Hypoxia-inducible Expression of Tumor-associated Carbonic Anhydrases 1

Charles C. Wykoff, Nigel J. P. Beasley, Peter H. Watson, Kevin J. Turner, Jaromir Pastorek, Amen Sibtain, George D. Wilson, Helen Turley, Kate L. Talks, Patrick H. Maxwell, Christopher W. Pugh, Peter J. Ratcliffe, and Adrian L. Harris

Institute of Molecular Medicine [C. C. W., N. J. P. B., K. J. T., A. L. H.] and the Nuffield Department of Clinical Laboratory Sciences [H. T., K. L. T.], John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom; Department of Pathology, University of Manitoba, Winnipeg, Manitoba, R3E 0W5 Canada [P. H. W.]; Institute of Virology, Slovak Academy of Sciences, 84246 Bratislava, Slovak Republic [J. F.]; Gray Laboratory Cancer Research Trust, Mount Vernon Hospital, Middlesex HA6 2JR, United Kingdom [A. S., G. D. W.]; Wellcome Trust Centre for Human Genetics, Oxford OX3 7BN, United Kingdom [P. H. M., C. W. P., P. J. R.]

ABSTRACT

The transcriptional complex hypoxia-inducible factor-1 (HIF-1) has emerged as an important mediator of gene expression patterns in tumors, although the range of responding genes is still incompletely defined. Here we show that the tumor-associated carbonic anhydrases (CAs) are tightly regulated by this system. Both CA9 and CA12 were strongly induced by hypoxia in a range of tumor cell lines. In renal carcinoma cells that are defective for the von Hippel-Lindau (VHL) tumor suppressor, up-regulation of these CAs is associated with loss of regulation by hypoxia, consistent with the critical function of pVHL in the regulation of HIF-1. Further studies of CA9 defined a HIF-1-dependent hypoxia response element in the minimal promoter and demonstrated that tight regulation by the HIF/pVHL system was reflected in the pattern of CA IX expression within tumors. Generalized up-regulation of CA IX in VHL-associated renal cell carcinoma contrasted with focal perinecrotic expression in a variety of non-VHL-associated tumors. In comparison with vascular endothelial growth factor mRNA, expression of CA IX demonstrated a similar, although more tightly circumscribed, pattern of expression around regions of necrosis and showed substantial although incomplete overlap with activation of the hypoxia marker pimonidazole. These studies define a new class of HIF-1-responsive gene, the activation of which has implications for the understanding of hypoxic tumor metabolism and which may provide endogenous markers for tumor hypoxia.

INTRODUCTION

Tumor hypoxia is an important indicator of cancer prognosis; it is associated with aggressive growth, metastasis, and poor response to treatment (1, 2). Of potential importance for understanding these effects is the role of hypoxia in regulating patterns of gene expression (3–6). Studies of gene expression have defined several classes of hypoxia-inducible genes that are up-regulated in hypoxic regions of tumors and demonstrated that activation of the transcriptional complex HIF-1 α is a key mediator of many of these effects (7–9).

Genes that are up-regulated by microenvironmental hypoxia through activation of HIF include glucose transporters, glycolytic enzymes, and angiogenic growth factors (5, 10, 11). For some HIF targets such as VEGF, a clear function in promoting tumor growth is established (12). However, the full range of HIF target genes has not yet been defined, and identification of additional genes responding to this pathway is likely to provide further insights into the consequences of tumor hypoxia and HIF activation. Indirect support for the importance of microenvironmental activation of HIF has also been provided by recent demonstrations of constitutive activation of HIF after inactivation of the VHL tumor suppressor gene (13) and amplification of the HIF response by other oncogenic mutations (14–17). Mutations in VHL cause the familial syndrome and are also found in the majority of sporadic RCCs (18). The gene product pVHL forms part of a ubiquitin-ligase complex (19, 20) that targets HIF-α subunits for oxygen-dependent proteolysis (13, 21). In VHL-defective cells, HIF-α is stabilized constitutively, resulting in up-regulation of hypoxia-inducible genes such as VEGF (13). Although the pVHL ubiquitin-ligase complex may have other targets (20) and other functions of pVHL have been proposed that may contribute to tumor suppressor effects (22, 23), these recent findings raise important questions as to the range of genes affected by constitutive HIF activation and the role of these genes in oncogenesis.

In this respect, one interesting group of genes is the tumor-associated transmembrane CAs CA9 (24–27) and CA12 (28, 29). CAs catalyze the reversible hydration of carbon dioxide to carbonic acid (30), providing a potential link between metabolism and pH regulation. The membrane-linked isoforms CA9 and CA12 were identified by RNA differential display as genes that are down-regulated by pVHL (29), although the effect of hypoxia was not examined and the mechanism of regulation was not defined. Interestingly, CA9 can confer a variety of features of the transformed phenotype when transfected into NIH 3T3 cells (24).

