ABSTRACT

The microenvironment of rapidly growing tumors is associated with increased energy demand and diminished vascular supply, resulting in focal areas of prominent hypoxia. A number of hypoxia-responsive genes have been associated with growing tumors, and here we demonstrate that the multidrug resistance (MDR1) gene product P-glycoprotein, a Mr ~170,000 transmembrane protein associated with tumor resistance to chemotherapeutics, is induced by ambient hypoxia. Initial studies using quantitative microarray analysis of RNA revealed an ~7-fold increase in MDR in epithelial cells exposed to hypoxia (pO₂, 20 torr, 18 h). These findings were further confirmed at the mRNA and protein level. P-Glycoprotein function was studied by analysis of verapamil-inhibitable efflux of digoxin and rhodamine 123 in intact T84 cells and revealed that hypoxia enhances P-glycoprotein function by as much as 7 ~0.4-fold over normoxia. Subsequent studies confirmed hypoxia-elicited MDR1 gene induction and increased P-glycoprotein expression in nontransformed, primary cultures of human microvascular endothelial cells, and analysis of multicellular spheroids subjected to hypoxia revealed increased resistance to doxorubicin. Examination of the MDR1 gene identified a binding site for hypoxia inducible factor-1 (HIF-1), and inhibition of HIF-1 expression by antisense oligonucleotides resulted in significant inhibition of hypoxia-inducible MDR1 expression and a nearly complete loss of basal MDR1 expression. Studies using luciferase promoter constructs revealed a significant increase in activity in cells subjected to hypoxia, and such hypoxia inducibility was lost in truncated constructs lacking the HIF-1 site and in HIF-1 binding site mutants. Extensions of these studies also identified a role for Sp1 in this hypoxia response. Taken together, these data indicate that the MDR1 gene is hypoxia responsive, and such results may identify hypoxia-elicited P-glycoprotein expression as a pathway for resistance of some tumors to chemotherapeutics.

INTRODUCTION

A major obstacle in the development of effective cancer chemotherapy is tumor development of the MDR phenotype (1). The MDR phenotype is generally considered to be acquired after administration of chemotherapeutic agents and is most prevalent in acute myelogenous leukemia and in aggressive carcinomas (e.g., breast and ovarian). The MDR phenotype is associated with the induction of the MDR1 gene, which encodes for and results in overexpression of P-gp. P-gp is a Mr ~170,000 member of the ABC-type transporter family and functions as an energy-dependent membrane efflux pump that transports a wide variety of structurally unrelated xenobiotics to maintain cytoplasmic concentrations at subtoxic levels (2). MDR1 is expressed in some normal cell types (e.g., intestinal epithelia and lymphocytes), but overexpression of P-gp has been shown to correlate with multidrug resistance, and increased P-gp expression has been demonstrated in numerous multidrug-resistant cell lines (3). At present, it is not fully understood how P-gp overexpression is mediated in cancer; however, a number of studies have suggested that transcriptional mechanism(s) of MDR1 induction in human tumors is complex (2, 3). For example, the cloned MDR1 promoter bears binding sites for a number of transcription factors, including Sp1, NF-Y, and YB-1 (4). Similarly, it was demonstrated recently that negative regulation of MDR1 is mediated by the p65 subunit of NF-κB in complex with c-fos (5).

Many types of human tumors are significantly oxygen deprived (6). Because of the combination of tissue mass and the particularly high rate of glycolysis in tumor cells (termed the Warburg effect), hypoxia is considered a property of many tumor types (6). Although most tissues of the body maintain an oxygen gradient spanning a distance of approximately 300–400 μm, studies assessing relative oxygen tensions within tumors have suggested that oxygen concentrations may be as much as 10–100-fold decreased at comparable distances from capillary blood supplies (6). Such an environment establishes a setting of concentric areas of chronic hypoxia within tumors and results in the transcriptional induction of numerous hypoxia-responsive genes, including glycolytic enzymes, proangiogenic factors, and proinflammatory genes (7).

Among other transcriptional pathways, hypoxia is known to induce HIF-1, a member of the rapidly growing Per-ARNT-Sim family of basic helix-loop-helix transcription factors (8). HIF-1 exists as an αβ heterodimer, the activation of which is dependent upon stabilization of an O₂-dependent degradation domain of the α subunit by the ubiquitin-proteasome pathway (9). Although not clear, HIF-1 appears to reside in the cytoplasm of normoxic cells, and similar to a number of other transcription factors (e.g., NF-κB and β-catenin), HIF-1 translocates to the nucleus to form a functional complex (10, 11). Binding of HIF-1 to the consensus domain of a number of genes results in the transcriptional induction of HIF-1-bearing gene promoters (7). HIF-1 is widely expressed, and consensus HIF-1 binding sequences exist in a number of genes and are termed HREs (7).

