Journal of Cellular Biochemistry 100:1081–1085 (2007)

FAST TRACKS

Phenotypic Expression of Human Hepatoma Cells in Culture

Kenneth R. Cutroneo,[1]* Sheryl L. White,[2] Thomas R. Buttolph,[2] Gretchen Allison,[3] and H. Paul Ehrlich[3]

[1]Department of Biochemistry, College of Medicine, 89 Beaumont Avenue, University of Vermont, Burlington, Vermont 05405
[2]Department of Anatomy and Neurobiology, College of Medicine, 89 Beaumont Avenue, University of Vermont, Burlington, Vermont 05405
[3]Division of Plastic Surgery—H071, Penn State University, College of Medicine, 500 University Avenue, Hershey, Pennsylvania 17033

Abstract Hepatomas thrive in a hypoxic environment resulting in the induction of a cluster of hypoxia related genes. The protein phenotypic expression include hypoxia inducible factor-α, prolyl-4-hydroxylase, vascular endothelear growth factor and erythropoietin. The present study was undertaken to determine if human hepatoma cells when cultured for 72 h in the presence of serum under normoxia would maintain their cancerous phenotypic expression of certain hypoxia inducible genes. Our positive results affords an in vitro model system to test hypoxia inhibitors on the expression and the intracellular compartmentalization or the secretion of these hypoxia-inducible proteins. J. Cell. Biochem. 100: 1081–1085, 2007.

Key words: human hepatoma cells (Hep 2G); β-subunit of prolyl-4-hydorxylase; hypoxia-inducible factor-1α; erythropoetin; vascular endothelial growth factor

A hypoxic microenvironment is of prime importance as a regulator of the expression of heat shock protein (HSP) and angiogenic factor genes by heat shock transcription factor (HSF) and hypoxia-inducible factor (HIF-1). The expression of these hypoxic genes are thought to contribute to the malignant progression of tumor cells. HIF-1 is composed of an oxygen-sensitive α-subunit (HIF-1 α or HIF-2 α) and a constitutively expressed beta-subunit. During normoxia HIF-1 α is destabilized by the post-translational hydroxylation of Pro-564 and Pro-402 catalyzed by oxygen-sensitive dioxygenases termed prolyl hydroxylases [Marxsen et al., 2004]. Hypoxia stimulates the synthesis of a cluster of enzymes via HIF-1 involved in post-translational modification of collagen polypeptide chains for the formation of the triple helix [Hofbauer et al., 2003]. HIF-1α is a transcription factor that senses low oxygen availability and enhances the activation of the hypoxia-inducible genes involved in energy/ion metabolism, angiogenesis, and erythropoiesis [Percy et al., 1997; Semenza, 2003; Lee et al., 2004]. Under normoxia, HIF-1α protein is known to be ubiquitinated by E3 ubiquitin ligase van hippe laudue (VHL) in various cell types [Huang et al., 1998; Lisztwan et al., 1999; Maxwell et al., 1999; Ohh et al., 2000]. Under hypoxia, VHL is unable to associate with and ubiquitinae HIF-1α. HIF-1α then escapes from degradation, translocates to the nucleus and activates the transcription of target genes. R59949, an inhibitor of diacylglycerol kinase enhances VHL binding to endogenous HIF-α and stimulates prolyl hydroxylase at both 21% O_2 as well as at 1% O_2 [Temes et al., 2005]. There is now an in vivo model to test the ability of agents on oxygen tension in particular organs.

Grant sponsor: NIH; Grant number: P20 RR16435.

*Correspondence to: Kenneth R. Cutroneo, Department of Biochemistry, College of Medicine, 89 Beaumont Avenue, University of Vermont, Burlington, VT 05405.
E-mail: kenneth.cutroneo@uvm.edu

Received 2 August 2006; Accepted 25 August 2006
DOI 10.1002/jcb.21150

A mouse line has been created that expresses the oxygen-dependent degradation domain of HIF-1α fused to the common firefly luciferase gene, under the control of a promoter that ensures organism-wide expression. In conditions of normoxia, the protein is quickly degraded, but in hypoxic situations, the protein is not degraded and administration of substrate illuminates the areas of expression [Schmid and Young, 2006]. The present study was carried out to determine if key proteins expressed in human hepatomas in vivo maintain their expression in *cell culture* under normoxic conditions.

MATERIALS AND METHODS

Cell Culture

Hep2G human hepatoma cells (ATCC #HB-8065) were obtained from the ATCC collection (Rockville, MD). The following materials were purchased from the designated companies: penicillin, streptomycin, L-glutamine, phosphate-buffered saline (PBS) (Gibco-BRL, Grand Island, NY); fetal bovine serum (FBS), (Hyclone Laboratories, Logan, UT); modified Eagle's media (MEM) (ATCC).