In this study, we demonstrate that in contrast to constitutive up-regulation in pVHL-defective cell lines, both CA9 and CA12 are strongly induced by hypoxia in a broad range of other cell types. The induction of CA9 by hypoxia was striking and has been studied in detail. We show that the CA9 promoter is tightly regulated by a HIF-responsive HRE close to its transcriptional start site, and that the gene product is expressed in a perinecrotic manner in many types of human cancer, overlapping with VEGF mRNA and the hypoxia marker pimonidazole. In keeping with constitutive activation of HIF after inactivation of pVHL, the focal pattern of expression observed in most tumors contrasted with that observed in RCCs, where CA IX was globally up-regulated. Our findings define a new biochemical pathway that is regulated by HIF, suggest that CA IX may be a useful marker for HIF activation either by microenvironmental hypoxia or genetic events such as VHL inactivation, and provide additional insights into mechanisms by which the HIF pathway might mediate effects on tumor metabolism.

MATERIALS AND METHODS

Cell Lines. 786-0 cells expressing pVHL or empty vector were a gift from W. G. Kaelin (Dana-Farber Cancer Institute, Boston, MA). RCC4 cells expressing pVHL or empty vector and other RCC lines were as described (13). RT112 human bladder carcinoma cells were a gift from M. Knowles (Imperial Cancer Research Fund, Leeds, United Kingdom). A549, NCI-H460, HeLa, EJ28, MDA-MB-468, MDA-MB-435S, MDA-MB-231, HBL-100, T-47D, and U2 O-S lines were from American Type Culture Collection (ATCC). Cells were grown under standard conditions in DMEM supplemented with 10% fetal bovine serum.
were grown in DMEM (Sigma) supplemented with 10% FCS (Globepharm), l-glutamine (2 µM), penicillin (50 IU/ml), and streptomycin sulfate (50 µg/ml). Studies of inducible gene expression were performed on cells approaching confluence. Parallel incubations were performed on aliquots of cells in normoxia (humidified air with 5% CO2) and either hypoxia or DFO mesylate (100 µM; Sigma). Hypoxic conditions were generated in a Naco 7001 incubator (Precision Scientific) with 0.1% O2, 5% CO2, and balance N2, unless otherwise specified. Experimental exposures were performed in normal growth medium for 16 h.

**RNA Analysis.** Total RNA was extracted by a modified acid/guanidinium thiocyanate/phenol/chloroform method (RNAzol B; Cinna/Biotech Laboratories), dissolved in hybridization buffer (80% formamide, 40 mM PIPES, 400 mM sodium chloride, and 1 mM EDTA, pH 8) and analyzed by RPA. To generate radiolabeled probe templates, cDNA fragments of human CA9 (nucleotides 3632–3771, accession number Z54349) and CA12 (nucleotides 301–450, accession number AF037335) were amplified by PCR and ligated into pSP72 (Promega). DNA templates for generating 32P-labeled RNA probes were linearized for 16 h with BglII and transcribed with SP6 RNA polymerase. For CA9 and CA12, RPA was performed on 30 µg of total RNA, using an internal control assay for U6 small nuclear RNA as described (13).

**Construction of Reporter Plasmids.** To generate plasmids p-506 and p-173, sequences of the CA gene between -506 and +43 relative to the transcriptional start site were amplified by PCR from genomic DNA. PCR products were ligated into pGL3-basic, a promoterless and enhancerless luciferase expression vector (Promega). To generate plasmids p-36, MUT1, and MUT2, complementary oligonucleotides with ends corresponding to the 5' restriction cleavage overhangs of BglII and MluI were annealed and ligated into BglII/MluI-digested pG3L3-basic. Oligonucleotides (sense strand) were: p-36 (forward), 5'-gctctccgcccaccagctctgtttccattgca-CGTAACGCGCCACCGACCAAGCTTACACCGGTTTACAGCCCGTACACACCG-3'; MUT1 (forward), 5'-gctctccgc-CACCACCGCTTTGCAGCTGTTACACGCGCTGTTTTTACAGCCCGTACACACCG-3'; MUT2 (forward), 5'-gctctccgcccaccagctctgtttccattgca-aCGTAACGCGCCACCGACCAAGCTTACACCGGTTTACAGCCCGTACACACCG-3'. Nucleotides introduced for cloning are lowercase; mutations are underlined. All CA9 promoter sequences were confirmed by dideoxy sequence analysis.

