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Candidate Genes for the Hypoxic Tumor Phenotype[1]

Albert C. Koong, Nicholas C. Denko, Karen M. Hudson, Cornelia Schindler, Lillian Swiersz, Cameron Koch, Sydney Evans, Hani Ibrahim, Quynh T. Le, David J. Terris, and Amato J. Giaccia[2]

Departments of Radiation Oncology [A. C. K., N. C. D., K. M. H., C. S., L. S., Q. T. L., A. J. G.] and Otolaryngology/Head and Neck Surgery [D. J. T., H. I.], Stanford University School of Medicine, Stanford, California 94305-5468, and Division of Oncology Research, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104 [C. K., S. E.]

Abstract

In this study, we have analyzed changes induced by hypoxia at the transcriptional level of genes that could be responsible for a more aggressive phenotype. Using a series of DNA array membranes, we identified a group of hypoxia-induced genes that included plasminogen activator inhibitor-1 (*PAI-1*), insulin-like growth factor-binding protein 3 (*IGFBP-3*), endothelin-2, low-density lipoprotein receptor-related protein (*LRP*), BCL2-interacting killer (*BIK*), migration-inhibitory factor (*MIF*), matrix metalloproteinase-13 (*MMP-13*), fibroblast growth factor-3 (*FGF-3*), *GADD45*, and vascular endothelial growth factor (*VEGF*). The induction of each gene was confirmed by Northern blot analysis in two different squamous cell carcinoma-derived cell lines. We also analyzed the kinetics of PAI-1 induction by hypoxia in more detail because it is a secreted protein that may serve as a useful molecular marker of hypoxia. On exposure to hypoxia, there was a gradual increase in PAI-1 mRNA between 2 and 24 h of hypoxia followed by a rapid decay after 2 h of reoxygenation. PAI-1 levels were also measured in the serum of a small group of head and neck cancer patients and were found to correlate with the degree of tumor hypoxia found in these patients.

Introduction

Within solid tumors, hypoxia develops at distances beyond the diffusion capacity of oxygen from blood vessels (typically 100–150 μM; Ref. 1). In addition, hypoxia can develop in areas of a tumor with compromised blood flow due to aberrant vasculature formation and high interstitial pressure (2). The tumor microenvironment is a critical component that influences the behavior of transformed cells and their response to therapeutic interventions. Evidence from recent laboratory studies suggests that tumor hypoxia contributes to the progression of a more malignant phenotype by selecting for cells with a diminished apoptotic potential. Hypoxic conditions will also reversibly inhibit cell-cycle progression under certain growth conditions (3). Because cells exposed to low oxygen conditions are relatively resistant to conventional radiotherapy and chemotherapy, this population of cells significantly impacts clinical response to anticancer therapies.

Tumor hypoxia has been directly measured in a variety of human cancers including head and neck carcinomas, cervical carcinomas, and soft tissue sarcomas. Brizel *et al.* and Nordsmark *et al.* showed that, in head and neck carcinomas, hypoxia correlated with a lower probability of disease-free survival (4, 5) and that, in soft tissue sarcomas, hypoxia was associated with increased incidence of distant metastases (6). Hockel *et al.* (7) also found that hypoxia in cervical carcinomas resulted in increased local and distant failures. Interestingly, hypoxia predicted for distant failure not only in patients treated with radiotherapy but also in those treated with surgery alone. These studies suggest that hypoxia alters fundamental, physiologically important pathways that result in more aggressive tumor behavior in a wide variety of tumors.

We hypothesized that the development of an increased malignant phenotype can at least partially be attributed to changes in hypoxic gene expression. Under hypoxic conditions, the major transcription factor affecting gene regulation is HIF-1[3] (8). This factor regulates a diverse family of genes including VEGF (9), the urokinase receptor (10), tyrosine hydoxylase (11), endothelin 1 (12), nitric oxide synthase (13), erythropoietin (14), and numerous glycolytic enzymes (15). HIF-1 binds as a heterodimer consisting of an oxygen-sensitive HIF-1α (helix-loop-helix protein, HLH) subunit (16–18) and a constitutively expressed oxygen insensitive ARNT/HIF-1β (aryl hydrocarbon receptor nuclear translocator) subunit (17, 18). HIF-1α-deficient embryonic stem (ES) cells that are null at this locus fail to induce *HIF-1* target genes when exposed to hypoxia (19, 20). The HIF-1 heterodimer binds to a 6-bp [5′-ACGTG(C/G)-3′] hypoxia responsive element (HRE) that functions as a transcriptional enhancer in hypoxia-responsive genes (21). Although the majority of hypoxia-regulated genes are dependent on HIF-1, other transcription factors such as nuclear factor κB (22, 23), AP-1 (24, 25), and c/EBPβ (26, 27) as well as Egr-1 (28) are also activated by hypoxia.