The Hep2G cells were maintained under air 5% CO_2 in ATCC complete growth medium, MEM with 2 mM L-glutamine and Eagle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate, 10% FBS, (v/v) and used at passage #38.

Protein Extracts

At 8, 12, 24, 48, and 72 h following normotoxic exposure, the cells were collected by scraping from six well plates in cold PBS and centrifuged at 500*g* for 3 min. Nuclear and cytoplasmic fractions were then isolated using the NE-PER Nuclear and Cytoplasmic reagent kits (Pierce Biotechnology, Rockford, IL), according to the manufacturer's protocols. The protein concentrations of the extracts were determined using the Coomassie-Plus reagent (Pierce).

Antibody Sources

Antibodies recognizing the following proteins were purchased: hypoxia inducible factor-1α, (HIF-α) 1:8,000 (cat#07-628, Upstate Biotech., Charlottesville, VA), vascular endothelial growth factor (VEGF), 1:8,000 (cat #sc-507, Santa Cruz Biotechnology, Santa Cruz, CA), erythropoietin (EPO), 1:8,000 (cat #sc1310, Santa Cruz Biotechnology) and human β-subunit of prolyl-4-hydroxylase (P-4-H) 1:8000 (cat # 63-164, ICN Biomedicals, Aurora, OH). These antibodies have all been shown to recognize their corresponding human antigens by their respective vendor. Five micrograms protein of nuclear or cytoplasmic extract was assayed per well. DAR (1:100,000) was used as secondary antibody for HIF-1α while DAM was used as secondary antibody for P-4-H. For media 10 μl/well was assayed. DAG at a 1:100,000 dilution was used as secondary antibody for VEGF and DAR at this same dilation was used as secondary antibody for EPO.

Dot Blot Hybridization

Hep2G cells were plated in 96 well dishes and grown to confluency in DMEM containing 10% (v/v) FBS. Equal proteins aliquots of the media, nuclear or cytoplasm were dot blotted onto a PVDF membrane (Millipore) using a dot blot template instrument to analyze a large number of samples. Proteins were transferred by electrophoresis onto a PVDF membrane which have any free protein binding sites blocked in a 5%(w/v) dried milk solution. The membrane is incubated with a primary antibody and after multiple washes, the membrane is incubated with peroxidase-conjugated secondary antibody, followed by another series of washes and the proteins are detected on X-ray film by BCA chemiluminescence (Pierce). To document equivalent protein loading an equal amount of protein of each fraction is dot blotted at each time of cell incubation. X-ray film is scanned and the protein dot density is used to quantify relative intracellular and extracellular protein levels and protein subcellular compartmentalization.

RESULTS

Hypoxia-inducible gene expression occurs in spontaneous, chemical-induced and transplantable hepatocarcinomas under an acidic and hypoxic environment maintained by anerobic glycolysis in vivo. As can be seen in Figure 1 HIF-1 and P-4-H are expressed in both the nuclear and cytoplasmic fractions of Hep2G human hepatoma cells up to 72 h in culture under normoxia. In addition at 8 h, the earliest time tested, under normoxia there is a decrease of P-4-H and an increase of HIF-1 in the nuclear fraction which represents a

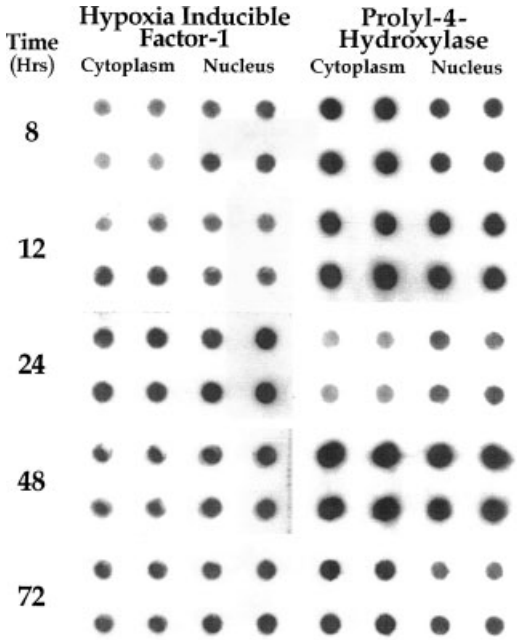

Fig. 1. Time course of hypoxia-inducible factor-1 and prolyl-4-hydroxylase in cultured Hep2G cells under normoxia in cytoplasmic (5 μg protein) and nuclear (5 μg protein) cellular fractions. All experiments were performed on duplicate cultures with duplicate dot blots of each subcellular fraction or media sample.

