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Research Article

HIF-2alpha-dependent PAI-1 induction contributes to angiogenesis in hepatocellular carcinoma

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ABSTRACT

Hypoxia promotes progression of hepatocellular carcinoma (HCC), not only affecting tumor cell proliferation and invasion, but also angiogenesis and thus, increasing the risk of metastasis. Hypoxia inducible factors (HIF)-1α and -2α cause adaptation of tumors to hypoxia, still with uncertainties towards the angiogenic switch. We created a stable knockdown of HIF-1α and HIF-2α in HepG2 cells and generated cocultures of HepG2 spheroids with embryonic bodies as an in vitro tumor model mimicking the cancer microenvironment. The naturally occuring oxygen and nutrient gradients within the cocultures allow us to question the role of distinct HIF isoforms in regulating HCC angiogenesis. In cocultures with a HIF-2α knockdown, angiogenesis was attenuated, while the knockdown of HIF-1α was without effect. Microarray analysis identified plasminogen activator inhibitor 1 (PAI-1) as a HIF-2α target gene in HepG2 cells. The knockdown of PAI-1 in HepG2 cells also lowered angiogenesis. Blocking plasmin, the downstream target of PAI-1, with aprotinin in HIF-2α knockdown (k/d) cells proved a cause–effect relation and restored angiogenesis, with no effect on control cocultures. Suggestively, HIF-2α increases PAI-1 to lower concentrations of active plasmin, thereby supporting angiogenesis. We conclude that the HIF-2α target gene PAI-1 favors the angiogenic switch in HCC.

Abbreviations: HCC, hepatocellular carcinoma; HIF, hypoxia-inducible factor; k/d, knockdown; PAI-1, plasminogen activator inhibitor type 1; VEGF, vascular endothelial growth factor; cv, control virus

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide [29]. Most cases arise from chronic hepatitis or liver-cirrhosis and are characterized as highly vascularized [60]. Key to the malignant outcome is the hypoxic microenvironment, causing changes in vascularization with an increased risk of invasion and metastasis by activating hypoxia-inducible factors (HIFs) [22,49].

HIFs facilitate adaptation to oxygen deprivation mainly by transcriptional programs [48]. They are heterodimers of an O_2-regulated α- subunit (HIF-1α, HIF-2α, encoded by *EPAS1*, or HIF-3α) and a constitutively expressed β-subunit (HIF-1β or aryl hydrocarbon receptor nuclear translocator, ARNT). HIF-α subunits dimerize with HIF-1β and bind hypoxia-responsive elements (HREs) that contain a conserved RCGTG core sequence within their target genes [31,47]. While HIF-1α and HIF-2α are frequently upregulated in HCC only HIF-2α is correlated with high patient lethality [2,53]. Though, recent studies have shown that HIF-1α and HIF-2α are structurally alike and regulate overlapping, but not identical sets of target genes, they promote highly divergent outcomes in cancer-progression and may even have counteracting roles [23,42,50]. In line, regulation of angiogenesis in HCC is controversially discussed [50]. Angiogenesis occurs through a multistep process, termed the "angiogenic switch", which ultimately tips the balance toward pro-angiogenic factors, including VEGF/VEGFR, plasminogen activator inhibitor type 1 (PAI-1), angiopoietins (ANG-1 and -2), or platelet-derived growth factor B (PDGF-B) [40]. VEGFA is the most prominent factor inducing angiogenesis and represents a HIF-1α and HIF-2α target gene in HCC [28,41]. This questions whether both isoforms have overlapping functions in regulating HCC angiogenesis.

In this study we knocked down HIF-1α and HIF-2α in HepG2 cells and generated cocultures of HepG2 spheroids and embryonic bodies derived from embryonic mouse stem cells as an *in vitro* tumor model mimicking the cancer microenvironment, to analyze HIF-isoform specific regulatory functions in HCC angiogenesis. We suggest that HIF-2α contributes to the angiogenic switch by facilitating PAI-1 expression.

Materials and methods

Materials

If not indicated otherwise, chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany). Primary antibodies were supplied by: anti-HIF-1α, BD Biosciences (Heidelberg, Germany); anti-HIF-2α, Abcam (Cambridge, UK); anti-PAI-1, American Diagnostica inc. (Pfungstadt, Germany); anti-CD31, Millipore (Darmstadt, Germany); APC anti-mouse CD144 (VE-Cadherin), BioLegend (San Diego, *CA*, USA). Secondary antibodies were from: Alexa Fluor®-fluorescence dye anti-rat/anti-rabbit 546, life technologies (Darmstadt, Germany); horseradish peroxidase-labeled anti-mouse/anti-rabbit, GE Biosciences (Freiburg, Germany). Nitrocellulose membrane was supplied by Amersham Biosciences (Freiburg, Germany). ECL detection system for Western analysis was from GE Healthcare (Little Chalfont, UK).

Cell culture

Medium, supplements, and fetal bovine serum (FBS) were purchased from PAA (Linz, Austria), if not indicated otherwise. HepG2 cells were grown in Minimal Essential Medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM non-essential amino acids. Medium of transfected cells additionally contained 2 μg/ml puromycin dihydrochloride.

The mouse embryonic stem cell line CGR8 (kindly provided by Prof. M. Wartenberg, University Hospital, Jena, Germany) was grown on gelatin-coated culture dishes with Glasgow Minimum Essential Medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 50 μM ß-mercaptoethanol and 10^3 U/ml leukemia inhibitory factor (LIF, Merck Millipore, Darmstadt, Germany) to prevent differentiation.

Tumor spheroid cell culture

HepG2 spheroids were grown from 1×10^6 cells in coated (Sigmacote) 250 ml-spinner flasks (Integra Biosciences, Fernwald, Germany) with 250 ml complete medium in a Cell Spin stirrer system (22.5 rotations/min, Integra Biosciences). 125 ml cell culture medium was replaced every second day. As spheroids develop a natural oxygen gradient, they were maintained under normoxia. Tumor spheroids were harvested using serological wide tip pipets® (VWR, Darmstadt, Germany) to reduce mechanical damage.

Cultivation of tumor spheroid-embryonic body (EB)-derived cocultures

Stem cells were transferred in Iscove Medium (Biochrom, Darmstadt, Germany) supplemented with 10% heat-inactivated FBS, 10% active FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 100 μM ß-mercaptoethanol. 35 μl containing 1000 single stem cells were dropped onto the lid of a 10 cm diameter dish. Each 8 day old HepG2 tumor spheroid was transferred from the spinner flask into a single drop containing stem cells. In order to avoid shearing forces we used cutted and flamed pipet tips. After preparing 30–40 coculture drops, the lid was turned over and placed on a 10 cm diameter dish, which was filled with 5 ml of sterile phosphate buffered saline (PBS). After 2 days, stem cells formed an EB fused with the tumor spheroid. These cocultures were transferred into 10 ml Iscove medium in a new 10 cm diameter dish to prevent adherence. Medium was changed daily. After additional 6 days of cultivation (corresponding to 8 days in coculture without LIF), cocultures were analyzed.

Lentiviral transduction

Lentiviral transfection with shRNA transfer vectors based on pLKO.1-puro containing shRNA sequences against HIF-1α, -2α, or PAI-1 was performed as described [35]. A non-target shRNA was analyzed as a control.