We sought to characterize global transcriptional changes in tumor cells after exposure to hypoxic stress with the goal of determining how hypoxia influences the regulation of defined sets of genes involved in metabolic regulation, cell-cycle control, angiogenesis, and tissue invasion. We used cDNA array membranes containing 588 genes and compared gene expression under normoxic and hypoxic conditions in a squamous cell carcinoma-derived cell line. These studies resulted in the identification of nine hypoxia inducible genes that were subsequently confirmed by Northern blot analysis to be hypoxia-inducible.

To demonstrate the potential clinical applicability of hypoxic gene expression, we analyzed PAI-1 in the serum of patients with squamous cell carcinomas. Previous reports have suggested that PAI-1 plays a role in tissue invasion/remodeling and its up-regulation may contribute to the development of a more malignant tumor phenotype (29–31). Furthermore, increased expression of PAI-1 in some human tumors has been correlated with poor prognosis (32, 33). Most importantly, because it is a secreted protein, serum levels are readily detectable and may be useful as a molecular marker of hypoxia. We obtained serum samples from head and neck carcinoma patients and investigated whether PAI-1 levels correlated with the degree of tumor

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[2] To whom requests for reprints should be addressed, at Stanford University Medical Center, Department of Radiation Oncology, Cancer Biology Research Laboratory, Stanford, CA 94305-5468. E-mail: giaccia@leland.stanford.edu.

[3] The abbreviations used are: HIF-1, hypoxia-induced factor-1; EF-5, [2-(2-nitro-1H-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide; PAI-1, plasminogen activator inhibitor-1; MIF, migration-inhibitory factor; BIK, BCL2 interacting killer; pO_2, partial pressure of oxygen; VEGF, vascular endothelial growth factor; DFO, desferroximine; IGFB-3, insulin-like growth factor-binding protein 3; Endo-2, endothelin-2; MMP-13, matrix metalloproteinase 13; LRP, low-density lipoprotein receptor-related protein; FGF-3, fibroblast growth factor 3.

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hypoxia. The use of larger gene arrays may yield other secreted proteins and provide additional serum markers that reflect tumor hypoxia.

Materials and Methods

Cell Lines. Two cell lines obtained from American Type Culture Collection were used in this study. FaDu cells were established in 1968 from a punch biopsy derived from a hypopharyngeal tumor. The morphology of FaDu cells *in vitro* is epithelial. FaDu cells form well-differentiated epidermoid carcinomas when transplanted into immune deficient mice. SiHa cells were established in 1975 from tissue fragments derived from a squamous cell carcinoma of the cervix. The morphology of SiHa cells *in vitro* is epithelial. SiHa cells form poorly differentiated epidermoid carcinomas when transplanted into immune deficient mice. SiHa cells possess one to two copies of human papilloma virus type 16 integrated in their genomes and FaDu cells are human papilloma virus-negative. These two cell lines were chosen because they were both derived from squamous cell carcinomas, a tumor type in which hypoxia has been thought to be an important physiological modulator of malignant progression. Both of the cell lines were not used past 10 passages in cell culture.

Clontech Atlas cDNA Expression Array Membranes. Hybridizations were carried out according to the manufacturer's specifications. The membranes were prehybridized at 68°C for 30 min in ExpressHyb solution. Message RNA was purified by binding to a poly(A) column and probe that was generated by reverse transcription in the presence of [α-^{32}P]dATP. The membranes were then hybridized overnight with 0.5×10^6 cpm/ml probe at 68°C with continuous agitation. Membranes were washed twice with 2× SSC/1% SDS and twice with 0.1× SSC/0.5% SDS. All of the washes were carried out for 30 min at 68°C. The membranes were then visualized by phosphorimaging, and quantitation was performed with ImageQuant software. Counts were normalized to M_r 23,000 highly basic protein (Accession Number P40429) for loading controls.

Northern Blot Analysis. Total RNA was isolated with Trizol according to the manufacturer's protocol. RNA samples (10 μg) were denatured in glyoxal for 1 h at 50°C and separated by agarose gel electrophoresis. The gel was then transferred by capillary action overnight to Nytran membrane and cross-linked by exposure to UV light. Probes were generated by reverse trasnscription PCR using the manufacturer's primers (Clontech), gel-purified, and labeled with ^{32}P by random priming. Hybridization to ^{32}P-labeled probes was carried out at 65°C using ExpressHyb solution (Clontech) according to the manufacturer's protocol and washed for 2 h to a stringency of 0.2× SSC/1% SDS. Equal loading and transfer between lanes was demonstrated by methylene blue staining of 28S and 18S ribosomal bands before probing. All of the membranes were exposed by phosphorimaging and quantitated with ImageQuant software.

