collagen deposition (Campbell et al., 2005). However, these results also suggest that other non-parenchymal cell types may be proliferating in PDGF-C Tg mice.

Given the extent of endothelial-like cell proliferation observed in tumors of older PDGF-C Tg mice (Fig. 2), we next examined whether liver endothelial cell proliferation was present in the livers of PDGF-C Tg mice by performing IHC for CD34. Sinusoidal CD34-positive IHC has been used as a marker for liver endothelial cells and for angiogenesis in human HCC (Cui et al., 1996;

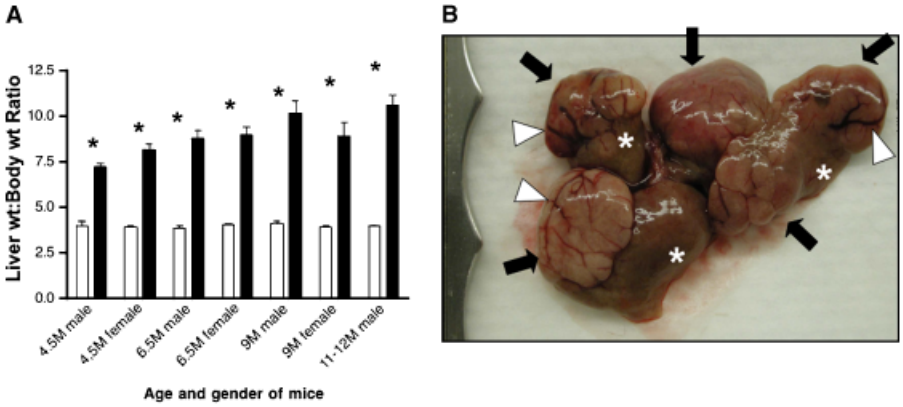

Fig. 1 Platelet-derived growth factor C (PDGF-C) Tg mice develop multifocal tumors by 12 months of age. (**A**) Increased ratio of liver weight to body weight in male and female PDGF-C Tg mice. Six or more male or female mice per genotype were analyzed ($^{*}p < 0.05$). (**B**) Multiple hepatic tumors develop in a 12-month-old PDGF-C Tg mouse. The asterisks indicate surrounding non-tumor tissue with black arrows indicating multiple macroscopic tumors and white arrowheads indicating angiogenesis.

Di Carlo et al., 2002). We performed double staining for CD34, as a marker of endothelial cells, and BrdU, as a marker of proliferation. Perisinusoidal CD34 expression is seen in the livers of 10-week-old PDGF-C Tg mice (Figs. 3C,3D, yellow arrows). BrdU staining is observed in sinusoidal cells that also co-stain with CD34 (Figs. 3C,3D, red arrows), suggesting that endothelial-like cells are also proliferating in the livers of Tg mice. Taken together, these results suggest that hepatic PDGF-C overexpression stimulates the proliferation of both stellate and sinusoidal endothelial cells. It is possible that these activated non-parenchymal cell populations each contribute to tumorigenesis seen in PDGF-C Tg livers by participating in fibrogenesis and/or neo-angiogenesis.

Imatinib blocks rat stellate cell proliferation *in vitro*

One potential strategy for the treatment of HCC would be to block or slow the development of tumors by targeting stromal cells rather than the hepatocytes themselves, a strategy used in the treatment of breast and prostate cancer (Zalatnai, 2006; Chu et al., 2007; Radisky and Radisky, 2007). The use of small-molecule inhibitors to block proliferation of activated stellate or endothelial cells in the liver (i.e., hepatic stromal cells) could thus be another therapeutic strategy for the treatment of HCC. The small-molecule inhibitor imatinib inhibits the catalytic activity of a number of protein kinases and has been used clinically to treat several cancers, such as chronic myeloid leukemia and gastrointestinal stromal tumors (GIST) (Dematteo et al., 2002; Roskoski, 2003; Steeghs et al., 2007). We began our investigations of the potential use of imatinib to treat stromal cell proliferation in the liver by treating activated stellate cell cultures (rat 2G cells). We found that imatinib blocks PDGF-CC-induced proliferation in a dose-dependent manner as determined by a DNA proliferation assay (Fig. 4A). Imatinib also inhibits serum- and PDGF-BB-induced proliferation in these cells