Because hypoxia is a feature of many tumor types and MDR1 gene induction occurs in this setting, we hypothesized that MDR1 may represent a hypoxia-responsive gene. Our analysis revealed that ambient hypoxia induces MDR1 gene expression and concomitant functional P-gp expression in both transformed epithelia and primary cultured endothelia. Moreover, these data revealed the existence of a previously unappreciated, functional HRE in the MDR1 gene. Taken together, these data suggest that hypoxia-induced P-gp expression may represent a pathway for resistance of some tumors to chemotherapeutics.

MATERIALS AND METHODS

Growth and Maintenance of Cell Lines. T84 cells and Caco-2/BE cells were grown as monolayers as described previously (12–14). For all experi-
ments, cells were grown to confluence on polycarbonate permeable supports, because studies of MDR1 mRNA and P-gp protein expression were maximal compared with those grown on either plastic or glass substrates (data not shown). It is likely that this reflects the polarized nature of P-gp expression [15]. HMVEC, a primary endothelial cell culture isolated from adult dermis, were obtained from Cascade Biologies (Portland, OR) and cultured as described previously [16]. Immunortalized human keratocytes (OKF6 cells) were grown and cultured as described previously [17] and were a kind gift from Dr. James Rheinwald (Brigham and Women’s Hospital and Harvard Medical School). Where indicated, bovine aortic endothelial cells were used as reported previously [18].

Monolayer Exposure to Hypoxia. Cultured cell exposure to hypoxia was performed as described previously [12]. Upon entry into the humidified hypoxic chamber (Coy Laboratory Products, Ann Arbor, MI), cell medium was exchanged with pre-equilibrated hypoxic medium. Standard hypoxic conditions [based on previous work (12, 19)] were pO2 20 torr, pCO2 35 torr, with the balance made up of nitrogen and water vapor. Normoxic controls were cells exposed to the same experimental protocols under conditions of atmospheric oxygen concentrations (pO2 147 torr and pCO2 35 torr) within a tissue culture incubator.

Analysis of mRNA Levels by RT-PCR. The transcriptional profile of epithelial cells exposed to ambient hypoxia was assessed in RNA derived from control or hypoxic epithelia (T84 cells at 6 or 18 h hypoxia) using quantitative genechip expression arrays (Affymetrix, Inc., Santa Clara, CA; Ref. 20). RT-PCR analysis of mRNA levels was performed using DNase-treated total RNA as described previously [14] using primers specific for MDR1 (forward primer 5′-AAG GGC CAG CAC ATT CC-3′ and reverse primer 5′-AGG CCT GGT GCA AAG AGG-3′, 180-bp fragment), HIF-1α (forward primer 5′-CTC AAA GTC GGA CAG CTC CA-3′ and reverse primer 5′-CCA TGC AGT TTT CTG CT-3′, 460-bp fragment), or control β-actin (forward primer 5′-ATG ACT TCC AAC CTG GCC GTG GGT C3′ and antisense primer 5′-TCT CAG CCC TCT TCA AAA ACT TCT C3′, 661 bp fragment). Each primer set was amplified using an optimized number of PCR cycles (25 cycles unless otherwise noted) of 94 °C for 1 min, 60 °C for 2 min, 72 °C for 7 min. The PCR reactions were then visualized on a 1.5% agarose gel containing 5 μg/ml of ethidium bromide.

Western Blotting. After experimental treatment, proteins were isolated from confluent monolayers from 100-mm Petri dishes as described before [14]. Proteins were measured (DC protein assay; Bio-Rad, Hercules, CA), and samples (25 μg/lane, as indicated) were resolved by reducing SDS-PAGE, transferred to nitrocellulose, and blocked overnight in blocking buffer (250 mM NaCl, 0.02% Tween 20, 5% goat serum, and 3% BSA). For Western blotting, anti-P-gp (rabbit polyclonal antibody; Biogenesis, Poole, United Kingdom), anti-Sp1 (mouse mAb; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-β-actin (Santa Cruz Biotechnology) was added for 3 h; blots were washed, and species-matched peroxidase-conjugated secondary antibody was added, as described previously [14]. Labeled bands from washed blots were detected by ECL (Amersham).