Hypoxic Treatment. FaDu and Siha cells were routinely cultured in DMEM + 10% FCS. Fresh media was exchanged 3–5 h before treating for varying amounts of time in a 37°C hypoxic incubator (Sheldon Manufacturing Inc.), which maintained an environment of less than 0.05% oxygen. The normoxic cells were maintained in a 37°C-incubator with 21% O_2. All of the experiments were performed at 70–80% cell confluency and the pH of the media remained between 7.0–7.4 for the duration of the experiment.

Immunohistochemical Staining of Tissues for EF-5 Binding; Photography and Analysis of Binding. The techniques used here were previously described (34, 35). For each patient, at least two tumor regions and two levels within each region (separated by 0.5 mm) were examined for regions of *in situ* EF-5 binding. The regions were imaged using a ×10 microscope objective (field size set electronically at 1.05×0.7 mm^2), and typically nine fields were examined for each section. To provide multiple pixels per cell while improving camera sensitivity, each image field consisted of 600×400 pixels each of which was a 2×2-bin of the actual camera chip pixels, with 12-bit gray-scale resolution.

Eppendorf pO_2 Histography and PAI-1 Determination. Eppendorf electrode measurements were taken through three tracks of neck nodes of patients with squamous cell carcinoma of the head and neck. Each pass with the probe recorded 50–100 measurements of oxygen concentration along the track. Measurements were also taken through one track of s.c. tissue of an uninvolved area in the neck to serve as a control. Serum levels of PAI-1 protein were measured using ELISA kits from biopool International (Ventura, CA) according to the instructions of the manufacturer. The PAI-1 ELISA has a detection limit of 0.5 ng/ml and measures latent (inactive) PAI-1, active PAI-1, and PAI-1 complexed with tPA/PAI and uPA/PAI. Using this assay and the manufacturer's protocol, the range of PAI-1 values found in individuals without pathophysiological conditions or in the third trimester of pregnancy is 4–43 ng/ml All of the human serum samples were obtained with the subjects' informed consent and were used for research purposes only. Total tumor burden (primary tumor and nodes) as assessed from computed tomography and magnetic resonance imaging scans indicated that there was no relationship between tumor burden and PAI-1 levels. Tumor burden ranged from 12.7 cm^2 to 60 cm^2. However, a relationship between median pO_2 values and PAI-1 levels in the serum was found. The graph represents data from eight patients with pathologically verified squamous cell carcinoma of the head and neck before any form of treatment.

Results

Fig. 1 shows a series of multiple gene array membranes that illustrate gene expression changes induced by hypoxia in a squamous cell carcinoma cell line (FaDu) originally derived from a pharyngeal wall tumor. These membranes were arrayed with 588 known genes categorized into six groups: (*a*) regulators of cell cycle; (*b*) apoptosis/tumor suppressors/oncogenes; (*c*) DNA damage/development; (*d*) cell

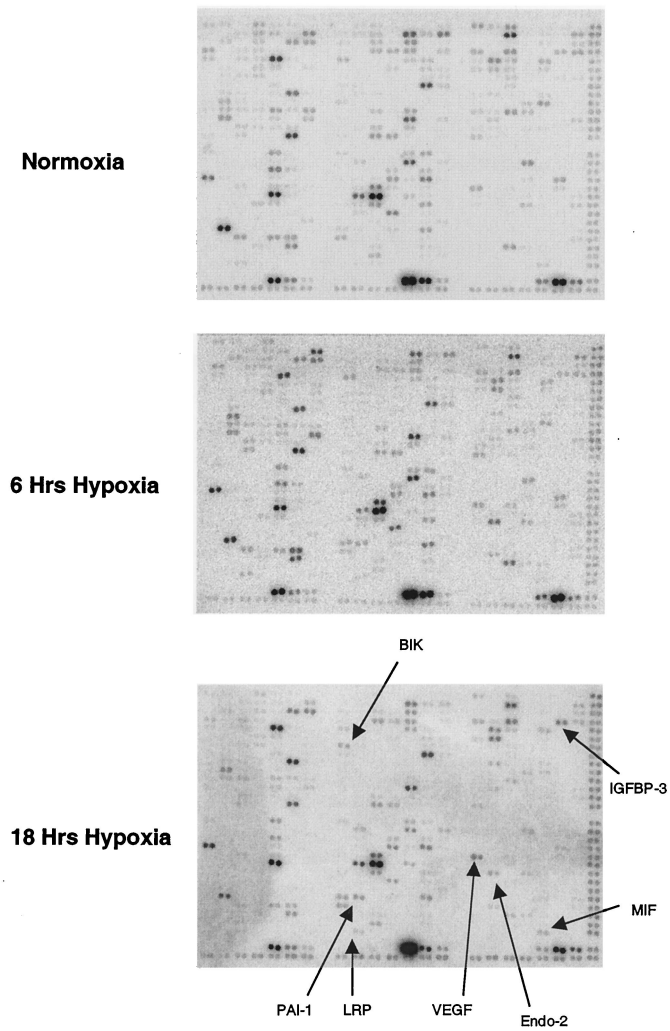

Fig. 1. Gene array membranes illustrating changes in gene expression induced by 6 and 18 h of hypoxia. Changes in the intensity of the target spot represent changes in the levels of mRNA expression in the hypoxia-treated samples as compared with the normoxic controls (*top panel*). The location of six hypoxia-inducible genes and VEGF are labeled in the 18 h hypoxia panel.

