Further Pharmacological and Genetic Evidence for the Efficacy of PlGF Inhibition in Cancer and Eye Disease

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SUMMARY

Our findings that PlGF is a cancer target and anti-PlGF is useful for anticancer treatment have been challenged by Bais et al. Here we take advantage of carcinogen-induced and transgenic tumor models as well as ocular neovascularization to report further evidence in support of our original findings of PlGF as a promising target for anticancer therapies. We present evidence for the efficacy of additional anti-PlGF antibodies and their ability to phenocopy genetic deficiency or silencing of PlGF in cancer and ocular disease but also show that not all anti-PlGF antibodies are effective. We also provide additional evidence for the specificity of our anti-PlGF antibody and experiments to suggest that anti-PlGF treatment will not be effective for all tumors and why. Further, we show that PlGF blockage inhibits vessel abnormalization rather than density in certain tumors while enhancing VEGF-targeted inhibition in ocular disease. Our findings warrant further testing of anti-PlGF therapies.

INTRODUCTION

Placental growth factor (PlGF) is a VEGF homolog. Studies in independently generated *PlGF*$^{-/-}$ lines identified a role for PlGF in ischemic, inflammatory, and malignant disease (Carmeliet et al., 2001; E. Cheung et al., IOVS 2009;50:ARVO E-Abstract 2943; Fischer et al., 2008; Luttun et al., 2002a; Van Steenkiste et al., 2009). PlGF induces responses in endothelial, malignant, immune, and other cells and binds to Flt1 (Fischer et al., 2008). Although Flt1 may act as a trap for VEGF, it also transmits signals in response to PlGF via its tyrosine kinase (TK) domains (Landgren et al., 1998). The role of Flt1 in cancer remains controversial, but most studies report that Flt1 inhibition/silencing reduces tumor growth in preclinical models. In mice expressing Flt1 without TK activity (*Flt1-TK*$^{-/-}$), tumor growth and metastasis

are inhibited (Hiratsuka et al., 2002) or not affected (Dawson et al., 2009).

Clinical studies show that PlGF levels correlate with poor prognosis of various cancers, including hepatocellular, colorectal, renal, and other cancers (Fischer et al., 2008; Ho et al., 2006), though PlGF is also epigenetically silenced (Xu and Jain, 2007). PlGF levels are upregulated in cancer patients treated with VEGF inhibitors (Willett et al., 2005). Moreover, PlGF expression in tumor or stroma promotes cancer (Marcellini et al., 2006).

We showed that the anti-PlGF monoclonal antibody (mAb) 5D11D4 slows tumor growth in preclinical models, in part by blocking angiogenesis and inflammation (Fischer et al., 2007). However, anti-PlGF mAbs generated by Bais et al. were ineffective in mouse tumor models (Bais et al., 2010), questioning whether the activity of 5D11D4 could be reproduced by independently generated anti-PlGF mAbs. Also, issues were raised about the dose and whether the effects of 5D11D4 were related to off-target activity. Furthermore, Bais et al. argued that insufficient genetic evidence supported a role for PlGF in cancer, overall questioning the therapeutic value of anti-PlGF strategies (Bais et al., 2010). Here, we provide genetic evidence for a disease-candidate role of PlGF and underscore the potential of anti-PlGF therapy in cancer by using complementary genetic and pharmacological tools to eliminate, silence, or inhibit PlGF in spontaneous tumor models. We also characterized via which underlying mechanisms loss/inhibition of PlGF blocked tumorigenesis. To obtain independent confirmation, other groups than those involved in our original study (Fischer et al., 2007) contributed autonomously.

RESULTS

Role of PlGF in Carcinogen-Induced Skin Epithelial Tumor Model

We first explored whether loss of PlGF inhibits growth and angiogenesis in a carcinogen-induced skin epithelial tumor model. PlGF levels were low in healthy skin, acutely upregulated by phorbol 12-myristate 13-acetate (PMA) (pg/mg: 13 ± 1 for control versus 55 ± 6 after PMA; n = 12–6; $p < 0.005$), and chronically elevated in small and large papillomas (pg/mg: 145 ± 49 and 192 ± 18; n = 5; $p < 0.001$). Formation of skin papillomas and associated neovessels was delayed in *PlGF*$^{-/-}$ mice (Figures 1A–1D). The average latency after the first PMA application was 3 weeks longer in *PlGF*$^{-/-}$ mice, and by 20 weeks, 94% of wild-type (WT) mice but only 59% of *PlGF*$^{-/-}$ mice developed tumors (Figures 1E and 1F). *PlGF*$^{-/-}$ mice formed 66% fewer papillomas per mouse. Similar effects were observed for large papillomas (>3 mm). In *PlGF*$^{-/-}$ mice, the first large papilloma developed 4 weeks later than in WT mice (Figures 1G and 1H). At 20 weeks, the incidence of large papillomas was 50% in WT mice but only 29% in *PlGF*$^{-/-}$ mice, and the number of large papillomas per mouse was decreased by 80%. Loss of PlGF impaired the growth of neovessels in healthy skin around the tumors (Figures 1B and 1D). Within papillomas, PlGF deficiency did not affect vessel densities but reduced their size by 65% and 55% in small and large papillomas, respectively (n = 5; $p < 0.05$), whereas the accumulation of macrophages, mast cells, or T cells was not affected (not shown).