P-gp Surface Expression. Surface expression of P-gp was analyzed by ELISA on intact endothelia or epithelia, using methodologies described previously [18]. Briefly, cells subjected to indicated periods of hypoxia or normoxia were washed with HBSS (Sigma Chemical Co., St. Louis, MO), blocked with medium for 30 min at 4°C. Anti-P-gp mAb (clone 3201, which recognizes a cell surface epitope; QED Biosciences; used as purified mAb at 20 μg/ml) was added to cells and allowed to incubate for 2 h at 4°C. Where indicated, mAb to MHC class I (Ref. 21; clone W6/32 obtained from the American Type Culture Collection, used as 1:200 diluted ascitic fluid) was used as a control. After washing with HBSS, a peroxidase-conjugated sheep antiserum secondary antibody (Cappel, West Chester, PA) was added. Secondary antibody (final dilution, 1:1000) was diluted in medium containing 10% fetal bovine serum. After washing, plates were developed by addition of peroxidase substrate [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 1 mM final concentration; Sigma] and read on a microtiter plate spectrophotometer at 405 nm ( Molecular Devices, Framingham, MA). Controls consisted of medium only and secondary antibody only. Data are presented as the mean ± SE absorbance at 405 nm (background subtracted).

P-gp Functional Analysis. P-gp function was assessed as verapamil-inhibitable efflux of digoxin. Briefly, epithelial monolayers were exposed to experimental conditions, washed with HBSS, and incubated with digoxin (final concentration, 6 μM; based on pilot experiments) in the absence or presence of verapamil (concentration range, 1–100 μM; Sigma). Cells were incubated for 60 min (based on pilot experiments; data not shown) at 37°C. Cells were washed with HBSS and cooled to 4°C. For digoxin determinations, cells were lysed in ice-cold H2O2, and lysates were cleared by Eppendorf centrifugation at 14,000 × g for 10 min. Digoxin levels in supernatants were assessed by HPLC analysis [22] using a Hewlett-Packard HPLC (model 1050) with a HP 1100 diode array detector and a reverse phase HPLC column (Luna 5 μm 150 × 4.60-mm; Phenomenex, Torrance, CA). Digoxin was measured with a 20–80% CH3C/H2O2 elution gradient over 30 min at 1 ml/min. Absorbance was measured at 220 nm. UV absorption spectra were obtained at chromatographic peaks. Digoxin was identified by chromatographic behavior (e.g., UV absorption maxima and co-chromatography standards; Ref. 22). Controls included monolayers incubated at 4°C for the entire period of the assay, and digoxin levels derived from these controls were used to normalize data.

In subsets of experiments, efflux of rhodamine 123 (Sigma) was used for functional analysis of P-gp (23). Experimentally treated cells were incubated with rhodamine 123 (final concentration, 10 μM) and washed three times with HBSS, and intracellular rhodamine fluorescence was assessed on a fluorescent plate reader (Cytofluor 2300, Millipore, Inc., Bedford, MA) after 1-h incubation at 37°C. Monolayers not exposed to rhodamine 123 were used to determine background fluorescence.

Multicellular Spheroid Model. A multicellular spheroid model was developed using KB cells grown at high density on membrane-permeable supports. Briefly, KB cells in suspension were plated on high density (−105 cells/cm2) on 0.33-cm2 collagen-coated permeable supports (Corning-Costar, Cambridge, MA) and allowed to grow as domes on these substrates for 2 weeks. Medium was replaced every other day and at 2 weeks, multicellular spheroids were subjected to a 24- or 48-h period of hypoxia or normoxia (as described above), in the presence or absence of the P-gp substrate, verapamil (Sigma; final concentration, 1 μM). At the termination of the experiment, the number of viable, intact cells was determined by measurement of the amount of esterase cleavable fluorescent marker BCECF-AM (final concentration, 5 μM; Calbiochem, San Diego, CA) retained over a 30-min period, a sensitive measurement cytotoxicity (24). Multicellular spheroids were washed three times in HBSS, and fluorescence intensity was measured on a fluorescent plate reader as described above. In subsets of experiments to examine the structure of multicellular spheroids, cultures were stained with rhodamine phalloidin, and confocal laser microscopy was used to image multicellular spheroids in the X-Y and X-Z planes, as described previously (25).