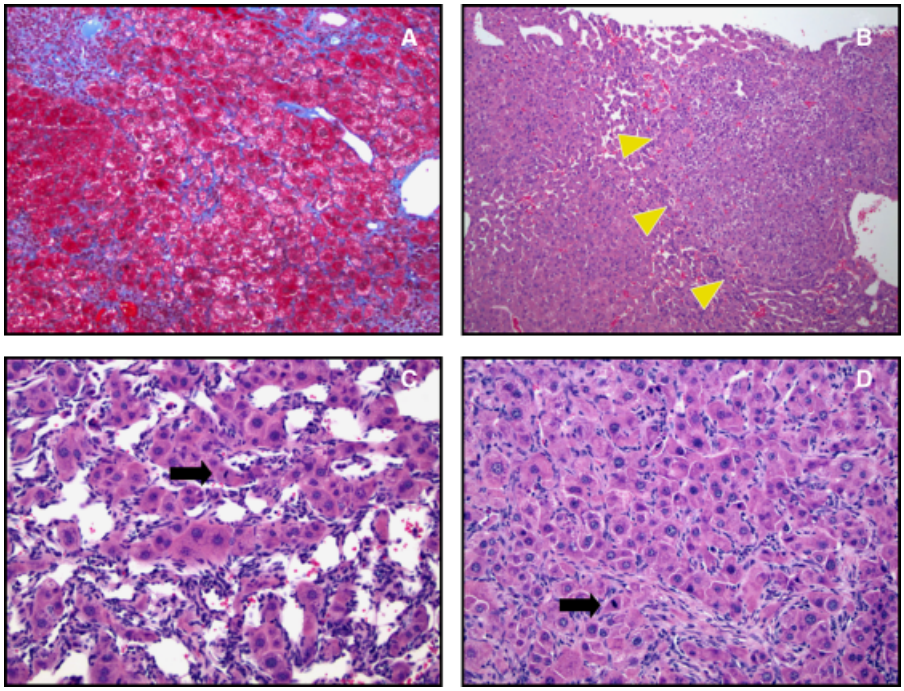

Fig. 2 Platelet-derived growth factor C (PDGF-C) Tg mice develop extensive fibrosis and hepatocellular carcinoma (HCC) with endothelial-like cell proliferation. (**A**) Extensive pericellular collagen deposition is seen in a 12-month-old PDGF-C Tg mouse liver (Masson trichrome, ×10). (**B**) HCC in a 12-month-old PDGF-C Tg mouse liver. There is loss of portal tracts and normal architecture in the area highlighted by yellow arrowheads, and trabecular structures are apparent (H&E, ×4). (**C**) HCC with proliferation of spindly cells that are morphologically reminiscent of sinusoidal endothelial cells in an 8-month-old PDGF-C Tg mouse (H&E, ×20). (**D**) Small HCC with loss of portal tracts and endothelial cell proliferation in a 12-month-old PDGF-C Tg mouse (H&E, ×20). Mitoses in the hepatocytes are present (arrow heads).