**Transient Expression Assays.** Cells at ~70% confluence in 60-mm dishes were transfected with 1 µg of a luciferase reporter construct and 0.4 µg of control plasmid, pCMV-β-gal (Promega), using FuGENE 6 (Roche Diagnostic) according to the manufacturer’s instructions. Cells were then incubated at 20% O2 for 8 h, followed by 20% or 0.1% O2 for 16 h. Luciferase activity was determined in cell lysates using a commercial assay system (Promega) and a TD-20e luminometer (Turner Designs). β-gal activity in cell lysates was measured using o-nitrophenyl-β-D-galactopyranoside as substrate in a 0.1 M phosphate buffer (pH 7.0) containing 10 mM KCl, 1 mM MgSO4, and 30 mM β-mercaptoethanol. To correct for variable transfection efficiencies between experimental conditions, the luciferase:β-gal ratio was determined for each sample. For cotransfection assays, cells were also transfected with 0.1–1 µg each of pCDNA3/HIF-1α or pCDNA3/HIF-2α containing the entire human HIF-1α or HIF-2α open reading frame, respectively. Transfections were balanced with various amounts of pCDNA3 (Invitrogen) and pCDNA3/HIF-α such that all cells received the same total quantity of DNA.

**Cell Lysis and Immunoblotting.** Whole-cell protein extracts were prepared from tissue culture cells by 10-s homogenization in denaturing conditions as described (31). Whole-cell protein extracts were prepared from tumors by fine section of frozen tissue and 30-s homogenization in denaturing conditions. Products were prepared from tissue culture cells by 10-s homogenization in denaturing conditions.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue specimens were collected by standard surgical oncology procedures were obtained from the Pathology Department, John Radcliffe Hospital (Oxford, United Kingdom). Immunostaining of paraffin sections was performed after dehydrating 4-µm sections. For CA IX detection, endogenous peroxidase was blocked with 0.5% hydrogen peroxide in water for 30 min. To block, 10% normal human serum in TBS was applied for 15 min. M75 (see “Immunoblotting”; 1:50) was applied for 30 min at room temperature. Secondary polymer from Envision kit (DAKO) was applied for 30 min at room temperature. For pimonidazole detection, sections were digested with 0.01% Pronase (Sigma) in PBS for 30 min at 37°C. Endogenous peroxidase was blocked with 0.1% hydrogen peroxide in water for 30 min. To block, Protein Block (DAKO) was applied for 5 min. Anti-pimonidazole IgG1 antibody (Natural Pharmacia; 1:100) was applied for 1 h at room temperature. Biotinylated rabbit antimonozyme secondary (DAKO; 1:200) was applied for 1 h at room temperature. Visualization of CA IX and pimonidazole staining was performed by diaminobenzidine substrate. Slides were counterstained with hematoxylin before mounting in Aquamount (BDH). Substitution of primary antibody with PBS was used as a negative control for both antibodies.

CA IX and pimonidazole were studied in semiserial tissue sections. The percentage of tumor cells showing positive staining for CA IX or pimonidazole and the extent of overlap between these regions within each tissue section was assessed by light microscopy at low magnification by three observers (C. C. W., P. H. W., and H. T.) and a consensus was determined.

**In Situ mRNA Hybridization.** Specific localization of VEGF mRNA was accomplished by in situ hybridization using an antisense riboprobe. Briefly, pBluescript (Stratagene) containing 517 consecutive complementary nucleotides of the VEGF (439 consecutive nucleotides of which are complementary to VEGF165, VEGF189, and VEGF206) was linearized with EcoRV for 16 h at 37°C. Labeled transcripts were synthesized using T7 (antisense) and SP6 (sense) polymerase in the presence of [35S]UTP (>800 Ci/mmol; Amersham Pharmacia). The methods for pretreatment, hybridization, washing, and dipping of slides in Ilford K5 for autoradiography were as described for formalin-fixed, paraffin-embedded tissue (33). The presence of hybridizable mRNA in tissue sections was established in semiserial sections using an antisense β-actin probe. Hybridizations using a sense probe were used to control for nonspecific signal. Autoradiography was at 4°C (two exposures per section for VEGF visualization at 10 and 18 days), before developing in Kodak D19 and counterstaining by Giemsa’s method. Sections were examined under conventional and reflected light-/dark-field conditions.

**Pimonidazole Administration.** Patients with squamous or basal cell carcinomas of the skin and patients with newly diagnosed transitional cell bladder carcinoma were studied. Signed informed consent was obtained in all cases. Pimonidazole hydrochloride was selected as the hypoxia marker because of its high water solubility, chemical stability, efficient tumor uptake, and low toxicity. Patients received 500 mg/m2 of pimonidazole hydrochloride, 1-[2-hydroxy-3-piperidinyl]propyl]-2-nitroimidazole hydrochloride (Hyoxprobe) in 100 ml of normal saline i.v. over 20 min. This dose is 25% of the maximum tolerated dose (34). Patients with tumors in skin underwent incisional or Trucut biopsy under local anesthetic 2–24 h after pimonidazole infusion. Patients with bladder carcinoma underwent transurethral resection of the tumor under general anesthetic 2–24 h after pimonidazole infusion. Tissue samples were immediately placed in 10% neutral buffered formalin, protected from light, and then processed in formalin blocks.

