Original Paper

Prolyl-4-Hydroxylase 2 Potentially Contributes to Hepatocellular Carcinoma-Associated Erythrocytosis by Maintaining Hepatocyte Nuclear Factor-4 α Expression

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Key Words

Erythropoietin • HCC • HNF

Abstract

Background: Increased red blood cell count (Erythrocytosis) is an important paraneoplastic syndrome of hepatocellular carcinoma (HCC) and is a significant risk factor for lethal lung artery thromboembolism. HCC-associated erythrocytosis is partially caused by the ability of several HCC cells to produce erythropoietin (EPO). Prolyl-4-hydroxylase 2 (PHD2) is an enzyme encoded by the gene EGLN1. The best-known function of PHD2 is to mediate the oxygen-dependent degradation of the labile α -subunit of hypoxia-inducible factor (HIF). However, there is increasing evidence that PHD2 also regulates HIF-independent pathways by interacting with other substrates. **Methods:** In the EPO-producing human HCC cell line HepG2, the expression of PHD2 gene was silenced with siRNA. EPO production was estimated using quantitative PCR and ELISA. **Results:** In HepG2 cells, PHD2 suppresses the activity of TGF- β 1 pathway and consequently maintains the expression of hepatocyte nuclear factor-4 α (HNF-4 α), an important transcription factor promoting the EPO expression in hepatocytes. PHD2 knockdown caused a marked reduction of EPO production. HIF seemed not to be involved in this biology. **Conclusion:** Our findings show that PHD2 represents a potential contributing factor for HCC-associated erythrocytosis. Selective inhibition of PHD2 in HCC cells might be considered as a new way to manage erythrocytosis in HCC patients.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer mortality [1]. Increased red blood cell count (Erythrocytosis) is an important paraneoplastic phenomenon in HCC patients [2-5]. Erythrocytosis generally leads to elevated blood viscosity

and is a significant risk factor for lung artery thromboembolism, a life-threatening condition [6]. The ability of some HCC cells to secrete erythropoietin (EPO), a glycoprotein hormone which promotes erythropoiesis [7], has been known to be a contributing factor for HCC-associated erythrocytosis [8, 9].

Prolyl-4-hydroxylase 2 (PHD2) is an enzyme encoded by the gene EGLN1. PHD2 requires oxygen as co-substrate. The best-known function of PHD2 is to regulate the cellular adaptation to oxygen deprivation (hypoxia) by mediating the proteasomal degradation of the oxygen-labile α -subunit of hypoxia-inducible factor (HIF) in an oxygen-dependent manner. PHD2 is therefore known as an oxygen sensor protein [10].

The expression level and the substrate preference of PHD2 vary between tissues [11-13]. Besides, PHD2 has been discussed to regulate several HIF-independent pathways by interacting with non-HIF substrates [14-18].

In this study, we found that in the EPO-producing human HCC cell line HepG2, PHD2 maintains the expression of hepatocyte nuclear factor-4 α (HNF-4 α), an important mediator of EPO expression in hepatocytes, by inhibiting the auto-/paracrine signalling of transforming growth factor- β 1 (TGF- β 1). An inhibition of PHD2 in HepG2 cells using siRNA caused a marked reduction of EPO production. HIF seemed to be not involved in this process. Our findings suggest that PHD2 potentially contributes to HCC-associated erythrocytosis. A selective PHD2 inhibition in HCC cells might be considered as a new way to manage paraneoplastic erythrocytosis in HCC patients.

Material and Methods

Cell culture and reagents

HepG2 cells (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 (Gibco, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS; Gibco), 100 IU/ml penicillin and 100 μ g/ml streptomycin (PAA Laboratories, Coelbe, Germany) in a humidified atmosphere with 5% CO₂. Human recombinant TGF- β 1 was purchased from PeproTech (Hamburg, Germany). TGF- β 1 neutralizing antibody was purchased from R&D Systems (Wiesbaden, Germany). For hypoxic treatments, cells were incubated in the hypoxia workstation (Ruskin invivo₂ 400) in the presence of 1% O₂, 5% CO₂ and 94% N₂.

Protein extraction and immunoblot analysis

Whole cell extraction and immunoblot analyses were performed as described previously [15]. The following primary antibodies were used: rabbit polyclonal antibodies against HIF-2 α (1:1000, Novus NB100-122, Cambridge, United Kingdom) and PHD2 (1:500, Novus NB100-137); goat polyclonal antibody against lamin A/C, actin and HNF-4 α (1:1000, Santa Cruz, Heidelberg, Germany).