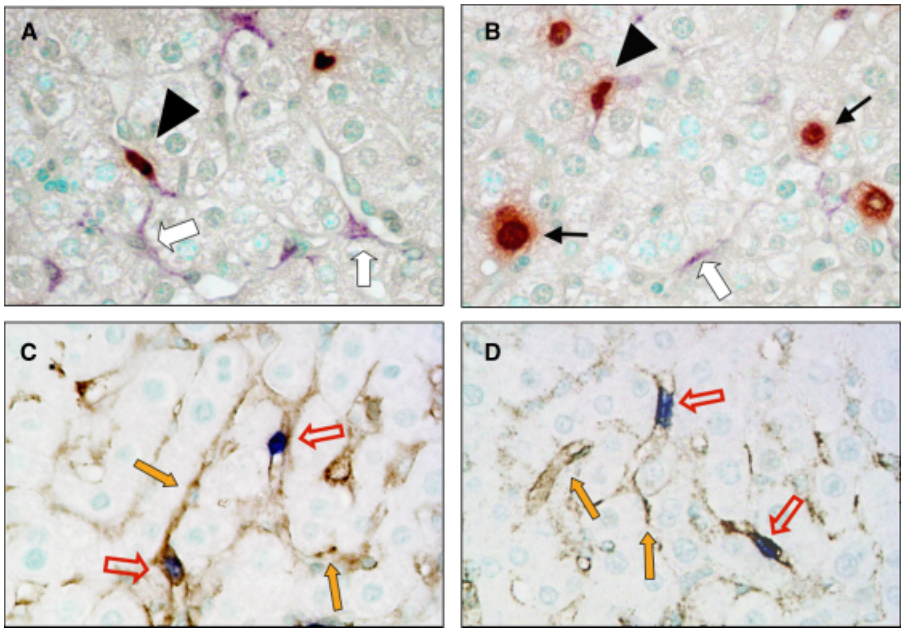

Fig. 3 Double staining for αSMA and bromodeoxyuridine (BrdU) (**A**, **B**) and CD34 and BrdU (**C**, **D**) in platelet-derived growth factor (PDGF)-C Tg mice. (A, B). Liver sections were stained with antibodies specific to αSMA (purple) and BrdU (brown). Single staining with αSMA is shown with white arrows, cells that stained with both markers are shown with black arrowheads, and BrdU-stained hepatocytes are shown with black arrows. (C, D). Liver sections were stained with antibodies specific for CD34 (brown) and BrdU (blue). Single staining with CD34 is shown with yellow arrows and cells stained with both CD34 and BrdU are shown with red arrows. For both sets of double staining, images from two different PDGF-C Tg livers are shown (× 400).

(Fig. 4A). Investigation of the signaling pathways downstream of the PDGF receptors demonstrated that imatinib treatment inhibits PDGF-CC-stimulated Akt activation, as demonstrated by immunoblotting (Fig. 4B, upper panel). These studies indicate that imatinib blocks PDGF-CC-induced stellate cell proliferation *in vitro*, possibly via inhibition of Akt.

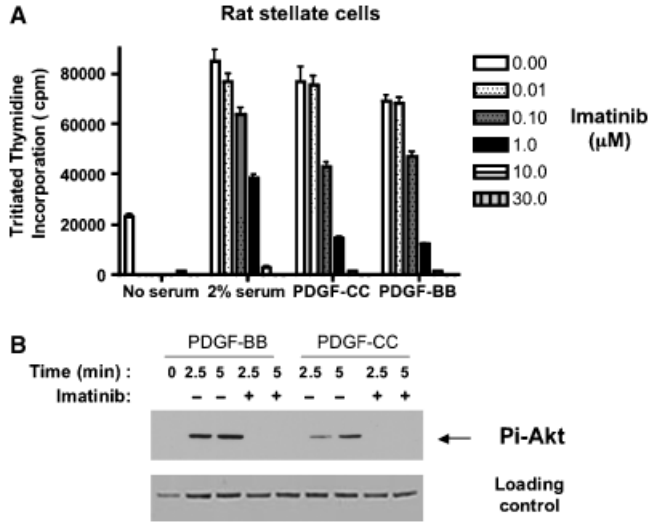

Fig. 4 Imatinib treatment inhibits (**A**) serum-, platelet-derived growth factor (PDGF)-CC- and PDGF-BB-stimulated DNA synthesis, and (**B**) PDGF-CC induced Akt activation in rat stellate cells. (A) DNA synthesis used as a measurement of proliferation was measured by ^{3}H-thymidine incorporation. Cells were pre-treated with the indicated concentration of imatinib 30 min before addition of growth factor (20 ng/ml) or serum (2%). (B) Activation of Akt of was determined by immunoblotting. The upper panel shows phospho-Akt (Ser473) and in the lower panel, a non-specific band demonstrates equal protein loading. All data are representative of at least three separate experiments, with four to six replicates in each ^{3}H-thymidine experiment. $^{*}p < 0.05$.