**RESULTS**

**VHL-dependent Regulation of CA9 and CA12 mRNAs by Hypoxia.** Expression of mRNAs encoding CA9 and CA12 was analyzed by RPA. To confirm the previous report of down-regulation by pVHL (29), we first examined expression in the VHL-defective RCC line RCC4 and a stable transfectant expressing a human VHL cDNA (RCC4/VHL). In normoxic cells, both mRNAs were down-regulated by pVHL. However, when cells were exposed in parallel to normoxia or hypoxia (0.1% oxygen), induction by hypoxia was observed in RCC4/VHL cells, whereas in RCC4 cells the high level of expression in normoxia was unchanged by hypoxia (Fig. 1A). We also examined expression in other RCC lines that are either defective (KRL40, SKRC28, UMR20, and 786-0) or wild type (Caki-1) for VHL, and in a stable transfectant of 786-O expressing wild-type pVHL (WT 8). Representative results from three cell lines are illustrated in Fig. 1A. In the VHL-defective cells, both CA9 and CA12 were constitutively expressed and unresponsive to changes in oxygen tension. In the
HYPOXIC INDUCTION OF CARBONIC ANHYDRASES 9 AND 12

Fig. 1. Induction of CA9 and CA12 mRNA by hypoxia. Cells were exposed to either normoxia (N; 20% O₂) or hypoxia (H; 0.1% O₂) for 16 h. CA9 and CA12 mRNA was examined by RPA. A, induction by hypoxia in renal carcinoma-derived cell lines is VHL dependent. RCC4, SKRC28, and UMRC2 are VHL defective. RCC4/VHL is a pHVLS stable transfectant of RCC4. Caki-1 is VHL competent. B, induction by hypoxia in nonrenal-derived cell lines from indicated tissue type. C, comparison of induction by hypoxia and DFO (each applied for 16 h) in A549 cells. LC, signal from internal control assay for the constitutively expressed U6 small nuclear RNA.

wild-type VHL cell lines, both genes, when expressed, were induced by hypoxia.

To examine regulation by hypoxia across a range of cell types, we performed RPAs for CA9 and CA12 on mRNA samples from normoxic and hypoxic cultures of 11 additional cell lines derived from five different tissue types: bladder (RT112 and EJ-28), bone (U2 O-S), breast (HBL-100, MDA-MB-435S, MDA-MB-468, MDA-MB-231, and T-47D), cervical (HeLa), and lung (A549 and NCI-H460). With the exception of bladder cell lines RT112 and EJ-28, every cell type expressed one or both CA isoforms, and in each case where expression was observed, it was induced by hypoxia. The amplitude of induction by hypoxia was particularly high for CA9; mRNA levels were at or below the limit of detection in normoxia, yet strikingly induced by hypoxia. Representative illustrations of one cell line from each tissue type are depicted in Fig. 1B. Because many hypoxia-inducible genes are up-regulated by treatment of cells with the iron chelator DFO (35), we also tested the effect of DFO and found a similar induction of both CA9 and CA12 mRNA (Fig. 1C).

CA9 Promoter Analysis. To investigate the unusually tight regulation of CA9 mRNA by hypoxia, we tested for oxygen-dependent function of the CA9 promoter. In the first set of experiments, we tested luciferase reporter genes containing ~0.5 kb of CA9 5′ flanking sequences (~506 to +43) and a deletion to nucleotide ~173 (~173 to +43) in transiently transfected HeLa cells. Both constructs showed very low levels of activity in normoxic cells but were induced strongly by hypoxia (Fig. 2A). By contrast, a similar reporter linked to a minimal SV40 promoter showed no induction by hypoxia.

To test whether these responses were dependent on HIF-1, we performed further transfections using a CHO mutant cell (Ka13) that is functionally defective for the HIF-1α subunit and cannot form the HIF-1 transcriptional complex (36). In the CHO wild-type parental subline C4.5, the ~173 nucleotide promoter conferred 17-fold transcriptional induction by hypoxia. In contrast, in the HIF-1α-deficient Ka13 subline, this hypoxic induction was absent (Fig. 2B). Cotransfection of human HIF-1α restored hypoxia-inducible activity to the CA9 promoter in the Ka13 cells and increased normoxic activity in both C4.5 and Ka13 (Fig. 2C). In C4.5 and Ka13 cells at 0.1% O₂, luciferase expression was increased 16- and 17-fold, respectively, by cotransfection of human HIF-1α. Thus, hypoxia-inducible activity of the CA9 promoter is completely dependent on HIF-1 and strongly influenced by the level of HIF-1α. Activity of the CA9 promoter in Ka13 cells could also be restored by cotransfection of HIF-2α, although normoxic activity was higher and fold induction by hypoxic stimulation was reduced (data not shown).