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adhesion/angiogenesis; (*e*) regulators of invasion/cell-cell interaction; and (*f*) growth factors/cytokines. Cells were exposed to 6 or 18 h of hypoxia prior to mRNA isolation, and gene expression was then compared with cells cultured under normoxia. Quantitative analysis of these membranes was performed with ImageQuant software.

Because numerous investigators have shown that VEGF mRNA levels are exquisitely sensitive to hypoxia, we used this level of gene induction as a cutoff point for assessing hypoxia-induced genes. We only analyzed genes that demonstrated a greater level of induction than found with VEGF. Using this criteria, we identified nine genes (Table 1) that exhibited a greater than 3-fold induction under hypoxic conditions. Interestingly, the level of mRNA induction as determined by gene array analysis did not always correlate with the level of induction as determined by Northern blot analysis (Table 1). However, all of the genes that we initially identified based on their hypoxic inducibility when compared with VEGF were indeed found to be induced by hypoxia as assessed by Northern blotting (Table 1).

Fig. 2 is a composite of Northern blots that demonstrates the increase in mRNA expression of the seven most-hypoxia-inducible genes derived from membrane analysis. In this figure, we compared the induction of each gene in two different squamous cell carcinoma-derived cells. The kinetics of induction of each gene after exposure to 6 and 18 h of hypoxia was similar between FaDu cells and Siha cells (a cell line derived from a squamous cell carcinoma of the uterine cervix). In each Northern blot, a lane representing the effects of a 6-h treatment of cells with 100 μM DFO (an iron-chelating, hypoxia-mimetic agent) is also included for comparison (36). The consistency between the patterns of induction by hypoxia and DFO in these two different cell lines suggests that the regulation of these genes may be similar in squamous cell carcinomas, at least *in vitro*.

Table 1 *Analysis of candidate genes*

Gene	Accession number	Fold induction by array analysis	Fold induction by northern analysis
MIF	25639M	9.3	2 to 3
BIK	X89986	7.6	2 to 3
PAI-1	X04429	7.4	>10
Collagenase-3 (MMP13)	X75308	6.3	<2
LDL receptor-related protein	X13916	6.0	5 to 10
IGFBP-3	M31159	5.6	5 to 10
FGF-3 (INT-2)	X14445	5.6	<2
Endo-2	M65199	4.4	>10
GADD45	M65199	4.3	<2
VEGF	M32977	3.3	>10

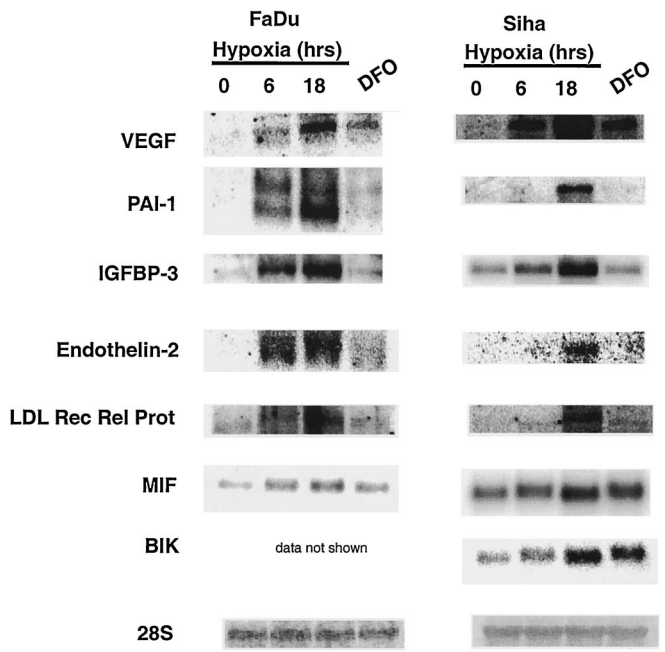

Fig. 2. Kinetics of *PAI-1*, *IGFBP-3*, *Endo-2* (*endothelin-2*), *LRP* (*LDL Rec Rel Protein*), *MIF*, *BIK*, and VEGF induction by hypoxia. The times for mRNA analysis were chosen so that membrane hybridization and Northern blotting could be directly compared. Northern blot analysis for two different squamous cell carcinoma cell lines, FaDu and Siha, is shown. In addition, the induction of each gene by the hypoxic mimetic agent DFO (6 h) is also included to further support the hypoxia-inducibility of each gene.