Imatinib treatment decreases NPC proliferation and Akt activation *in vivo*

Given that PDGF-C Tg mice demonstrate proliferation of stellate cells and endothelial cells, and that the activation and proliferation of these stromal cells appears to precede tumor development, we used this animal model of hepatocarcinogenesis to test whether stromal cell proliferation would be blocked by imatinib treatment, which could subsequently block HCC progression. We described previously that PDGF-C overexpression induces both PDGFRα and PDGFRβ expression, which are targets of imatinib (Buchdunger et al., 2000; Capdeville et al., 2002). In the present experiments, we treated PDGF-C Tg mice and WT littermates daily with 100 mg/kg imatinib (i.p.). After 7 days of treatment, we injected BrdU 2 hr before necropsy to determine whether imatinib treatment would attenuate proliferation of various cell types in the liver. Imatinib partially inhibits BrdU labeling of NPC in PDGF-C Tg mice, but does not significantly alter the proliferation of hepatocytes in Tg mice (Fig. 5A). We determined the extent of apoptosis using a flurogenic caspase 3 assay (Pierce et al., 2000), but did not observe an increase in caspase 3 activity in either WT or Tg mice after inhibitor treatment (data not shown). These results suggest that 7-day treatment with imatinib did not decrease NPC proliferation by increasing cell death in the liver.

To determine whether imatinib treatment blocks PDGF-C-induced signaling pathways *in vivo*, we injected the inhibitor into PDGF-C Tg and WT littermates and removed the livers 2 hr later. Phospho-Akt is elevated in the livers of Tg mice compared with WT (Fig. 5B) as described previously (Campbell et al., 2005). Two-hours imatinib treatment inhibits Akt activation

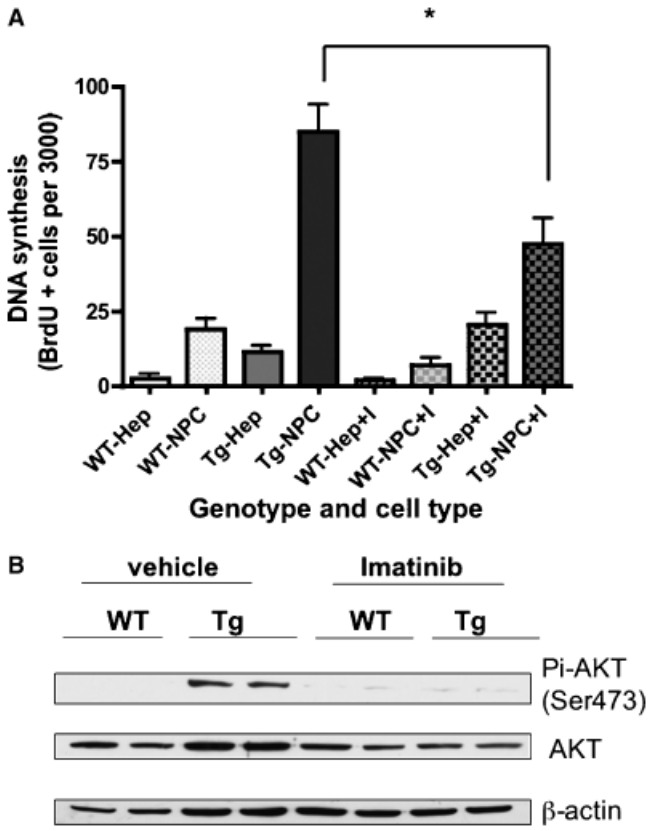

Fig. 5 Imatinib treatment decreases the proliferation of non-parenchymal cells (NPC) in platelet-derived growth factor (PDGF)-C Tg mice after 7 days. (**A**) WT or Tg mice were injected with imatinib once a day for 1 week (i.p., 100 mg/kg). Bromodeoxyuridine (BrdU) (50 mg/kg, i.p.) was injected 2 hr before necropsy. BrdU staining and counting were performed as described in Materials and methods. "I" indicates imatinib treatment, and untreated WT and Tg mice received saline. (**B**) Two-hour imatinib treatment decreases phospho-Akt. WT or Tg mice were injected with imatinib (i.p. 50 mg/kg). Two hours later, the livers were excised and liver protein lysates were prepared. Activation of Akt was determined by immunoblotting for phospho-Ser473-Akt. Re-probing blots with β-actin was used to confirm equal loading.

in vivo (Fig. 5B) similar to its effect *in vitro* (Fig. 4B). We did not observe any difference in the extent of apoptosis between imatinib treated- and untreated-PDGF-C Tg mice (data not shown), indicating that 2-hr treatment with imatinib did not facilitate cell death. These results suggest that imatinib rapidly blocks PDGF-C-induced activation of Akt in the livers of Tg mice.