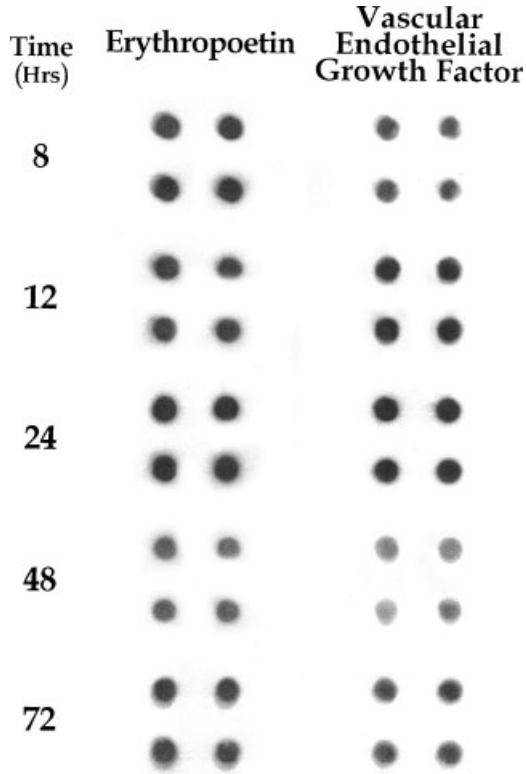

Fig. 2. Time course of media (10 μl) vascular endothelial growth factor and erythropoietin of Hep2G cells cultured under normoxia.

decreased translocation of this enzyme from the cytoplasm and an increased translocation of this latter factor from the cytoplasm. This enzyme is involved in the degradation of the HIF-1 through VHL binding and 26S proteosome degradation. This post-translational modification enzyme also is involved in collagen synthesis in the cytoplasm. The only other time point under normoxia that the Hep2G cells demonstrated a translocation of P-4-H from the cytoplasm to the nuclear fraction was at 24 h in culture with the HIF-1 remaining unchanged in both the cytoplasmic and nuclear factions. As seen in Figure 2 no changes were observed for the hypoxia-inducible expressed gene phenotypes of VEGF and EPO secreted into the media of Hep2G carcinoma cells cultured up to 72 h under normoxia. However, both of these factors in the media of 48 h Hep2G cultures were significantly decreased, possibly due to turnover.

DISCUSSION

Tumors exist in vivo in an acidic, hypoxic microenvironment undergoing anaerobic glycolysis, angiogenesis with the HIF-1 induction of VEGF [Jung et al., 2005; Kim et al., 2005; Zhang et al., 2005] and EPO [Percy et al., 1997]. Collagen prolyl hydroxylases are required for proper collagen helix synthesis. These enzymes belong to the group of four oxoglutarate and iron-dependent oxygenases. P-4-H has been characterized as an alpha2 beta 2 tetramer in which protein disulfide isomerase (PDI) is the beta subunit isoforms (EC 5.3 4.1) with two different alpha subunit (EC 1.14.11.2) isoforms. The enzymatically active alpha subunit (64 kDa) of both the human and chick enzymes are glycosylated [Guzman et al., 1992]. PDI, the β-60 kDa subunit is a constituent of P-4-H which catalyses the co- and posttranslational hydroxylation of certain sequences specific collagen and HIF-1α prolyl residues. The vertebrate P-4-Hβ subunit is identical to the multifunctional enzyme PDI. Moreover, unassembled α-subunits are maintained in an assembly competent inactive form by interaction with the molecular chaperone BiP [John and Bulleid, 1996].

PDI has two interrelated activities, that is, the ability to catalyze the formation, reduction,

and isomerization of disulfide bonds and the ability to bind to polypeptide chains [Freedman et al., 1994; Pirneskoski et al., 2001]. This latter activity enables PDI to function as a molecular chaperone and to assist in the folding of polypeptides. It has been suggested that PDI forms more permanent associations with specific proteins, for example, to become a subunit of P-4-H and microsomal triglyceride transfer protein. Inhibition of P-4-H activity can be obtained with a monoclonal antibody to the beta subunit of this enzyme [Kaska et al., 1987].

We recently published a comprehensive review on the relationship of tissue fibrosis to the final outcome of carcinogenesis in various tissues [Cutroneo et al., 2006]. Hypoxia is the microenvironment in which carcinomas thrive. Hypoxia also increases ProCOL1A1 mRNA [Falanga et al., 1993], COL1A1 transcription and collagen synthesis in normal fibroblasts [Falanga et al., 2002; Papakonstantinou et al., 2003].

In the present study a human hepatoma cell culture model of key proteins elevated in carcinomas maintained in an acidic hypoxic environment in vivo were shown to be phenotypically expressed in cells cultured under normoxia for at least 72 h. This affords an in vitro model to access the effects of inhibitors of HIF-1 synthesis and the subsequent effects on its cluster of hypoxia inducible proteins.

ACKNOWLEDGMENTS

This publication was made possible by NIH Grant Number P20 RR16435 from the COBRE Program of the National Center for Research Resources.

