Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

The placental growth factor as a target against hepatocellular carcinoma in a diethylnitrosamine-induced mouse model

Femke Heindryckx[1], Stephanie Coulon[1], Ellen Terrie[1], Christophe Casteleyn[2], Jean-Marie Stassen[3], Anja Geerts[1], Louis Libbrecht[4], Joke Allemeersch[5], Peter Carmeliet[6,7], Isabelle Colle[1], Hans Van Vlierberghe[1,*]

[1]*Department of Gastroenterology and Hepatology, Ghent University Hospital, Ghent, Belgium;* [2]*Applied Veterinary Morphology, Department of Veterinary Sciences, University of Antwerp, Wilrijk, Belgium;* [3]*ThromboGenics, Leuven, Belgium;* [4]*Department of Pathology, Ghent University Hospital, Ghent, Belgium;* [5]*VIB, Nucleomics Core, Leuven, Belgium;* [6]*Laboratory of Angiogenesis & Neurovascular Link, Vesalius Research Center, VIB, Leuven, Belgium;* [7]*Laboratory of Angiogenesis & Neurovascular Link, Vesalius Research Center, KU Leuven, Leuven, Belgium*

Background & Aims: The placental growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family known to stimulate endothelial cell growth, migration and survival, attract angiocompetent macrophages, and determine the metastatic niche. Unlike VEGF, genetic studies have shown that PlGF is specifically involved in pathologic angiogenesis, thus its inhibition would not affect healthy blood vessels, providing an attractive drug candidate with a good safety profile.

Methods: We assess whether inhibition of PlGF could be used as a potential therapy against hepatocellular carcinoma (HCC), by using *PlGF* knockout mice and monoclonal anti-PlGF antibodies in a mouse model for HCC. In addition, the effect of PlGF antibodies is compared to that of sorafenib, as well as the combination of both therapies.

Results: We have found that both in a transgenic knockout model and in a treatment model, targeting PlGF significantly decreases tumor burden. This was achieved not only by inhibiting neovascularisation, but also by decreasing hepatic macrophage recruitment and by normalising the remaining blood vessels, thereby decreasing hypoxia and reducing the prometastatic potential of HCC.

Conclusions: Considering the favourable safety profile and its pleiotropic effect on vascularisation, metastasis and inflammation, PlGF inhibition could become a valuable therapeutic strategy against HCC.

Keywords: Angiogenesis; Sorafenib; Vascular normalisation; Antiangiogenic treatment; Metastasis; PlGF; VEGF.

Received 29 May 2012; received in revised form 25 September 2012; accepted 28 September 2012; available online 6 October 2012

* Corresponding author. Address: De Pintelaan 185, 9000 Gent, Belgium. Tel.: +32 9 332 23 70; fax: +32 9 332 26 74.

E-mail address: Hans.VanVlierberghe@UGent.be (H. Van Vlierberghe).

Abbreviations: PlGF, placental growth factor, VEGF, Vascular endothelial growth factor; HCC, hepatocellular carcinoma; VEGFR, vascular endothelial growth factor receptor; TAM, tumor-associated macrophages; WT, wild type; αPlGF, murine anti-PlGF monoclonal antibody; DEN, diethylnitrosamine; PGF, gene encoding PlG; H&E, haematoxilin–eosin staining; qPCR, quantitative real-time PCR; IHC, immunohistochemistry; ROI, region of interest; ELISA, enzyme-linked immunosorbent assay; HSC, hepatic stellate cell; SEM, scanning electron microscopy.

Introduction

Every year approximately 500,000 patients are diagnosed with hepatocellular carcinoma (HCC), making it the 5th most common and 3rd most deadly cancer worldwide. Currently, no curative option exists for advanced HCC and systemic treatment is limited due to the high multidrug resistance [1].

Significant progress on the treatment of HCC has been made by the introduction of sorafenib [2]. Sorafenib is a small molecular inhibitor targeting tyrosine protein kinases, causing both an antiproliferative and antiangiogenic effect [3]. It is currently used as the standard-of-care for advanced HCC patients, yet it often causes severe adverse effects including diarrhoea, weight loss, hand–foot skin reaction, and fatigue [4,5].