Imatinib treatment decreases CD34 expression *in vivo*

Given that imatinib blocks PDGF-C-induced NPC proliferation, we next determined whether endothelial cell proliferation was blunted by performing CD34 IHC on livers from WT and Tg treated mice. CD34 perisinusoidal staining is seen in the livers of 10-week-old PDGF-C Tg mice treated with saline (Fig. 6A). In WT mice, little to no staining is seen in the parenchyma, although CD34 staining can be seen in vessels in the portal tracts (Fig. 6C). Imatinib treatment decreases CD34 staining in the liver of PDGF-C Tg mice after 1 week of treatment (Fig. 6B). The decrease in parenchymal CD34 staining in imatinib-treated PDGF-C Tg mice was statistically significant (Fig. 6D) as determined by computer-assisted morphometry. These results indicate that imatinib treatment blunts the sinusoidal expression of CD34 in PDGF-C Tg livers, which correlates with decreased NPC proliferation.

Imatinib blocks ERK1/2 and decreases PDGFRα expression

To determine whether 1-week treatment with imatinib blocks PDGF-CC-induced signaling pathways *in vivo*, livers were removed 2 hr after the last inhibitor injection and protein lysates were prepared. We examined the status of phosphorylated ERK1/2 by immunoblotting, and found that phospho-ERK1/2 is elevated in the livers of Tg mice compared with WT (Fig. 7, top panel) as described previously (Campbell et al., 2005). ERK1/2 phosphorylation is blocked by imatinib treatment without altering the total levels of these kinases (Fig. 7). Imatinib treatment for 1 week also markedly reduces the total levels of PDGFRα in PDGF-C Tg livers (Fig. 7, middle panel). Imatinib treatment decreases the amount of active ERK1/2 and the expression of PDGFRα, which coincides with blunted CD34 expression and NPC proliferation. Taken together, these data suggest that imatinib inhibits CD34 expression and NPC proliferation, possibly via PDGFRα-signaling pathways.

Discussion

Despite the knowledge that fibrosis and cirrhosis are the greatest risk factors for developing HCC, very few therapeutic options are currently available (Llovet et al., 2004; Avila et al., 2006; Bruix et al., 2006). Ongoing studies on the molecular pathogenesis indicate that HCCs have multiple "genetic signatures" (Smith et al., 2003; Lee and Thorgeirsson, 2004, 2005; Lemmer et al., 2006; Luo et al., 2006; Villanueva et al., 2007) and that these genetic profiles may vary as precursor nodules progress to HCC (Libbrecht et al., 2005; Llovet et al., 2006). Recent sequencing studies show over 100 different tumor signatures in breast and prostate cancer (Sjoblom et al., 2006), suggesting that the underlying molecular complexity of HCC has yet to be appreciated. HCC genetic and epigenetic heterogeneity is due in part to the non-clonal nature of hepatic tumors as well as diverse contributions from non-hepatocytes or stromal cells in tumorigenesis. Thus, therapeutic strategies to treat HCC will need to incorporate the heterogeneity of

