ROLES OF HYPOXIC STRESS PROTEINS IN SOLID TUMORS PRIMING FOR REOXYGENATION?

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RESUMEN: Rol de la Proteinas de Estrés en los Tumores Sólidos: Preparación para la Reoxigenación?

Existen actualmente evidencias de que la hipoxia juega un rol central en la progresión maligna de una amplia variedad de tumores sólidos. Por ejemplo, los microambientes hipóxicos de muchos tumores humanos afectan la capacidad de respuesta terapéutica y pueden también contribuir a las tasas incrementadas de supervivencia de las células más malignas luego de la reoxigenación. La naturaleza detallada de estos fenotipos de resistencia dependientes de hipoxia todavía no están claros, pero pueden incluir un grupo de proteinas diversas que son inducidas en respuesta a los estímulos hipóxicos. Este reporte revisa la evidencia que implica a tres proteinas de estrés hipóxico importantes (*p53*, hemooxigenasa-1 y metalotioneina-IIA) en la protección contra la apoptosis, estrés oxidativo y drogas antineoplásicas luego de la hipoxia/reoxigenación.

Palabras Claves: Hipoxia, Proteinas de estrés hipóxico, Proteinas reguladas por oxígeno, Hemo-oxigenasa, Metalotioneina, Microambiente tumoral.

RÉSUMÉ: Rôle des protéines de stress dans les tumeurs solides. Préparation pour la réoxygénation ?

Il apparaît maintenant évident que l'hypoxie joue un rôle important dans la progression maligne d'une grande variété de tumeurs solides. Un exemple en sont les micro-environnements hypoxiques de nombreuses tumeurs humaines qui affectent la capacité de réponse thérapeutique et peuvent contribuer également à l'élévation des taux de survie des cellules les plus malignes après réoxygénation. La nature détaillée de ces phénotypes de résistance dépendants de l'hypoxie n'est pas encore très claire mais elle peut impliquer un groupe de protéines variées qui sont induites en réponse aux stimuli hypoxiques. Ce rapport examine l'intervention manifeste de 3 protéines importantes de stress hypoxique (*p53*, hémo-oxygénase -1 et métallothionéine-IIA) dans la protection contre l'apoptose, le stress oxydatif et les drogues antinéoplasiques, après hypoxie/réoxygénation.

Mots-clés : Hypoxie, Protéines de stress hypoxique, Protéines régulées par l'oxygène, Hémo-oxygénase, Métallothionéine, Micro-environnement tumoral.

SUMMARY: It is now apparent that hypoxia has a central role in the malignant progression of a wide range of solid tumors. For example, hypoxic microenvironments within many human tumors directly affect therapeutic responsiveness and may also contribute to increased survival rates of the more malignant cells following reoxygenation. The detailed nature of these hypoxiadependent resistance phenotypes remains unclear, but it may involve a set of diverse proteins that are induced in response to hypoxic insults. This report reviews the evidence implicating three important hypoxic stress proteins ((*p53*, heme oxygenase-l, and metallothionein-IIA) in protection against apoptosis, oxidative stress, and antineoplastic drugs following hypoxia/ reoxygenation.

Key Words Hypoxia, Hypoxic stress proteins, Oxygen regulated proteins, Heme oxygenase, Metallothionein, Tumor microenvironment.

HYPOXIA IN HUMAN TUMORS

Hypoxia is known to have roles in a number of physiological and pathophysiological processes, including erythroid development, angiogenesis, wound repair, fibrosis, ischemia, and neoplasia. A growing body of evidence suggests that many human tumors contain a significant fraction of hypoxic cells which can directly affect therapeutic responsiveness and possibly malignant progression. The mean oxygen concentrations within most normal tissues exceed 40 mm Hg, while those of many malignant tumors, including breast, cervical, and squamous cell carcinomas, contain regions of very low oxygen concentrations, with PO_2 levels reaching as low as 0-10 mm Hg (Gatenby et al., 1985; Sutherland et al., 1996; Vaupel et al., 1991; Vaupel and Hockel 1995) . These regions are characterized as either chronic or transient (Hockel et al., 1996a; Overgaard and Horsman 1996). Chronic hypoxia is thought to arise from the inability of aberrant vascular networks to deliver an adequate blood supply (Rak et al., 1995a), while microregions of transient hypoxia are associated with deregulated constriction of the tumor blood vessels (Overgaard and Horsman, 1996). *In vitro* and experimental tumor studies showed that hypoxia and subsequent reoxygenation appear to affect on malignant progression in terms of the development of metastasis and resistance to therapy (see Hockel et al., 1996a for a review). The existence of hypoxic microenvironments within these tumors is believed to be correlated with a poorer prognosis independent of treatment, compared with the case for well oxygenated tumors.