Inspection of the CA9 5′ flanking sequences revealed a consensus HRE beginning 3 bp 5′ to the transcriptional start site, orantayed on the antisense strand, reading 5′-TACGTGCA-3′ (Fig. 2, left). To test the importance of this site, we constructed a CA9 minimal promoter containing this sequence (~36 to +14). This minimal promoter retained hypoxia-inducible activity in C4.5 cells but had no inducible activity in Ka13 cells (Fig. 2C). Absolute levels of activity were lower in comparison to the ~173 nucleotide promoter construct, being reduced ~8-fold, indicating that although sequences ~173 to ~36 amplified promoter activity, responsiveness to hypoxia was conveyed by the minimal sequence containing the HRE. To confirm the importance of this HRE, two mutations were made within its core (antisense strand): a 3-bp substitution from CGT → AAA (MUT1), and a single substitution of G → T (MUT2; Fig. 2, left). Both mutations completely ablated hypoxia-inducible activity, although basal activity was preserved or slightly increased for MUT1 (Fig. 2C).

Regulation of CA IX Protein by Oxygen. As a first step toward understanding the significance of hypoxia-inducible expression of CA9 mRNA, the effect of hypoxia was examined on CA IX protein levels in whole-cell extracts. Immunoblots of representative cells using anti-CA IX monoclonal antibody M75 are illustrated in Fig. 3A. Strikingly, induction of CA IX protein by hypoxia was observed in multiple cell lines, whereas the VHL-defective RCC4 cells showed constitutive up-regulation of CA IX protein. Thus, hypoxic up-regulation of CA9 mRNA is clearly reflected at the protein level. We next examined the response of CA IX to increasing degrees of hypoxia (Fig. 3B). The level of CA IX hypoxic induction after 16 h of exposure increased with decreasing oxygen tensions from 5 to 0.1%. Because the original description of CA IX was as an antigen induced by culture of cells at high density (37), we also compared the effects of culture at high density with those of hypoxia. In normoxic cultures of A549 cells, high density clearly induced CA IX; although the effect was considerably smaller than that of hypoxia (Fig. 3C).

CA IX Expression in Human Tumors. We next sought to determine whether regulation of CA9 by hypoxia in tissue culture cells was reflected in patterns of expression within naturally occurring human tumors. To confirm the specificity of M75 immunostaining in our laboratory, we first compared immunohistochemical staining with CA IX immunoblot signals in pellets of cultured cells. Pellets were prepared from normoxic cultures of RCC4 and RCC4/VHL cells and processed in parallel for whole-cell protein extraction and immunohistochemistry. In keeping with the immunoblotting results, immunostaining of these sections with M75 revealed strong membrane expression of CA IX in RCC4 cells (Fig. 4A) and no staining in...
normoxic RCC4/VHL cells (Fig. 4B). Then, we compared immuno-

staining and immunoblots of tissue extracts from similar regions of
tumor and normal tissue in four sets of paired samples from surgical
excisions of head and neck tumors. By immunostaining, CA IX
expression was low or absent in normal tissue surrounding the tumors
but was expressed at significant levels in each tumor specimen.
Results of immunoblot analysis correlated closely with immuno-

staining, signals being very low or undetectable in each normal tissue
sample, and correlated with the extent of CA IX immunostaining in
tumor samples (data not shown).

Of particular interest to regulation by hypoxia is the relationship of
CA IX expression to zones of tumor necrosis. This was first examined
in a series of nine tumors of head and neck, breast, and ovary, each of
which showed well-defined zones of necrosis. Three tumors of each
type were analyzed. In each specimen, a predominantly or even
exclusively perinecrotic expression pattern was observed for CA IX.
Representative sections from each tumor type are illustrated in Fig. 4.
Expression was localized to the cellular membrane. Tracing a line
from the necrotic center to adjacent viable cells revealed a gradient of
CA IX expression, with the highest levels observed in cells closest to
or within necrotic regions (Fig. 4, C–F).