Antisense Oligonucleotide Treatment of Epithelia. HIF-1α depletion in epithelial cells was accomplished by using antisense oligonucleotide loading as described previously (26) using phosphorothiate derivatives of antisense (5′-GCC GGC GCC CTC CAT-3′) or control sense (5′-ATG ACT TCC AAG CTG GCC GTG GGT C3′) oligonucleotides. Sp1 depletion by antisense was accomplished as described before using antisense (5′-ATA TTA GGC ATC ACT TCA AGG CC-3′) or control sense (5′-CCT GGA GTG ATG CCT AAT AT-3′) oligonucleotides (27). Epithelial cells were washed in serum-free medium and then in medium containing 20 μg/ml Effectene transfection reagent (Qiagen, Inc., Valencia, CA) with 2 μg/ml HIF-1α antisense or sense oligonucleotide. Cells were incubated for 4 h at 37°C and then replaced with serum containing growth medium. Treated cells were exposed to hypoxia or normoxia for indicated periods of time. As indicated, MDR1 or HIF-1α mRNA were quantified by RT-PCR as described above (see “Transcriptional Analysis”) or by Western blot.

MDR1 Reporter Assays. Caco2 or BAE cells, as indicated, were used here to assess MDR1 inducibility by hypoxia. Plasmids expressing sequence corresponding to wild-type MDR1 (−189 to +133), truncations at the 3′ end (−189 to +4), truncations at the 5′ end (−2 to +133), or internal truncations (−119 to +4) of the MDR1 promoter (a kind gift from Drs. Martin Haas and Bryan Strauss, University of California, San Diego, CA) were cotransfected with β-galactosidase plasmids (p-Hook-2; Invitrogen) using standard methods of overnight transfection using Effectene transfection reagent (Qiagen). In subsets of experiments, cells were cotransfected with a promoterless vector (pGL3-Basic; Promega Corp., Madison, WI) to control for background luciferase activity. After transfection, cells were subjected to hypoxia or normoxia for 24 h. Luciferase activity was assessed (Topcount-
HYPOXIA INDUCES MDR1

RESULTS

Hypoxia Induces MDR1 and Functional P-gp. A transcriptional profiling approach was used to identify potential hypoxia-regulated gene expression in model epithelia (T84 cells). Microarray analysis (20) identified a hypoxia time-dependent induction of the MDR1 gene (2.2- and 7.1-fold increase over control normoxia at 6 and 18 h, respectively). Similar exposure to the proinflammatory cytokine IFN-γ, which we have shown to regulate a number of genes in a fashion similar to hypoxia (14, 30), did not induce MDR1 (Fig. 1A), thus providing some degree of hypoxia selectivity. Semiquantitative RT-PCR analysis (comparison of MDR1 and control NXT; Hewlett-Packard) using a luciferase assay kit (Stratagene). All luciferase activity was normalized with respect to a constitutively expressed β-galactosidase reporter gene.

In subsets of experiments, HIF-1 binding site mutations were introduced in 3' end (−189 to +4) truncations of the wide-type promoter using the GeneEditor in vitro site-directed mutagenesis system (Promega). Briefly, mutations encoding a three-nucleotide mutation in the MDR1 HIF-1 binding site (consensus motif 5'-GCCGTG-3' mutated to 5'-GCCAT-3') within HIF-1 site located at positions −49 to −45 relative to the transcription start site (28, 29)] by PCR introduced a unique NCO1 cleavage site and allowed us to screen mutations based on enzymatic cleavage of plasmid DNA. Oligonucleotides used for the three-nucleotide mutation were (mutated sequence in lowercase) 5'-AGG ACA AGC GCC GGG GGC atG GCT GAG CAC AGC GCC-3'. A deletional mutation of the HIF-1 site was also generated using the oligonucleotide 5'-AGG AAG GCC GCC GCC CTG AGC ACA GCC-3'. All mutations were confirmed by sequencing using pGL2-basic primers. Hypoxia inducibility in transient transfectants using such mutated luciferase constructs was exactly as described above.