Recent observations confirm the clinical relevance of tumor oxygenation. In one study of patients with advanced carcinoma of the cervix who were treated with radiotherapy with or without chemotherapy, patients with a median PO_2 • 10 mm Hg had a 50%

survival of only 8 months, whereas patients with a median PO_2 >10 mm Hg had a 50% survival rate of more than 3 years after treatment (Hockel et al. 1993). Subsequent studies by Hockel's group, using a larger patient cohort and longer follow-up (including a subgroup of patients who underwent primary surgery), confirmed the earlier studies. Specifically, the data indicated that the poorer outcome of patients with hypoxic tumors was mainly due to regional failures with and without distant metastases, regardless of whether surgery or radiation was applied as primary treatment (Hockel et al., 1996b).

The underlying phenotypic alterations involve hypoxia-associated increases in radio- and chemotherapeutic resistance (Coleman, 1988; Overgaard Horsman, 1996; Sakata et al., 1991; Shen et al., 1989; Vaupel Hockel, 1995), gene amplification (Rice et al., 1986), genomic instability (Anderson and Stoler, 1993), increased metastatic variants (Brizel et al., 1996; Ginis and Faller, 1996), and diminished apoptotic potential (Graeber et al., 1996). The precise mechanisms of these hypoxia-associated phenotypes are not well delineated, but some may involve one or more of a set of hypoxia-related stress proteins, termed oxygen regulated proteins or ORPs, that can be upregulated during periods of significant depression in total cellular protein synthesis and cell cycle arrest (Giaccia, 1996; Graeber et al., 1996; Heacock and Sutherland, 1986; Heacock and Sutherland, 1990) .

Oxygen Regulated Proteins (ORPs)

Our previous studies have shown that many cancer and normal mammalian cells respond to hypoxia by increasing the synthesis of ORPs. Figure 1 shows autoradiograms of (35 S-methionine/cysteine) labeled proteins from aerobic and hypoxic A431 human squamous cell carcinoma cultures in two-dimensional SDS-polyacrylamide denaturing gels. Maximum inductions of ORP synthesis and optimal cell survival are observed under conditions of <0.1% O_2 (4-12 h). This experiment not only demonstrates the enhanced synthesis of proteins with masses and isoelectric points similar to those of the originally documented ORPs (260, 150, 100, 80, and 33 kDa) (Heacock and Sutherland, 1990) but also reveals other prominent ORPs, including 52, 57, and 60. Although the inductions of many ORPs can be cell specific, this pattern is typical of human and rodent cell cultures. It should be also noted that this method detects only a small fraction of the approximately 50,000 different cellular protein species and, therefore, likely represents a small fraction of the actual number of proteins induced by hypoxia. Our group is actively involved in studies designed to deterrnine the identities and roles of some of these ubiquitous ORPs represented in Fig. 1. To date, we have determined that ORPs 100 and 80 are identical to glucose regulated proteins (GRPs) 78 and 94, respectively (Roll et al., 1991); ORP 33 is heme oxygenase (HO-1) (Murphy et al., 1991); and ORP 7 is metallothionein IIA (MT-IIA) (Murphy et al., 1994).