Because pVHL inactivation leads to loss of CA9 regulation by
oxygen in cultured cells and is common in clear cell renal carcinoma
but not other renal tumors, we next compared expression patterns in
a second series of 35 clear cell renal tumors and eight papillary renal
tumors. Representative sections from a clear cell and a papillary tumor
are illustrated in Fig. 4, G and H. Expression patterns were markedly
different. In 33 of 35 clear cell tumors, (both sporadic and derived
from VHL syndrome patients), CA IX was expressed throughout

Fig. 3. Regulation of CA IX protein by hypoxia and cell density. Western blots of
whole-cell extracts using anti-CA IX monoclonal antibody M75 are shown. A, cells
exposed to either normoxia (N; 20% O2) or hypoxia (H; 0.1% O2) for 16 h. Expression is
constitutive in the VHL-defective cell line, RCC4, but inducible by hypoxia in RCC4/
VHL transfectants and a range of nonrenal cell lines. B, effects of graded hypoxia; A549
cells, 16 h exposure to the indicated oxygen concentration. C, comparison of induction by
increasing cell density and hypoxia; A549 cells, 16 h exposure to hypoxia.

Fig. 2. Functional analysis of human CA9 5′-flanking sequences in transient expression assays. Left panel, schematic diagram of reporter genes; the indicated CA9 wild-type and
mutant sequences were inserted 5′ to a promoterless luciferase reporter gene. Arrow, 5′
transcriptional initiation site. Underlined sequence, CA9 putative HRE. Right panels, reporter
gene activities in transiently transfected cells. CA9 promoter sequences are indicated to the
left of each column. SV40, control minimal SV40 promoter. A, activities in normoxic and
hypoxic HeLa cells. B and C, activities in wild-type CHO (C4.5) cells (columns 1) and HIF-1α-deficient CHO (Ka13) cells (columns 2). A, hypoxia-inducible activity of the CA9
promoter. B, hypoxia-inducible activity of the CA9 promoter is ablated in Ka13 cells. Cotransfection of HIF-1α restores induction by hypoxia in Ka13 cells and augments CA9 promoter
activity in both wild-type and Ka13 cells. In comparison, minimal effects are seen on the SV40 promoter. C, a minimal CA9 promoter retains HIF-1α-dependent, hypoxia-inducible activity.
Two mutations within the putative HRE, MUT1 and MUT2, completely ablate hypoxia-inducible activity, whereas basal transcription is preserved. Columns, mean luciferase activities
corrected for transfection efficiency from a typical experiment performed in duplicate. Each duplicate experiment was repeated two to six times. Numbers to the right are the ratios of hypoxic to normoxic expression of the indicated reporter construct. Transfected cells were incubated at 20% O2 for 8 h and then incubated at 20% O2 (normoxia) or 0.1%
O2 (hypoxia) for 16 h.
tumor tissue; strong membrane staining was observed in tumor cells, regardless of proximity to necrosis or vessels (G). In contrast, in papillary renal tumors CA IX immunostaining was much less evident but was observed in tumors containing areas of necrosis, where, as with the nonrenal tumors, staining was strikingly focal and perinecrotic (four of eight papillary tumors contained necrosis, and all four showed focal CA IX positivity; Fig. 4H). Thus, the tight regulation of CA9 expression by oxygen observed in cell culture appeared to be reflected in strikingly focal patterns of expression around areas of necrosis.

Relationship of CA IX Expression with an Endogenous and an Administered Hypoxia Marker in Human Tumors. To compare CA IX expression with potential markers of tumor hypoxia, we examined expression of VEGF mRNA and activation of the bioreductive hypoxia marker pimonidazole in relationship to CA IX staining. Serial sections of a subset of our first series of tumors were analyzed for VEGF mRNA expression by in situ hybridization, and CA IX expression was analyzed by immunostaining. Representative views from an ovarian and head and neck tumor sample are illustrated in Fig. 5. VEGF mRNA was expressed at varying levels throughout tumor tissue but was increased greatly in regions adjacent to necrosis. CA IX immunostaining showed strong overlap but was more tightly limited to perinecrotic regions.

For comparison of pimonidazole staining with CA IX expression, a series of 14 transitional cell bladder carcinomas and 6 squamous or basal cell skin carcinomas derived from patients who had received pimonidazole prior to surgical excision of tumor tissue was analyzed. Representative views of pimonidazole- and CA IX-stained sections are illustrated in Fig. 6, and assessment of pimonidazole and CA IX staining with corresponding overlap for each tumor biopsy are indicated in Table 1. In most tumors (16 of 20), pimonidazole staining was more extensive across tumor sections than CA IX staining, being primarily banded around necrotic areas (Fig. 6, C and E) and the periphery of papillary structures in bladder carcinomas (Fig. 6A).
Although less extensive, the large majority of CA IX immunostaining localized within regions of pimonidazole adduct formation and was also associated with necrosis (Fig. 6, D and F) or the periphery of papillary structures in bladder carcinomas (Fig. 6 B). Some regions containing CA IX were observed that were slightly farther removed from necrosis than regions staining positive for pimonidazole (Fig. 6, C and D). In 4 of 20 cases, CA IX staining was more extensive than pimonidazole staining. In these cases, in addition to the characteristic perinecrotic and peripheral papillary expression, a proportion of CA IX expression was not obviously associated with such regions in the plane of the section. Nevertheless, within these four tumors the pimonidazole-positive regions were consistently localized within regions of CA IX positivity, again demonstrating the overlap between these markers. Despite the relationship between pimonidazole and CA IX at the microscopic level in all tumors, we did not observe an overall correlation between the percentage of tumor stained for pimonidazole and CA IX (Table 1).