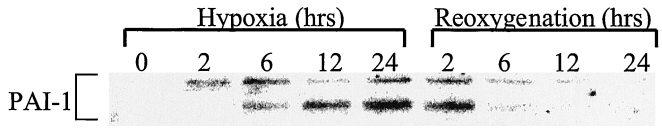

Fig. 3. Kinetics of PAI-1 mRNA induction by hypoxia and mRNA decay after reoxygenation in FaDu squamous carcinoma cells. PAI-1 mRNA is sensitive to changes in oxygen levels inasmuch as it exhibits a rapid increase under hypoxic conditions and decay on reoxygenation.

Fig. 3 shows an extended time course of PAI-1 induction in FaDu cells. Minimal PAI-1 mRNA is present under aerobic conditions, and an increase is seen 2 h after exposure of FaDu cells to hypoxia. These levels continue to increase for at least 24 h of hypoxia and return to near baseline levels after 6 h of reoxygenation. The changes in *PAI-1* gene expression were normalized to changes in *28S ribosome* gene expression which varied less than 10% in this experiment (data not shown). The sensitivity of *PAI-1* gene expression to levels of hypoxia makes it an ideal marker of *in vivo* hypoxia. In addition, because it is a secreted protein, serum levels can be monitored in a relatively noninvasive manner to determine the response to treatment and to detect early subclinical recurrence.

Fig. 4 is a comparison of PAI-1 protein levels and tumor hypoxia in serum from patients with squamous cell carcinomas. The patients are divided into two groups according to the degree of hypoxia found in their tumors. Hypoxia was determined either by immunohistochemical staining of EF-5 adducts, a nitroimidazole that is given by i.v. infusion in which it selectively and irreversibly binds to hypoxic cells (34, 35) or by intratumor Eppendorf pO_2 histography (37). In patients who received EF-5, PAI-1 levels were substantially greater in tumors that had EF-5 staining in more than 10% of the staining of the specimen (data not shown). However, because Eppendorf measurements are the most widely accepted method of estimating tumor oxygenation, we compared the levels of PAI-1 in the serum of oxic tumors with hypoxic tumors. PAI-1 values were plotted as a function of median pO_2 values (<4 mm Hg or >4 mm Hg) derived from Eppendorf readings. When compared with normal controls, there is a substantial increase in serum PAI-1 levels in both the low and high hypoxia groups and a trend toward higher PAI-1 levels in patients with more hypoxic tumors. This preliminary data on a small group of patients supports the initiative for a more extensive clinical analysis to investigate the relationship between PAI-1, tumor hypoxia, and outcome.

Discussion

When tumor cells are exposed to hypoxic stress, transcription of a discrete set of genes is initiated to serve a variety of cellular functions. It has been proposed that within solid tumors, hypoxia functions as a selective pressure leading to an apoptosis-resistant population of cells (38). In addition, other investigators have demonstrated that hypoxia increases the ability of tumor cells to metastasize (39, 40). These laboratory studies suggest that hypoxia influences tumor development by modulating gene transcription. This hypothesis is also supported by clinical data that correlates hypoxic tumors in soft tissue sarcomas,

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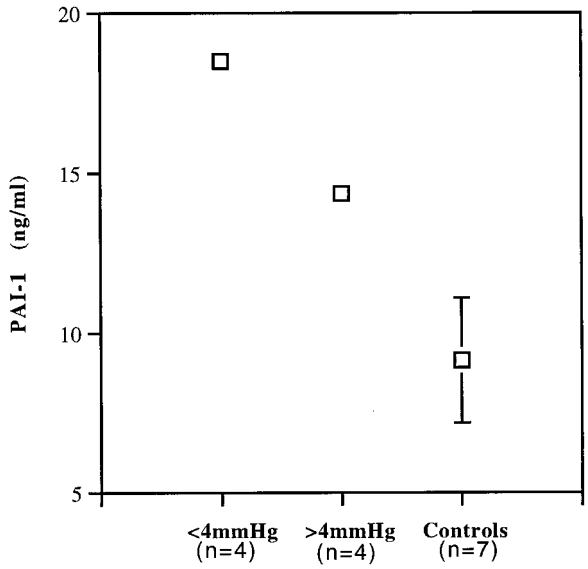

Fig. 4. Correlation of PAI-1 secreted protein levels in the serum of patients with tumor oxygenation. PAI-1 serum protein levels were detected by ELISA in triplicate. Tumor hypoxia was determined by Eppendorf histography. Patients were divided into two groups based on median pO_2 levels (>4 mm Hg or < 4 mm Hg). *Error bars*, two SEs.

head and neck carcinomas, and cervical carcinomas with worse overall survival (4–7). In this study, we sought to characterize gene expression changes that occur in response to hypoxia because we hypothesized that changes in gene expression might be responsible in part for the more aggressive phenotype of the hypoxic tumor cell.