Other documented ORPs include erythropoietin, vascular endothelial growth factor (VEGF), interleukin-6, platelet derived growth factor, endothelin 1, transforming growth factor ß, DT-diaphorase, ϒ-glutamylcysteine synthetase, xanthine oxidase, ornithine decarboxylase, adenylate kinase-3, and the glycolytic enzymes (Bodi et al., 1995; O'Rourke et al., 1996; Sutherland et al., 1996; Yan et al., 1995). Furthermore, a wide range of transcription factors, including hypoxia inducible factor-1 (HIF-1), ciun, cFos, pS3 (Gracber et al., 1994), $C/EBP_ß$, HSF-1, AP-1, $NF\text{-}_κB$, ATF-2, and ATF-4, are associated with hypoxia and anoxia responses in both normal and cancer cells (Estes et al., 1995; Graeber et al., 1994; Laderoute et al., 1996; Sutherland et al., 1996). Transcriptional and/or posttranslational modifications of these factors may occur in response to hypoxia, but in many cases the significance of the changes is not clear.

ORPs and Malignant Phenotypes

Recent research has demonstrated fundamental contributions of hypoxia-induced proteins to processes that favor malignant progression (Giaccia, 1996; Rak et al., 1995b). For example, VEGF, which mediates tumor angiogenesis (Plate et al., 1992; Shweiki et al., 1992, 1995) correlates directly with areas of hypoxia present in *in vitro* tumor models (Waleh et al., 1995) and in solid tumors (Plate et al., 1992; Shweiki et al., 1992). These studies support the theory that VEGF isoforms generated in hypoxic microregions of tumors stimulate host endothelial cells to assemble new vasculature, thus driving tumor growth by a feedback loop. Interestingly, the hypoxia-associated induction of VEGF may be also positively modulated by mutant ras oncogenes (Mazure et al., 1996; Rak et al., 1995a).

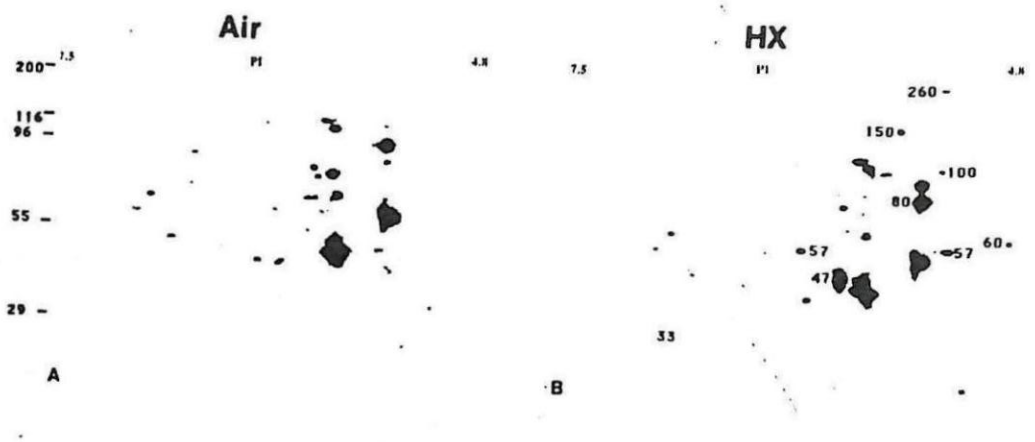
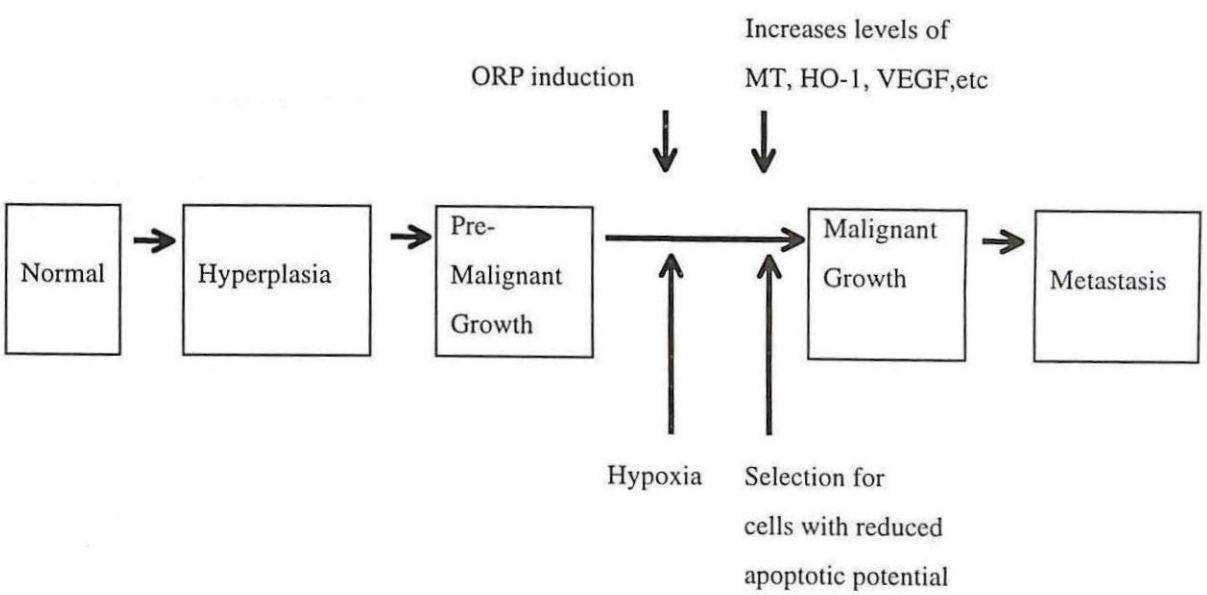