DISCUSSION

In this work, we have demonstrated that the tumor-associated CAs CA9 and CA12 are strongly inducible by hypoxia in a broad range of tumor cells. Our findings also explain up-regulation of these CA isoforms in VHL-defective renal tumors, indicating that they are expressed constitutively at a high level in VHL-defective cells as a consequence of constitutive activation of HIF. The work therefore extends the range of HIF target genes to a new class of molecule that may have important implications for understanding the consequences of microenvironmental tumor hypoxia, as well as the tumor-promoting effects of VHL inactivation. The regulation of CA9 was particularly tightly controlled by oxygen, and we analyzed the hypoxia-inducible response of this gene in detail.

Studies of the CA9 promoter demonstrated that sequences close to the transcriptional initiation site were sufficient to convey a hypoxia-inducible response, that this activity was mediated by HIF, and that it was dependent on a consensus HRE lying adjacent to the initiation site. The CA9 promoter contains neither a TATA box nor a consensus initiator sequence at the cap site (38). The association of this unusual anatomy with tight regulation by hypoxia is therefore of interest and suggests that it may be informative to pursue the mechanism by which HIF interacts with the basal transcriptional machinery operating on this gene. Furthermore, irrespective of the mechanism, the strong inducibility conveyed by the minimal CA9 promoter is unusual and may itself be of utility, for instance in the refinement of gene therapy vectors seeking to target therapeutic gene expression to hypoxic regions of tumors (39, 40).

Our findings also raise a number of issues relevant to recently published analyses of the CA9 promoter that did not examine the effect of hypoxia: (a) they provide an explanation for the remarkably low levels of CA9 promoter activity recently reported under standard culture conditions (41), because promoter activity is so strongly dependent on hypoxia; (b) they are consistent with the positive activity demonstrated for sequences −173 to +31 (41) and show that the transcriptional effects mediated by these sequences interact with the HRE in the minimal promoter to amplify the response to hypoxia; (c)
they are consistent with the absence of a DNase I footprint in the region of the HRE (41), because even in hypoxia it has been shown that HIF-1 binding characteristics are such that an in vitro footprint is not demonstrated (42); (d) they provide a potential explanation for the repressive effects of p53 expression on the activity of the CA9 promoter in some cells (43), because it has been suggested that p53 can interact with the regulation of HIF-1α stability so as to reduce activity of the HIF/HRE complex (15, 16).

In tissue culture, CA9 demonstrated a very marked difference between constitutive expression in VHL-defective RCC cells and strong induction by hypoxia in cells known or presumed to be VHL competent. This provided an opportunity to determine the extent to which these contrasting patterns of regulation in culture were reflected in patterns of expression within native tumors. In our series of renal tumors, we found a striking contrast between generalized expression in clear cell carcinomas, which are usually defective in VHL, and focal perinecrotic expression in papillary renal tumors, which are usually wild type for VHL. Notwithstanding the absence of direct ascertainment of VHL genotype in all of the tumors analyzed, this strongly suggests that effects of VHL status on HIF-dependent, hypoxia-inducible gene expression are reflected in patterns of expression within native tumors. Up-regulation by constitutively active HIF therefore provides an explanation for the utility of CA9 as a marker for clear cell carcinoma. The pattern of diffuse expression in clear cell carcinoma is in agreement with findings of a previous analysis of CA IX expression in which the authors focused on high levels of expression in clear cell carcinoma versus absent expression in a variety of benign lesions and postulated that CA IX expression might be useful as a marker of malignant change (25). That study also noted focal expression in papillary renal carcinoma, although the authors did not comment on the relation to necrosis. In our studies, we found that the striking localization of focal CA IX expression to zones of necrosis is not just observed in papillary renal carcinoma but also in several series of nonrenal tumors. The pattern is similar to that first described for VEGF mRNA (5), and we compared directly the pattern of CA IX immunostaining with that of in situ mRNA hybridization for VEGF in several types of tumors. In this work, we used in situ mRNA hybridization for VEGF to localize the site of production, because, in contrast with CA IX, some isoforms of VEGF are secreted. Patterns of expression for CA IX and VEGF mRNA were clearly concordant. However, CA IX expression was more strikingly delimited, being essentially limited to regions surrounding zones of necrosis.