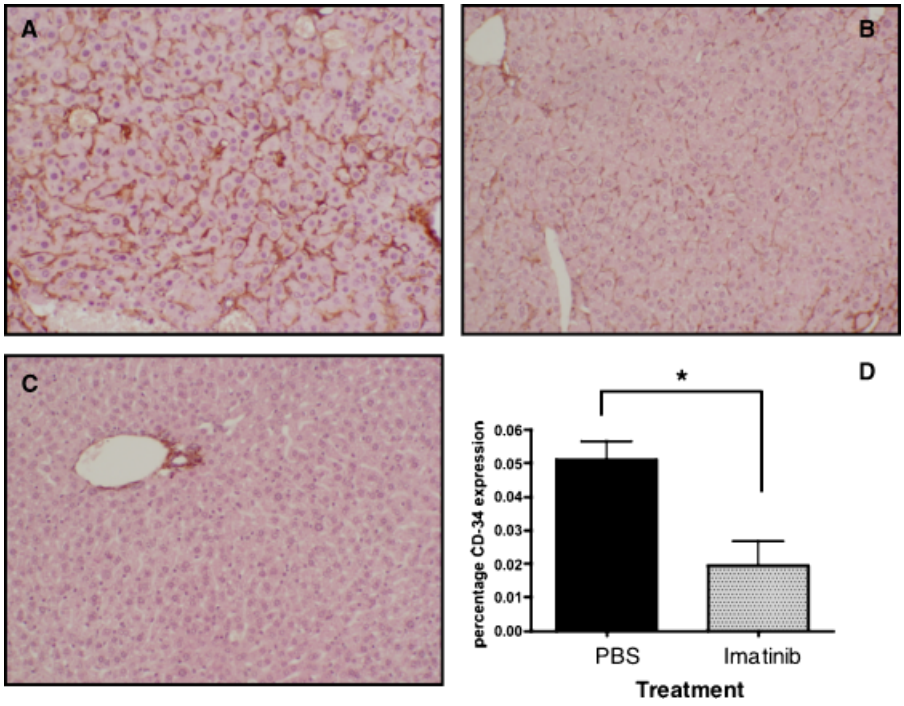

Fig. 6 Imatinib treatment decreases CD34 expression in platelet-derived growth factor (PDGF)-C Tg mice. WT or Tg mice were injected with imatinib once a day for 1 week. Hepatic CD34 staining was analyzed in (**A**) saline-treated PDGF-C Tg, (**B**) Imatinib-treated PDGF-C Tg, and (**C**) saline-treated WT mice. (**D**) Eight-$\times 20$ fields were analyzed using morphometry as described in Materials and methods. "UnTxt" indicates untreated Tg mice and "+imatinib" received inhibitor (i.p.100 mg/kg/day). For control treatment, WT and Tg mice received saline instead of inhibitor ($^{*}p < 0.05$).

liver cell types, stages of carcinogenesis, and the tumors themselves.

One possible therapeutic strategy for HCC would be to target protein kinases, which regulate key signaling pathways that control specific stages of the step-wise progression to HCC. The development of pharmacological inhibitors that block angiogenesis associated with solid tumors such as HCC is an active area of research (Semela and Dufour, 2004; Avila et al., 2006). However, there are few animal models that mimic this aspect of HCC. We have developed a Tg mouse model where overexpression of PDGF-C results in the development of HCC in a step-wise fashion (Campbell et al., 2005). We now show that proliferation of endothelial-like cells that also appear to express CD34 precedes the development of hepatic tumors. In the present study, we have utilized this model to determine whether treatment with a small-molecule tyrosine kinase inhibitor, imatinib, blocks NPC proliferation. Imatinib treatment decreases NPC or stromal cell proliferation, which coincides with decreased perisinusoidal CD34 expression, and reduced levels of active Akt, ERK1/2, and total levels of PDGFRα. These results indicate that imatinib demonstrates some efficacy in PDGF-C-induced angiogenesis and that PDGF-C Tg mice are a useful pre-clinical model that allows the testing of therapeutic strategies that may block the progression to HCC.

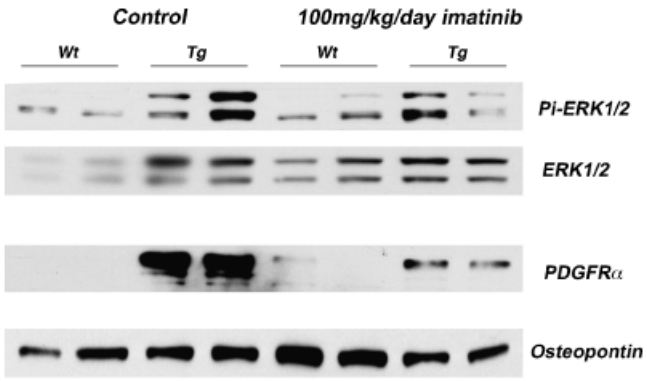

Fig. 7 Imatinib treatment decreases phospho-ERK and total platelet-derived growth factor receptor (PDGFR)α levels in the livers of PDGF-C Tg mice. WT or Tg mice were injected with imatinib once a day for 1 week (i.p., 100 mg/kg). Two hours after the last imatinib injection, the livers were excised and liver protein lysates were prepared. Activation of ERK1/2 and total levels of ERK1/2, and PDGFRα were determined by immunoblotting. Equal loading was confirmed be re-probing immunoblots with osteopontin.