With the use of a multiple gene array membrane, we screened 588 genes that had previously been identified to play a role in oncogenesis, for their response to changes in oxygenation. Because hypoxia had been previously shown to be a potent transcriptional activator of VEGF, we chose to use it as a cutoff point for identifying additional hypoxia-regulated genes. Using this criteria, we found nine genes that demonstrated greater hypoxic induction than VEGF as determined by ImageQuant analysis. The level of hypoxic induction when analyzed by Northern blot did not always correlate with the level of induction by array analysis because of differences in both the quantitative and qualitative aspects of probe and target gene hybridization. Such differences have been previously reported for p53-regulated genes (41). Furthermore, although the gene array screening was performed in FaDu cells, a similar level of induction was found by Northern blot analysis in Siha cells. It is noteworthy that Table 1 is not an exhaustive list of hypoxia-induced genes because the squamous carcinoma cells do not express or express at varying levels the genes on the array membrane. The gene array represents only a small fraction of expressed genes, and we analyzed only genes that were more-hypoxia-inducible than VEGF.

Table 1 is a ranked list of hypoxia-inducible genes that compares their induction by gene array analysis and Northern blot analysis. These genes can be broadly categorized into two groups: those involved in apoptosis (*BIK* and *IGFBP-3*) and those involved in local tissue/tumor response (*MIF*, *PAI-1*, *Endo-2*, *MMP-13*, *FGF-3*, *LRP*, and VEGF).

BIK and *IGFBP-3* are both proapoptotic genes (42, 43) that are transcriptionally up-regulated during hypoxia. Apoptosis is a complex process that reflects a shift in the delicate balance between pro- and antiapoptotic genes. During the time in which these genes are induced, we did not see any significant increase in apoptosis, which makes the function of these genes during hypoxia unclear. Perhaps other antiapoptotic pathways have become activated during hypoxia, which then negates the effects of these pro-apoptotic genes, or these genes may play other roles in growth regulation under hypoxic conditions.

The second and larger category of genes that we have identified by gene array analysis are involved in tissue remodeling and invasion. Young *et al.* have demonstrated that when tumor cells are exposed to hypoxia and reoxygenation, it results in an increased rate of metastasis as determined by lung colony formation of metastatic foci (39). Studies presented here and elsewhere suggest that many of the genes involved in the breakdown of the basement membrane and the eventual establishment of metastatic tumor foci are hypoxia-inducible (10, 44). Thus, the induction of tissue-remodeling genes by hypoxia undoubtedly contributes to the development of a more malignant phenotype.

A more detailed analysis of PAI-1 revealed that its regulation is exquisitely sensitive to hypoxia. Under normoxic conditions, there are undetectable levels of PAI-1 and between 2–24 h of hypoxia there is a gradual increase in PAI-1 mRNA. Reoxygenation of 2–6 h under normoxic conditions results in a marked decrease in PAI-1 expression to near-normoxic levels. Several groups have reported that PAI-1 is hypoxia-inducible in cell lines *in vitro* [(45, 46). Furthermore, analysis of the 5′ genomic sequence from the transcriptional start site of the *PAI-1* gene reveals a putative hypoxia responsive element (HRE) that provides a possible mechanism for PAI-1 regulation by hypoxia.[4]

As discussed above, increased PAI-1 staining of tumor sections has been correlated with a worse prognosis. However, the link between PAI-1, tumor hypoxia, and outcome has yet to be made. Because PAI-1 is a secreted protein, its serum levels can be easily measured and may serve as a surrogate marker of tumor hypoxia. Although we found a relationship between serum PAI-1 levels in head and neck cancer patients and the extent of hypoxia found in the tumors of these patients, a more thorough study is warranted to investigate whether other genes involved in plasminogen metabolism are also associated with tumor aggressiveness. It is also important to note that other pathophysiological conditions may elevate serum PAI-1 including pregnancy, cardiac ischemia, and blood clotting disorders, making a thorough clinical examination a necessity. In summary, PAI-1 represents but one hypoxia-regulated secreted protein that may eventually aid in cancer diagnosis, prognosis, and surveillance.

References

1. Thomlinson, R. H., and Gray, L. H. The histological structure of some human lung cancers and the possible implications for radiotherapy. Br. J. Cancer, *9:* 539–549, 1955.
2. Brown, J. M., and Giaccia, A. J. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res., *58:* 1408–1416, 1998.
3. Green, S. L., and Giaccia, A. J. Tumor hypoxia and the cell-cycle: implications for malignant progression and response to therapy. Cancer J., *4:* 218–223, 1998.
4. Brizel, D. M., Sibley, G. S., Prosnitz, L. R., Scher, R. L., and Dewhirst, M. W. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int. J. Radiat. Oncol. Biol. Phys., *38:* 285–289, 1997.
5. Nordsmark, M., Overgaard, M., and Overgaard, J. Pretreatment of oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother. Oncol., *41:* 31–40, 1996.
6. Brizel, D. M., Scully, S. P., Harrelson, J. M., Layfield, L. J., Bean, J. M., Prosnitz, L. R., and Dewhirst, M. W. Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res., *56:* 941–943, 1996.
7. Hockel, M., Schlenger, K., Aral, B., Mitze, M., Schaffer, U., and Vaupel, P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res., *56:* 4509–4515, 1996.
8. Semenza, G. L. Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. Curr. Opin. Genet. Dev., *8:* 588–594, 1998.
9. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelail growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature (Lond.), *359:* 843–845, 1992.
10. Graham, C. H., Fitzpatrick, T. E., and McCrae, K. R. Hypoxia stimulates urokinase receptor expression through a heme-dependent pathway. Blood, *91:* 3300–3307, 1998.