The concordance of hypoxia-inducible versus constitutive patterns of expression in tissue culture with focal perinecrotic versus diffuse patterns of expression in tumors strongly supports the view that the focal perinecrotic pattern of expression is driven by microenviron-
mental hypoxia. Furthermore, the particularly tight regulation of CA9 by hypoxia suggested that it might be useful as a hypoxia marker. It was, therefore, of interest to compare the pattern of CA IX immunostaining with staining for the hypoxia marker pimonidazole (44–46). Our analysis demonstrated clear overlap of the staining patterns, supporting expression of CA IX in hypoxic regions. Previous studies have compared the distribution of immunodetectable pimonidazole adducts with VEGF immunostaining. One study concluded that pimonidazole and VEGF displayed the same pattern of staining on adjacent sections during the angiogenesis associated with a model of liver fibrogenesis (47), whereas an earlier study emphasized the discrepancies between pimonidazole and VEGF staining, although regions of overlap were demonstrated (48). Among the explanations considered for the differences between the distribution of VEGF staining and pimonidazole adducts were regulation of VEGF by nonhypoxic stimuli and diffusion of VEGF from hypoxic sites of production. For CA9, basal expression in normoxic cells was lower than we have observed for VEGF, induction by hypoxia was more striking, and the protein was not secreted. Despite this, we also observed differences in pimonidazole and CA IX staining. The substantial regions of overlap presumably reflect regions where tumor hypoxia was of sufficient duration and severity to activate both markers. Regions of nonoverlap could reflect the operation of additional positive or negative influences on expression or activation or different time frames of induction or activation. For instance, pimonidazole adducts are formed over a relatively short period of time and are then long-lived (45, 46), whereas we have found that CA IX is a stable protein that, in tissue culture, accumulated over a long period of hypoxia (data not shown). Thus, CA IX induction might only be expected in regions of relatively chronic tumor hypoxia and would reflect a different hypoxic time frame from pimonidazole activation. Correlation of focal CA IX expression with direct measurements of tumor oxygenation and with clinical parameters of outcome will be of interest.

The demonstration that an extracellular CA is up-regulated by microenvironmental tumor hypoxia has potentially important implications for understanding the regulation of tumor pH and the response to hypoxia. It has been widely held that lactate production by glycolysis is a major cause of the acidic extracellular pH of tumors (49), and indeed glycolytic enzymes are induced by hypoxia (11), as is lactate production (50). However, tumors grown from mutant cells with glycolytic defects show a similar extracellular acidosis in the absence of lactate accumulation (51, 52), indicating that other mechanisms must be involved. Recently, it has been proposed that extracellular CAs could convert CO2 diffusing from oxygenated areas to carbonic acid and promote the generation of bicarbonate and hydrogen ions (29, 52). Bicarbonate might then be exchanged for intracellular chloride, providing a mechanism for maintaining the characteristic extracellular acidosis and intracellular alkalosis that is postulated to promote tumor growth (53). Thus, it is likely that the hypoxia-inducible behavior of tumor-associated CAs could exert important biological effects through an influence on microenvironmental pH. This could have therapeutic implications because CA inhibitors have been shown to inhibit the invasion of renal cell carcinoma lines in model culture systems (54) and have synergistic effects with other chemotherapeutic agents in animal models (55). The potential for strong induction by hypoxia will now need to be considered in assessing the diagnostic and therapeutic implications of tumor-associated extracellular CAs.

ACKNOWLEDGMENTS

We thank Richard Poulsom, Rosemary Jeffery, and Jan Longcroft (Imperial Cancer Research Fund, Lincoln’s Inn Fields, London, United Kingdom) for assistance with the in situ hybridization studies.

REFERENCES


7083
Hypoxia-inducible Expression of Tumor-associated Carbonic Anhydrases

Charles C. Wykoff, Nigel J. P. Beasley, Peter H. Watson, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://cancerres.aacrjournals.org/content/60/24/7075">http://cancerres.aacrjournals.org/content/60/24/7075</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Cited articles</th>
<th>This article cites 53 articles, 28 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/60/24/7075.full.html#ref-list-1">http://cancerres.aacrjournals.org/content/60/24/7075.full.html#ref-list-1</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Citing articles</th>
<th>This article has been cited by 100 HighWire-hosted articles. Access the articles at: /content/60/24/7075.full.html#related-urls</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Reprints and Subscriptions</th>
<th>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Permissions</th>
<th>To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a>.</th>
</tr>
</thead>
</table>