HIF-1 site-locating region was identified by microarray analysis and the mutant designations M and H (reflected as a decrease in intracellular rhodamine over time) was used as an external epitope on P-gp (32). As shown in Fig. 3, cellular P-gp expression is reflected at the functional level. To examine whether cell surface expression of P-gp was evident, a whole cell ELISA technique was adopted using mAb 3201, which recognizes an external epitope on P-gp (3). As shown in Fig. 2D, Caco2 cells subjected to hypoxia demonstrated an enhanced efflux (reflected as decreased accumulation) of digoxin (Fig. 2, A and C; P < 0.025 by ANOVA). The addition of increasing concentrations of verapamil (range, 10–100 μM) resulted in increased intracellular accumulation of digoxin but to a lesser extent in cells pre-exposed to 48 h of hypoxia. As an additional functional assay, efflux of the P-gp substrate rhodamine 123 was used. As shown in Fig. 2D, Caco2 cells subjected to hypoxia demonstrated significantly increased efflux capacity (reflected as a decrease in intracellular rhodamine 123 over those in normoxia; P < 0.01). Taken together, such data indicate that increased expression of P-gp is reflected at the functional level.

To examine whether cell surface expression of P-gp was evident, a whole cell ELISA technique was adopted using mAb 3201, which recognizes an external epitope on P-gp (32). As shown in Fig. 3A, increasing periods of time in hypoxia resulted in increased detectable P-gp (P < 0.01 by ANOVA) but no obvious change in MHC class I
expression ($P = $ not significant). Similarly, staining of endothelial cells subjected to hypoxia revealed a time-dependent increase in P-gp expression (Fig. 3B).

To better approximate the hypoxia response in a three-dimensional structure (33), a multicellular spheroid model was developed and examined for sensitivity to the P-gp cytotoxic substrate doxorubicin (3). As shown in Fig. 4, confocal imaging of multicellular spheroids (X-Y quadrant shown in Fig. 4A) revealed a dome-like appearance of cell clusters in the X-Z orientation (Fig. 4B). Such multicellular spheroids were then used to examine toxicity of the chemotherapeutic agent and P-gp substrate doxorubicin. As shown in Fig. 4C, the degree of cell death (measured as the number of intact cells and reflected by increased BCECF labeling) was significantly decreased in cells subjected to either 24-h ($P < 0.01$) or 48-h ($P < 0.001$) periods of hypoxia. Similar results were obtained in monolayer grown cells (41 ± 8.2% increase in BCECF labeling in 48-h hypoxia-exposed KB cells treated with doxorubicin; $P < 0.025$). Taken together, these data indicate that hypoxia provides a stimulus for the induction of the $MDR1$ gene as well as an increase in functional, surface-expressed P-gp.

Role of HIF-1 in $MDR1$ Induction. In an attempt to gain specific insight into the mechanisms of $MDR1$ induction, we began examining induction pathways from hypoxia response genes. In the course of our experiments, we identified a previously unappreciated HIF-1 binding site in the $MDR1$ gene promoter (DNA consensus motif 5'-GCGTG-3' located at positions −49 to −45 relative to the transcription start site; Refs. 28, 29) and a HIF-1 ancillary sequence (5'-CACAG-3'; Ref. 34) located 8 bp in the 3' direction (nucleotides −37 to −33). Two approaches were used to analyze the role of HIF-1 in hypoxia-inducible $MDR1$ expression:

(a) Antisense oligonucleotides directed against HIF-1α were used to block HIF-1α expression (Fig. 5A), and the influence on $MDR1$ induction in hypoxia was assessed. As can be seen in Fig. 5, the directed loss of HIF-1α resulted in the significant down-regulation of basal i.e., (normoxic cells) $MDR1$ mRNA expression (97% loss by densitometry) and a nearly complete loss of $MDR1$ hypoxia inducibility (98 and 91% decrease at 6 and 18 h of hypoxia, respectively; Fig. 5B).

(b) As a second approach, luciferase reporter constructs expressing varied lengths of the $MDR1$ promoter were used to address hypoxia inducibility, and specifically, the role of HIF-1. As shown in Fig. 6, truncations in the 5' end (inclusive sequence of −189 to +133) showed a 2.5 ± 0.3-fold increase in luciferase activity over normoxic controls when compared with cells subjected to 24-h hypoxia ($P < 0.01$). Truncations at the 3' end (inclusive sequence of −189 to +4, 3'Δ$MDR$) did not significantly influence luciferase expression in normoxia and did not influence hypoxia inducibility ($P = $ not significant). Likewise, truncations at both the 3' and 5' end (inclusive sequence of −119 to +4, 4Δ$MDR$) did not influence expression in normoxia or hypoxia inducibility. However, as shown in Fig. 6, truncations in the 5' region (inclusive sequence of −2 to +133, 5'Δ$MDR$), which deletes the HIF-1α binding site at positions −49 to −45, resulted in a nearly complete loss of hypoxia inducibility ($P < 0.001$ compared with the wild-type