RNA isolation and quantitative PCR

RNA isolation and quantitative PCR were performed as described previously [15]. 500-1000 ng total RNA was reverse transcribed with M-MuLV Reverse Transcriptase (New England Biolabs, Frankfurt, Germany). The following primers and assays were used: human L28 forward, 5'-ATG GTC GTG CGG AAC TGC T-3'; human L28 reverse, 5'-TTG TAG CGG AAG GAA TTG CG-3'; Taqman Gene Expression Assay for EPO (Hs04183508), HNF-4 α (Hs00230853), TGF- β 1 (Hs00998133) and TGF- β receptor1 (Hs00610320).

RNA interference

Cells cultured to 60-70% confluence were transfected with 100 nM siRNA or MISSION siRNA universal negative control (Sigma-Aldrich, Seelze, Germany) using Lipofectamine RNAiMAX (Invitrogen, Darmstadt, Germany) as described previously. Pre-designed siRNA against PHD2 (Dharmacon, Darmstadt, Germany) was used [15].

Chromatin immunoprecipitation

Proteins were cross-linked to DNA using 1% formaldehyde for 10 min at room temperature. Cells were lysed in lysis buffer (0.05 M HEPES, 0.14 M NaCl, 1 μ M EDTA, 10% glycerine, 0.5% NP40, 0.25% Triton

X-100) for 15 min on ice. Nuclei were collected in buffer containing 1 μ M EDTA, 0.5 μ M EGTA and 10 μ M Tris. Chromatin was sonicated, incubated with rabbit polyclonal antibody against HIF-2 α (1 μ g, Novus NB100-122) and precipitated with protein G Sepharose (GE Healthcare, Freiburg, Germany). Eluted DNA was purified with QIAquick Extraction Kit (Qiagen, Hilden, Germany). HRE-containing region of the EPO enhancer was amplified and analysed using agarose gel electrophoresis. Following primers were used [19]: forward, 5'-TCG TTT TCT GGG AAC CTC CA -3'; reverse, 5'-GGA GCC ACC TTA TTG ACC AG-3'.

Assay of EPO and TGF- β 1

The concentrations of EPO and TGF- β 1 in cell-culture supernatants were measured by enzyme-linked immunosorbent assays (Quantikine IVD ELISA Kit, R&D Systems, Wiesbaden, Germany).

MTT assay

The cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, Seelze, Germany) assay. Cells were incubated with MTT solution (5 g/l) for 2 hours at 37°C and then lysed with MTT lysis buffer (10 % SDS, 1 % HCl, 0.46 % isopropanol). The optical density was detected with a microplate reader (Thermo Scientific, Bonn, Germany) at 570 nm.

Statistical analysis

Data are shown as means $\pm$ standard error (SEM). Statistical comparison between two groups was performed by two-tailed Student's t- or Mann-Whitney-test, as applicable. Differences were considered significant when $P < 0.05$. All statistics were calculated using GraphPad Prism 5 (GraphPad Software, Wittenhausen, Germany).

Results and Discussion

PHD2 promotes EPO production and HNF-4 α expression in HepG2 cells

HepG2 cells were transiently transfected with siRNA against PHD2. The knockdown efficiency was determined using immunoblotting (Fig. 1A). As shown in Fig. 1B and 1C, PHD2 knockdown led to a marked reduction of EPO mRNA and EPO secretion. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay showed that the viability of PHD2 knockdown cells was not impaired at this time point (Fig. 4).

The expression of EPO in hepatocytes was reported to be maintained by the transcription factor HNF-4 α [20, 21]. As shown in Fig. 1D and 1E, the levels of both HNF-4 α mRNA and protein were reduced in PHD2 knockdown cells. These results indicate that PHD2 promotes the EPO production by HepG2 cells by maintaining the expression of HNF-4 α .