Another weakness of current antiangiogenic therapies is that tumors tend to escape their treatment by upregulating alternative angiogenic pathways. As a result, several cases have been reported of resistance against sorafenib [6,7], sunitinib [8,9], and vascular endothelial growth factor (VEGF) inhibitors [10–14]. Furthermore, hypoxic conditions created by antiangiogenic therapy may select for more invasive tumor variants, better adapted to survive, and proliferate [15–17]. Hypoxia is linked to metastasis, regulating several steps of the metastatic process, such as invasion through the basement membrane and extracellular matrix, increasing production of fibronectin and regulation of genes that promote the pre-metastatic niche [18]. Thus, there is a vast need of new angiogenic targets that could prevent these negative effects.

The placental growth factor (PlGF) is a member of the VEGF family, known to stimulate endothelial cell growth, migration, and survival [19–21]. PlGF is expressed in several cell types, including endothelial cells, hepatic stellate cells, and activated macrophages [22–24]. It attracts angiocompetent macrophages and bone marrow progenitor cells and determines the metastatic niche [19,20]. Unlike VEGF, PlGF binds to VEGF-receptor-1

Cancer

(VEGFR1), and its co-receptors neuropilin-1 and 2 [25,26]. Besides its effect on tumor vascularisation, PlGF can alter tumor-associated macrophage (TAM) polarisation [27]. Tumors tend to attract M2 macrophages, which produce angiogenic and proliferative factors, enhancing tumor progression, while M1 macrophages are associated with an antitumoral response [28].

Genetic studies have shown that PlGF is specifically involved in pathologic angiogenesis [19,21]. Therefore, its inhibition would not affect healthy blood vessels, providing an attractive drug candidate with a good safety profile [29]. In this study, we assess whether inhibition of PlGF could serve as a therapeutic agent in a diethylnitrosamine (DEN)-induced mouse model for HCC, in which well-vascularised tumors occur after 25 wk and a time-dependent increase of PlGF is observed [30]. In our study, we focus on the effect of PlGF inhibition on angiogenesis, metastasis, inflammation, and vascular morphology.

Materials and methods

HCC induction

Mice received intraperitoneal injections of DEN (Sigma–Aldrich, Bornem, Belgium) once a week or saline as previously described [31]. This model induces HCC after 25 wk.

PlGF inhibition

An anti-PlGF monoclonal antibody (clone 5D11D4; referred to as αPlGF) specifically recognising mouse PlGF-2 was obtained from Thrombogenics, Belgium. Mice that received DEN for 25 wk developed HCC and were subsequently treated with 25 mg/kg αPlGF diluted in saline (2×/week, intraperitoneal, n = 12), 10 mg/kg sorafenib diluted in saline with 20% polysorbate 80 (daily, intragrastric n = 12), combination of αPlGF + sorafenib (n = 12), or 25 mg/kg IgG (2×/week, intraperitoneal n = 12) for 5 wk. *Pgf* knockout mice (*PlGF*KO) (n = 29) were obtained from the laboratory of Angiogenesis and Neurovascular link (Leuven) and were injected with DEN for 20, 25, and 30 wk after which they were sacrificed and compared to their WT counterparts (n = 32). Healthy control mice (n = 40) were subjected to the same treatment regimes (n = 8 per group).

Sampling and histology

Animals (n = 8 per group) were sacrificed under isoflurane (Forene®, Hoofddorp, The Netherlands) anaesthesia while blood was obtained from the ophthalmic artery. After macroscopic evaluation and quantification of hepatic tumors, organs were sampled as previously described [31]. Haematoxilin–eosin staining (H&E) and reticulin staining were performed to identify HCC nodules, as previously described [30,32,33]. Stainings were done using standard histology protocols and evaluated by an experienced pathologist.

Additional methods

Methods describing laboratory animal ethics, survival analysis, immunohistochemistry, ELISA, vascular corrosion casting, quantitative real-time PCR, whole genome microarrays and statistics have been provided as Supplementary Material.