[4] L. Swiersz *et al.*, unpublished data.

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11. Czyzyk-Krzeska, M. F., Furnari, B. A., Lawson, E. E., and Millhorn, D. E. Hypoxia increases rate of transcription and stability of tyrosine hydroxylase mRNA in pheochromocytoma (PC12) cells. J. Biol. Chem., *269:* 760–764, 1994.
12. Bodi, I., Bishopric, N. H., Discher, D. J., Wu, X., and Webster, K. A. Cell-specificity and signaling pathway of *endothelin-1* gene regulation by hypoxia. Cardiovasc. Res., *30:* 975–984, 1995.
13. Melillo, G., Musso, T., Sica, A., Taylor, L. S., Cox, G. W., and Varesio, L. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promotor. J. Exp. Med., *182:* 1683–1693, 1995.
14. Jelkmann, W. Erythropoietin: structure, control of production, and function. Physiol. Rev., *72:* 449–489, 1992.
15. Semenza, G. L., Roth, P. H., Fang, H-M., and Wang, G. L. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J. Biol. Chem., *269:* 23757–23767, 1994.
16. Wang, G. L., and Semenza, G. L. Purification and characterization of hypoxia-inducible factor 1. J. Biol. Chem., *270:* 1230–1237, 1995.
17. Huang, L. E., Arany, Z., Livingston, D. M., and Bunn, H. F. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. J. Biol. Chem., *271:* 32253–32259, 1996.
18. Kallio, P. J., Pongratz, I., Gradin, K., McGuire, J., and Poellinger, L. Activation of hypoxia-inducible factor 1α: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proc. Natl. Acad. Sci. USA, *94:* 5667–5672, 1997.
19. Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y., and Semenza, G. L. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. Genes Dev., *12:* 149–162, 1998.
20. Maltepe, E., Schmidt, J. V., Baunoch, D., Bradfiedl, C. A., and Simon, M. C. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature (Lond.), *27:* 403–407, 1997.
21. O'Rourke, J. F., Dachs, G. U., Gleadle, J. M., Maxwell, P. H., Pugh, C. W., Stratford, I. J., Wood, S. M., and Ratcliffe, P. J. Hypoxia response elements. Oncol. Res., *9:* 327–332, 1997.
22. Koong, A. C., Chen, E. Y., and Giaccia, A. J. Hypoxia causes the activation of nuclear factor κB through the phosphorylation of IκBα on tyrosine residues. Cancer Res., *54:* 1425–1430, 1994.
23. Karakurum, M. S. R., Chen, J., Pinsky, D., Yan, S. D., Anderson, M., Sunouchi, K., Major, J., Hamilton, T. K. K., Rot., A., Nowygrod, R., and Stern, D. M. Hypoxic induction of *interleukin-8* gene expression in human endothelial cells. J. Clin. Investig., *93:* 1564–1570, 1994.
24. Ausserer, W. A., Bourrat-Floeck, B., Green, C. J., Laderoute, K. R., and Sutherland, R. M. Regulation of c-jun expression during hypoxic and low glucose stress. Mol. Cell. Biol., *14:* 5032–5042, 1994.
25. Yao KS, X. S., Curran, T., and O'Dwyer, P. J. Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. Mol. Cell. Biol., *14:* 5997–6003, 1994.
26. Yan, S. F., Tritto, I., Pinsky, D., Liao, H., Huang, J., Fuller, G., Brett, J., May, L., and Stern, D. Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. Central role of the binding site for nuclear factor-IL-6. J. Biol. Chem., *270:* 11463–11471, 1995.
27. Yan, S. F., Zou, Y. S., Mendelsohn, M., Gao, Y., Naka, Y., Du Yan, S., Pinsky, D., and Stern, D. Nuclear factor interleukin 6 motifs mediate tissue-specific gene transcription in hypoxia. J. Biol. Chem., *272:* 4287–4294, 1997.
28. Yan, S. F., Lu, J., Zou, Y. S., Soh-Won, J., Cohen, D. M., Buttrick, P. M., Cooper, D. R., Steinberg, S. F., Mackman, N., Pinsky, D. J., and Stern, D. M. Hypoxia-associated induction of early growth response-1 gene expression. J. Biol. Chem., *274:* 15030–15040, 1999.
29. Schmitt, M., Harbeck, N., Thomssen, C., Wilhelm, O., Magdolen, V., Reuning, U., Ulm, K., Hofler, H., Janicke, F., and Graeff, H. Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. Thromb. Haemostasis, *78:* 285–296, 1997.