Figure 1. Two-dimensional electrophoretic patterns of Triton-XlOOsoluble polypeptides from aerobic (AIR; A) and hypoxic (HX; B) A431 cells. Approximately 7 x 10^5 cpm of sample was loaded for the first dimension. The x-axis represents pI range (pH 4.8 to 7.5), and the y-axis represents molecular weight (x1000 Da).

ORPs and Reoxygenation

We suggest that hypoxia also influences malignant progression through effects of specific ORPs on cellular survival and function during subsequent reoxygenation. This review focuses on three such proteins: *p53*, HO-l, and MT-IIA. Studies by our group (Murphy et al., 1993, 1994; Sutherland et al., 1996) and by Dr. Amotto Giaccia's laboratonS (Giaccia, 1996; Graeber et al., 1996) suggest the involvement of these ORPs in cell survival responses of tumor cells experiencing hypoxia and reoxygenation episodes. Specifically, pS3 has been implicated in apoptotic selection following hypoxia, and we postulate that the hypoxia-induced levels of HO-1 and MT-IIA proteins act as anti-oxidants during reoxygenation events. The following schematic summarizes our current understanding of how hypoxia, in particular induction of the abovementioned ORPs, may enhance malignant growth.



As adapted from Giaccia (1996).

p53

The *p53* gene encodes a tumor suppressor protein that is believed to be an important mediator of cell cycle control and a genetically encoded program of cell death, or apoptosis (Yonish-Rouach, 1991). It is believed that p53, acting as a transcription factor (Prives al., 1994), activates expression of proteins to induce cell cycle arrest or apoptosis in response to DNA damaging agents and oxidative stress (Arrowsmith and Morin, 1996; Donehower and Bradley, 1993; Lee and Berstein, 1995; Vogelstein and Kinzler, 1992). Other studies now indicate that hypoxic stress also induces the accumulation and activity of this protein (Graeber et al., 1994). This induction by low oxygen conditions suggests a potential model for hypoxia in tumorigenesis (Giaccia, 1996). The hypothesis is based, in part, on the fact that mutations of p53 occur in diverse tumor types and affect protein binding to DNA (Kinzer and Vogelstein, 1996). Most tumors, however, are initiated by genes other than *p53* and go through clonal evolution resulting in expansion. As tumorigenesis progresses, a tumor will possess both wild-type and mutant p53 cells. At this stage, many tumors will have outgrown their blood supply, the result being microregions of hypoxia or anoxia where *p53* activation occurs. Although VEGF expression is also activated by hypoxia, resulting in angiogenesis, the tumor eventually reaches a stage where both chronic and transient hypoxia (see above) will occur. As these regions experience hypoxia/reoxygenation cycles, cells harboring wild-type *p53* initiate apoptosis, while cells with mutant *p53* will begin to multiply uncontrollably.