Our results suggest that imatinib may be an effective therapy for stages of HCC when neovascularization is present, because inhibitor treatment of PDGF-C Tg mice results in decreased expression of CD34 perisinusoidal cells associated with fewer proliferating NPC. Although imatinib was effective in decreasing NPC proliferation, this drug did not significantly alter hepatocyte proliferation after 7 days of treatment, possibly because normal hepatocytes do not express PDGF receptors (Pinzani et al., 1996). This treatment regime did not increase cell death, indicating that in this model, imatinib appears to be cytostatic rather than cytotoxic. Moreover, the inhibitor was effective in a model in which endothelial cell proliferation was ongoing and fibrosis was already established. Future studies will investigate whether long-term treatment with imatinib can block or delay the development of HCC in this model.

Imatinib has been used in recent studies to block fibrosis and cholestatic injury in animal models of repeated pig serum injection and bile duct ligation (BDL) (Kinnman et al., 2001; Yoshiji et al., 2005; Neef et al., 2006). In these studies, the rationale for using imatinib was based on evidence that expression of PDGFRβ, another target of imatinib, is elevated in these injury models (Kinnman et al., 2001; Yoshiji et al., 2005). Kinnman et al. (2001) first showed that imatinib blocked PDGF-BB-induced proliferation of activated stellate cells *in vitro*. *In vivo* studies by the same group demonstrated that imatinib treatment decreased the proliferation of hepatic stellate cells and desmin staining in sinusodial cells during acute BDL injury. Pig serum-induced fibrogenesis was also blocked by imatinib during the 6-week injury and treatment period (Yoshiji et al., 2005). Neef et al. (2006) found that imatinib was partially effective in blocking fibrogenesis in the rat BDL model during the first 3 weeks of injury . However, during the more chronic phase of BDL injury (days 22–35), imatinib treatment was not effective in decreasing fibrosis that was already established. In the present study, we show that imatinib also blocks PDGF-CC-induced proliferation of activated stellate cell cultures *in vitro*. For our *in vivo* study we focused on whether imatinib was effective on a different liver cell type, liver endothelial cells. After 1 week of imatinib treatment, the livers of PDGF-C Tg mice expressed less CD34 and PDGFRα in addition to decreased NPC proliferation, suggesting that this inhibitor was effective in targeting liver endothelial cells. We are currently investigating whether imatinib treatment also attenuates PDGF-C-induced fibrosis in our Tg model.

In clinical studies where patients with advanced-stage HCC were treated with imatinib, this inhibitor showed no therapeutic effect (Eckel et al., 2005). However, very few patients in this small study had hepatic expression of c-kit or PDGF receptors. Imatinib treatment of other cancers is effective when c-kit or PDGF receptors are expressed (Pietras et al., 2003; Roskoski, 2003; Steeghs et al., 2007). In our studies, imatinib treatment decreased the levels of PDGFRα expression after 1 week of treatment. In PDGF-C Tg mice, PDGFRα expression is perisinusodial, possibly on endothelial cells (data not shown), suggesting that the inhibitor may be targeting this receptor isoform in liver sinusoidal cells. These results are consistent with the growing consensus that imatinib effects are more robust when the targeted protein kinases are expressed in the diseased tissue. This notion is supported by the results from the animal studies above. Imatinib was effective during the acute or early injury phase, a time when PDGFRβ is detected. It should also be noted that the timing, dosage, and route of inhibitor delivery will alter the efficacy of imatinib (Nakagawa et al., 2006). Moreover, inhibitor efficacy may also depend on the type of hepatic injury.

In summary, we show that hepatic overexpression of PDGF-C results in proliferation of endothelial cells in the liver, which may contribute to neovascularization associated with HCC development. Moreover, PDGF-C Tg mice progress in a step-wise fashion from fibrosis and angiogenesis to HCC development, mimicking hepatocellular carcinogenesis in humans. In this model, imatinib treatment inhibits the proliferation of NPC that also appear to express CD34. These studies suggest that imatinib may be effective in blocking angiogenesis, a later stage in the stepwise progression of hepatic tumorigenesis. It will be of interest to determine whether the use of imatinib and other multi-kinase inhibitors that may attenuate fibrosis or angiogenesis would prevent subsequent HCC development in PDGF-C Tg mice. Importantly, these studies indicate that the different liver cell types, including stromal cells, are susceptible targets for small molecule inhibitors. Clearly, multi-pronged therapeutic treatment strategies including multi-kinase inhibitors will likely be necessary for the treatment of HCC, given the genetic complexity of hepatic tumors themselves, and the fact that cirrhosis, dysplastic nodules, and tumors often co-exist in patients with HCC.

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