Fig. 3. P-gp induction by hypoxia. In A, T84 cells were grown to confluence on polycarbonate supports. Anti-P-gp monoclonal Ab or control anti-MHC class I was added to the, and P-gp surface levels were assessed by ELISA. Data are means from three separate experiments; bars, SE. B, immunofluorescence localization of P-gp on permeabilized endothelial cells exposed to the indicated periods of hypoxia (0–48 h). CTL, an isotype-matched control for autofluorescence. Representative experiment from three.

Fig. 4. Influence of hypoxia on multicellular spheroids. KB cell multicellular spheroids were developed on polycarbonate permeable supports as described in “Materials and Methods.” A, a quadrant X-Y axis image of rhodamine phallolidin-labeled multicellular using confocal microscopy. B, X-Z axis image of rhodamine phallolidin-labeled spheroid. The location of the polycarbonate support is also indicated. In C, established multicellular spheroids were exposed to 24 or 48 h ambient hypoxia (pO2, 20 torr) or normoxia (pO2, 147 torr; II, normoxia; III, hypoxia) and in the presence or absence of doxorubicin. Cell death was assessed by accumulation of BCECF as described in “Materials and Methods.” Data are means from three separate experiments; bars, SE.

Fig. 5. HIF-1 antisense oligonucleotides block $MDR1$ hypoxia inducibility. In A, confluent Caco2 epithelial monolayers were exposed to mock treatment, HIF-1α sense oligonucleotides, or HIF-1α antisense oligonucleotides for 24 h. Total RNA was isolated, and HIF-1α or β-actin transcripts were assessed by RT-PCR. In B, HIF-1α sense oligonucleotides or HIF-1α antisense oligonucleotide-loaded cells were assessed for $MDR1$ inducibility by hypoxia (0, 6, or 18 h, as indicated). Representative experiment from three.

Fig. 6. Effect of truncations in the $MDR1$ promoter length on HIF-1α binding site. A, oligonucleotides with HIF-1 binding sites at positions −49 to −45 (HIF-1α) or −189 to +133 (5'Δ$MDR$) were cloned with a G-Crich linker to create the constructs shown. B, luciferase reporter constructs expressing varied lengths of the MDR1 promoter were used to address hypoxia inducibility, and specifically, the role of HIF-1. As shown in Fig. 6, truncations in the 5' end (inclusive sequence of −189 to +133) showed a 2.5 ± 0.3-fold increase in luciferase activity over normoxic controls when compared with cells subjected to 24-h hypoxia ($P < 0.01$). Truncations at the 3' end (inclusive sequence of −189 to +4, 3'Δ$MDR$) did not significantly influence luciferase expression in normoxia and did not influence hypoxia inducibility ($P = $ not significant). Likewise, truncations at both the 3' and 5' end (inclusive sequence of −119 to +4, 4Δ$MDR$) did not influence expression in normoxia or hypoxia inducibility. However, as shown in Fig. 6, truncations in the 5' region (inclusive sequence of −2 to +133, 5'Δ$MDR$), which deletes the HIF-1α binding site at positions −49 to −45, resulted in a nearly complete loss of hypoxia inducibility ($P < 0.001$ compared with the wild-type
The HIF-1 binding site of the MDR1 promoter (5’-GCGTG-3’ at positions -99 to -45) also encodes a consensus site for binding of the transcription factor Sp1 (55). Because Sp1 can also be hypoxia-responsive (36), we determined whether decreased activity related to our mutagenesis strategy resulted from diminished Sp1 binding. To do this, we used the 3’ΔMDR construct (Fig. 6A), for which the only Sp1 binding site is also the putative HIF-1 binding site, and examined activity after Sp1 or HIF-1α depletion with antisense oligonucleotides. As shown in Fig. 7A, loading of KB cells with Sp1 antisense oligonucleotides resulted in a nearly complete loss of Sp1 protein expression by Western blot analysis. Similar to previous work (27), this Sp1 sense oligonucleotide also partially diminished Sp1 expression. Promoter activity was analyzed using these conditions (Fig. 7B) and indicated that depletion of Sp1 expression significantly diminished MDR1 promoter activity (31% ± 7% decrease; P < 0.05) but to a lesser extent than depletion of HIF-1α (86 ± 11% decrease; P < 0.01). These studies suggest that in addition to HIF-1, Sp1 may also contribute to MDR1 hypoxia inducibility.