It is believed that these cells harboring the mutant *p53* gene expand their mutant genomes through gene amplification and gross chromosomal abnormalities and utimately become metastatic (Kinzer and Vogelstein, 1996). This model has been partially confirmed in both tissue culture and animal studies. Giaccia's group (Graeber et al., 1996) demonstrated that ElA/Ha-ras transformed mouse embryonic fibroblast cells (MEFs) possessing the null genotype for *p53* exhibited greatly diminished apoptosis rates following hypoxia (0.02% O_2 for 48 h) as compared with their wild-type counterparts (Fig. 3). Studies using mixed cultures of wild-type and mutant *p53* cells (1000: 1 ratio) exposed to multiple rounds of hypoxia also demonstrated that transient hypoxia can select for apoptosis-resistant cells for example, the mutant cells had overtaken the wild-type cells following only 7 rounds of hypoxia/reoxygenation. *In vivo* evidence of this process was also obtained using tumors grown from these wild-type and null *p53* cells (Graeber et al., 1996). Specifically, apoptotic regions of tumors derived from either *p53* wild-type or null MEFs were correlated with hypoxic regions within the same tumors. However, the incidence of apoptosis was 3- to 4-fold greater in hypoxic regions of *p53* wild-type tumors than in hypoxic regions of *p53*-deficient tumors, whereas no difference was observed in aerobic regions from the same tumors.

ORPs and Redox Control

Cells have developed very intricate strategies to maintain an intracellular reduced state in response to not only an oxidizing extracellular environment but also oxidants from normal metabolism, reductive biosynthesis, and many environmental stresses. The cell apparently utilizes fluctuations of redox levels (or ratios) to control protein activity in a manner similar to posttranslational phosphorylation. It is this redox control that is believed to be an important determinant in the regulation of many animal and plant processes, including transcriptional regulation, cell division, meiosis, DNA replication, cell division, and protein assembly and repair (Buchanan et al., 1994; Powis et al., 1995). The main defenses, or modulators of redox, include three groups: (1) the glutathione (GSH) and thioredoxin redox buffer systems that maintain protein thiol homeostatasis (Meister, 1991; Powis et al., 1995); (2) superoxide dismutase, catalase, and glutathione peroxidase, which are involved in superoxide anion and H_2O_2 metabolism; and (3) protein disulfide isomerase, which regulates protein folding. A wide variety of stresses and toxins can readily result in an intracellular oxidized state that gives rise to the formation of reactive oxygen species and thus leads to lipid peroxidation, DNA crosslinking, and formation of disulfide bonds in proteins (Powis et al., 1995). In this report we focus on hypoxia with subsequent reoxygenation, an event common in many solid tumors experiencing both transient and chronic ischemia-like insults.

Hypoxia is believed to result in severely compromised oxidative defense systems upon subsequent reoxygenation. It is believed that the generation of reactive oxygen radicals upon reperfusion is responsible for tissue damage in pathophysiological processes such as stroke or ischemic infarctions of the heart. Recent studies by our group and others have begun to unravel cellular strategies of cancer cells that allow them to survive oxidative stress associated with reoxygenation. We hypothesize that it is the hypoxia/reoxygenation-resistant cells within transient and chronic hypoxic

micro-regions of a solid tumor that contribute to malignant progression. The effect of hypoxia on the redox state of human cancer cells has been determined by direct measurement of the principal cellular antioxidant thiol, GSH, in HT29 colonic carcinoma cells (O'Dwyer et al., 1994) and squamous cell carcinoma SiHa cultures (Laderoute et al., 1996). Both studies showed that total GSH content was depleted to approximately 20-30% of the level found in aerobic control cells. These reductions are thought to be caused, in part, by the inhibition of GSH-synthesizing enzymes. Interestingly, hpoxia/reoxygenation apparently has no effect on the steady state levels of either thioredoxin or protein disulfide isomerase (B. Murphy, unpublished observations).