PHD2 maintains HNF-4 α expression in HepG2 cells by inhibiting TGF- β 1 signalling

We attempted to study the mechanism by which PHD2 maintains HNF-4 α expression. The expression of HNF-4 α in hepatocytes was shown to be inhibited by TGF- β 1 [22]. We confirmed the inhibitory effect of TGF- β 1 on HNF-4 α expression in HepG2 cells (Fig. 2A, 2B). TGF- β 1 is a secreted protein ubiquitously expressed in humans and functions in an auto-/paracrine manner [23]. As shown in Fig. 3A, the expression of TGF- β 1 in HepG2 cells was significantly up-regulated by PHD2 knockdown. The concentration of secreted TGF- β 1 was also elevated (Fig. 3B). The expression of TGF- β receptor 1 (TGF- β R1), the most important receptor subtype for TGF- β 1 [24], was also up-regulated in the PHD2 knockdown cells (Fig. 3C). TGF- β 1 neutralizing antibody attenuated the effect of PHD2 on HNF-4 α expression (Fig. 3D). TGF- β 1 pathway has been shown to inhibit cell proliferation [24]. As shown in Fig. 4, the viability of PHD2 knockdown cells was significantly lower than the control cells 96 hours after transfection, which was in line with the up-regulation of TGF- β 1 and TGF- β receptor 1 expression 48 hours after transfection. These data suggest that the down-regulation of HNF-4 α expression in the PHD2 knockdown HepG2 cells was the consequence of up-regulation of TGF- β 1 and TGF- β 1 receptor expression, and the following activation of the auto-/paracrine signalling of TGF- β 1. In the murine osteosarcoma cell line LM8, PHD2 was also observed to

Fig. 1. PHD2 maintains EPO and HNF-4 α expression. HepG2 cells were transfected with water (WT), non-specific siRNA (neg.Control) or siRNA against PHD2 (siPHD2). A+E: 48 h after transfection, PHD2 (A) and HNF-4 α (E) were detected by immunoblotting. Actin was used as loading control. The results are representative for 3 independent experiments. B+D: 48 h after transfection, total mRNA was analysed for EPO, HNF-4 α and ribosomal protein L28 mRNA expression by qPCR. Normalised EPO/L28 (B) and HNF-4 α /L28 (D) ratios are shown (mean $\pm$ SEM, n = 3; *P < 0.05, Student's t-test). C: 72 h after transfection, the concentration of EPO in the cell-culture supernatants was analysed using ELISA detection (mean $\pm$ SEM, n = 4; *P < 0.05, Mann-Whitney-test).

Fig. 2. TGF- β 1 suppresses HNF-4 α expression. A: HepG2 cells were incubated with or without TGF- β 1 for 24 h. HNF-4 α was detected by immunoblotting. Lamin A was used as loading control. The results are representative for 3 independent experiments. B: HepG2 cells were incubated with or without TGF- β 1 (10 ng/ml) for 24 h. Total mRNA was analysed for HNF-4 α and ribosomal protein L28 mRNA expression by qPCR. Normalised HNF-4 α /L28 ratios are shown (mean $\pm$ SEM, n = 3).

inhibit TGF- β 1 signalling [18]. Since the regulation of TGF- β 1 and TGF- β R1 expression has not been clearly investigated, the mechanism of this biology remains to be further studied.

The promoting effect of PHD2 on EPO expression in HepG2 cells does not involve HIF

The enhancer of EPO was reported to contain binding sites for HIF. HIF consists of an oxygen-labile α -subunit (HIF-1 α , -2 α) and a stable β -subunit (HIF- β) [19, 25-27]. Although

Fig. 3. PHD2 influences the activity of TGF- β 1 pathway. A+C: HepG2 cells were transfected with water (WT), non-specific siRNA (neg.Control) or siRNA against PHD2 (siPHD2). 48 h after transfection, total mRNA was analysed for TGF- β 1, TGF- β receptor 1 (TGF- β R1) and ribosomal protein L28 mRNA expression by qPCR. Normalised TGF- β 1/L28 (A) and TGF- β R1/L28 (C) ratios are shown (mean $\pm$ SEM, n = 3; *P < 0.05, Student's t-test). B: HepG2 cells were transfected with non-specific siRNA (Control) or siRNA against PHD2 (siPHD2). 48 h after transfection, the concentration of TGF- β 1 in the cell-culture supernatants was analysed using ELISA detection (mean $\pm$ SEM, n = 6; *P < 0.05, Student's t-test). D: HepG2 cells were transfected with non-specific siRNA or siRNA against PHD2 (siPHD2). 24 h after transfection, TGF- β 1 neutralizing antibody was added. After 24 h further incubation, total mRNA was analysed for HNF-4 α and ribosomal protein L28 mRNA expression by qPCR. Normalised HNF-4 α /L28 ratios are shown (mean $\pm$ SEM, n = 3; *P < 0.05, Student's t-test).