30. Robert, C., Bolon, I., Gazzeri, S., Veyrenc, S., Brambilla, C., and Brambilla, E. Expression of plasminogen activator inhibitors 1 and 2 in lung cancer and their role in tumor progression. Clin. Cancer Res., *5:* 2094–2102, 1999.
31. Bajou, K., Noel, A., Gerard, R. D., Masson, V., Brunner, N., Holst-Hansen, C., Skobe, M., Fusenig, N. E., Carmeliet, P., Collen, D., and Foidart, J. M. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat. Med., *4:* 923–928, 1998.
32. Chambers, S. K., Ivins, C. M., and Carcangiu, M. L. Plasminogen activator inhibitor-1 is an independent poor prognostic factor for survival in advanced stage epithelial ovarian cancer patients. Int. J. Cancer, *79:* 449–454, 1998.
33. Kuhn, W., Schmalfeldt, B., Reuning, U., Pache, L., Berger, U., Ulm, K., Harbeck, N., Spathe, K., Dettmar, P., Hofler, H., Janicke, F., Schmitt, M., and Graeff, H. Prognostic significance of urokinase (uPA) and its inhibitor PAI-1 for survival in advanced ovarian carcinoma stage FIGO IIIc. Br. J. Cancer, *79:* 1746–1751, 1999.
34. Lord, E. M., Harwell, L., and Koch, C. J. Detection of hypoxic cells by monoclonal antibody recognizing 2-nitroimidazole adducts. Cancer Res., *53:* 5721–5726, 1993.
35. Evans, S. M., Joiner, B., Jenkins, W. T., Laughlin, K. M., Lord, E. M., and Koch, C. J. Identification of hypoxia in cells and tissues of epigastric 9L rat glioma using EF5 [2-(2-nitro-1H-imidazol-1-yl)-*N*-(2,2,3,3,3- pentafluoropropyl) acetamide]. Br. J. Cancer, *72:* 875–882, 1995.
36. Wang, G. L., and Semenza, G. L. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood, *82:* 3610–3615, 1993.
37. Adam, M. F., Gabalski, E. C., Bloch, D. A., Oehlert, J. W., Brown, J. M., Elsaid, A. A., Pinto, H. A., and Terris, D. J. Tissue oxygen distribution in head and neck cancer patients. Head Neck, *21:* 146–153, 1999.
38. Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W., and Giaccia, A. J. Hypoxia mediated selection of cells with diminished apoptotic potential in solid tumors. Nature (Lond.), *379:* 88–91, 1996.
39. Young, S. D., and Hill, R. P. Effects of reoxygenation on cells from hypoxic regions of solid tumors: anticancer drug sensitivity and metastatic potential. J. Natl. Cancer Inst., *82:* 371–380, 1990.
40. Rofstad, E. K., and Danielsen, T. Hypoxia-induced metastasis of human melanoma cells: involvement of vascular endothelial growth factor-mediated angiogenesis. Br. J. Cancer, *80:* 1697–1707, 1999.
41. Amundson, S. A., Bittner, M., Chen, Y., Trent, J., Meltzer, P., and Fornace, A. J., Jr. Fluorescent cDNA microarray hybridization reveals complexity and heterogeneity of cellular genotoxic stress responses. Oncogene, *18:* 3666–3672, 1999.
42. Han, J., Sabbatini, P., and White, E. Induction of apoptosis by human Nbk/Bik, a BH3-containing protein that interacts with E1B 19K. Mol. Cell. Biol., *16:* 5857–5864, 1996.
43. Rajah, R., Valentinis, B., and Cohen, P. Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-β1 on programmed cell death through a p53- and IGF-independent mechanism. J. Biol. Chem., *272:* 12181–12188, 1997.
44. Graham, C. H., Forsdike, J., Fitzgerald, C. J., and Macdonald-Goodfellow, S. Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. Int. J. Cancer, *80:* 617–623, 1999.
45. Fitzpatrick, T. E., and Graham, C. H. Stimulation of plasminogen activator inhibitor-1 expression in immortalized human trophoblast cells cultured under low levels of oxygen. Exp. Cell Res., *245:* 155–162, 1998.
46. Pinsky, D. J., Liao, H., Lawson, C. A., Yan, S. F., Chen, J., Carmeliet, P., Loskutoff, D. J., and Stern, D. M. Coordinated induction of plasminogen activator inhibitor-1 (PAI-1) and inhibition of plasminogen activator gene expression by hypoxia promotes pulmonary vascular fibrin deposition. J. Clin. Investig., *102:* 919–928, 1998.

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