Our group has previously identifled two ORPs that are strongly implicated in cellular protection against oxidative stress. We suggest that both MT-IIA and HO-1 function as antioxidants which may partially replace the depleted GSH buffering system and therefore offer hypoxic tumor cells protection during reperfusion. Furthermore, it is likely that inductions of the two ORPs result in increased resistance to chemotherapy in reoxygenated cells and may also have roles in cell cycle regulation.

Heme Oxygenase-1 (HO-1)

Heme oxygenase catalyzes the rate-limiting step in the oxidative degradation of heme to biliverdin (Tenhunen et al., 1968), which is enzymatically reduced to bilirubin by biliverdin reductase (Tenhunen et al., 1969). There are two documented isoforms of the enzyme, HO-1 and HO-2 (Maines,1988). HO-2 is a constitutive protein found in the central nervous system, while HO-1 is the inducible form that is found in most animal tissues. Transcription of HO-1 is induced by its substrate, heme, as well as by heavy metals, cytokines, endotoxin, hormones, and oxidative and hypoxic stresses (Lee et al., 1996a; Murphy et al., 1991). The functional significance of HO-1 induction following oxidative stress is only now becoming apparent. For example, within the last five years, a number of groups have reported that increased HO-1 provided cellular protection against heme-mediated oxidant injury. Nath et al. (1992) observed that prior induction of HO- 1 with hemoglobin prevented kidney failure and reduced mortality in the rat. Furthermore, tin protoporphyrin, a competitive inhibitor of heme oxygenase, exacerbated kidney dysfunction. Others have reported similar protective effects of HO-1 against hememediated oxidant injury in a rat model of endotoxic shock and lung injury (Otterbein et al., 1995) and in cultured endothelial cells (Abraham et al., 1995). More recently, Lee et al. (1996a) provided strong evidence indicating that overexpression of HO-1 in human pulmonary epithelial cells (A549 cells stably transfected with rat HO-1 cDNA) facilitated cellular protection against non-heme-mediated oxidant insults, (e.g., hyperoxia), as determined by comparing their reaction with that of wild-type cells. This is consistent with other studies suggesting that oxidative stresses result in elevated expression of HO-1 (Applegate et al., 1991; Choi et al., 1995; Lee et al., 1996b; Vile et al., 1994). Lee's group further showed that the overexpression of HO-1 in the A549 culture model may be associated with cell growth arrest, which may facilitate cellular protection against non-heme-mediated oxidant insults.

The precise mechanism of cellular protection by HO-1 remains unclear. However, an examination of the products of the HO enzyme and subsequent reactions in the heme degradation pathway may offer some insights. For example, it has been reported that regulation of the enzymatic rates of the HO reaction (e.g., by oxidative stress, antisense molecules, methemoglobin, and tin mesoporphyrin) directly regulates ferritin protein levels as a result of iron release-from heme (Eisentein et al., 1991; Vile et al., 1994). Ferritin constitutes the major storage site for nonmetabolized intracellular iron and therefore plays a critical role in regulating the availability of iron to catalyze reactions such as the Fenton reaction and the peroxidation of lipids (Vile et al., 1994). It is believed that the increased ferritin concentrations resulting from increased HO activities result in a sequestration of iron and thus impact on free radical reactions, reducing the oxidant burden on the cell (Balla et al., 1992). Indeed, previous studies have implicated ferritin in the protection of rat kidney, and cultured aortic and skin fibroblast cells from oxidant-induced damage (see Vile et al., 1994). Therefore, ferritin, regulated by the response of HO to inducers such as hypoxic and oxidative stresses, probably acts as an important stress-inducible antioxidant.

Another interesting by-product of heme degradation by HO- 1 is bilirubin, which is the product of the biliverdin reductase reaction. This end product of heme metabolism has generally been considered a potentially cytotoxic, lipid-soluble waste product that must be excreted. However, it now appears that bilirubin, at micromolar concentrations *in vitro*, displays potent antioxidant properties (Stocker et al., 1987).