Fig. 4. Influence of PHD2 on cell viability. HepG2 cells were transfected with non-specific siRNA (Control) or siRNA against PHD2 (siPHD2). 48 h or 96 h after transfection, cell viability was analyzed using MTT assay (mean $\pm$ SEM, n = 5-6; *P < 0.05, ns: not significant, Student's t-test).

sharing several overlapping functions, HIF-1 α and HIF-2 α differ in their binding affinity to EPO enhancer. Rankin et al. [19] showed that HIF-2 α , but not HIF-1 α , binds to EPO enhancer in the HCC cells Hep3B. Warnecke et al. observed that HIF-1 α contributed to EPO expression in HCC cells under treatment with 2, 2'-dipyridil [28]. However, there was no evidence that HIF-1 α contributes to EPO expression in normoxic HCC cells without 2, 2'-dipyridil treatment.

We found that the mRNA level of EPO was markedly reduced in HIF-2 α knockdown HepG2 cells (Fig. 5A, B). Knockdown of HIF-1 α did not cause a down-regulation of EPO expression

Fig. 5. HIF-2 α is not involved in the regulation of EPO expression by PHD2. **A:** HepG2 cells were transfected with non-specific siRNA (Control) or siRNA against HIF-2 α (siHIF-2 α). 48 h after transfection, cells were incubated in hypoxia for 2 h. HIF-2 α was detected by immunoblotting. Actin was used as loading control. The results are representative for 3 independent experiments. **B:** HepG2 cells were transfected with non-specific siRNA (Control) or siRNA against HIF-2 α (siHIF-2 α). 24 h after transfection, cells were transfected with water (WT), non-specific siRNA (neg.Control) or siRNA against PHD2 (siPHD2). After 24 h further incubation, total mRNA was analysed for EPO and ribosomal protein L28 mRNA expression by qPCR. Normalised EPO/L28 ratios are shown (mean $\pm$ SEM, $n = 3$; * $P < 0.05$, Student's t -test). **C+D:** HepG2 cells were transfected with non-specific siRNA (neg.Control) or siRNA against PHD2 (siPHD2). 48 h after transfection, HIF-2 α was detected by immunoblotting. Lamin A was used as loading control (**A**). The binding of HIF-2 α to the EPO enhancer was detected using ChIP (**B**). Hypoxia (Hox, 24h 1% O₂) was used as a positive control. The results are representative for 3 independent experiments.

in normoxic HepG2 cells (data not shown). As shown in Fig. 5C, PHD2 knockdown did not cause a detectable accumulation of HIF-2 α protein in normoxic HepG2 cells. In chromatin immunoprecipitation (ChIP) assay, an increased binding of HIF-2 α to the EPO enhancer was not observed in PHD2 knockdown HepG2 cells (Fig. 5D). In HIF-2 α knockdown cells, PHD2 knockdown also caused a significant down-regulation of EPO expression (Fig. 5B). These data indicate that HIF-2 α does not play a major role in the regulation of EPO expression by PHD2. We could not exclude the possibility that the accumulation of HIF-2 α could not be detected due to insufficient sensitivity of immunoblotting and ChIP. However, even if some HIF-2 α accumulates in PHD2 knockdown cells, the effect of HIF-2 α accumulation on EPO expression is probably less important than the effect of HNF-4 α down-regulation.

In conclusion, we show that PHD2 promotes the EPO biosynthesis in the human HCC cell line HepG2 by inactivating TGF- β 1 pathway. Selective inhibition of PHD2 in HCC cells using liver-specific gene therapy techniques (e.g. mediated by adeno-associated viral gene transfer vectors) might be considered as a new way to manage erythrocytosis in HCC patients. Since no specific PHD2 inhibitor has been characterized, we were not able to confirm our results by inhibiting PHD2 pharmacologically. Hepatitis B virus was observed to interact with TGF- β 1/HNF-4 α axis [29]. Since HepG2 is the only well-established EPO-producing HCC cell line without viral infection [30], all our experiments were performed with this cell line. *In vivo* experiments and isolation of novel EPO-producing HCC cell lines would be

indispensable. It would also be worthwhile to characterize the interaction partner of PHD2 in HCC cells contributing to the inhibition of TGF- β 1 signaling and to study the reaction of PHD2 knockdown cells to hypoxia and hypoxic mimetics.

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Disclosure Statement

The authors declare no conflict of interest.

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