Results

PlGF knockout study

Silencing of *Pgf* significantly improved survival (p <0.01) (Fig. 1A). Macroscopically, *PlGF*KO-mice developed less tumors compared to their WT counterparts, respectively 0.11 ± 0.11 in 20 wk

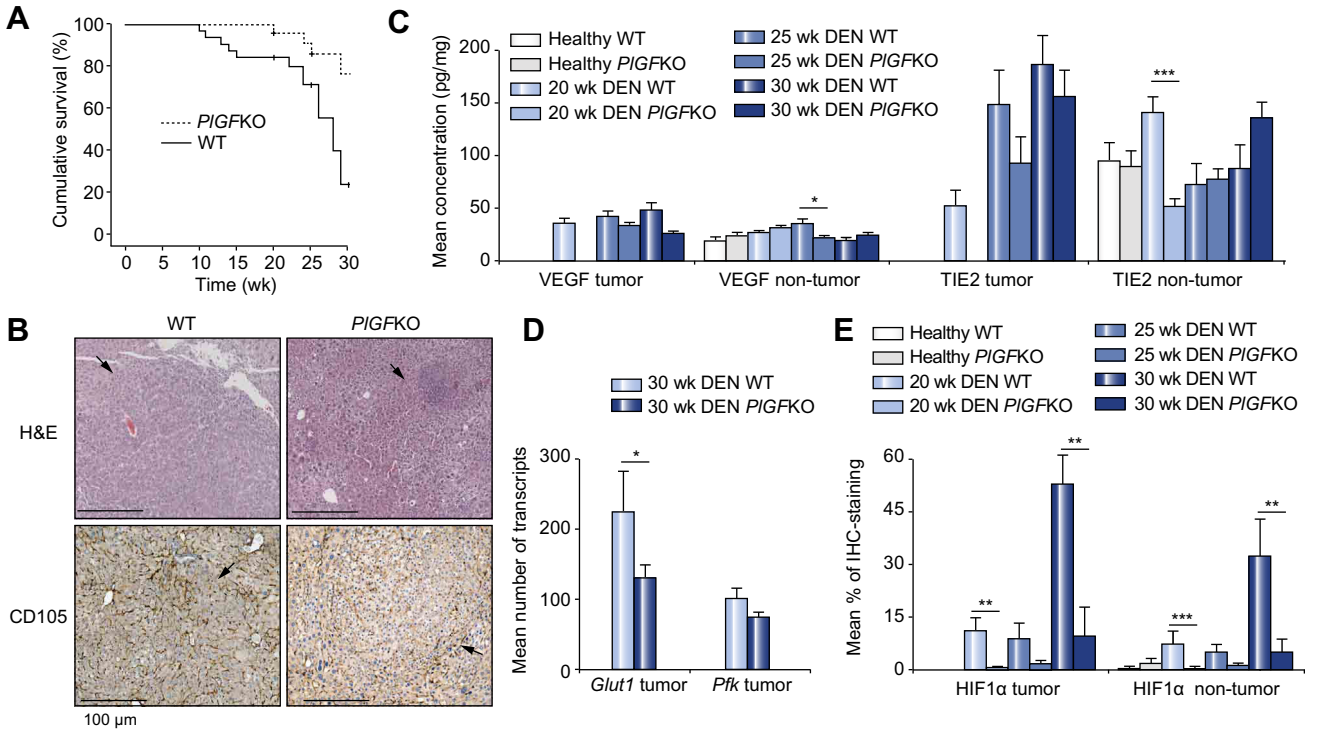

Fig. 1. Tumor burden, vascularisation, and hypoxia in *PlGF*KO mice. (A) Silencing of *PlGF* improved survival. (B) Upper panel: H&E stained livers of *PlGF*KO and WT mice (200×). Lower panel: CD105-stained livers of *PlGF*KO and WT mice (200×). (C) Concentration of VEGF was decreased in the surrounding of *PlGF*KO livers at 25 wk DEN, but not in tumors. (D) *Glut1* transcription was significantly decreased at 30 wk DEN in *PlGF*KO tumors. (E) HIF1α was decreased in tumor and non-tumor tissues of 20 and 30 wk DEN (*p <0.05, **p <0.01, and ***p <0.001; n = 8 in all groups). Arrows show HCC lesions.