A comparison of basal expression of MDR1 (i.e., normoxia) indicated no differences between mock-treated cells and cells depleted of HIF-1α (P = not significant). Given our mRNA results suggesting a loss of MDR1 expression in HIF-1α-depleted cells (see Fig. 5) and the loss of promoter activity with HIF-1 mutations (Fig. 6C), it is thus likely that other cis-acting regulatory elements spanning positions -2 to -119 contribute to basal expression of MDR1. One possibility in this region includes regulatory elements binding to the CCAAT box located at positions -72 to -81 of the MDR1 promoter (4). To test this idea, the wild-type promoter was compared with CCAAT-box mutants (C→G at position -75 and T→C at position -77). The wild-type promoter spanning -1202 to +118 was hypoxia inducible compared with normoxia (4.0 ± 0.6-fold increase over normoxia; P < 0.01), and CCAAT-box mutants did not influence such hypoxia inducibility (3.8 ± 0.4-fold increase over normoxia; P < 0.01). Such data suggest that CCAAT-box binding proteins do not influence basal expression of MDR1 or MDR1 hypoxia inducibility. Taken together, these reporter construct data provide strong evidence for a functional HRE, mediated by HIF-1, within the MDR1 promoter.
**HYPOXIA INDUCES MDR1**

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**DISCUSSION**

A mounting body of evidence has demonstrated that a hypoxic microenvironment is coincidental with the development and maintenance of tumors (37). It is clear that both nontranscriptional and transcriptional pathways relevant to tumor formation may be activated by ambient hypoxia (6). Similarly, tumor resistance to chemotherapeutics is likely acquired via transcriptional and nontranscriptional pathways. We report here that both transformed and nontransformed cell types subjected to hypoxia results in transcriptional induction of MDR1, as well as induced expression of the MDR1 gene product P-gp, a predominant membrane transporter associated with chemotherapeutic resistance (2). Subsequent studies identified a functional HIF-1 binding site within the MDR1 promoter. Such results define a pathway for the hypoxia-induced expression of MDR1 as well as functional P-gp.

As a result of numerous factors (cell mass, increased cellular glycolytic rates, irregular vessel formation, and others), tumor microenvironments are considered chronically hypoxic. Whereas normal tissues typically maintain O_2_ levels in the 40–60 mm Hg range, oxygen electrode studies have demonstrated that median tumor O_2_ levels are typically less than 10 mm Hg (37). Moreover, significant evidence also indicates that hypoxic cells are more resistant to chemotherapy (38). Contributing factors to hypoxia resistance in tumors have generally been attributed to: (a) tumor distance from the source of chemotherapy, and thus a diffusion barrier to delivery of drug; and (b) the fact that hypoxic cells generally divide slower that fully oxygenated cells, making therapies targeting rapidly growing cells less effective (37). The present findings in cultured cell monolayers and in multicellular spheroids provide a third option, that the hypoxic microenvironment may directly influence expression of membrane xenobiotic transporters, such as MDR1/P-gp. These findings are consistent with the recent findings of Wartenberg et al. (39), who demonstrated hypoxia-elicited induction of a number of genes in multicellular spheroids, including P-gp. In should be noted, however, that others have examined hypoxia inducibility of MDR1 with different results (40–42). For example, Liang (40) and Sakata et al. (41) demonstrated a hypoxia-associated drug resistance phenotype in human glioma cell lines but did not detect increased expression of MDR1 by Northern blot analysis. It is difficult to directly compare these studies, because significantly different protocols were used. For instance, Kalra et al. (42) looked at gene expression at later periods of hypoxia than those here and also incorporated an additional period of reoxygenation before analysis of gene expression. Although we have not examined this latter aspect directly, it is probable that the increase in MDR1 elicited by hypoxia would be lost with reoxygenation (e.g., reoxygenation results in rapid and complete HIF-1α degradation; Ref. 9). Thus, it is likely that differences between these studies are technical in nature.