Specifically, bilirubin has been demonstrated to scavenge peroxyl radicals as efficiently as a-tocopherol, which is regarded as the best antioxidant of lipid peroxidation. Interestingly, carbon monoxide (CO) is also an end-product of the HO reaction. In fact, HO is believed to be the sole intracellular source of CO. This small molecule is postulated to behave as a messenger molecule much like nitric oxide (NO), perhaps by activating guanylyl cyclase (Marks et al., 1991; Verma et al., 1993). CO has been demonstrated to inhibit platelet aggregation and cause relaxation of femoral, carotid, and rat coronary and aortic smooth muscle (see Marks et al, 1991, for a review) as well as function as a neurotransmitter (Verma et al., 1993). More recent studies suggest that HO may modulate intracellular signaling by NO through CO effects on cyclic GMP, a second messenger for NO. In particular, Maulik and coworkers (1996) used rat hearts to demonstrate that inducers of NO (L-arginine) and inhibitors of HO (protoporphyrins) reduced free radical formation (malonaldehyde) and increased specific myocardial functions, as compared with levels in untreated hearts, following ischemic insults of 30 min. Although NO can behave as an oxidant under certain conditions, in vitro studies by Maulik' s group demonstrated that NO reduced the reactive oxygen species produced by myoglobin and oxoferrylmyoglobin, which are present in high concentrations during the reperfusion of the ischemic heart. Our laboratory is presently developing a similar tumor model to study the effects of HO-derived CO on cell survival in transient hypoxia and thus in malignant progression.

Metallothioneine-IIA

Metallothioneins are a family of ubiquitous low molecular weight proteins (6 -7 kDa) enriched in cysteine residues. The metallothionein (MT) family consists of two major isoforms (MT-I and -II) and a brain-specific isoform, MT-III. There are at least 12 distinct genes of the MT-I and -II family, of which 6 or 7 are believed to be functional. The expression and regulation of four of these proteins, MT-IA, MT-IB, MT- 1G, and MTIIA, have been extensively studied (Samson et al.. 1995: Skroch et al.. 1993). All four isoforms are constitutivelv expressed in most cultured human cells, with MT-IIA accounting for at least 50% of the total cellular MT protein (Skroch et al., 1993), and it is this isoform which is responsive to a wide variety of inducers. MTs have well-established regulatory roles in metal ion homeostasis and in the detoxification of heavy metals (Leyshon-Sorland et al., 1993a). Other inducers include hormones (glucocorticoids and progesterone), cytokines (interferon-α, interleukin-I, and tumor necrosis factor), phorbrol ester tumor promoters, and environmental stresses such as hypoxia, oxidative stress, UV irradiation, and DNA damaging agents (e.g., cisplatin) (Lazo and Pitt, 1995; Leyshon-Sorland et al., 1993b; Murphy et al., 1994; Skroch et al., 1993).

Because MT is the major intracellular protein thiol- and zinc-binding protein, it may assume an important regulatory role in Zn homeostasis and thus affect the activity of Zn dependent proteins, including transcription factors TF-IIA and SP-1 (Woo et al., 1996). It is also possible that the metal response transcription factors (MRFs) are regulated by their target genes, since preliminary reports showed two of these proteins to contain zinc finger domains of the cysteine-histidine type (Inouye et al., 1994; Radtke et al., 1996; Woo et al., 1996). MT may also contribute copper to key antioxidants, including Zn/Cu superoxide dismutase, and therefore have an antioxidant role. The nucleophilic nature of the protein also implies a direct antioxidant role. For example, purified MT protein was reported to act as a scavenger of free hydroxyl radicals (Thornalley and Vasak, 1985) and has been shown to be approximately 39-fold more effective at protection of DNA from hydroxyl radical attack than glutathione cysteine (Abel and de Ruiter, 1989). Furthermore, a high nuclear content of MT in V79 Chinese hamster cells was shown to confer protection from hydroxyl radical attack (Chubatsu and Menrghini,1993). This ORP also protects against the toxic effects of tert-butyl hydroperoxide (Schwartz et al., 1994), NO (Schwartz et al., 1995), and some electrophilic mutagens (Kaina et al., 1990; Kelley et al., 1988; Lohrer AND Robson, 1989; Schwartz et al., 1995).