REFERENCES

Cutroneo KR, White SL, Chiu J-F, Ehrlich HP. 2006. Tissue fibrosis and carcinogenesis: Divergent or successive pathways dictate multiple molecular therapeutic targets for oligo decoy therapies. J Cell Biochem 97:1161–1174.

Falanga V, Martin TA, Takagi H, Kirsner RS, Helfman T, Pardes J, Ochoa MS. 1993. Low oxygen tension increases mRNA levels of alpha 1 (I) procollagen in human dermal fibroblasts. J Cell Physiol 157:408–412.

Falanga V, Zhou L, Yufit T. 2002. Low oxygen tension stimulates collagen synthesis and COL1A1 transcription through the action of TGF-β1. J Cell Physiol 191:42–50.

Freedman RB, Hirst TR, Tuite MF. 1994. Protein disulphide isomerase: Binding bridges in protein folding. Trends Biochem Sci 19:331–336.

Guzman NA, Ascari WQ, Cutroneo KR, Desnick RJ. 1992. Comparison between avian and human prolyl 4-hydroxylases: Studies on the holomeric enzymes and their constituent subunits. J Cell Biochem 48:172–189.

Hofbauer KH, Gess B, Lohaus C, Meyer HE, Katschinski D, Kurtz A. 2003. Oxygen tension regulates the expression of a group of procollagen hydroxylases. Eur J Biochem 270:4515–4522.

Huang LE, Gu J, Schau M, Bunn HF. 1998. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci 95:7987–7992.

John DC, Bulleid NJ. 1996. Intracellular dissociation and reassembly of prolyl 4-hydroxylase: The alpha-subunits associated with the immjunoglobulin-heavy-chain binding protien (BiP) allowing reassembly with the beta-subunit. Biochem J 317:659–665.

Jung JE, Lee HG, Cho IH, Chung DH, Yoon SH, Yang YM, Lee JW, Choi S, Park JW, Ye SK, Chung MH. 2005. STAT3 is a potential modulator of HIF-1 mediated VEGF expression in human renal carcinoma cells. FASEB J 19:1296–1298.

Kaska DD, Gunzler V, Kivirikko KI, Myllyla R. 1987. Characterization of a low-relative-molecular-mass prolyl 4-hydroxylase from the green alga chlamydomonas reinhardii. Biochem J 241:483–490.

Kim K, Yoshida D, Teramoto A. 2005. Expression of hypoxia-inducible factor 1 alpha and vascular endothelial growth factor in pituitary adenomas. Endocrine Pathol 16:115–121.

Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW. 2004. Hypoxia-inducible factor (HIF-1)α: Its protein stability and biological functions. Exp Mol Med 36:1–12.

Lisztwan J, Imbert G, Wirbelauer C, Gstaiger M, Krek W. 1999. The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. Genes Dev 13:1822–1833.

Marxsen JH, Stengel P, Doege K, Heikkinen P, Jokilehto T, Wagner T, Jelkmann W, Jaakkola P, Metzen E. 2004. Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases. Biochem J 381:761–767.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. 1999. The tumor suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275.

Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, Kaelin WG. 2000. Ubiquitinization of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel-Lindau protein. Nat Cell Biol 2:423–427.

Papakonstantinou E, Aletras AJ, Roth M, Tamm M, Karakiulakis G. 2003. Hypoxia modulates the effects of transforming growth factor-beta isoforms on matrix-formation by primary human lung fibroblasts. Cytokine 24:25–35.

Percy MJ, McMullin MF, Lappin TRJ. 1997. Sequence analysis of the hypoxia-responsive element of the human erythropoietin gene in patients with erythrocytosis. Biochem Mol Med 62:132–134.

Pirneskoski A, Ruddock LW, Kappa P, Freedman RB, Kivirikko KI, Koivunen P. 2001. Domains b′ and a′ of protein disulfide isomerase fulfill the minimum requirement for function as a subunit of prolyl 4-hydroxylase. The N-terminal domains a′ and b′ enhances this function

and can be substituted in part by those of ERp57. J Biol Chem 276:11287–11293.

Schmid T, Young MR. 2006. Lights on for low oxygen: A noninvasive mouse model useful for sensing oxygen deficiency. Mol Interv 6:20–22.

Semenza GL. 2003. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3:721–732.

Temes E, Martin-Puig S, Acosta-Iborra B, Castellanos MC, Feijoo-Cuaresma M, Olmos G, Aragones J, Landazuri MO. 2005. Activation of the HIF-prolyl hydroxylases by R59949, an inhibitor of the diacylglycerol kinase. J Biol Chem 280:24238–24244.

Zhang Q, Tang X, Lu QY, Zhang ZF, Brown J, Le AD. 2005. Resveratrol inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1 alpha and VEGF expression in human tongue squamous cellcarcinoma and hepatoma cells. Mol Cancer Ther 4:1465–1474.