Antitumor Activity of the Mouse Anti-Human PlGF mAb 16D3

To extend the genetic evidence for a role of PlGF in cancer, we also sought additional pharmacological evidence for a therapeutic potential of anti-PlGF in cancer and therefore evaluated if the mouse anti-human PlGF mAb 16D3 (dissociation constant [K_D] = 12 pM for human PlGF-1) could reproduce the anticancer activity of 5D11D4; this mAb was generated independently of 5D11D4 by immunizing *PlGF*$^{-/-}$ mice with human PlGF-2. Delivery of 16D3 dose-dependently elevated plasma 16D3 levels (Figure S1A available online). At 12.5 mg/kg, 2×/wk, 16D3 inhibited growth of MDA-MB-435 tumors in immunocompromised mice by 40% (mm^3: 1670 ± 194 for control versus 998 ± 210 for 16D3; n = 12; $p = 0.03$). 16D3 also inhibited the growth of human pancreatic DanG xenografts (Figures S1B–S1D) and cancer-associated cachexia (loss in body weight: 14% in control versus 7% in 16D3; n = 9–10; $p = 0.04$). Both tumors produced human PlGF (pg/mg protein in tumor lysates: 19 ± 5 for MDA-MB-435; n = 12 and 103 ± 37 for DanG; n = 11).

PlGF Blockage Retards Hepatocellular Carcinoma Growth

We then used two hepatocellular carcinoma (HCC) models to characterize more extensively the effects and mechanisms of PlGF blockage. To exclude that germline PlGF deficiency induces compensatory changes that favor tumor inhibition independently, we tested whether conditional PlGF silencing inhibited growth of HCC. In the first model, transgenic expression of an SV40 T-antigen oncogene induced hyperplasia/dysplasia (4–8 weeks), nodular adenoma (12 weeks), and diffuse carcinoma (>16 weeks) (Dupuy et al., 2003). PlGF was undetectable in healthy hepatocytes but apparent in HCC, whereas Flt1 was upregulated in macrophages, Kupffer cells, and vessels (not shown). By RT-PCR, *PlGF* transcripts were upregulated to 295% ± 28% of normal levels in adenomas (n = 4; $p < 0.05$). Hepatic PlGF was silenced by treating HCC mice from 8 to 15 weeks with *PlGF*-specific siRNA, reducing PlGF protein levels by 60% ± 8% (n = 5; $p < 0.05$). Despite incomplete PlGF silencing, hepatomegaly was reduced, with the largest inhibition when PlGF expression and macrophage infiltration were maximal (see below) (Figure 1I); fewer and smaller tumor nodules were present at 15 weeks (superficial nodules 0.5–1 cm and >1.0 cm: 12.7 ± 2.9 and 6.0 ± 0.7 in untreated versus 12.0 ± 1.4 and 4.7 ± 1.1 in control siRNA, and 1.0 ± 0.7 and 0 ± 0 in *PlGF*-specific siRNA, n = 3; $p < 0.005$; Figures 1J–1M).

We also used a carcinogen-induced HCC model by treating mice with diethylnitrosamine (DEN), which causes fibrosis and dysplastic lesions at 16 weeks and hypervascularized tumors by 25 weeks. Hepatic PlGF levels were not acutely upregulated by DEN (not shown) and remained initially low (pg/mg: 2.9 ± 0.7 in healthy versus 1.5 ± 0.4 after 4 weeks DEN; n = 6; p = not significant [NS]) but increased to 423 ± 77 pg/mg in end-stage tumor nodules (n = 6; $p < 0.05$). After 30 weeks of DEN treatment, 58% of WT mice (n = 24) but only 5% of *PlGF*$^{-/-}$ mice (n = 21) succumbed ($p = 0.002$, log rank) and fewer tumor nodules were present in *PlGF*$^{-/-}$ mice (nodules/liver: 17.9 ± 2.2 in WT versus 5.9 ± 2.4 in *PlGF*$^{-/-}$; n = 18; $p = 0.002$). When WT mice with established HCC were treated with 5D11D4 (20 mg/kg;