MT is of particular interest in the study of malignant progression primarily because of its suspected involvement in protection against anticancer drugs, radiation, oxidative stress, and apoptosis (Deng et al., 1996; Kaina et al., 1990; Lazo and Pitt, 1995; Lohrer and Robson, 1989; Sato et al., 1995; Schwartz et al., 1995). Overexpression of this thiol-rich protein has been implicated in increased resistance to electrophilic and alkylating antineoplastic agents including cisplatin, doxorubicin, melphalan, bleomycin, N-methyl-N-nitrosurea (MNU), and N-methyl-N'nitro-N-nitrosoguanidine (MNNG). It has also been suggested that MT plays a physiological role in the cellular proliferative (and malignant) phenotype of human colonic and breast cancer cells (Nagel and Vallee, 1995). Clinical studies reinforce

these f1ndings, since high expression of MT correlate with poor prognosis in human breast cancer (Goulding et al., 1995) and with poorly vascularized, malignant human lung tumors (Koomagi et al., 1995).

Our ongoing research appears to confirm the importance of MT in the protection of tumor cells against the deleterious effects of hypoxia/reoxygenation and subsequent drug exposure. For example, our finding that hypoxia increases hMT-IIA expression in A431 cells suggested that increased protein expression in hypoxic microenvironments could cause transient drug resistance. We investigated the ability of hypoxia to cause cisplatin resistance by comparing the clonogenic survival of aerobic and reoxygenated A431 SC cells exposed to various doses of cisplatin for 1 h at 37°C before plating. Hypoxic pretreatment caused an 18-fold increase in survival at the highest cisplatin dose at which survival could be measured (16 Ag/ml; 53 llM). It is worth emphasizing that this cisplatin resistance was a transient phenomenon that cannot be directly compared with the greater degree of resistance associated with prior selection in the presence of the drug (Kelley et al., 1988). Furthermore, it is likely that the survival differences would be greater after an in vivo-like reoxygenation, where the hypoxic cells would not experience the sudden enormous change in oxygen exposure (an approximately 200-fold increase) associated with our normal hypoxic protocol (0.01% O_2 for 8 to 14 h).

To establish a stronger link, we initiated studies using transgenic MT knockout mice. We examined the effect of the loss of MT expression on the cytotoxicity of the drug by using homologous embryonic fibroblast cells from transgenic mice with targeted disruptions of MT-1 and MT-II genes (MT -/-; null) (Kondo et al., 1995). Our initial studies focused on the transformed (SV40 large tumor antigen) knockout and wild-type cells (MT +/+). This type of transformation eliminates functional *p*53 function. Clonogenic survival assays showed that the cisplatin resistance of hypoxic MT +/+ cells was 400- and 2400-fold greater than that of hypoxic and aerobic null cells, respectively (B. Murphy et al., unpublished results). In sum, these data support the hypothesis that endogenous and induced MT levels affect the sensitivity of mammalian cells to clinically important anticancer drugs. We are presently studying primary homozygous null and wild-type cells to investigate the role of *p*53-dependent and -independent apoptosis pathways in cisplatin-mediated cytotoxicity following hypoxia reoxygenation. Preliminary studies also indicate a protective role for MT, in *p*53 wild-type cells, against oxidative stress followinz ischemia-like events.

Summary.

In summary, the evidence reviewed here implicates specific hypoxic stress proteins in the process of malignant progression. Specifically, three ORPs-p53, HO-1, and MT-IIA_appear to be intimately involved in the development of phenotypes associated with increased resistance to apoptosis, oxidative stress, and antineoplastic drugs in response to hypoxia/reoxygenation insults. Future research is needed to confirm these postulates and to further delineate the precise mechanisms of action. Ongoing studies using improved cDNA difference libraries and protocols will almost certainly identify other important proteins associated with hypoxic microenvironments of solid tumors.

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