The human MDR1 promoter is complex, with multiple binding elements and complex regulation. Previous studies have suggested that MDR1 is positively regulated by a number of transcription factors (4, 35, 43–48), negatively regulated by coupled NF-κB/p65 and c-fos (5), and p53 can either positively or negatively regulate MDR1 (49, 50). We demonstrate here a positive regulatory influence for HIF-1 and to a lesser extent for Sp1 in hypoxia inducibility. Initial insight was gained from microarray analysis of epithelia subjected to defined periods of hypoxia. Although epithelia are known to constitutively express MDR1/P-gp, our studies suggested a graded increase to >7-fold induction with hypoxia. Although these initial observations were made in a transformed cell type (T84 cells), this pathway is not likely to be a result of an oncogenic transformation, since similar levels of mRNA induction were evident in nontransformed, primary endothelial cell cultures. Subsequent experiments indicated a temporal induction of the MDR1 gene product P-gp. After cis-activation, P-gp can remain intracellular or be transported to the cell surface as a functional transmembrane protein (51). Our studies with both endothelia and epithelia indicated that mature protein resulting from hypoxia induction is transported to the cell surface and can be measured both at the protein level (cell surface ELISA using an ecto-epitope mAb) and at the functional level (verapamil-inhibitable efflux of digoxin and rhodamine 123).

Given the temporal and robust hypoxia response observed in the induction of MDR1, a candidate regulator was HIF-1. A search of the cloned gene promoter revealed a classic HIF-1 binding site at positions −49 to −45 relative to the transcription start site (28, 29) and a HIF-1 ancillary sequence (5′-CACAG-3′; Ref. 34) located 8 bp in the 3′ direction (nucleotides −37 to −33). However, the existence of a HIF-1 binding consensus is not evidence for a HIF-1-mediated response; instead, the HRE is defined as a cis-acting transcriptional regulatory sequence located within 5′-flanking, 3′-flanking, or intervening sequences of target genes (7). Three approaches were used to define a role for HIF-1 in the induction of MDR1: (a) the use of previously published antisense oligonucleotides (26), but not sense controls, resulted in a nearly complete blockade of MDR1 induction; (b) the combination of antisense oligonucleotides and transient reporter construct transfections were used to add further evidence for HIF-1 and revealed a complete blockade of MDR1 induction; and (c) a third approach using luciferase reporter constructs was used to identify the hypoxia-responsive region of the promoter. Results from these studies narrowed the region to −119 to −2, and mutations of this HIF-1 site resulted in a >80% decrease in hypoxia inducibility. Taken together, strong evidence is provided that the flanking regions surrounding the HIF-1 binding site at positions −49 to −45 function as a classic HRE. Of note, it is interesting that depletion of HIF-1α via antisense oligonucleotides, truncation of the 5′ region of the promoter,
as well as mutations of the HIF-1 binding site diminish expression of MDR1 mRNA in nonhypoxic cells, suggesting that HIF-1 may function in basal expression of MDR1. Such findings are consistent with previous work indicating that targeted disruption of HIF-1α in murine embryonic stem cells results in diminished basal expression of lactate dehydrogenase and phosphoglycerate kinase-1 (52). However, it is likely that additional 5′ regulatory elements exist, since HIF-1 depletion failed to modulate expression of the wild-type reporter construct under normoxic conditions.

Our results may have broad clinical implications for patients with MDR1-expressing tumors and in those patients who develop chemotherapy-resistant tumors. MDR1 expression is most prominent in a number of solid tumors (3), and MDR1 positively correlates with the propensity of tumors for lymph node spread and metastases (53). Consistent with these observations in solid tumors, it is clear that the degree of hypoxia within the tumor microenvironment is sufficient to induce HIF-1 (54). Moreover, it was recently shown that the majority of solid tumors, particularly those with a propensity for P-gp expression, stain positive for nuclear HIF-1α (55). As such, combination therapies aimed at modulating HIF-1 expression [e.g., targeting prolyl-4-hydroxylases that hydroxylate Pro-564 of HIF-1α (56, 57)] in concert with standard chemotherapy may provide a strategy to overcome tumor resistance. However, some caution may be warranted in systemic down-regulation of MDR1, because MDR1/P-gp is expressed in a number of normal tissues and provides normal physiological function (51).

In summary, a model is proposed that the MDR1 gene is hypoxia-responsive, and such results may identify hypoxia-eicted P-glycoprotein expression as a pathway for resistance of some tumors to chemotherapeutics. Moreover, it is likely that both basal and hypoxia-induced MDR1 expression parallel expression of functional HIF-1α.

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